17β-Hydroxysteroid Dehydrogenase Type 12 is Responsible for Maturation-Inducing Steroid Synthesis During Oocyte Maturation in Nile Tilapia

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Abstract

17α, 20β-Dihydroxy-4-pregnen-3-one (DHP) is a maturation-inducing steroid in many teleost fish. Carbonyl reductase-like 20β-hydroxysteroid dehydrogenase (CR/20β-HSD) is a candidate enzyme responsible for DHP production during oocyte maturation in various fish, including Nile tilapia. However, a novel type of 17β-hydroxysteroid dehydrogenase, type 12-like (17β-HSD12L), is responsible for DHP production during oocyte maturation in masu salmon. 17β-HSD12 (presumably orthologous to salmon 17β-HSD12L) has been detected in Nile tilapia; however, its enzymatic activity and specific ability to convert the DHP substrate 17α-hydroxyprogesterone (17OHP) have not been examined. This study aimed to determine whether CR/20β-HSD or 17β-HSD12 is responsible for DHP production during oocyte maturation in the Nile tilapia. Mammalian expression vectors containing tilapia *hsd17b12* or *CR/20bhsd* were transfected into HEK293T cells, followed by incubation with 17OHP. HEK293T cells transfected with *hsd17b12* exhibited a strong ability to convert exogenous 17OHP to DHP (73.8% yield). Cells transfected with *CR/20bhsd* or the control vector converted only 7.4% and 7.5% of 17OHP to DHP, respectively. In addition, based on LC-MS/MS analyses, 17β-HSD12 did not convert any substrates other than 17OHP, including DHP, adrenosterone, androstenedione, estrone, testosterone, 11-ketotestosterone, and estradiol-17β. CR/20β-HSD showed strong 17β-HSD oxidoreductase activity especially with adrenosterone and androstenedione. Tissue-specific *hsd17b12* expression analyzed by RT-PCR showed that *hsd17b12* mRNA was strongest amplification in full-grown follicles. Finally, full-grown ovarian follicles were incubated with salmon pituitary extract (SPE, 100 μg/mL) or human chorionic gonadotropin (HCG,100 IU/mL) to induce 20β-HSD activity *in vitro*, and enzyme activity was assessed by co-incubation with 100 ng/mL 17OHP for 2, 4, 8, and 16 h. Conversion of 17OHP to DHP by ovarian follicles incubated with SPE and HCG peaked at 16 h, subsequent with increased follicular *hsd17b12* mRNA levels, which were significantly higher than those in control incubations. However, the levels of *CR/20bhsd* mRNA remained low and did not differ among time points. The present study strongly suggests that 17β-HSD12, and not CR/20β-HSD, is the 20β-HSD responsible for DHP production by ovarian follicles during oocyte maturation in Nile tilapia.

Keywords: 17β-HSD12; Oocyte maturation; Maturation-Inducing Steroid; Nile tilapia

1. Introduction

In several fish species, 17α, 20β-dihydroxy-4-pregnen-3-one (DHP), has been identified as a maturation-inducing steroid (Nagahama 1988; Adachi *et al*., 2003). Gonadotropin is of primary

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42 importance in regulating the synthesis of maturation-inducing steroids during oocyte
43 maturation. Follicle-enclosed post vitellogenic oocytes of some teleost undergo germinal
44 vesicle breakdown *in vitro* when they are incubated with gonadotropin (Nagahama 1987a).
45 Gonadotropin influences DHP production by inducing the activity of the enzyme 20β-
46 hydroxysteroid dehydrogenase (20β-HSD) (Nagahama 1987b) to catalyze the reduction of 20-
47 carbonyl groups to 20β-hydroxylated products (Nagahama and Yamashita 2008). An enzyme
48 with 20β-HSD activity was first isolated from the neonatal pig testis, showing strong 17α-
49 hydroxyprogesterone (17OHP) conversion activity to produce DHP (Nakajin *et al*., 1987), and
50 its cDNA sequence shows high similarity to human carbonyl reductase (Tanaka *et al*., 1992),
51 which is thought to catalyze the reduction of carbonyl compounds with NAD(P)H-dependent
52 oxidoreductase activity (Tanaka *et al*., 2002). The significance of the identity between 20β-
53 HSD and carbonyl reductase is unclear, since enzymes with similar functions catalyze the
54 reduction of carbonyl groups to form corresponding products (Kazeto *et al*., 2001). Guan *et al*.
55 (1999) isolated cDNAs that encode proteins with high homology to pig 20β-HSD from rainbow
56 trout (*Oncorhynchus mykiss*) ovarian follicles, named carbonyl reductase-like 20β-HSD
57 (CR/20β-HSD). CR/20β-HSD type A has both 20β-HSD and carbonyl reductase activities.

58 However, it is not clear whether CR/20β-HSD functions in DHP production during oocyte
59 maturation. A study of Nile tilapia (*Oreochromis niloticus*) has reported that human chorionic
60 gonadotropin (HCG) could induce *CR/20bhsd* mRNA expression in post-vitellogenic
61 immature follicles within 1–2 h, suggesting that *CR/20bhsd* is responsible for 20β-HSD activity
62 prior to oocyte maturation (Senthilkumaran *et al*., 2002). However, its role in DHP production
63 was not investigated. *In vitro* studies of ovarian follicles from zebrafish incubated with HCG
64 have shown no effect on *CR/20bhsd* induction (Wang and Ge, 2002). Similarly, in masu salmon
65 studies with gonadotropin induction, CR/20β-HSD has not been linked to DHP production (Ijiri
66 *et al*., 2017).

17β-Hydroxysteroid dehydrogenases (17β-HSDs) are a family of multifunctional enzymes that modulate the synthesis and metabolism of steroids by catalyzing the reduction of 17-ketosteroids or the oxidation of 17β-hydroxysteroids using NAD(P)H or $NAD(P)^+$ as a cofactor (Labrie *et al*., 1997). 17β-HSD controls the formation and conversion of several reproductive hormones, such as androgen and estrogen (Mindnich *et al*., 2004), with important roles in sex differentiation and gametogenesis in fish (Nagahama 2005; Ijiri *et al*., 2008). There are multiple types of 17β-HSDs with several functions. Isozymes of 17β-HSD differ from each other in selective substrate affinity, reaction direction, and tissue distribution. Therefore, different isozymes might possess different physiological functions in the steroidogenic pathway and in the different steps of the biosynthesis of sex steroids from cholesterol (Poirier *et al*., 2001). However, most studies of 17β-HSDs have focused on mammals. Therefore, the mechanism by which steroidogenic enzymes control the reproductive cycle in fish is not well understood.

Recently, Ijiri *et al*. 2017 identified and characterized 17β-HSD12-like as a key enzyme catalyzing the final steps in the synthesis of DHP in masu salmon and showed that it is involved in DHP production in the granulosa cell layer during oocyte maturation. An *in vitro* investigation has shown a close relationship between the induction of 20β-HSD activity and up-regulation of *hsd17β12-like* (*hsd17β12l*) mRNA by forskolin and chum salmon pituitary extract (SPE) at one or two months before ovulation. A phylogenetic analysis placed the full-length *hsd17β12l* sequence between Atlantic salmon (*Salmo salar*) *hsd17β3* and *hsd17β12,* but it was not highly orthologous to either. Furthermore, Nile tilapia 17β-HSD type 12 (17β-HSD12), which is expressed exclusively in the testis of immature fish (Zhou *et al*., 2005), is phylogenetically positioned between zebrafish 17β-HSD3 and human 17β-HSD12 in the same clade with *Tetraodon* 17β-HSD12B; it was therefore termed tilapia 17β-HSD12, encoded by *hsd17b12*. Enzymatic activity of tilapia *hsd17b12* expressed in HEK293 cells showed no

conversion from androstenedione to testosterone and between estrone to estradiol or vice versa. However, 17OHP was not tested as a substrate. Hence, it was categorized as an enzyme with unknown substrate specificity and is a potential ortholog to masu salmon 17β-HSD12L, which participates in DHP production.

Based on these previous studies, it is unclear whether the gene encoding the enzyme responsible for the conversion of 17OHP to DHP during oocyte maturation is *CR/20bhsd* or *hsd17b12.* Both may be involved in steroid metabolism, but gonadotropin induction and their substrate specificities and 20β-HSD activity during oocyte maturation are not known. This study aimed to determine the physiological roles and enzymatic activities of Nile tilapia *hsd17b12* by focusing exclusively on its role in DHP production. Owing to the close relationship between 17β-HSD type 3 and 17β-HSD type 12, we investigated the potential activities of Nile tilapia *hsd17b12* to ascertain its major enzymatic activities, i.e., 17β-HSD or 20β-HSD activity.

2. Materials & Methods

2.1. Animals

Nile tilapia were reared in a temperature-controlled (26 ± 1°C) re-circulating freshwater tank under natural light conditions. Adult females were maintained separately in an isolated tank, with continual spawning every two weeks. Four females two days before estimated spawning day were sampled for ovarian follicle isolation used in the incubation experiment. In the other experimental group, fish were randomly sampled from the pooled fish tank and four fish who had large follicles were used for experiments. Ovarian follicles were isolated from ovaries by careful dissection in ice-cold tilapia Ringer solution (140 mM NaCl, 10 mM $NaHCO_3$, 4 mM KCI, 2 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5.5 mM glucose, adjusted to pH 7.8 by 0.1N NaOH) and separated into the largest follicles and remaining ovarian fragments, which

were separated into small pieces containing 30-40 small oocytes with intact connective tissues. The average diameter of 10–30 follicles from each female was measured using ImageJ (http://rsb.info.nih.gov/ij/). Ovaries were fixed in Bouin's solution, dehydrated in an ascending series of graded ethanol, and embedded in paraffin. Sections of 5 μm in thickness were stained with hematoxylin and eosin to observe the developmental stage of each ovary. Experimental procedures complied with the guidelines of the Animal Research Committee of Hokkaido University.

2.2. Cloning of hsd17b12 and CR/20bhsd cDNAs

Nile tilapia testes were immersed in ice-cold tilapia Ringer's solution. Total RNA was extracted using ISOGEN II (Nippon Gene Co., Tokyo, Japan). cDNA was synthesized using Oligo(dT)15 primer (Promega, Madison, WI, USA) and ReverTra Ace RT enzyme (Toyobo Co., Ltd., Osaka, Japan) following the manufacturer's recommendation. GenBank accession numbers for the aligned nucleotide sequences were as follows: *hsd17b12,* AY663854.2 and *CR/20bhsd*, AF439713.1. Primers for an additional nested polymerase chain reaction (PCR), including two pairs of inner primers with adapter sequences for the vector pIRES2-ZsGreen1, were designed to cover all open reading frames of target genes. *Hsd17b12* was amplified by PCR using Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) with outer forward and reverse primers, and the amplicons were subjected to nested PCR with inner forward and reverse primers. *CR/20bhsd* was amplified using its primer sets following the same method. *Hsd17b12* and *CR/20bhsd* open reading frames (ORF) were inserted into the pIRES2-ZsGreen1 vector (Takara) containing the CMV promoter and ZsGreen fluorescence protein (ZsGreen) by Gibson Assembly Master Mix (New England Biolabs). All clones were sequenced bidirectionally using the Applied Biosystem 3130 xl Genetic Analyzer (Foster City, CA, USA). *Cyp19a1a* (aromatase; accession number

AF472620) was used as a negative control for the 20β-HSD activity analysis. The ORF of tilapia aromatase was sub-cloned into the vector pIRES2-ZsGreen1.

2.3. 20β-HSD activity analysis

Hsd17b12 and *CR/20bhsd* expression vectors were transfected into HEK293T cells using ScreenFect A Transfection Reagent (Wako Pure Chemical Inc., Osaka, Japan) according to the manufacturer's protocol. Approximately 20,000 HEK293T cells were cultivated in 96-well cell culture plates containing 80 μL of DMEM (Dulbecco's Modified Eagle medium; Nissui Pharmaceutical Co., Tokyo, Japan) with 10% FBS (fetal bovine serum), supplemented with 100 U/mL penicillin (Wako) and 100 μg/mL streptomycin (Wako) at 37 °C and 5% CO_2 in a humidified atmosphere. At 24 h after transfection, the cells were incubated in DMEM without FBS supplemented with 100 ng/mL 17OHP for 20 h. Empty pIRES2-ZsGreen1 was used as a control, *cyp19a1a* expression vector was used as a negative control, and three independent replicates were conducted for each treatment. ZsGreen intensity was measured using the Wallac 1420 Multi-label Counter (Perkin Elmer Life Sciences, Waltham, MA, USA) and used for the normalization of transfection efficiency.

2.4. DHP measurement

After incubation, DHP and other steroid metabolites were extracted from the media using three extractions with ethyl ether, according to the methods of Kagawa *et al*. (1981). The DHP concentrations of the extracted steroid metabolites were then measured using time-resolved fluoroimmunoassays (TR-FIA), according to the method introduced by Yamada *et al*. (2002).

2.5. Tissue-specific hsd17b12 expression

Total RNA was isolated from brain, pituitary, head-kidney, trunk-kidney, liver, spleen, heart, ovarian fragments and follicle layers from ovaries of adult female two days before spawning,

and sperm-containing testis of adult male, using ISOGEN II. Total RNA (500 ng) from each tissue was reverse transcribed (RT) as described above in 20 μL reactions volume, according to the manufacturer's instructions. The diluted RT reactions (10%, v/v; 2 μL) were then used as initial cDNA templates for PCR amplification (20 μL reactions) of both *hsd17b12* and *beta-actin* (*β-actin*) as an internal control. The primers were designed to amplify fragments that included an intron/exon boundary, in order to distinguish the amplification of genomic contaminants. Primers were designed as follows: *hsd17b12;* 5′-TGTTGCCAGAGAAATCGAGGG-3′ (forward) and 5′-AGGAAGGGAGCCACACACTGG-3′ (reverse), *β-actin;* 5′-GTCGCCCCAGGCATCAGGGTG-3′ (forward) and 5′-TAGCCACGCTCTGTCAGGATC-3′ (reverse). The RT-PCR cycles were as follows: 94 °C for 1 min, followed by 30 cycles of 98 °C (15 s), annealing at 60 °C (15 s) and 56 °C (15 s) for *hsd17b12* and *β-actin*, respectively, and 72 °C (30 s), ending with 2 min of extension at 72 °C. Negative control was set up with water as templates. Ten microliters of the PCR reactions were electrophoresed on 2% agarose gels, stained with ethidium bromide, and digitally photographed under UV light.

2.6. Incubation of follicles

Ovaries were divided into two groups; the largest follicles and the remaining ovarian fragments were separated under a binocular stereo microscope. Ten follicles (about 0.8g) or remaining ovarian fragments (about 2g) were independently incubated in 1 mL of Leibovitz's L-15 culture medium (GIBCO®, Rockville, MD, USA), with 100 ng/mL 17OHP alone (control), or 17OHP and 100 μg/mL SPE (from the pituitaries of post-ovulation chum salmon), or 17OHP and 100 IU/mL HCG (Puberogen®, Novartis Animal Health, Tokyo, Japan) in 24-well non-coated plastic culture plates. The incubations were maintained in a humidified incubator at 27 °C for 2, 4, 8 and 16 h. Four replicated incubations were conducted from four fish for each time point and treatment. Each experiment used follicles from a single female to exclude individual

variation in follicle responsiveness. After each incubation, yolk was removed from follicles or ovarian fragments and they were immersed in RNA*later* Solution (Ambion) and stored at -80 °C until used for RNA extraction. At the same time, medium was collected to measure DHP concentration.

2.7. Quantitative PCR

Total RNA was extracted from the group of 10 yolkless follicles from each treatment or ovarian fragments using ISOGEN II (Nippon Gene Co.). Total RNA (200 ng) from each incubated tissue was reverse transcribed (RT) using random hexamer primers (Invitrogen, Life Technologies Co., Carlsbad, CA, USA) and ReverTra Ace RT enzyme reactions, according to the manufacturer's instructions. The diluted RT reactions (10%, v/v; 2 μL) were used as templates for 20-μL quantitative PCR (qPCR) reactions using PowerUp™ SYBR® Green Master Mix (Applied Biosystems) and the StepOnePlus Real-Time PCR system with StepOne™ Software (Applied Biosystems). The qPCR primer sets included one primer that flanked intron–exon boundaries to prevent genomic amplification (Table 1).

2.8. Liquid chromatography mass spectrometry (LC-MS/MS)

Approximately 30,000 HEK293T cells were cultivated in 24-well cell culture plates containing 1 mL of DMEM following the method described above. After transfection with the *hsd17b12* expression vector, incubation was performed with other potential substrates for 17β-HSD, include androstenedione (A4), adrenosterone (11KA4), estrone (E_1), testosterone (T), 11-ketotestosterone (11KT), and estradiol-17β (E_2). DHP was also used to evaluate 20β-dehydrogenase activity. In addition, cells transfected with the *CR/20bhsd* expression vector were incubated with 17OHP, A4 and 11KA4. Each experiment included three independent replicates, and non-transfected cells were used as controls. The concentration of all tested substrates were 1000 ng/mL.

The steroids extracted using 5 mL of dichloromethane were added to 1 mL of culture medium. Then, dichloromethane was gently evaporated at 45 °C in a heating bath, followed by resuspension in 300 µL of methanol and filtering through a 0.22-µm micropore filter (Starlab Scientific Co., Ltd., Xi'an, China). Liquid chromatography was conducted using a DGU-20A-5R degassing unit (Shimadzu Corp., Kyoto, Japan). Steroids were separated on a polar C18 column (2.6 µm, 100 × 2.1 mm; Phenomenex) with methanol and 0.1% (v/v) formic acid in water for running in gradient mode with elution at 0.25 mL/min. Mass spectrometry analyses were performed using the TripleTOF 5600^{+} system (AB SCIEX, Concord, ON, CA) with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) ion sources. Total ion chromatograms were recorded using Analyst® TF software (SCIEX). Fragmentation of steroids was achieved by applying a mass range of 10 V and collision energy of 40 V.

2.9. Statistical analyses

Multiple comparisons were performed to evaluate differences among groups with Tukey–Kramer tests. All quantitative mRNA data were converted to log 10 values before analyses and are reported as means ± standard error of the mean (SEM). Statistically significant differences were calculated at $P < 0.05$, and StatView for Windows version 5.0.1 (SAS Institute Inc., Cary, NC, USA) was used for analyses.

3. Results

3.1. Diameter and histological observation of follicles

Follicles obtained from four females two days before spawning with average diameters of 2.14 ± 0.16 mm (n = 10 from each female) exhibited a germinal vesicle migration to almost attaching to the oocyte membrane, while the other four females selected from the pooled tank contained follicles with average diameter of 1.81 ± 0.07 mm (n = 10 from each female), showing germinal vesicle migration at the central intermediate position to the oocyte

237 membrane within the oocyte. Based on follicle size and location of the germinal vesicle, we
238 define the former as a full-grown follicle and the latter as late-vitellogenic. After the largest
239 follicles were removed, ovarian fragments included various stages of oocytes, including the
240 chromatin nucleolus stage, perinucleolar stage, cortical alveolar stage, and mid-vitellogenic
241 stage with a diameter of 0.70 ± 0.14 mm (n = 10) (Figure 1).

242 *3.2. 20β-HSD activity analysis*

243 The conversion rates of 17OHP to DHP for *hsd17b12* and *CR/20bhsd* transfected into
244 HEK293T cells are shown in Figure 2. HEK293T cells transfected with *hsd17b12* converted
245 17OHP to DHP at the highest rate of 73.82%, which was significantly different from those of
246 cells transfected with other genes ($P < 0.05$). HEK293T cells transfected with *CR/20bhsd*
247 showed a low conversion rate of 7.41%, which was not significantly different from 7.53% for
248 HEK293T cells transfected with *cyp19a1a*. The control group with empty vectors showed the
249 lowest DHP production (5.13%). When compared with the control, values for *cyp19a1a* and
250 *CR/20bhsd* were not statistically different (Figure 2).

251 *3.3. Tissue-specific hsd17b12 expression*

252 RT-PCR analysis revealed that *hsd17b12* mRNA was barely present in ovarian fragments and
253 strongest amplification was found in full-grown follicles at germinal vesicle migration stage,
254 followed by testis. No amplification was detected from the mRNA of pituitary, head-kidney,
255 liver and spleen, however, a very faint amplifications were presented in brain, trunk-kidney
256 and heart. (Figure 3)

257

258

259 *3.4. DHP concentration in incubation media*

Figure 4 shows the levels of DHP in incubated media of full-grown follicles with a 2.14 mm follicle average dimeter. The DHP in the incubation media of the control group (17OHP alone) was constantly low throughout the experimental period until 16 h. HCG induced greater DHP production from exogenous 17OHP than 17OHP alone (control) at 2 h of incubation followed by SPE at 4 h ($P < 0.05$). The DHP concentrations in the 17OHP plus HCG groups were 0.64, 1.57, 2.04, 4.04, and 10.40 ng/mL at 0, 2, 4, 8, and 16 h of incubation, respectively. The levels were always greater than those for 17OHP alone. The highest DHP levels were observed for 17OHP plus SPE induction, observing 11.81 ng/mL production at 16 h. These levels were 11 times higher than those for 17OHP alone.

Figure 5 shows the levels of DHP in the media after the incubation of late-vitellogenic follicles with a 1.81 mm follicle average dimeter. At 0 h (before incubation), the concentration of DHP was lowest among all experimental time points. The DHP concentration in the incubation media of the control group gradually increased until 8 h and decreased slightly at 16 h of incubation, but there were no statistically significant differences between incubation periods. SPE or HCG induced greater DHP production from exogenous 17OHP than 17OHP alone at 4 h of incubation ($P < 0.05$). The DHP concentrations in the 17OHP plus HCG groups were 0.72, 1.19, 1.80, 1.91, and 2.01 ng/mL at 0, 2, 4, 8, and 16 h of incubation, respectively. The levels were always greater than those for 17OHP alone, i.e., 0.72, 0.73, 1.02, 1.81, and 1.32 ng/mL, respectively. The highest DHP levels were observed for 17OHP plus SPE induction, i.e., 2.16 ng/mL at 8 h, but these levels were not significantly different from those for 17OHP alone. Both HCG and SPE stimulated higher DHP production from exogenous 17OHP in full-grown follicles than late-vitellogenic follicles at any experimental time point.

3.5. Hsd17b12 mRNA levels in incubated follicles

No significant difference was observed between *hsd17b12* mRNA levels in full-grown or late-vitellogenic follicles incubated in 17OHP alone for each incubation period (Figures 6 and 7). *Hsd17b12* mRNA levels from full-grown follicles were increased in the presence of SPE or HCG until exceeding the levels observed in the control at 2 h of incubation ($P < 0.05$; Figure 6). In full-grown follicles, the highest *hsd17b12* mRNA level was detected in the presence of SPE at 8 h, which was 78 times higher than that of 17OHP alone. At 16 h, the mRNA level of *hsd17b12* was 17 times higher than that of 17OHP alone.

Hsd17b12 mRNA levels from late-vitellogenic follicles were lowest at 0 h, before incubation, and increased in the presence of SPE or HCG until exceeding the levels observed in the control at 4 h of incubation ($P < 0.05$; Figure 7). In late-vitellogenic follicles, the highest *hsd17b12* mRNA level was detected in the presence of SPE at 8 h, which was 27 times higher than that of 17OHP alone. At 16 h, mRNA expression was 13 times higher than that of 17OHP alone. (Figure 7). Remarkably, *hsd17b12* mRNA levels induced by SPE or HCG were approximately 100 times higher in full-grown follicles than in late-vitellogenic follicles.

Figure 8 shows the levels of *hsd17b12* mRNA in ovarian fragments after removal of full-grown follicles from females at two days before spawning. *Hsd17b12* mRNA levels did not differ significantly among experimental time points, and SPE or HCG did not stimulate *hsd17b12* mRNA levels. Overall, *hsd17b12* mRNA levels in ovarian fragments were far lower than those in full-grown and late-vitellogenic follicles. The results obtained in ovarian fragments when late-vitellogenic follicles were removed were similar as those obtained two days before spawning; non-significant differences in mRNA levels were found throughout experimental periods and treatments (data not shown).

3.6. CR/20bhsd mRNA levels in incubated follicles

307 *CR/20bhsd* mRNA levels in full-grown follicles were not elevated at any experimental time
308 point when incubated with SPE or HCG. In the presence of 17OHP alone, *CR/20bhsd* mRNA
309 levels did not show significant differences throughout all time points (Figure 9). *CR/20bhsd*
310 mRNA levels from late-vitellogenic follicles showed a similar pattern as full-grown follicles
311 such that significant differences were not observed among experimental periods and
312 treatments (data not shown). In ovarian fragments from females two days before spawning,
313 *CR/20bhsd* mRNA levels at all experimental time points and treatments showed no
314 significant differences (Figure 10). The results obtained in ovarian fragments from females
315 when late-vitellogenic follicles were removed were similar as those obtained two days before
316 spawning; non-significant differences were found (data not shown).

317 *3.7. Analyses of 17β-HSD activity*

318 To analyze the 17β-HSD activity of *hsd17b12*, we examined transfected HEK293T cells for
319 the conversion of several steroids that might be substrates for oxidoreductase activity at
320 position C-17, including androstenedione (A4), adrenosterone (11KA4), and estrone (E_1).
321 Steroid conversions were not observed using E_1 and 11KA4 as substrates. Both non-
322 transfected and *hsd17b12* transfected HEK293T cells could convert a small amount of A4 to
323 T. Dehydrogenase activity at position C-17 was evaluated using testosterone (T), 11-
324 ketotestosterone (11KT), and estradiol-17β (E_2), which exhibited no conversion to A4,
325 11KA4, and E_1, respectively, compared with non-transfected HEK293T cells. LC-MS/MS
326 analysis revealed that *hsd17b12* only showed oxidoreductase activity at position C-20, with
327 specific conversion of 17OHP to DHP (Figure 11) and no 20β-dehydrogenase activity to
328 convert DHP to 17OHP (Table 2). The results assessing enzymatic activity of *CR/20bhsd*
329 oxidoreductase activity was observed at position C-17, showing 63.5% and 12.2% conversion
330 from 11KA4 and A4 to 11KT and T, respectively. HEK293T cells transfected with

331 *CR/20bhsd* showed no oxidoreductase activity at position C-20 to convert 17OHP to DHP in
332 comparison with non-transfected cells. (Table 2).

333 **4. Discussion**

334 The present study clearly demonstrated that HEK293T cells transfected with Nile tilapia
335 *hsd17b12* exhibit considerable 20β-HSD activity converting 17OHP to DHP, suggesting that
336 the enzyme encoded by *hsd17b12* contributes to DHP production. Furthermore, there was no
337 evidence that CR/20β-HSD is responsible for DHP production in Nile tilapia. The effect of
338 SPE or HCG on *hsd17b12* mRNA expression and DHP production from 17OHP was
339 enhanced in full-grown follicles compared to that of late-vitellogenic follicles, similar to the
340 results of a previous study on masu salmon demonstrating that 17β-HSD12-like exhibits 20β-
341 HSD activity before ovulation. These results suggest that Nile tilapia *hsd17b12* encodes an
342 ortholog of the masu salmon 17β-HSD12-like enzyme.

343 In masu salmon, the expression of *hsd17β12l* mRNA was limited in testis and follicle layers
344 at final maturation (Ijiri *et al*., 2017). In tilapia, *hsd17b12* mRNA showed the same
345 expression pattern. Zhou *et al*. (2005) did not find *hsd17b12* mRNA expression in the tilapia
346 ovary, probably because they did not test ovarian preparation at just before oocyte
347 maturation. Although we detected very faint amplification of *hsd17b12* mRNA in the tilapia
348 brain, trunk-kidney and heart, such limited low expression may not have physiological
349 importance.

350 The full-grown follicles incubated with SPE or HCG in this study were involved in the
351 induction of 20β-HSD activity and increases in *hsd17b12* mRNA levels. HCG had similar
352 effects to those of SPE, suggesting that HCG and SPE mimic luteinizing hormone (LH) to
353 stimulate the induction of *hsd17b12* synthesis, causing an elevation in 20β-HSD activity to
354 convert 17OHP to DHP.

355 The late-vitellogenic follicles also exhibited *hsd17b12* mRNA expression in response to LH,
356 but DHP production was maintained at low levels. The *hsd17b12* mRNA level induced by
357 LH was approximately 100 times lower than those in full-grown follicles. The induced
358 expression level may not have been sufficient to detect differences in 20β-HSD activity when
359 compared with the controls. This suggests that ability to induce sufficient amounts of 20β-
360 HSD for DHP production in response to LH would be acquired at full-grown follicles just
361 before oocyte maturation. Ovarian fragments that were separated from large follicles included
362 pre-vitellogenic and mid-vitellogenic stages. These ovarian fragments do not exhibit elevated
363 *hsd17b12* mRNA expression in response to LH, unlike full-grown and late-vitellogenic
364 follicles. Altogether, those data support the assumption that DHP is mainly produced in full-
365 grown follicles, and LH is unable to effectively stimulate the synthesis of *hsd17b12* mRNA
366 in the ovary in early spawning cycles.

367 In the present study, full-grown follicles acquired substantial ability to convert 17OHP to
368 DHP in response to LH concomitant with elevated *hsd17b12* mRNA expression. This
369 suggests that the activity of 20β-HSD is involved in *hsd17b12* transcriptional events in
370 response to LH. The induction of *hsd17b12* transcription by LH occurred within 2 h and
371 declined before 16 h, which led to continuous DHP production into high yield until 16 h. It is
372 suggested that effects of LH on stimulation of *hsd17b12* mRNA expression are transient.

373 A 17β-HSD12-like ortholog was previously annotated in zebrafish by Tokarz *et al*. (2012)
374 and was named 20β-HSD type 2 (*hsd20β2*); it catalyzes the reduction of cortisone to 20β-
375 hydroxycortisone at position C-20. The 20β-HSD activity of zebrafish *hsd20β2* towards
376 17OHP has not yet been examined; nevertheless, the authors suggested that zebrafish
377 *hsd20β2* is not likely to play a role in reproduction-related steroid biosynthesis based on its
378 ubiquitous expression in all tissues examined in both adult fish and throughout
379 embryogenesis. Subsequently, masu salmon *hsd17β12l,* which is positioned between 17β-

HSD3 and 17β-HSD12 in a phylogenetic tree, belongs to the same sub-cluster as zebrafish *hsd20β2* and is considered orthologous. 17β-HSD3 is mainly responsible for conversion of A4 to T (Mindnich *et al*., 2005). 17β-HSD12 mainly catalyzes E_1 to E_2 and is also involved in lipid metabolism (Luu-The *et al*., 2006; Lima *et al*., 2013), while zebrafish *hsd20β2* and masu salmon *hsd17β12l* catalyze at position C-20. Nile tilapia *hsd17b12* is positioned between 17β-HSD3 and 17β-HSD12 according to a phylogenetic analysis by Zhou *et al*. (2005). However, 17β-HSD12s of zebrafish and *Tetraodon* together with all 17β-HSD12s from tetrapods belonged to one clade, whereas tilapia *hsd17b12* was clustered into another subclade, suggesting that tilapia *hsd17b12* differs from 17β-HSD12 of zebrafish, *Tetraodon*, and tetrapods. Despite these findings, they named this enzyme 17β-HSD12 because one group of *hsd17β12l* (*hsd20β2*) had not been identified at that time, and other cDNAs close to 17β-HSD12 had not yet been isolated from the tilapia. Indeed, Tokarz *et al*. (2012) re-conducted phylogenetic analysis using *hsd17b3*, *hsd17b12* and *hsd20β2*, and they demonstrated Nile tilapia *hsd17b12* was included in the *hsd20β2* (*hsd17β12l*) clade independent from the *hsd17b12* and *hsd17b3* clade.

The results of LC-MS/MS analyses clearly support the idea that tilapia 17β-HSD12 catalyzes the reduction of carbonyl groups at position C-20, specifically converting 17OHP to DHP, and shows no dehydrogenase activity against DHP. Nile tilapia 17β-HSD12 is not able to catalyze A4, 11KA4, and E_1 as steroid substrates into T, 11KT, and E_2, respectively, or vice versa. Thus, the enzyme exhibited neither oxidoreductase activity nor dehydrogenase activity at position C-17. This implies that tilapia 17β-HSD12 differs in substrate specificity from 17β-HSDs and is highly similar to masu salmon 17β-HSD12-like belonging to the group of 20β-HSD enzymes.

In this study, we found that *CR/20bhsd* transfected into HEK293T cells did not show greater conversion activity from 17OHP to DHP than that of controls from the data generated by

405 both TR-FIA and LC-MS/MS analyses. *CR/20bhsd* mRNA expression levels in follicles
406 showed no response to SPE or HCG at all follicle stages. This result was similar to those of a
407 previous study in which two forms of *CR/20β-HSD* were identified in masu salmon and the
408 mRNA levels of both *CR/20β-HSD typeA* and *typeB* in the forskolin-induced granulosa were
409 not greater than the levels observed in the control group (Ijiri *et al*., 2017). However, another
410 study of Nile tilapia reported that full-grown (post-vitellogenic) immature follicles incubated
411 with HCG, with centrally located germinal vesicles, induced the short-term expression of
412 *CR/20bhsd* mRNA within 1-2 h, suggesting that an increase in *CR/20bhsd* mRNA expression
413 prior to oocyte maturation is responsible for 20β-HSD activity (Senthilkumaran *et al*., 2002).
414 The expression of *CR/20bhsd* in full-grown follicles was significantly lower than that in
415 ovarian fragments. These results, together with the observation that CR/20β-HSD was not
416 induced by SPE or HCG in the present study, suggest that *CR/20bhsd* does not encode an
417 20β-HSD enzyme involved in DHP production during oocyte maturation in Nile tilapia.

418 In addition, we found a novel activity of Nile tilapia CR/20β-HSD that possesses strong
419 oxidoreductase activity to convert 11KA4 to 11KT. To our knowledge, this conversion
420 activity was only reported once before in 17β-HSD isolated from *Pseudomonas testosteroni*,
421 which showed high yields of conversion (Lokman *et al*., 1997). There is a possibility that
422 tilapia CR/20β-HSD and a specific type of 17β-HSD play a role for 11KT synthesis in the
423 testis. We found tilapia CR/20β-HSD also possesses conversion activity to convert A4 to T,
424 similar to tilapia 17β-HSD type 1 showing activity of inter-conversion between A4 and T
425 (Zhou *et al*., 2005). Those data indicate tilapia CR/20β-HSD possesses 17β-HSD activity to
426 reduce the carbonyl group at the C-17 position for 11KA4 and A4 conversion. We ruled out a
427 role of tilapia CR/20β-HSD for DHP production in the present study; however, there is still a
428 possibility that it plays essential roles in steroidogenesis through mechanisms which are
429 currently unknown.

Based on the finding that tilapia *hsd17b12* had strong 20β-HSD activity but lacked any 17β-HSD activities, combined with the results showing tilapia *CR/20bhsd* does not have 20β-HSD activity, we propose that Nile tilapia *hsd17b12* should be re-named as *hsd20β*, but not *hsd20β2.* In a previous study from masu salmon, there was still a possibility that this orthologous gene may possess some 17β-HSD activities; therefore, it was named as *hsd17b12l*. However, if 17β-HSD activities are not found and only 20β-HSD activity is detected, the name should be 20β-HSD encoded by *hsd20β* to avoid confusion between *hsd17b12l* (*hsd20β2*) and *CR/20bhsd*.

In summary, the present study demonstrated that tilapia CR/20β-HSD is not responsible for DHP production. Furthermore, stimulation by SPE or HCG for post-vitellogenic follicles induces *hsd17b12* to produce an enzyme with strong 20β-oxidoreductase activity to convert 17OHP to DHP. This enzyme does not have 17β-HSD activity under the conditions tested in this study. These data suggest that tilapia *hsd17b12* encodes 20β-HSD, which is responsible for DHP production to induce oocyte maturation and ovulation.

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Conflict of Interests

The authors declare no conflict of interests.

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Tables

Table 1: Sequences of primers used for cloning, subcloning, and quantification of mRNA levels in Nile tilapia.

Gene names	Directions	Sequence (5'-3')
nested-PCR		
hsd17b12	Forward	TGAGCTGGAAACAAAGGAGCT
(1st round)	Reverse	GTGCAGCTGGAGTCCCAT
hsd17b12	Forward	TACCGGACTCAGATCGATGTCATTTGCTGACCTACTG
(2nd round)	Reverse	ATTCGAAGCTTGAGCCTACTCCCCCTTTTCACCGGT
CR/20bhsd	Forward	AGCTCTGTGACTGTGACCTCT
(1st round)	Reverse	AACGCTTCGATCTCACACGCT
CR/20bhsd	Forward	TACCGGACTCAGATCCATGGTCTCTTTTTACATGTCA
(2nd round)	Reverse	ATTCGAAGCTTGAGCTCACCACTTCTGAACGGTCT
cyp19a1a	Forward	AGCGCTACCGGACTCAGATCCA
(subcloning)	Reverse	GCAGAATTCGAAGCTTGAGCCT
qPCR		
hsd17b12	Forward	ATCACACGCAGTCCAGAACG
	Reverse	CCCTTTTCACCGGTCCAATC
CR/20bhsd	Forward	TCACCAGCTGGACATCGACG
	Reverse	GAGTCGTGTCTGCCACTTTG
β-actin	Forward	CCCCAGGCATCAGGGTGT
	Reverse	TTGCTCTGGGCCTCATCAC

1st round primers were used for the amplification of ORFs of target genes, 2nd round primers were used for the amplification and cloning of target genes. Subcloning primers were used to insert ORFs of *cyp19a1a* into the vector pIRES2-ZsGreen1.

Table 2: 17β-HSD activity of Nile tilapia 17β-HSD12, CR/20β-HSD and non-transfected HEK293T.

Substrate	Product	Conversion Rate		
		17β-HSD12	CR/20β-HSD	Non-transfected HEK293T
17OHP	DHP	14%	0.4%	0.5%
A4	T	2.6%	12.2%	2.8%
11KA4	11KT	n.d.	63.5%	n.d.
E_1	E_2	n.d.	-	n.d.
DHP	17OHP	n.d.	-	n.d.
T	A4	1.6%	-	2.0%
11KT	11KA4	1.3%	-	1.4%
E_2	E_1	5.8%	-	6.0%

Six reproductive steroids were used as substrates. DHP was using for the inter-conversion of 20β-HSD activity.

n.d.; not determined owing to low activity. Hyphen; not apply in experiment

Abbreviation: 17α-Hydroxyprogesterone; 17OHP, 17α, 20β-Dihydroxy-4-pregnen-3-one; DHP, Androstenedione; A4, Testosterone; T, Adrenosterone; 11KA4, 11-Ketotestosterone; 11KT, Estrone; E_1, Estradiol-17β; E_2

Figures

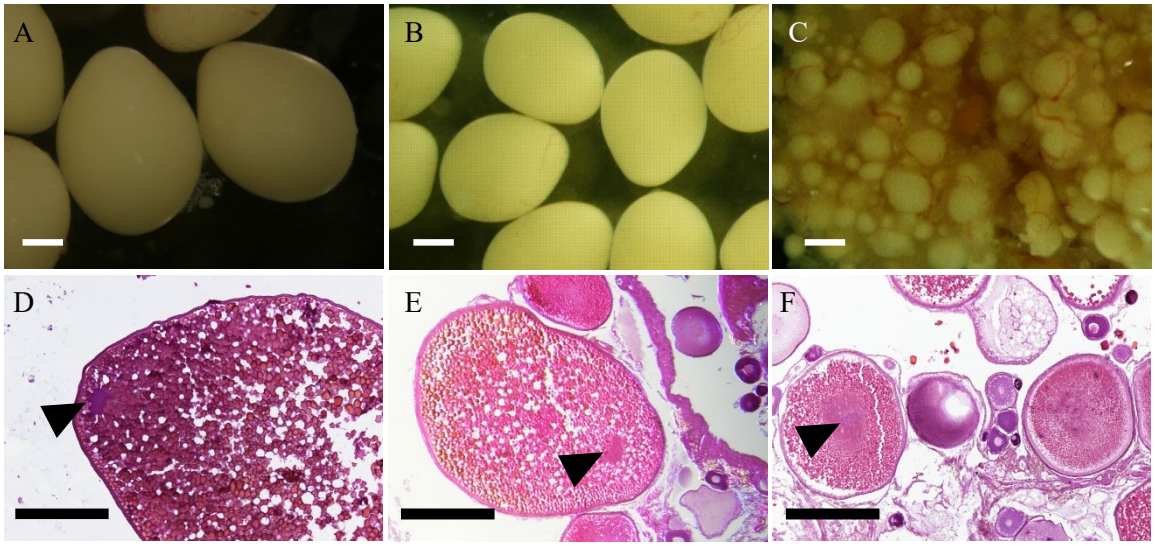


Fig. 1. Follicles of Nile tilapia. A) Image of full-grown follicles in Nile tilapia. B) Image of late-vitellogenic follicles in Nile tilapia. C) Image of ovarian fragments in Nile tilapia. D) Full-grown follicles with germinal vesicle migration at almost attaching to oocyte membrane. E) Late-vitellogenic follicles with germinal vesicle at intermediate position from the center to oocyte membrane. F) Ovarian fragments of Nile tilapia after the largest follicles were removed, stained by H&E. Arrow indicated germinal vesicle. Bar 500 µm.

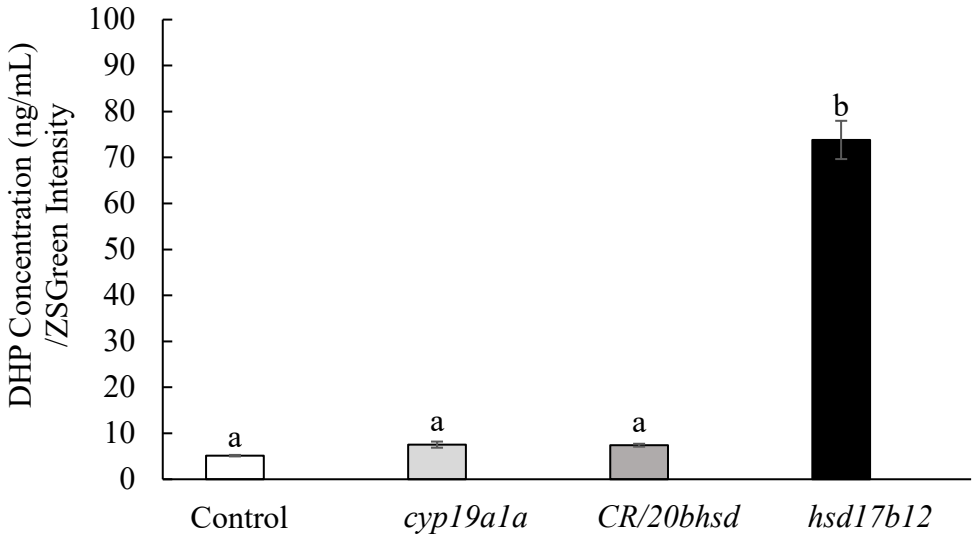


Fig. 2. DHP production by HEK293T cells transfected with the empty vector (control; open column), *cyp19a1a* (light-gray column), *CR/20bhsd* (dark-gray column), and *hsd17b12* (black column) in the presence of 100 ng/mL 17OHP after normalization against ZSGreen intensity. In each experiment, three transfection replicates were performed for each treatment. Each vertical bar represents the mean ± SE. Different letters indicate a significant difference at $P < 0.05$.

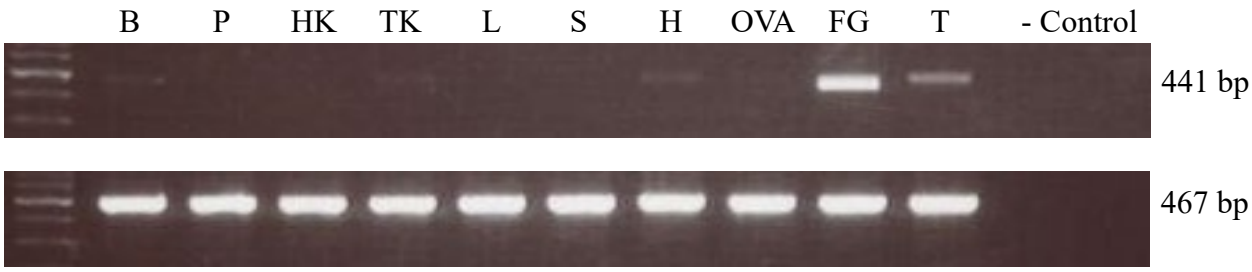


Fig. 3. Tissue-specific expression in ten tissues of Nile tilapia *hsd17b12* transcript in adult female 2 days before spawning and adult male testis, as determined by RT-PCR (upper panel). Distilled water was used as a negative control. Lower panel is for *β-actin* as internal control. B; brain, P; pituitary, HK; head-kidney, TK; trunk-kidney, L; liver, S; spleen, H; heard, OVA; ovarian fragments, FG; full-grown follicles, T; testis

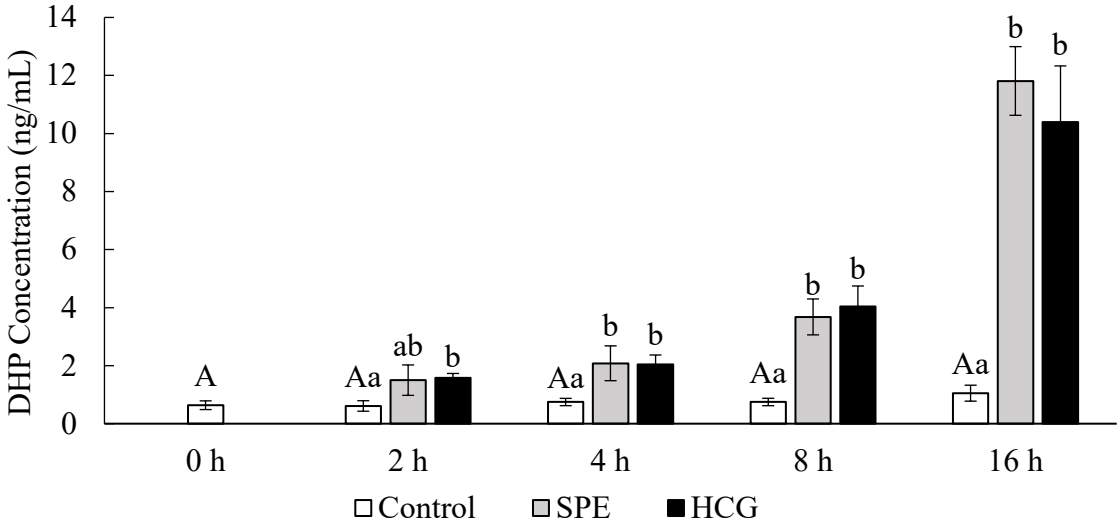


Fig. 4. Conversion to DHP by full-grown follicles for different incubation periods. The concentration of DHP in the media after incubation; 10 full-grown follicles were incubated with 17OHP alone (control) at 100 ng/mL (open column) and with 17OHP (100 ng/mL) with SPE (100 μg/mL) (gray column) or 17OHP (100 ng/mL) with HCG (100 IU/mL) (black column). Each vertical bar represents the mean ± SEM from four experiments using different females. Different letters indicate a significant difference at $P < 0.05$

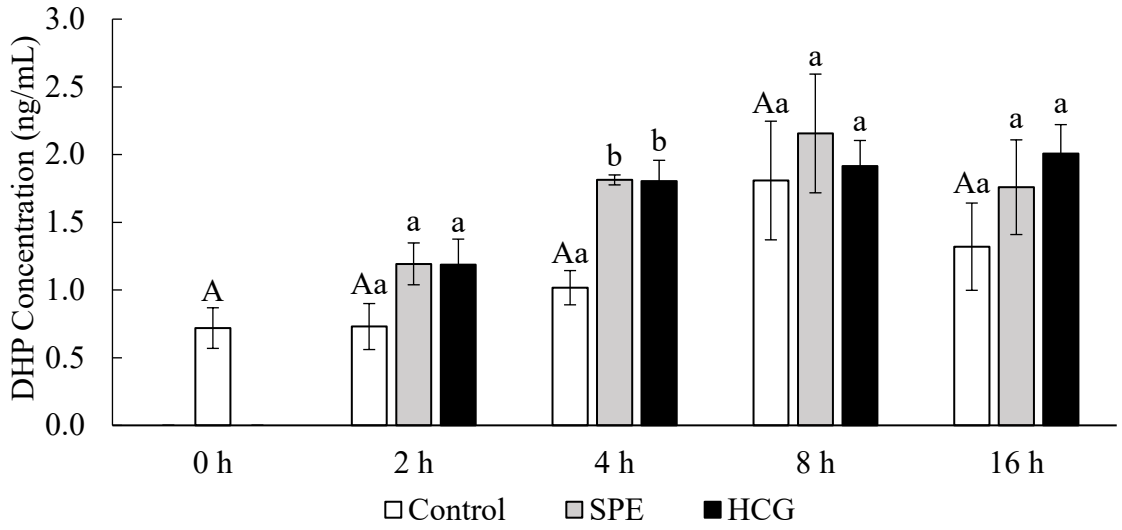


Fig. 5. Conversion to DHP by late-vitellogenic follicles for different incubation periods. The concentration of DHP in the media after incubation; 10 full-grown follicles were incubated with 17OHP alone (control) at 100 ng/mL (open column) and with 17OHP (100 ng/mL) with SPE (100 μg/mL) (gray column) or 17OHP (100 ng/mL) with HCG (100 IU/mL) (black column). Each vertical bar represents the mean ± SEM from four experiments using different females (females 1, 2, and 3). Different letters indicate a significant difference at $P < 0.05$

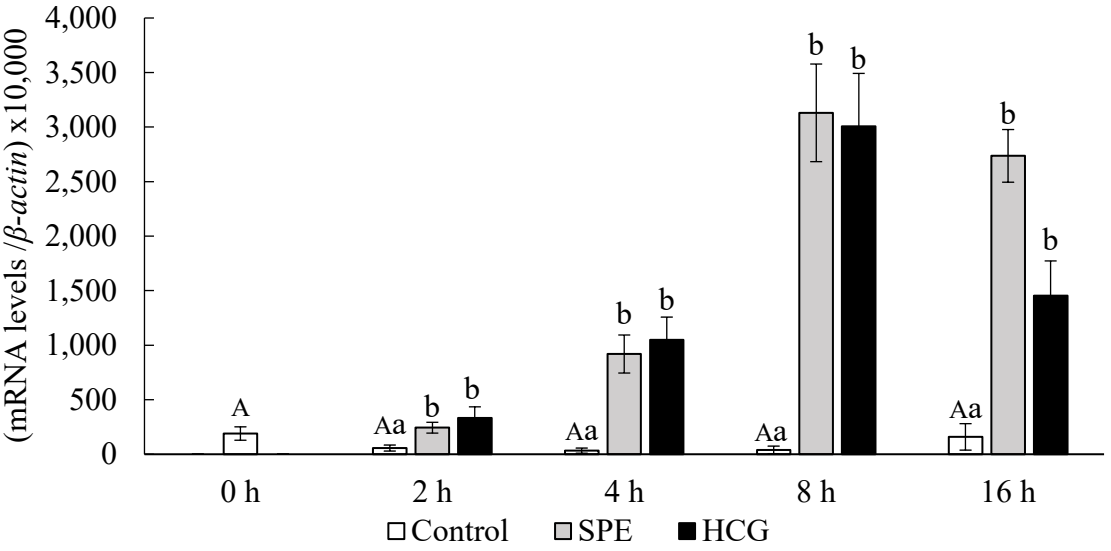
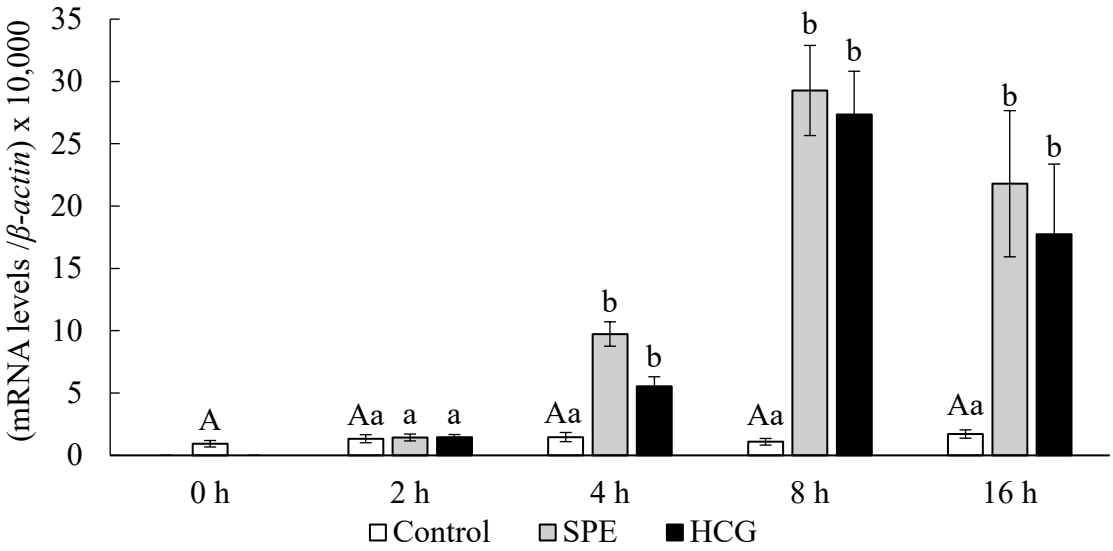


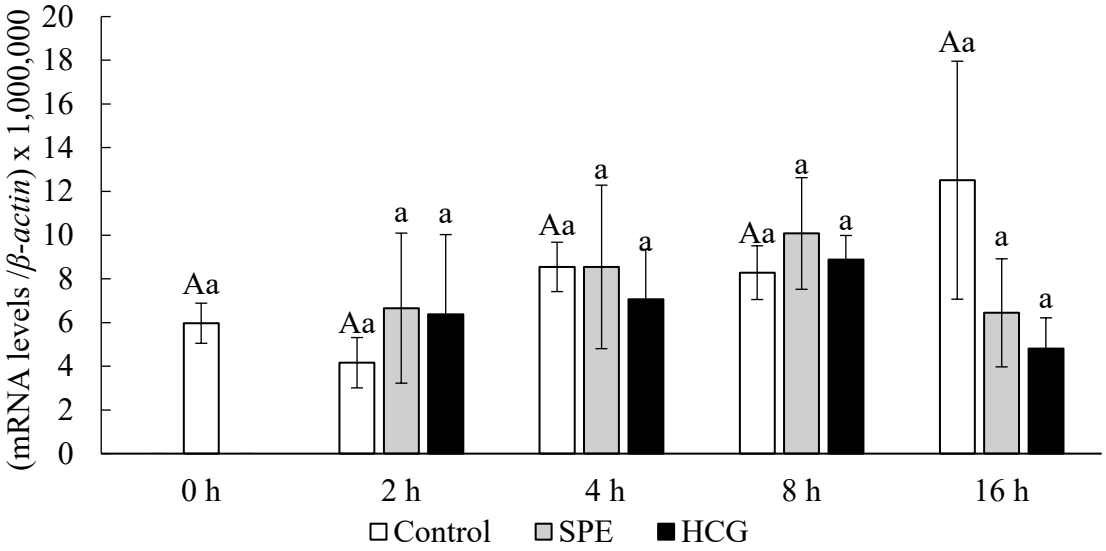
Fig. 6. *Hsd17b12* mRNA levels in incubated full-grown follicles. Levels of *hsd17b12* mRNA from full-grown follicles incubated with 17OHP alone (control) at 100 ng/mL (open column), 17OHP (100 ng/mL) with SPE (100 μg/mL) (gray column), or 17OHP (100 ng/mL) with HCG (100 IU/mL) (black column). Each vertical bar represents the mean ± SEM from four experiments using full-grown follicles from different females. Different letters indicate a significant difference at $P < 0.05$



649

650 Fig. 7. *Hsd17b12* mRNA levels in incubated late-vitellogenic follicles. Levels of *hsd17b12*
651 mRNA from follicles incubated with 17OHP alone (control) at 100 ng/mL (open column),
652 17OHP (100 ng/mL) with SPE (100 µg/mL) (gray column), or 17OHP (100 ng/mL) with
653 HCG (100 IU/mL) (black column). Each vertical bar represents the mean ± SEM from four
654 experiments using follicles from different females. Different letters indicate a significant
655 difference at $P < 0.05$

656



657

658 Fig. 8. *Hsd17b12* mRNA levels in incubated ovarian fragments (about 2 g). Levels of
659 *hsd17b12* mRNA from ovarian fragments incubated with 17OHP alone (control) at 100
660 ng/mL (open column), 17OHP (100 ng/mL) with SPE (100 µg/mL) (gray column), or 17OHP
661 (100 ng/mL) with HCG (100 IU/mL) (black column). Each vertical bar represents the mean ±
662 SEM from four experiments using ovarian fragments from different females. Different letters
663 indicate a significant difference at $P < 0.05$

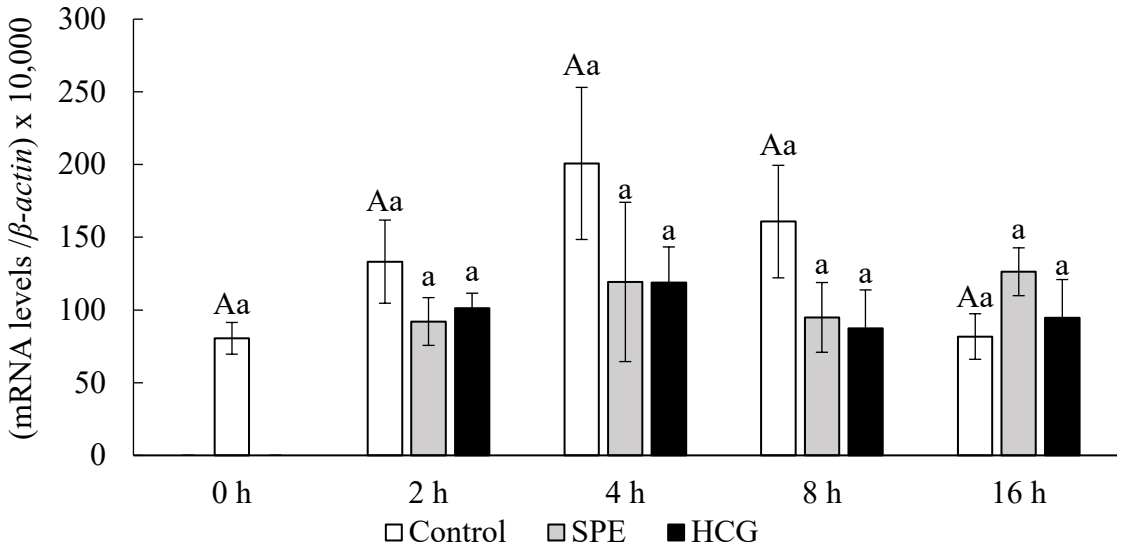


Fig. 9. *CR/20bhsd* mRNA levels in incubated full-grown follicles. Levels of *CR/20bhsd* mRNA from full-grown follicles incubated with 17OHP alone (control) at 100 ng/mL (open column), 17OHP (100 ng/mL) with SPE (100 µg/mL) (gray column), or 17OHP (100 ng/mL) with HCG (100 IU/mL) (black column). Each vertical bar represents the mean ± SEM from four experiments using full-grown follicles from different females. Different letters indicate a significant difference at $P < 0.05$

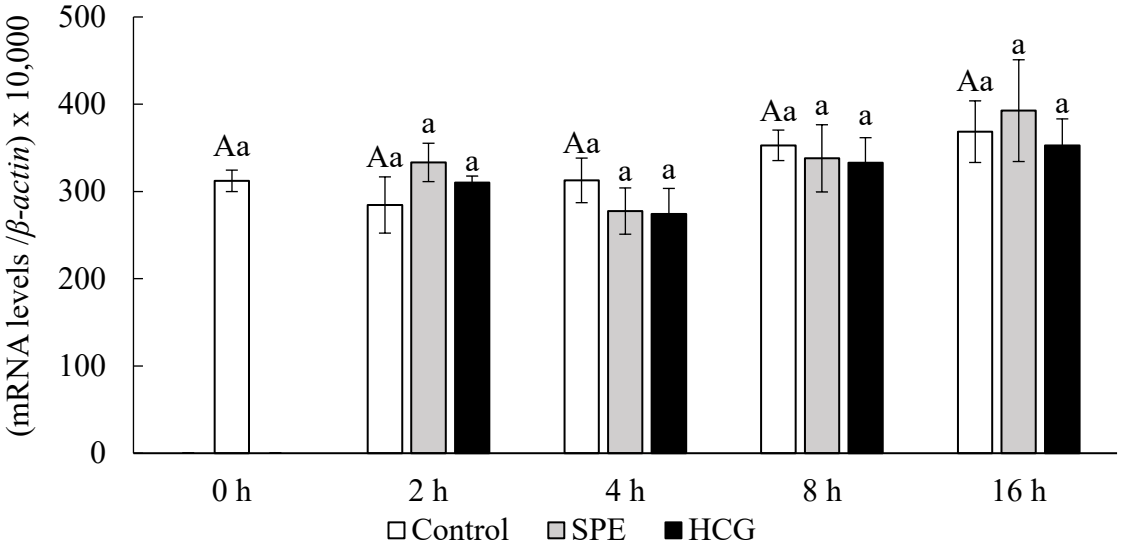


Fig. 10. *CR/20bhsd* mRNA levels in incubated ovarian fragments (about 2 g). Levels of *CR/20bhsd* mRNA from ovarian fragments incubated with 17OHP alone (control) at 100 ng/mL (open column), 17OHP (100 ng/mL) with SPE (100 µg/mL) (gray column), or 17OHP (100 ng/mL) with HCG (100 IU/mL) (black column). Each vertical bar represents the mean ± SEM from four experiments using ovarian fragments from different females (females 1, 2, and 3). Different letters indicate a significant difference at $P < 0.05$

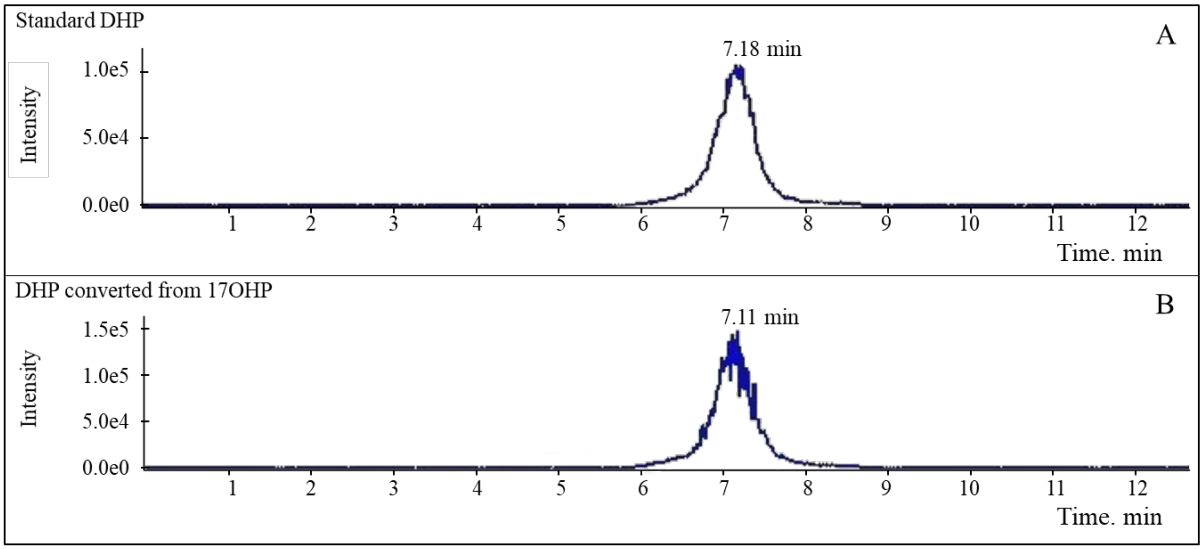


Fig. 11. Ion chromatogram of enzymatic conversion of tilapia *hsd17b12* transfected into HEK293T cells using 17OHP as a substrate, detected by LC-MS/MS as DHP. A) Standard DHP shows a peak retention time of 7.18 min. B) DHP converted from 17OHP shows a peak at retention time of 7.11 min.