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

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Abstract

17 α , 20 β -Dihydroxy-4-pregnen-3-one (DHP) is a maturation-inducing steroid in many teleost fish. Carbonyl reductase-like 20 β -hydroxysteroid dehydrogenase (CR/20 β -HSD) is a candidate enzyme responsible for DHP production during oocyte maturation in various fish, including Nile tilapia. However, a novel type of 17 β -hydroxysteroid dehydrogenase, type 12-like (17 β -HSD12L), is responsible for DHP production during oocyte maturation in masu salmon. 17 β -HSD12 (presumably orthologous to salmon 17 β -HSD12L) has been detected in Nile tilapia; however, its enzymatic activity and specific ability to convert the DHP substrate 17 α -hydroxyprogesterone (17OHP) have not been examined. This study aimed to determine whether CR/20 β -HSD or 17 β -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, 17 β -HSD12 did not convert any substrates other than 17OHP, including DHP, adrenosterone, androstenedione, estrone, testosterone, 11-ketotestosterone, and estradiol-17 β . CR/20 β -HSD showed strong 17 β -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 μ g/mL) or human chorionic gonadotropin (HCG, 100 IU/mL) to induce 20 β -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 17 β -HSD12, and not CR/20 β -HSD, is the 20 β -HSD responsible for DHP production by ovarian follicles during oocyte maturation in Nile tilapia.

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

1. Introduction

In several fish species, 17 α , 20 β -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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importance in regulating the synthesis of maturation-inducing steroids during oocyte maturation. Follicle-enclosed post vitellogenic oocytes of some teleost undergo germinal vesicle breakdown *in vitro* when they are incubated with gonadotropin (Nagahama 1987a). Gonadotropin influences DHP production by inducing the activity of the enzyme 20 β -hydroxysteroid dehydrogenase (20 β -HSD) (Nagahama 1987b) to catalyze the reduction of 20-carbonyl groups to 20 β -hydroxylated products (Nagahama and Yamashita 2008). An enzyme with 20 β -HSD activity was first isolated from the neonatal pig testis, showing strong 17 α -hydroxyprogesterone (17OHP) conversion activity to produce DHP (Nakajin *et al.*, 1987), and its cDNA sequence shows high similarity to human carbonyl reductase (Tanaka *et al.*, 1992), which is thought to catalyze the reduction of carbonyl compounds with NAD(P)H-dependent oxidoreductase activity (Tanaka *et al.*, 2002). The significance of the identity between 20 β -HSD and carbonyl reductase is unclear, since enzymes with similar functions catalyze the reduction of carbonyl groups to form corresponding products (Kazeto *et al.*, 2001). Guan *et al.* (1999) isolated cDNAs that encode proteins with high homology to pig 20 β -HSD from rainbow trout (*Oncorhynchus mykiss*) ovarian follicles, named carbonyl reductase-like 20 β -HSD (CR/20 β -HSD). CR/20 β -HSD type A has both 20 β -HSD and carbonyl reductase activities. However, it is not clear whether CR/20 β -HSD functions in DHP production during oocyte maturation. A study of Nile tilapia (*Oreochromis niloticus*) has reported that human chorionic gonadotropin (HCG) could induce *CR/20bhsd* mRNA expression in post-vitellogenic immature follicles within 1–2 h, suggesting that *CR/20bhsd* is responsible for 20 β -HSD activity prior to oocyte maturation (Senthilkumaran *et al.*, 2002). However, its role in DHP production was not investigated. *In vitro* studies of ovarian follicles from zebrafish incubated with HCG have shown no effect on *CR/20bhsd* induction (Wang and Ge, 2002). Similarly, in masu salmon studies with gonadotropin induction, CR/20 β -HSD has not been linked to DHP production (Ijiri *et al.*, 2017).

17 β -Hydroxysteroid dehydrogenases (17 β -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 17 β -hydroxysteroids using NAD(P)H or NAD(P)⁺ as a cofactor (Labrie *et al.*, 1997). 17 β -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 17 β -HSDs with several functions. Isozymes of 17 β -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 17 β -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 17 β -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 20 β -HSD activity and up-regulation of *hsd17 β 12-like* (*hsd17 β 12l*) mRNA by forskolin and chum salmon pituitary extract (SPE) at one or two months before ovulation. A phylogenetic analysis placed the full-length *hsd17 β 12l* sequence between Atlantic salmon (*Salmo salar*) *hsd17 β 3* and *hsd17 β 12*, but it was not highly orthologous to either. Furthermore, Nile tilapia 17 β -HSD type 12 (17 β -HSD12), which is expressed exclusively in the testis of immature fish (Zhou *et al.*, 2005), is phylogenetically positioned between zebrafish 17 β -HSD3 and human 17 β -HSD12 in the same clade with *Tetraodon* 17 β -HSD12B; it was therefore termed tilapia 17 β -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 17 β -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 20 β -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 17 β -HSD type 3 and 17 β -HSD type 12, we investigated the potential activities of Nile tilapia *hsd17b12* to ascertain its major enzymatic activities, i.e., 17 β -HSD or 20 β -HSD activity.

2. Materials & Methods

2.1. Animals

Nile tilapia were reared in a temperature-controlled ($26 \pm 1^\circ\text{C}$) 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 NaHCO₃, 4 mM KCl, 2 mM NaH₂PO₄, 1 mM MgSO₄, 1 mM CaCl₂, 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 μ m 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 20 β -HSD activity analysis. The ORF of tilapia aromatase was sub-cloned into the vector pIRES2-ZsGreen1.

2.3. 20 β -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 μ L 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 μ g/mL streptomycin (Wako) at 37 °C and 5% CO₂ 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 RNAlater 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 (E₁), testosterone (T), 11-ketotestosterone (11KT), and estradiol-17β (E₂). 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

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

3.2. *20 β -HSD activity analysis*

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

3.3. *Tissue-specific hsd17b12 expression*

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

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 diameter. 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 diameter. 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

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

3.7. Analyses of 17 β -HSD activity

To analyze the 17 β -HSD activity of *hsd17b12*, we examined transfected HEK293T cells for the conversion of several steroids that might be substrates for oxidoreductase activity at position C-17, including androstenedione (A4), adrenosterone (11KA4), and estrone (E₁). Steroid conversions were not observed using E₁ and 11KA4 as substrates. Both non-transfected and *hsd17b12* transfected HEK293T cells could convert a small amount of A4 to T. Dehydrogenase activity at position C-17 was evaluated using testosterone (T), 11-ketotestosterone (11KT), and estradiol-17 β (E₂), which exhibited no conversion to A4, 11KA4, and E₁, respectively, compared with non-transfected HEK293T cells. LC-MS/MS analysis revealed that *hsd17b12* only showed oxidoreductase activity at position C-20, with specific conversion of 17OHP to DHP (Figure 11) and no 20 β -dehydrogenase activity to convert DHP to 17OHP (Table 2). The results assessing enzymatic activity of *CR/20bhsd* oxidoreductase activity was observed at position C-17, showing 63.5% and 12.2% conversion from 11KA4 and A4 to 11KT and T, respectively. HEK293T cells transfected with

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

4. Discussion

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

In masu salmon, the expression of *hsd17 β 12l* mRNA was limited in testis and follicle layers at final maturation (Ijiri *et al.*, 2017). In tilapia, *hsd17b12* mRNA showed the same expression pattern. Zhou *et al.* (2005) did not find *hsd17b12* mRNA expression in the tilapia ovary, probably because they did not test ovarian preparation at just before oocyte maturation. Although we detected very faint amplification of *hsd17b12* mRNA in the tilapia brain, trunk-kidney and heart, such limited low expression may not have physiological importance.

The full-grown follicles incubated with SPE or HCG in this study were involved in the induction of 20 β -HSD activity and increases in *hsd17b12* mRNA levels. HCG had similar effects to those of SPE, suggesting that HCG and SPE mimic luteinizing hormone (LH) to stimulate the induction of *hsd17b12* synthesis, causing an elevation in 20 β -HSD activity to 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 20 β -HSD activity when
359 compared with the controls. This suggests that ability to induce sufficient amounts of 20 β -
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 20 β -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 17 β -HSD12-like ortholog was previously annotated in zebrafish by Tokarz *et al.* (2012)
374 and was named 20 β -HSD type 2 (*hsd20 β 2*); it catalyzes the reduction of cortisone to 20 β -
375 hydroxycortisone at position C-20. The 20 β -HSD activity of zebrafish *hsd20 β 2* towards
376 17OHP has not yet been examined; nevertheless, the authors suggested that zebrafish
377 *hsd20 β 2* 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 *hsd17 β 12l*, which is positioned between 17 β -

HSD3 and 17 β -HSD12 in a phylogenetic tree, belongs to the same sub-cluster as zebrafish *hsd20 β 2* and is considered orthologous. 17 β -HSD3 is mainly responsible for conversion of A4 to T (Mindnich *et al.*, 2005). 17 β -HSD12 mainly catalyzes E₁ to E₂ and is also involved in lipid metabolism (Luu-The *et al.*, 2006; Lima *et al.*, 2013), while zebrafish *hsd20 β 2* and masu salmon *hsd17 β 12l* catalyze at position C-20. Nile tilapia *hsd17b12* is positioned between 17 β -HSD3 and 17 β -HSD12 according to a phylogenetic analysis by Zhou *et al.* (2005). However, 17 β -HSD12s of zebrafish and *Tetraodon* together with all 17 β -HSD12s from tetrapods belonged to one clade, whereas tilapia *hsd17b12* was clustered into another subclade, suggesting that tilapia *hsd17b12* differs from 17 β -HSD12 of zebrafish, *Tetraodon*, and tetrapods. Despite these findings, they named this enzyme 17 β -HSD12 because one group of *hsd17 β 12l* (*hsd20 β 2*) had not been identified at that time, and other cDNAs close to 17 β -HSD12 had not yet been isolated from the tilapia. Indeed, Tokarz *et al.* (2012) re-conducted phylogenetic analysis using *hsd17b3*, *hsd17b12* and *hsd20 β 2*, and they demonstrated Nile tilapia *hsd17b12* was included in the *hsd20 β 2* (*hsd17 β 12l*) clade independent from the *hsd17b12* and *hsd17b3* clade.

The results of LC-MS/MS analyses clearly support the idea that tilapia 17 β -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 17 β -HSD12 is not able to catalyze A4, 11KA4, and E₁ as steroid substrates into T, 11KT, and E₂, respectively, or vice versa. Thus, the enzyme exhibited neither oxidoreductase activity nor dehydrogenase activity at position C-17. This implies that tilapia 17 β -HSD12 differs in substrate specificity from 17 β -HSDs and is highly similar to masu salmon 17 β -HSD12-like belonging to the group of 20 β -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

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

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

Based on the finding that tilapia *hsd17b12* had strong 20 β -HSD activity but lacked any 17 β -HSD activities, combined with the results showing tilapia *CR/20bhsd* does not have 20 β -HSD activity, we propose that Nile tilapia *hsd17b12* should be re-named as *hsd20 β* , but not *hsd20 β 2*. In a previous study from masu salmon, there was still a possibility that this orthologous gene may possess some 17 β -HSD activities; therefore, it was named as *hsd17b12l*. However, if 17 β -HSD activities are not found and only 20 β -HSD activity is detected, the name should be 20 β -HSD encoded by *hsd20 β* to avoid confusion between *hsd17b12l* (*hsd20 β 2*) and *CR/20bhsd*.

In summary, the present study demonstrated that tilapia CR/20 β -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 20 β -oxidoreductase activity to convert 17OHP to DHP. This enzyme does not have 17 β -HSD activity under the conditions tested in this study. These data suggest that tilapia *hsd17b12* encodes 20 β -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

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

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

565 1st round primers were used for the amplification of ORFs of target genes, 2nd round primers
566 were used for the amplification and cloning of target genes. Subcloning primers were used to
567 insert ORFs of *cyp19a1a* into the vector pIRES2-ZsGreen1.

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570 Table 2: 17β-HSD activity of Nile tilapia 17β-HSD12, CR/20β-HSD and non-transfected
571 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%

572 Six reproductive steroids were used as substrates. DHP was using for the inter-conversion of
573 20β-HSD activity.

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

575 Abbreviation: 17α-Hydroxyprogesterone; 17OHP, 17α, 20β-Dihydroxy-4-pregnen-3-one;

576 DHP, Androstenedione; A4, Testosterone; T, Adrenosterone; 11KA4, 11-Ketotestosterone;

577 11KT, Estrone; E₁, Estradiol-17β; E₂

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 μ m.

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 $\pm$ 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 μ g/mL) (gray column) or 17OHP (100 ng/mL) with HCG (100 IU/mL) (black column). Each vertical bar represents the mean $\pm$ 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 $\pm$ 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 $\pm$ SEM from four experiments using full-grown follicles from different females. Different letters indicate a significant difference at $P < 0.05$

Fig. 7. *Hsd17b12* mRNA levels in incubated late-vitellogenic follicles. Levels of *hsd17b12* mRNA from 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 follicles from different females. Different letters indicate a significant difference at $P < 0.05$

Fig. 8. *Hsd17b12* mRNA levels in incubated ovarian fragments (about 2 g). Levels of *hsd17b12* 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. Different letters 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 $\pm$ 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 $\pm$ SEM from four experiments using ovarian fragments from different females (females 1, 2, and 3). Different letters indicate a significant difference at $P < 0.05$

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