Studies on testicular steroidogenesis in Japanese eel Anguilla japonica

Doctoral Dissertation

STUDIES ON TESTICULAR STEROIDOGENESIS IN JAPANESE EEL *Anguilla japonica*

March 2021

Graduate School of Marine Science and Technology

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Doctoral Course of Applied Marine Biosciences

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Chapter 1.

General introduction

Japanese eel (*Anguilla japonica*) is a highly valued species in aquaculture industry and almost all eels for commercial use are raised in farms from glass eels caught in estuaries. So far, glass eels for aquaculture totally rely on wild-caught glass eels. Accordingly, fisheries catch of glass eels has declined since the 1970s and the soaring prices of glass eel occurred (Han et al., 2008; Kagawa et al., 2001). In 2014, the specie has been listed as endangered on the International Union for Conservation of Nature (IUCN) Red List (Jacoby and Gollock, 2014). Therefore, the establishment of artificial large-scale glass-eel production, which can provide seedings stably, is strongly desired.

To establish the eel production, the gonadal maturation of eels is essential to obtain their gametes. However, this species has immature gonad and does not undergo further gonadal development under captive conditions, suggesting that the condition of cultivation may be lacked the factors to induce gonadal maturation and/or may be suppressed the maturation. Therefore, the artificial induction of gonadal development has been conducted to obtain their gametes. As a way of that, it has been attempted to bring cultivate conditions close to natural conditions. Many researchers have identified the spawning migration of silver eels, the behavior in their spawning area, the spawning ground, and the spawning time (Higuchi et al., 2018; Higuchi et al., 2020; Tsukamoto et al., 2011). However, artificial gonadal development of Japanese eel based on environmental manipulations has not been sufficient to obtain the haploid gametes (Mikawa et al., 2019). Hence, the artificial induction of gonadal maturation has relied on the administration of exogenous gonadotropic hormones, as the powerful method.

The control of sexual maturation in Japanese eel has been conducted by repeated injection of chum salmon pituitary homogenates (SPH) in females and human chorionic gonadotropin (hCG) in male, as exogenous hormones (Ijiri et al., 2011a). However, in

male, many problems including inadequacy of semen and variation in gonadosomatic index occur (Tanaka, 2015). The unstable quality and quantity of gametes have been considered to be caused by the use of the heterologous hormones (Ohta et al., 2017). Moreover, repeated injections of hCG have a possibility to produce antibodies against hCG and reduce the effects. Thus, it has been believed that bioactive Japanese eel gonadotropins (Gths), as homologous gonadotropic hormones, are required for the production of gametes with stable quality and quantity.

In general, Gths, follicle-stimulating hormone (Fsh), luteinizing hormone (Lh), and chorionic gonadotropin (CG), are members of the glycoprotein hormone family and heterodimeric hormones that consist of one common α subunit and one hormone-specific β subunit (Levavi-Sivan et al., 2010). The glycosylation of each subunit and their dimerization, which occurs in the rough endoplasmic reticulum and Golgi body, is essential for their bioactivity (Molés et al., 2008). In particular, CG has a C-terminal peptide with a number of O-linked glycosylation sites and the O-linked oligosaccharides bound in the site play a role prolonging the life of the hormone in circulation (Keay et al., 2004; Legardinier et al., 2005; Menzer and Schams, 1979). It has been well known that Fsh and Lh are expressed in the pituitary gland of vertebrate including teleost and CG is produced in placental trophoblasts of primates and equids (Pierce and Parsons, 1981). Therefore, it is desired that Japanese eel Gths are purified from the pituitary for the artificial induction of sexual maturation, but high-purified Gths are still not available because it is difficult to purify suitable amounts of native Gths from pituitaries, unlike CG which is obtainable from urine of pregnant women (Keay et al., 2004).

Currently, advancements in biotechnology have enabled the production of bioactive recombinant Japanese eel Gths (reGths; reFsh and reLh) using mammalian cell lines, such

as FreeStyle 293-F cells (Kazeto et al., 2019). The recombinant hormones have potential as a new tool of the artificial induction of sexual maturation, but effects of reFsh and reLh on Japanese eel testis have not yet been well clarified, as well as other fish species. Hence, accumulation of knowledge on mechanism of the testicular development by reGths is required as a clue to the development of reliable methods to induce sexual maturation using homologous hormones in Japanese eel.

In vertebrate, spermatogenesis is dependent on the Fsh and Lh stimulation through their receptors. Previous reports in teleost demonstrated that serum Fsh and Lh levels increased in primarily early gamete development stage and final maturation stage, respectively (Campbell et al., 2003; Kusakabe et al., 2006; Swanson et al., 1991, 2003). Moreover, it is known that their actions are mediated by sex steroid hormones (Tokarz et al., 2015). Especially, 11-ketotestosterone (11KT) has been established in teleost as a unique and the most potent androgen that plays important roles in secondary sexual characteristic and spermatogenesis. The previous analyses indicated that the effects of Gths on testicular 11KT production were species-specific (Chauvigné et al, 2010, 2012; García-López et al., 2009, 2010; Molés et al., 2011, 2020; Zmora et al., 2007). Therefore, in order to understand on the physiological roles of Gths in Japanese eel, it is important to elucidate the effects of Gths on testicular steroidogenesis.

The biosynthesis of 11KT from cholesterol is catalyzed by several steroidogenic enzymes (Fig. 1) and steroidogenic acute regulated protein (Star) (Tokarz et al., 2015). In Japanese eel, cDNAs encoding several steroidogenic enzymes, cytochrome P450 side-chain cleavage enzyme (Cyp11a1), 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (Hsd3b), cytochrome P450 17α-hydroxylase/C_{17-21} lyase (Cyp17a1; the protein was previously known as Cyp17), cytochrome P450 11β-hydroxylase (Cyp11b), 11β-

hydroxysteroid dehydrogenase type 2 (Hsd11b2), have been cloned (Ozaki et al., 2006; Jiang et al., 1996, 1998; Kazeto et al., 2000, 2003, 2006). However, their expression regulation by Gths is poorly demonstrated in immature testis. In addition, Hsd17b with 17-ketosteroid reducing activity (17KSR activity) responsible for 11KT production has not yet been isolated and characterized in Japanese eel. In mammal, Hsd17b type3 (Hsd17b3), exhibiting 17KSR activity, is an important enzyme that involved in the transformation of C17-reduced androgen. Therefore, the Hsd17b3 is thought to be a strong candidate with 17KSR activity responsible for 11KT production in teleost. In addition, previous studies reported that two other Hsds, Hsd17b type12 (Hsd17b12) (Mindnich et al., 2004a) and 20β-hydroxysteroid dehydrogenase type2 (Hsd20b2) (Tokarz et al., 2012), are closely related with Hsd17b3. However, it remains to be elucidated whether these proteins exist in Japanese eel and relate with testicular steroidogenesis.

Moreover, a decade ago, a novel tentative of HSD17B, HSD17B15, was first described in human in a review (Luu-The et al., 2008). However, the detailed assessment of Hsd17b15, such as the methods for the analysis, the nucleotide/protein sequences, and the gene expression levels, was not described in the review and Hsd17b15 with 17KSR activity involved in sex steroid hormones has not been reported in vertebrate including teleost. Since then, HSD17B15 was described as a prostate short-chain dehydrogenase reductase 1 (PSDR1) (Bertin et al., 2014), retinal reductase 1 (RalR1) or retinol dehydrogenase 11 (RDH11) (Lhor and Salesse, 2014). In contrast to Luu-The et al. (2008), Kedishvili et al. (2002) found no significant oxidation or reduction of steroids by human RDH11 expressed in Sf9 cell lines. Thus, it remains to be elucidated whether *rdh11* gene encodes the protein with enzymatic activity involved in sex steroid hormones and Japanese eel Rdh11 involved in 11KT synthesis in testis

In this study, effects of reGths on the production of 11KT and steroidogenic enzymes involved in 11KT synthesis were investigated to accumulate knowledge on the physiological role of Gths and testicular steroidogenesis. In chapter 2, the Gths-mediated 11KT production was analyzed using the immature testicular organ culture methods. In chapter 3, for the deeper understanding of testicular 11KT biosynthesis, the steroidogenic pathway for 11KT production and the 17KSR activity crucial for 11KT synthesis were identified. Subsequently, cDNAs encoding candidate enzymes potentially exhibiting 17KSR activity were isolated and characterized. Furthermore, the effect of Gths on the 17KSR activity and the expression of the candidate genes were examined. Finally, in chapter 4, to identify a new type of Hsd17b related with 11KT synthesis encoded by *rdh11* gene, cDNAs encoding the protein with 17KSR activity involved in the synthesis of sex steroid hormones were isolated and characterized.

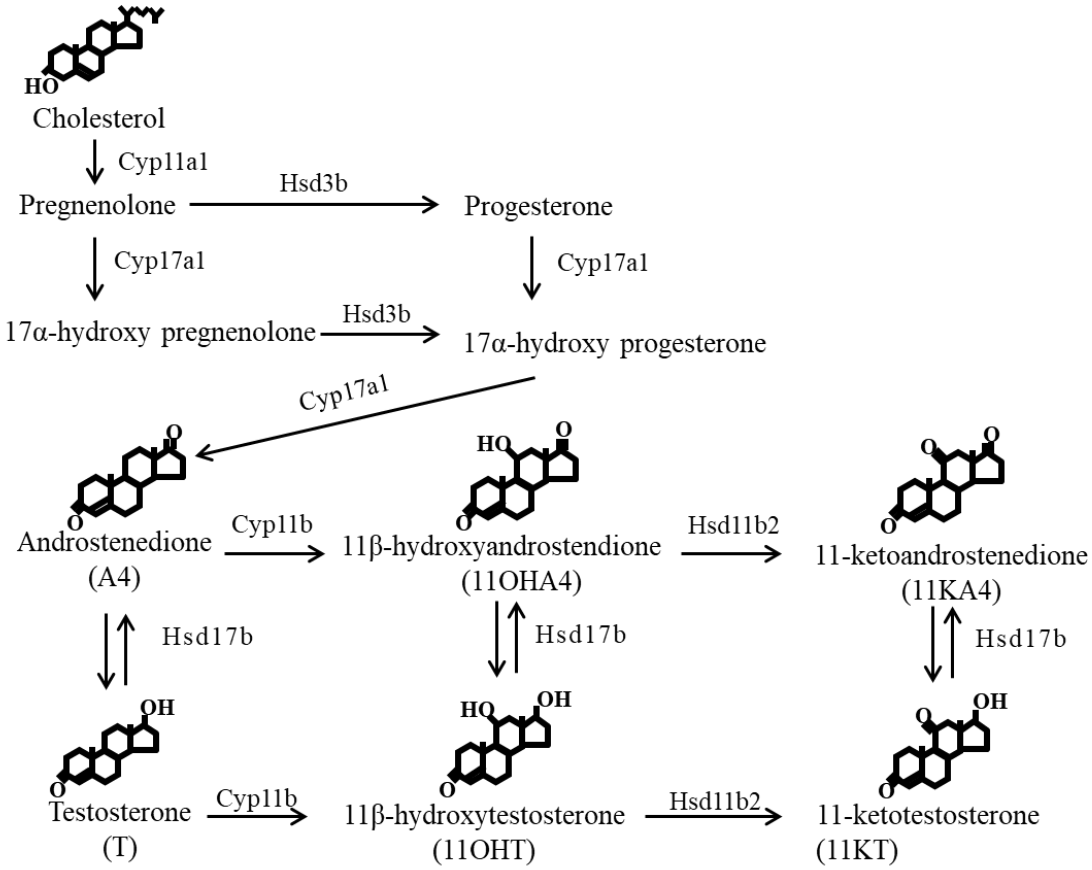

Figure 1. General steroidogenic pathway.

Chapter 2.

Functional analysis of recombinant single-chain Japanese eel Fsh and Lh produced in FreeStyle 293-F cell lines: binding specificities to their receptors and differential efficacy on testicular steroidogenesis

1. Introduction

Pituitary gonadotropins (Gths), follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh), are primary regulators of gonadal maturation across all vertebrate species. In mammals, Fsh and Lh exclusively bind to their respective cognate receptors, Fsh receptor (Fshr) and Lh /choriogonadotropin receptor (Lhcgr), and in turn induce signal transductions and follow specific gene expressions. The physiological functions of mammalian Fsh and Lh on spermatogenesis are well established. Fsh control Sertoli cell activities such as Sertoli cell proliferation and paracrine of growth factors while Lh regulates sex steroid production in Leydig cells (Huhtaniemi and Themmen, 2005).

The functional analyses of fish Gths are inferior to those in mammalian species as methods for reverse genetics and gene targeting were lacking, although there have been recent advancements in methods used for teleosts (Chu et al., 2014, 2015; Li and Cheng, 2018; Murozumi et al., 2014; Xie et al., 2017; Zhang et al., 2015a, 2015b). Interestingly, several studies using Gths and Gth receptors (Gthrs) knockout fishes produced by genome editing technologies revealed that male fishes that lost either Fsh or Lh signals were fertile and suggested highly overlapping of Fsh and Lh functions in spermatogenesis (Li and Cheng, 2018). Thus, the differences of Fsh and Lh functions have been not well understood in fish. Furthermore, it has been difficult to purify suitable amounts of native Gths from pituitaries and to produce bioactive recombinant Gths. These hormones are members of the glycoprotein hormone family and form a heterodimer consisting of a common α-subunit (common glycoprotein α, Cga) and a hormone-specific β-subunit (Fshb and Lhb). The glycosylation of each subunit and heterodimerization are essential for their biological activities (Combarnous 1992).

Recent advancements in biotechnology have enabled the production of bioactive recombinant fish Gths, mainly with two different strategies. One strategy is to co-express both subunit genes to produce heterodimeric forms (Chen et al., 2012; Kazeto et al., 2008; Molés et al., 2011; Zmora et al., 2007). Another method using a fusion cDNA, in which Cga and β-subunit cDNA are connected with a sequence encoding an anchor peptide, has been used to produce single-chain Gths (Chauvigné et al., 2012, 2014a, 2017; Chen et al., 2012; Kobayashi et al., 2010; Molés et al., 2011; Peñaranda et al., 2018). The half-life of recombinant Gths produced by the fusion cDNA method seems to be longer, possibly because the single-chain form prevents inactivation by dissociation of the heterodimer (Boime and Ben-Menahem, 1999; Garcia-Campayo and Boime, 2001; Molés et al., 2011).

Japanese eel is a highly valued species in Japan and almost all eels for commercial use are raised in farms from glass eels caught in estuaries. Eels do not mature in normal aquaculture conditions and thus many trials have been conducted to raise juveniles by artificial maturation using various hormones. Currently, human chorionic gonadotropin (hCG) for males has been widely used to induce sexual maturation (Kagawa et al., 2005). However, in male, many problems including inadequacy of semen and variation in gonadosomatic index occur (Tanaka, 2015). The unstable quality of gametes has been considered to be caused by the use of the heterologous hormones (Ohta et al., 2017). Moreover, repeated injections of hCG have a possibility to produce antibodies against hCG and to reduce the effects. Accordingly, farmed eels are still fully dependent on wild stock, required to produce sufficient amounts of bioactive recombinant eel Gths and to better understand the physiological functions of each Gth in fish.

The recombinant Japanese eel Fsh and Lh (reFsh and reLh) have been developed using insectan and mammalian recombinant protein expression system (Kazeto et al., 2008,

2019). Bioactive reFsh and reLh by co-expressing both subunits were produced using *Drosophila* S2 cells (Kazeto *et al*., 2008). However, the Gths were essentially ineffective in *vivo* even though the Gths were very effective *in vitro*, probably because the glycosylation patterns of Gths produced by insect cells were lacking sialylated oligosaccharides that prolong the half-life of glycoproteins in vivo. Recently, Kazeto et al.(2019) established FreeStyle 293-F cell lines producing reFsh and reLh using constructs encoding a single-chain molecule consisting of Cga and each β-subunit, because mammalian cells are able to express glycoproteins with complex sialylated oligosaccharides and the single-chain Gths seem to be more stable as described above. It is strongly desired to reveal the physiological function of reFsh and reLh.

In this chapter, functional analyses of reFsh and reLh were performed to confirm their bioactivities, focusing on the binding specificities to their receptors and effects on testicular steroidogenic activities.

2. Materials and Methods

2.1.Gonadotropin

Recombinant single-chain Fsh (reFsh) and Lh (reLh) produced by FreeStyle 293-F cells (Life Technologies, Carlsbad, CA) were used in the present study. Production and purification of the recombinant Gths are described elsewhere (Kazeto et al. 2019; Nyuji et al. 2016). In brief, expression vectors encoding either Fshb or Lhb, tethered with a GS spacer (GGGSGGGSGGGGGG), Cga and a His-tag were transfected into FreeStyle 293-F cells (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. After incubation for 5-7 days, the media were collected by centrifugation. The recombinant Gths were purified from the media by immobilized metal affinity chromatography using Ni-NTA agarose (Qiagen, Valencia, CA). The purity and glycosylation of the resultant Gth were confirmed by SDS-PAGE and Western blot analysis using rabbit antisera against Japanese eel Fshb or Lhb according to Kazeto et al. (2008). The concentration of recombinant Gths was determined by a BCA protein assay kit (Pierce, Rockford, IL).

2.2.Reporter assays

The bioactivities of reFsh and reLh were analyzed using the reporter assay system, as described by Kazeto et al. (2012). Briefly, COS-7 cells were cultured at 37℃ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and co-transfected with an eel Gth receptors expression vector constructed with pSI (Promega) in the presence of pRL-Null plasmid (Promega, Madison, WI) as well as a reporter vector, pGL4.29 (Promega), using X-tremeGENE 9 DNA Transfection Reagent (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. Empty

pSI vector was used as the control.

One day after transfection, the cells were trypsinized and seeded in 96-well culture plates. The following day, the medium was replaced with a serum-free medium supplemented with 0.1% bovine serum albumin to starve the cells. Thereafter, the cells were treated with several concentrations of reFsh and reLh. Activities of firefly and *Renilla luciferase* were quantified using a PicaGene Dual Pansy Luminescent kit (Wako Pure Chemical Industries, Osaka, Japan) and a TECAN Infinite 200 multimode plate reader (Tecan, Grodig, Austria). Firefly luciferase activity was normalized to the *Renilla luciferase* activity as an internal control. Each experiment was performed in six replicates.

2.3.In vitro assay on testicular fragments

Cultivated male Japanese eels (400–600 g body weight) raised from glass eels and kept in a 1-ton tank at 15°C were used in this study. Eels were euthanized in 0.05% 2-phenoxyethanol and the testes were removed by dissection. After cutting the testes into small pieces, the testicular fragments were incubated at 20°C in 48-well plates (30 mg/well) containing 300 μl of L-15 medium (Gibco, Gaithersburg, MD) supplemented with 0.5% bovine serum albumin (BSA) and 10 mM of HEPES buffering agent (pH 7.4).

To examine the effects of the reGths on 11KT production in the testis, testicular fragments were incubated with different doses of reFsh and reLh (0, 30, 100, 300, and 1000 ng/ml) for 72 h. Testicular fragments were also incubated with reFsh and reLh (0, 300 ng/ml) for various durations (1, 3, 6, 12, 24, 72, and 120 h).

To confirm whether 11KT production by Fsh and Lh is mediated through induction of any gene expressions, testicular fragments were pre-incubated with a transcriptional inhibitor (actinomycin D; Enzo Life Science, Farmingdale, NY; 0, 0.1, 1, 10 μg/ml), and a

translational inhibitor (cycloheximide; Sigma, Poole, UK; 0, 0.1, 1, 10 μg/ml) for 1 h. Next, reFsh and reLh (0, 300 ng/ml) were added and the testicular fragments further incubated for 24 h and 72 h.

Vehicle (phosphate buffered saline: PBS) only was used as the zero-dose control in all experiments. All treatments were conducted in triplicate. After incubations, the testicular fragments and media were collected and kept at –80ºC until use to prepare total RNA from the testicular fragments and to extract steroids from the incubation media.

2.4. Measurement of 11KT concentration in incubation media

The concentration of 11KT in incubation media was measured by time-resolved fluoroimmunoassay according to the methods of Yamada et al
. (1997). In brief, steroids were extracted three times in diethylether (5 times the volume of incubation media). The extracted steroids were reconstituted in an assay buffer (0.05 M Tris, 0.9% NaCl, 0.5% BSA, 0.05% NaN3, 0.01% Tween 40, 20 μM diethylenetriamine-N, N, N', N'', N''-penta-acetic acid, pH 7.75) and incubated with an antiserum against 11KT (Cosmo Bio, Tokyo, Japan) in 96-well plates coated with BSA-conjugated 11KT as the competitor.

The BSA-conjugated 11KT was prepared following the method reported by Asahina *et al*. (1995). After incubation at 20ºC for 4 h and following washes with PBS including 0.05% Tween 20 (PBST), europium (Eu)-labeled goat anti-rabbit IgG (Perkin-Elmer, Waltham, MA) was added to each well and incubated at 20ºC for 1 h.

After washing with PBST, an enhancement solution (Perkin-Elmer) was added and the intensity of fluorescence from dissociated Eu was measured by the Infinite F200 (Tecan, Grodig, Austria)

2.5. Quantitative real-time PCR

Total RNA was extracted from testicular fragments cultured with different doses of reFsh and reLh for 72h as described above using TRIzol Reagent (Invitrogen, Carlsbad, CA), and 1 μg of total RNA was reverse-transcribed using Omniscript Reverse Transcription (QIAGEN, Hilden, Germany) with random primers according to the manufacturer's protocol. The transcript abundance of genes encoding cytochrome P450 side-chain cleavage (*cyp11a1*), 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (*hsd3b*), cytochrome P450 17α-hydroxylase/C_{17-21} lyase (*cyp17a1;* the gene was previously known as *cyp17*), cytochrome P450 11β-hydroxylase (*cyp11b*), 11β-hydroxysteroid dehydrogenase type2 (*hsd11b2*), steroidogenic acute regulatory protein (*star*), *fshr*, *lhcgr1* and *lhcgr2* was determined by quantitative real-time PCR (qPCR) using TaqMan Universal Master Mix2 with UNG (Applied Biosystems, Carlsbad, CA) or Quantifast SYBR Green PCR Kit (QIAGEN) on the Applied Biosystems 7500 Real-Time PCR System according to the manufacturer's instructions. Specific primers and probes were based on the past literature (Matsubara et al., 2003) and designed with Primer Express v3.0 software (Applied Biosystems) (Table 1). The detailed conditions and the validation of qPCR assay were according to the handbook (ABI PRISM Sequence User Guide) and the previous report (Matsubara et al., 2003).

2.6. Localization of fshr and *lhcgr1*

To identify target cells of Fsh and Lh, localization of *fshr* and *lhcgr1* transcripts was examined by qPCR using three types of cell fractions: enriched Leydig cells, Sertoli cells, or germ cells. The testicular cell fractions were prepared in quadruplicate as follows. Testicular fragments were digested with 0.2% collagenase (Gibco, Gaithersburg, MD), 1

IU/ml dispase (Gibco), and 0.5 μg/ml DNase (Roche). During digestion, gentle pipetting was applied every 30 min. After 2 hours, TryPLE express (Gibco) was added to the digestive solution and incubated for 30 min. Subsequently, KnockOut Serum Replacement (KSR, Gibco) was added to stop the digestive reaction, then allowed to stand for 5 min. The supernatant was collected and filtered through a 40 μm nylon mesh and then through 20 μm nylon mesh. The digested product was centrifuged at 1000 rpm for 10 min. Cell pellets were then washed three times with DPBS. The cell suspension was put into percoll gradient 10, 30, 35, and 40% and then centrifuged at 3000 rpm for 30 min. The formed cell layer was collected and washed three times with DPBS.

Testicular somatic cells and germ cells were separated by utilizing characteristic differences to adhere to culture plates according to previous reports (Miura, et al., 1996; Wu et al, 2019). Cells obtained from between percoll layers 35% and 40%, in which most of Leydig cells and germ cells were fractioned, were placed in 0.2% gelatin-coated dishes at a concentration of 0.5–0.9 × 10^5 cells/cm^2 and incubated at 25°C in a humidified atmosphere (5% CO_2 in air). The culture medium consisted of DMEM/F-12 GlutaMAX™ (Gibco) with 50 mM NaCl, antibiotics, and 10% KSR. After 1 day, the suspended cells (i.e. germ cells) were collected and transferred to newly prepared 0.2% gelatin-coated dishes. The suspended cells were additionally incubated for 1 day and collected as germ cell fractions. The attached cells (i.e. Leydig cells) were also incubated for a further 1 day. After incubation, the attached cells were washed three times with DPBS to remove remained germ cells and dead cells, and then used for RNA extraction as Leydig cell fractions.

Sertoli cells migrated to the interface between the 10% and 30% percoll layers were placed in 0.2% gelatin-coated dishes at a concentration of 0.5 × 10^5 cells/cm^2 and briefly

incubated for 30 min. After discarding suspended cells, the adhering cells were cultured as described above. After 2 days, the adhering cells were washed three times with DPBS to remove contaminated germ cells and dead cells, and then used as Sertoli cell fractions.

Total RNA was extracted from each cell fraction using ISOGEN (Nippon Gene Co., Ltd., Toyama, Japan) and the cDNA was synthesized from 250 ng of total RNA using ReverTra Ace qPCR Master Mix with gDNA Remover (TOYOBO). The purity of the cell fractions was confirmed by qPCR using marker genes for each cell (*cyp11a1* for Leydig cells, *gsdf* for Sertoli cells, and *vasa* for germ cells). Data were represented as relative mRNA levels to mRNA levels of an internal standard gene, *rps15* as in a previous report (Rolland et al., 2009). The primers for *rps15* were designed based on Japanese eel genome sequence (Henkel et al., 2012) and shown in Table 1.

2.7. Statistical analysis

All data are expressed as means ± SEM. Data analyses were carried out using one-way ANOVA followed by Fisher's PLSD *post hoc* test or unpaired *t* test.

Table 1. Sequence of primers and probes used for qPCR

Gene	Primer/Probe	Sequence	Product size (bp)	GenBank Accession No.	the past literatures
star	Forward primer	5'-TGCCAGACCTTGGGAAAGTG-3'	146	AB095110.1	
	Probe	5'-CTGGACCAGCGACCTGACAACCTGTATG-3'			
	Reverse primer	5'-GCCAATCCTCTGCAGAATCTTAA-3'			
cyp11a1	Forward primer	5'- GGAGTCCTTCTGAAGGATGGG-3'	119	AY654741.1	
	Probe	5'-AAGTCCACTCGATTGGTCCTAAACAAGCAAG-3'			
	Reverse primer	5'-TCCTGTCCCACCTCGTCCAA-3'			
hsd3b	Forward primer	5'-TCTATGGTGTTAACGTGAAAGGGA-3'	71		Matsubara et al., 2003
	Probe	5'-AGCTCCTGCTGGAAGCCTGCATCC-3'			
	Reverse primer	5'-GAAGGACGCCACGTTCTCC-3'			
cyp17a1	Forward primer	5'-TGTCGCCCCTCCTCATACC-3'	79		Matsubara et al., 2003
	Probe	5'-ACGTGGCCCTCGCTGACTCCA-3'			
	Reverse primer	5'-ACTCTGGCCCCTTTTCCAACT-3'			
fshr	Forward primer	5'-GCTGAGGCAGATCCACAGTCA-3'	114		Kazeto *et al*., 2012
	Probe	5'-CCCGTTGTTCTGGACATCTCGCG-3'			
	Reverse primer	5'-CGCTTCAGCCTCCACAGAAT-3'			
lhcgr1	Forward primer	5'-GAAACACAGGGAGAACGCTTTC-3'	134	EU635883.1	
	Probe	5'-ACCACAGGGGATTGGGTACCCCTCT-3'			
	Reverse primer	5'-CGAAGTCGTCGAGGCTGTACT-3'			
lhcgr2	Forward primer	5'-AGAGAAGCTGATGTCTTTGCTGA-3'	185		Maugars and Dufour, 2015
	Reverse primer	5'-CTGGAGTGGCAGAAATCAAACT-3'			
cyp11b	Forward primer	5'-GAACACAGTGAAAGAGATTCTCAGGAT-3'	97		Jiang *et al*., 1996
	Reverse primer	5'-AGGGATATGGTAGTTCTGAATGATAATG-3'			
hsd11b2	Forward primer	5'-AGAGGCTGCATGGAGGTCAA-3'	130	AB252646.1	
	Reverse primer	5'-GGCAAGGAAATGGCTGCTC-3'			
vasa	Forward primer	5'-ACCTCCCGCCATAATGAAATT-3'	79	LC466010	
	Reverse primer	5'-CACGTAGCCCGACTTGCTAAC-3'			
gsdf	Forward primer	5'-CACCGAAGTCTCAGGAATCCA-3'	150	LC466011	
	Reverse primer	5'-TTATCCAGTTCTCCCAACCAAGA-3'			
actb	Forward primer	5'- ACGGGCAGGTCATCACCAT -3'	111		Kazeto *et al*., 2012
	Probe	5'-CCTTCCTTCCTGGGTATGGAGTCCTGC -3'			
	Reverse primer	5'-AGTTGAAGGTGGTCTCGTGGATT -3'			
rps15	Forward primer	5'- ACCAGGTGGAAATCAAGCC-3'	121	LC485293	
	Reverse primer	5'- GGAATGAAGCGGGAAGAATG-3'			

3. Results

3.1.Reporter assay

Stimulation of the intracellular cAMP-productive pathway by reFsh and reLh was analyzed using COS-7 cells transiently expressing the recombinant eel Gthrs. Both reFsh and reLh were able to stimulate Fshr (Fig. 2A). The reFsh dose, which indicated a significant activated Fshr level compared with the control, was 10 ng/ml, whereas the minimum effective dose of reLh was 1 ng/ml (Fig. 2A). Regarding EC_{50} (concentration that gives half-maximal response), reFsh (266.0 ng/ml) and reLh (300.3 ng/ml) had similar efficacy. On the other hand, reFsh did not enhance cAMP production through the Lhcgr1, while the effective doses of reLh to enhance cAMP production were over 3 ng/ml and the EC_{50} was 1752.0 ng/ml (Fig. 2B).

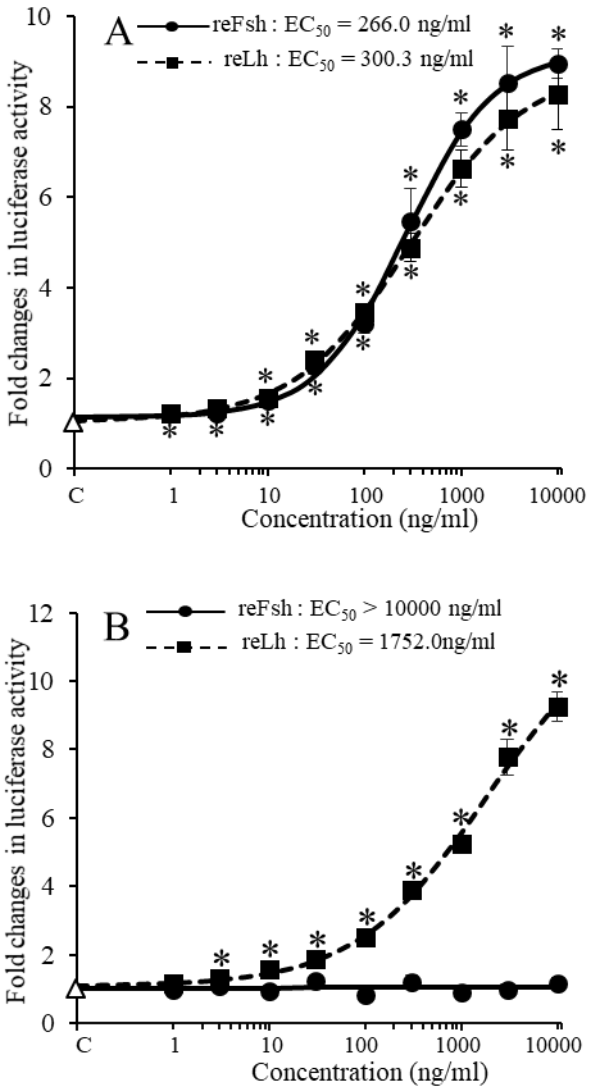

Figure 2. Concentration-dependent effects of reFsh or reLh on the activation of eel Gthrs. COS-7 cells expressing the eel recombinant Gthrs were incubated with increasing doses of eel reGth (n=6 per treatment). The values are expressed as fold change in abundance relative to the values in the control group. (A) Fshr, (B) Lhcgr1. The asterisk indicates significant differences ($P<0.05$) from the control group (open triangle).

3.2.Effects of reFsh and reLh on testicular 11KT production

After incubation for 72 h, reFsh and reLh resulted in a dose-dependent promotion of 11KT production in the testicular fragments. The dose of reFsh, which showed a significant increase of 11KT production compared with control, was 300 ng/ml, whereas the minimum effective dose of reLh was 30 ng/ml (Fig. 3A).

ReFsh and reLh appeared to increase 11KT production along with the incubation time. In particular, 11KT concentrations in media incubated with reLH were remarkably elevated after 72 h, and significantly higher than those with reFsh after 3 h, 72 h, and 120 h (Fig. 3B).

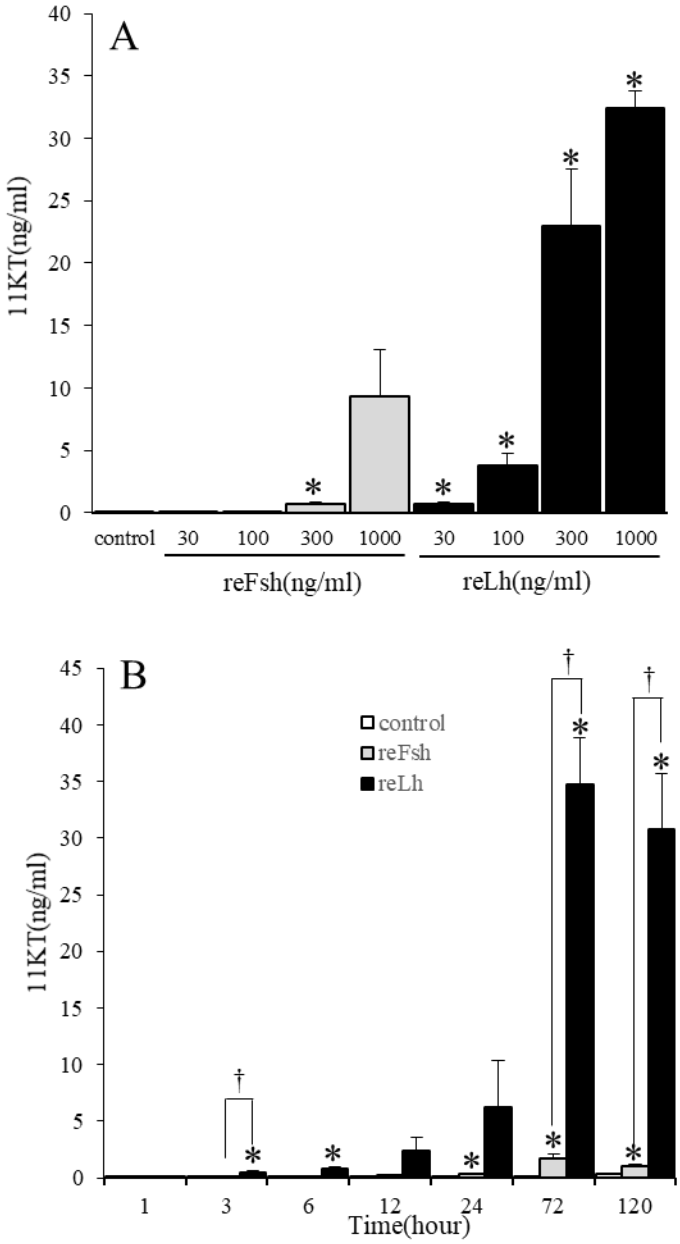

Figure 3. Effects of different doses (A) and time-course (B) of reFsh and reLh on testicular 11KT production. Bars represent the mean ± SEM of the measurements in triplicate. The asterisk indicates significant differences from the control group ($P<0.05$). The dagger represents significant differences between the reFsh group and the reLh group ($P<0.05$).

3.3. *Effects of reFsh and reLh on transcript abundances of genes encoding steroidogenic enzymes, star, fshr,* and *lhcgr*

ReFsh and reLh increased transcript abundances of genes encoding steroidgenic enzymes in a dose-dependent manner. The minimum effective dose of reGths in the significant upregulation of *cyp17a1* transcripts was 100 ng/ml. With regards to *cyp11a1*, *hsd3b*, *cyp11b*, and *hsd11b2* transcripts, low doses of reLh were more effective compared with those of reFsh. The significant increase of *star* transcripts was observed only in high doses of reFsh treatments (300 and 1000 ng/ml). *Lhcgr1* transcripts were significantly increased by high doses of reFsh treatments (300 and 1000 ng/ml) and low doses of reLh treatments (30ng/ml). While, the effects of reFsh and reLh on *lhcgr2* expression were ignorable. *Fshr* transcripts were markedly increased by 300 and 1000 ng/ml of reLh (Fig. 4).

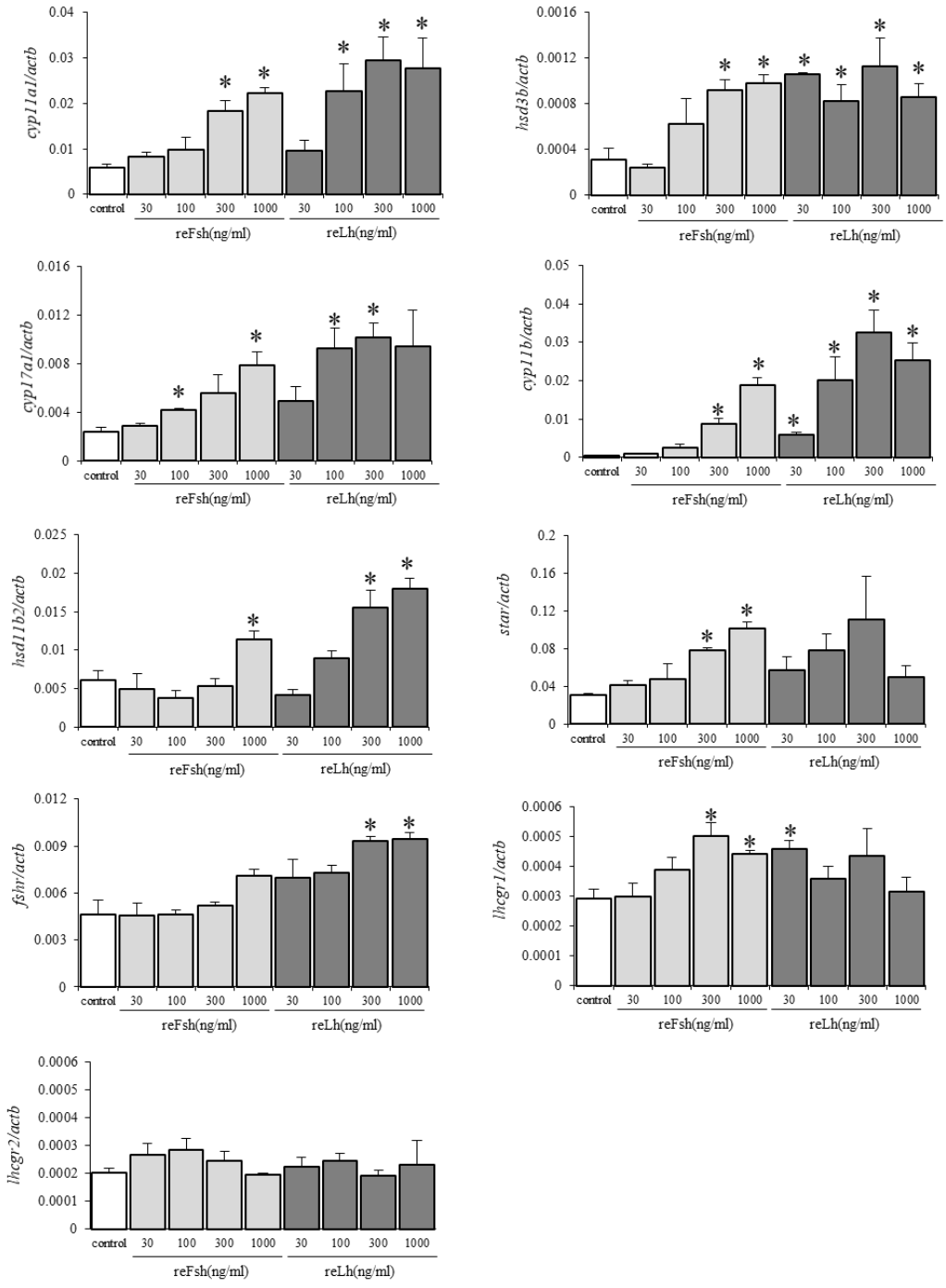

Figure 4. Effects of reFsh and reLh on transcript abundances of genes encoding *cyp11a1*, *hsd3b*, *cyp17a1*, *cyp11b*, *hsd11b2*, *star*, *fshr*, *lhcgr1* and *lhcgr2*. Bars represent the mean ± SEM of the measurements in triplicate. The asterisk indicates significant differences from the control group ($P<0.05$).

3.4.Regulation mechanisms on 11KT production under reFsh and reLh controls

After 24 h, a translational inhibitor, cycloheximide, significantly inhibited 11KT production induced by reFsh and reLh whereas no effects of a transcriptional inhibitor, actinomycine D, on 11KT production induced by reFsh was observed. Although actinomycine D tends to decrease 11KT production by reLh in a dose-dependent manner, the effects were not significant (Fig. 5A). After 72 h, both inhibitors remarkably decreased 11KT production induced by reFsh and reLH with a dose-response (Fig. 5B).

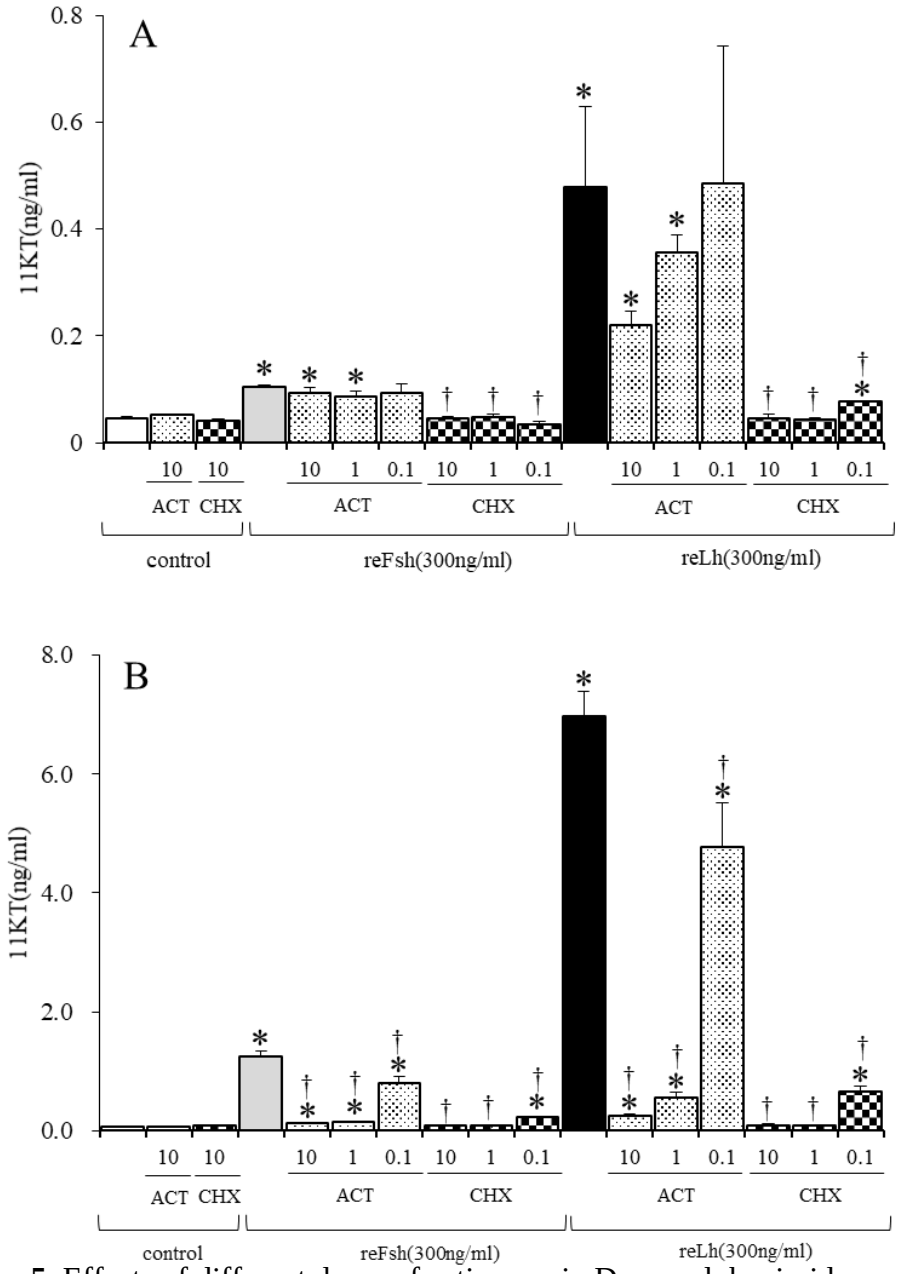

Figure 5. Effects of different doses of actinomycin D or cycloheximide on reFsh and reLh-stimulated testicular 11KT production. 11KT level in medium measured at differing time points (A: 24 h, B: 72 h) after addition of the reGths. Bars represent the mean ± SEM of the triplicate measurements. The asterisk indicates significant differences from the control group ($P<0.05$). The dagger represents significant differences between the present and absence of the inhibitors ($P<0.05$).

3.5.Localization of fshr and lhcgr1 transcripts in the testis

To confirm the efficiency of cell fractionations, transcript abundances of marker genes for each cell type were examined. Transcript levels of *cyp11a1* and *gsdf* were significantly higher in the Leydig cell and Sertoli cell fractions, respectively, compared with other fractions (Fig. 6A and B). While, *vasa* mRNA levels in the germ cells fraction was remarkably higher than other fractions (Fig. 6C).

Transcripts of *fshr* and *lhcgr1* were detected in the Sertoli and Leydig cell fractions; those transcripts in the germ cell fraction were not detectable. Transcripts of *lhcgr1* seem to be abundant in Leydig cell fractions compared with Sertoli cell fractions (Fig.7A and B).

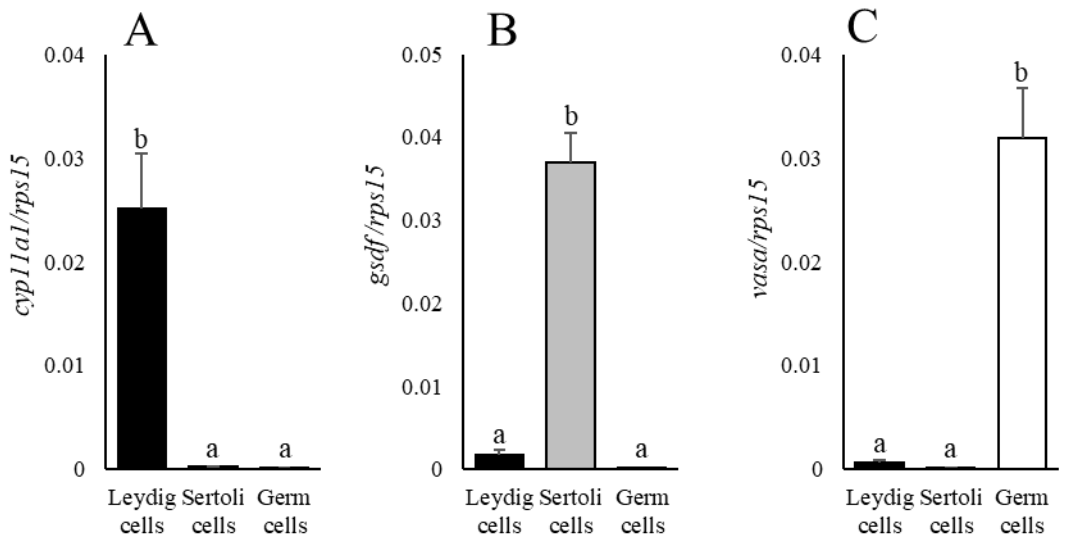

Figure 6. Relative mRNA levels of marker genes (A: *cyp11a1*, B: *gsdf*, C: *vasa*) in cell fractions. Bars represent the means ± SEM of the quadruplicate measurements. Different letters (a and b) indicate significant differences in each graph ($P<0.05$).

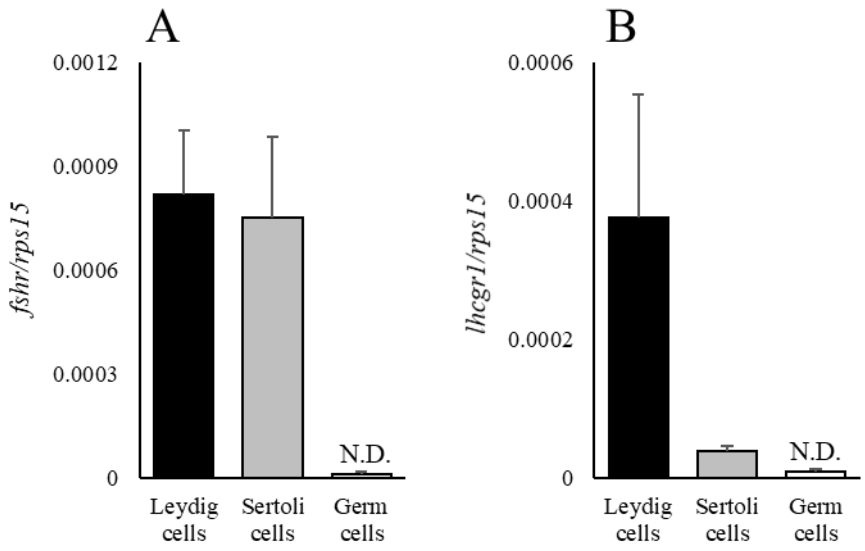

Figure 7. Cellular localization of *fshr* (A) and *lhcgr1* (B) transcripts in Japanese eel testis. The relative mRNA levels were normalized with *rps15*. Bars represent the means ± SEM of the quadruplicate measurements. N.D., not detectable, values were below a limit of detection for qPCR analysis.

4. Discussion

4.1.Binding specificities of reFsh and reLh to their receptors

Among fish species, ligand recognition of Fshr and Lhcgr differs. In sea bass (*Dicentrarchus labrax*) (Molés et al., 2011) and mummichog (*Fundulus heteroclitus*) (Ohkubo et al., 2013), both gonadotropins are only able to enhance their cognate receptor whereas African catfish (*Clarias gariepinus*) (Vischer et al., 2003) and Senegalese sole (*Solea senegalensis*) (Chauvigné et al., 2012) have indicated promiscuous activation of Fshr by Lh.

In the present study, the interaction between Japanese eel Gths and their receptors was examined using the reporter assay system. The reFsh activated Fshr, but not Lhcgr1, which appears to be a common mechanism in teleosts. On the other hand, reLh activated Lhcgr1 as well as Fshr. Interestingly, EC_{50} for the reLh-stimulated cAMP production on Lhcgr1 (EC_{50} = 1752.0 ng/ml) was higher than that on Fshr (EC_{50} = 300.3 ng/ml). In sea bass (*Dicentrarchus labrax*) (Rocha et al. 2009), Lh plasma and Lhcgr mRNA levels reportedly increase during spermiogenesis and spermiation. Consequently, our results suggested that activation of Lhcgr1 by high Lh concentration affected the final stages of gametogenesis.

Kazeto et al. (2012) demonstrated that hCG activated the Lhcgr, but not Fshr. On the other hands, in this study, the reLh stimulated the Fshr and Lhcgr, as well as the reLh produced using insect cell line (Kazeto et al., 2008), suggesting that the activation of both receptors by the reLh was not dependent on the expression systems. Therefore, these results suggest that the Lh and hCG have distinct effects on spermatogenesis in Japanese eel.

Whereas the testicular culture using reFsh and reLh was conducted at 20 ℃, the reporter assay was performed at 37℃ for mammalian cell lines expressing eel *fshr* and *lhcgr1*. Moreover, differences of oligosaccharide chains of recombinant Gths depending on host systems have been reported to affect the ligand-receptor interaction (Molés et al., 2011). Thus, the binding ability of these hormones to the receptors might be slightly different in the eel testicular culture system.

4.2.Testicular 11KT production by reFsh and reLh

In the present study, the functional analyses of reFsh and reLh bioactivities, particularly testicular steroidogenic activities, were performed using *in vitro* culture system. Cultivated Japanese eels have an immature testis and spermatogenesis can be induced by exogenous Gths via 11KT production *in vitro* (Kazeto et al., 2008; Ohta et al., 2007). Furthermore, cDNAs encoding genes related to spermatogenesis such as Gth receptors and steroidogenic enzymes have been isolated (Jiang et al., 1996; Kazeto et al., 2000, 2003, 2006, 2012; Li et al., 2003; Maugars and Dufour, 2015; Ozaki et al., 2006; Ohta et al., 2007) thus enabling us to readily analyze hormonal activities.

The biological functions of piscine Gths seem to be separated less clearly compared with mammalian Gths (Schulz et al., 2010). Previous studies on salmonids have suggested that Fsh and Lh regulate early gametogenesis and final maturation, respectively (Campbell et al., 2003; Kusakabe et al., 2006; Swanson et al., 1991, 2003). In Japanese eels, previous *in vitro* studies demonstrated that Fsh induced spermatogenesis via 11KT production as well as Lh (Kazeto et al., 2008; Ohta et al., 2007).

In this study, reLh had remarkably higher potency on 11KT production, although reFsh induced 11KT production as in previous reports. In a previous study by Kazeto et al.

(2008), the differential efficacy on 11KT production could not be found, possibly due to shorter duration of incubations (18 h); 11KT production induced by reLh was remarkably elevated after 72 h in the present study. In addition, both Fsh and Lh exert steroidogenic activities through Fshr and Lhcgr in Leydig cells in several fish species. Previous reports demonstrated that Fsh possessed an equivalent activity to Lh on testicular androgen production *in vitro* in channel catfish (*Ictalurus punctatus*) (Zmora et al., 2007), African catfish (*Clarias gariepinus*) (Gacía-López et al., 2009), and Senegalese sole (*Solea senegalenis*) (Chauvigné et al., 2012). In zebrafish (*Danio rerio*), Fsh showed more potent activities on androgen production *in vivo* and *in vitro* (Gacía-López et al., 2010). Fsh, but not Lh, upregulated transcripts of several genes related with steroidogenesis, *star*, and *cyp17a1*. Conversely, Lh was more effective on 11KT production in sea bass (*Dicentrarchus labrax*) (Molés et al., 2011) as seen in eels (this study). The efficacy of Fsh and Lh on androgen production seems to be species-specific and/or depends on the developmental stages of gonads, which is related to abundances of Fshr and Lhcgr.

In the present study, Lhcgr1 appears to express at lower levels than Fshr in the immature testis. In reporter assay, reFsh and reLh activated Fshr with similar EC_{50}s and activation of Lhcgr1 required higher Lh concentration. Nevertheless, reLh showed stronger and quicker effects on androgen production. In eels, Lh expression in the pituitary was negligible at immature stages (Saito et al., 2003).

Collectively, these results suggest distinct roles of Fsh and Lh; Fsh induces early spermatogenesis through 11KT production at proper and lower levels, and then Lh triggers spermatogenic events such as meiosis and spermiogenesis through steroid production at higher levels. These Gths control mechanisms resemble those seen in salmonids. Repeated injections of hCG are widely used to induce spermatogenesis in eels. The previous report

demonstrated that hCG specifically binds to eel Lhcgr1 (Kazeto et al., 2012) suggesting that hCG injections cause excess amount of androgen production, possibly resulting in abnormal early gonadal development.

In mammals, the stability of Fsh and Lh has been reported to be different and related with *in vivo* bioactivity such as receptor binding activity and pulsatile variation in serum concentration (Baenziger et al., 1992; Smith et al., 1992). Previous studies in teleost have reported that in vivo half-life of Lh was shorter than that of Fsh (Chauvigné et al., 2017; Molés et al., 2011), suggested that short-term effects of Lh were necessary for a certain phase of reproductive cycle (Mazón et al., 2015). The differential stability of Fsh and Lh might cause in their distinct functions.

The differential efficacy of reFsh and reLh on the induction of transcription of reproductive genes could be found; reFsh significantly promoted transcription of *star* and *lhcgr1* genes. The Fsh-only star transcript induction corresponds to results in zebrafish reported by García-López et al. (2010). Star is a protein that mediates cholesterol delivery across the mitochondrial membrane to Cyp11a1 (Stocco, 2000). In salmonids, increases in transcript abundances of *star* gene during early stages of spermatogenesis have been observed (Kusakabe et al., 2006; Maugars and Schmitz, 2008; von Hofsten et al., 2002). Additionally, Fsh, but not androgen (11KT and MT), stimulated *star* mRNA expression in testicular explants (Sambroni et al., 2013). These findings suggest that Fsh induces cholesterol delivery by Star for steroidogenesis during early spermatogenesis, and the accumulated cholesterol or metabolized steroids sustain subsequent steroidogenesis induced by Lh. Upregulation of *Lhcgr* gene by Fsh is consistent with a previous study by Sambroni et al. (2012). The *lhcgr1* expression augmented by reFsh suggests that one of Fsh's roles is increasing testicular sensitivity to Lh.

In zebrafish, Lh induced androgen release without augmenting transcripts of steroidogenic enzymes, thus implying the existence of the nongenomic control mechanisms (García-López et al., 2010). Androgen production by Gths is reportedly dependent on protein synthesis rather than mRNA synthesis in carp (*Cyprinus carpio*) (Chang and Huang, 1982). In eels, significant increases of *cyp11a1* and *hsd3b* mRNA were only detected in Fsh-treated testis (Kazeto et al., 2008). Our results unexpectedly showed that higher steroidogenic activities of reLh resulted in higher transcript levels of steroidogenic enzymes; reLh significantly induced transcription of steroidogenic enzymes (*cyp11a1*, *cyp17a1*, *hsd3b*, *cyp11b,* and *hsd11b2*) related with 11KT production at a lower dose than reFsh. These conflicting results may be due to the different incubation time (18 h) in Japanese eels and developmental stages (mature stage) of zebrafish.

Thus, the present study was performed to examine the regulation mechanisms of 11KT production by reFsh and reLh using transcriptional and translational inhibitors, to confirm whether 11KT production by reFsh and reLh is mediated through induction of any gene expressions. In the present study, a translational inhibitor prevented 11KT production by reFsh and reLh both in short (24 h) and long (72 h) treatment. While short (24 h) incubations with transcriptional inhibitors on 11KT production induced by reFsh and reLh had no effects; however, long (72 h) incubations had suppressive effects thus suggesting that mRNAs encoding steroidogenic enzymes and Gth receptors were stored in the testis and/or have a long half-life. Moreover, Fsh and Lh induce those expressions by acting on the post-transcriptional process, although Fsh and particularly Lh further induced those *de novo* transcriptions. This possibly explains the results of the previous report (Kazeto et al., 2008), which showed that Fsh and Lh have equivalent activities on 11KT production during short (18 h) incubation.

Fsh is undoubtedly a potent steroidogenic hormone in fish, however its physiological roles (except for androgen production) such as Sertoli cell activation seen in mammalian species remain unclear. A recent study reported that *fshr* mutant male medaka (*Oryzias latipes*) possessed normal testis and were fertile, while mutant females had a small ovary without vitellogenesis and thus were infertile, possibly due to low expression of ovarian aromatase, *cyp19a1a* (Murozumi et al., 2014). Xie et al. (2017) have demonstrated that testicular development in *fshb* and *fshr* knockout male zebrafish did not show significant differences compared with their heterozygous controls. Therefore, Fsh functions may not be critical and may overlap with Lh functions in male fish reproduction.

4.3.*Cellular localization of fshr and lhcgr1*

In the present study, results of qPCR using testicular cell fractionations of Japanese eel showed that *fshr* and *lhcgr1* were expressed both in Sertoli and Leydig cells, which is unlikely in mammals. Localization of *lhcgr2* has not been cleared, because *lhcgr2* was not detectable in most of samples (data not shown). Recently, Ozaki et al. (2019) performed *in situ* hybridization to confirm localization of *fshr* and *lhcgr1* mRNA in the testis of New Zealand shortfinned eel, which is a very closely related species to Japanese eel. As results, *lhcgr1* mRNA was not detectable in the immature testis due to the low expression levels. Then, the present study was employed the alternative method to confirm localization of *fshr* and *lhcgr1* mRNA in the immature testis of Japanese eel. In the previous study, however, *fshr* mRNA was detected both in Sertoli cells and Leydig cells. In the testis of shortfinned eels matured by hCG injections, *lhcgr1* mRNA was also detectable in those somatic cells. The localization of Fshr protein in Sertoli and Leydig cells has been also demonstrated by immunohistochemical observations using an antibody against Fshr in

Japanese eels (Ohta et al., 2007). These reports strongly reinforce our results in this study. To our knowledge, there is no report that localization of *fshr* and *lhcgr* mRNA was detected in other cell types except for germ cells, Sertoli cells and Leydig cells in the testis of fish including eels. Therefore, the present experiment demonstrated that germ cells, Sertoli cells and Leydig cells were highly separated using marker genes of those cells, although other cell types in the connective tissue might be contaminated. The co-expression of *fshr* and *lhcgr* in Leydig cells seems to be conserved in teleosts (Chauvigné et al.,2012; García-López et al., 2009, 2010), thus complicating our understanding of the physiological roles of Fsh and Lh in fish.

In the present study, the reporter assay showed that eel Fsh binds to Fshr in a specific manner, which was also seen in a previous report (Kazeto et al., 2008). Moreover, Fsh directly induces 11KT production through Fshr expressed in testicular somatic cells, implying the overlap of signal transduction mediated with Fshr and Lhcgr1 in testis to produce 11KT. However, 11KT production may be driven predominantly by Fsh at least during early spermatogenesis until Lh production is initiated. Androgen production by Fsh mediated with Fshr in Leydig cells has also been suggested in a previous study (García-López et al., 2009).

Our results showed that Sertoli cells also expressed both *fshr* and *lhcgr1*. The co-localization has been reported in zebrafish, which might be due to its undifferentiated gonochoristic mode of gonadal sex differentiation (García-López et al., 2010). However, the effects of Fsh and Lh mediated with Sertoli cells are not clear from this study. Hence further experiments are required to better understand the cellular localization of spermatogenesis-related genes in Japanese eel testis.

A recent report demonstrated that some fish *lhcgr* were also expressed in

spermatids; Lh directly induces spermiogenesis through Lhcgr localized on spermatid cell membranes (Chauvigné et al., 2014b). The localization of *lhcgr* was examined using cellular fractions from immature testis and future studies will address whether eel spermatid expresses *lhcgr*.

In conclusion, the present studies provided evidence showing that single-chain reFsh and reLh produced in FreeStyle 293-F cell lines were biologically active and involved with steroidogenic activities. Indeed, reFsh and reLh will be useful in future studies. Our data showed that both Fsh and Lh acted as steroidogenic hormones through their receptors in the immature testis; however, Lh was more potent in 11KT production, suggesting differential roles in spermatogenesis.

Chapter 3

17β-hydroxysteroid dehydrogenase type 12a responsible for testicular 11-ketotestosterone synthesis in the Japanese eel, *Anguilla japonica*

1. Introduction

Androgens play critical roles in the reproductive cycle of vertebrates. The biosynthesis of androgen from cholesterol is catalyzed by several steroidogenic enzymes under the control of pituitary gonadotropins (Gths), follicle-stimulating hormone (Fsh), and luteinizing hormone (Lh) (Payne and Youngblood, 1995). Among these, 17β-hydroxysteroid dehydrogenases (Hsd17bs) are oxidoreductive steroidogenic enzymes that catalyze the interconversion of 17-keto and 17β-hydroxysteroids and are essential for the formation of the sex steroids in gonads and other tissues (Labrie et al., 1997). In mammals, 14 types of Hsd17bs have been characterized (Mindnich and Adamski, 2009; Prehn et al.,2009). The number of isoenzymes of Hsd17bs differs according to animal species, and the tissue-distribution, substrate specificity, and oxidoreductive activity are highly dependent on the type of enzymes (Luu-The, 2001; Mindnich et al., 2004b).

11-ketotestosterone (11KT) has been established in teleost as a unique and the most potent androgen that plays important roles in sex determination, secondary sexual characteristic, and spermatogenesis (Brog, 1994). It is well-known in teleost that the plasma levels and the production of androgens increase with progression of reproductive status (Cavaco et al., 1996; García-López et al, 2006; Kusakabe et al., 2006; Rocha et al., 2009). Miura *et al*. (1991) have reported that an injection of gonadotropic hormone, such as human chorionic gonadotropin (hCG), activated 11KT production and spermatogenesis in the immature male Japanese eel. In addition, it has also been reported that testicular gene expression of steroidogenic enzymes, which are essential for 11KT synthesis, was regulated by hCG treatment in this species (Ijiri et al., 2006; Kazeto et al., 2008). Moreover, in chapter 2, the treatment of reGth induced 11KT synthesis though the

expression regulation of steroidogenesis related genes in the testis. However, Hsd17bs with 17-ketosteroid reducing activity (17KSR activity) responsible for 11KT production has not yet been well clarified in Japanese eel as well as in the other species of teleost.

Hsd17b type3 (Hsd17b3), exhibiting 17KSR activity, is an important enzyme that catalyzes the transformation of androstenedione (A4) into testosterone (T), as a precursor of dihydrotestosterone (DHT: a potent androgen in mammals) in mammalian testis (Geissler at al., 1994; Sha et al., 1997; Tsai-Morris et al., 1999). It is also known as a causative gene of male pseudohermaphroditism because the mutation of the enzyme leads to this disease (Geissler at al., 1994). Therefore, this Hsd17b is thought to be a strong candidate with 17KSR activity responsible for 11KT production in teleost and the cDNA encoding Hsd17b3 was recently isolated and characterized in zebrafish (Mindnich et al., 2005). However, zebrafish *hsd17b3* mRNA was detected in various tissues and the gene expression in the ovary was much higher than that in the testis unlike in mammals in which the testis is the predominant source of the transcript of *hsd17b3* gene (Geissler at al., 1994; Sha et al., 1997; Tsai-Morris et al., 1999; Mindnich et al., 2005). In addition, regulation of the *hsd17b3* gene expression by Gths remains to be examined (Mindnich et al., 2005). Two other Hsds, Hsd17b type12 (Hsd17b12) (Mindnich et al., 2004a) and 20β-hydroxysteroid dehydrogenase type2 (Hsd20b2) (Tokarz et al., 2012), are closely related with Hsd17b3, and thus, could be candidate for the genes with 17KSR activity responsible for 11KT production.

Two forms of Hsd17b12 (Hsd17b12a and Hsd17b12b) were identified by *in silico* screen of zebrafish expressed sequence tags (EST) database and phylogenetic analysis showed close relationship with the Hsd17b3 (Mindnich et al., 2004a). These enzymes catalyzed the conversion of estrone (E1) to 17β-estradiol (E2); however, their involvement

in 11KT synthesis is still unclear (Mindnich et al., 2009). More recently, catfish Hsd17b12, which exhibits high homology with zebrafish Hsd17b12b, was identified (Rajakumar et al., 2014). In the catfish, expression of *hsd17b12* gene increased together with T levels after hCG administration (Rajakumar et al., 2014). However, 17KSR activity of the Hsd17b12 has not yet been examined.

Zebrafish Hsd20b2 was initially isolated as a homolog of Hsd17b3 (Tokarz et al., 2012). However, the enzyme expressed in human cervical carcinoma cell line and in zebrafish fibroblast cell line did not transform E1 into E2 and A4 into T, while they converted cortisone into 20β-hydroxycortisone (Tokarz et al., 2012). Therefore, the enzyme was named Hsd20b2. Recently, Ijiri *et al*. (2017) demonstrated that a cognate gene of Hsd20b2 (named as Hsd17b12-like) in masu salmon catalyzed the transformation of 17α-hydroxyprogesterone (17OHP) to 17α, 20β-dihydroxy-4-pregnen-3-one (DHP); however, 17KSR activity of the Hsd20b2 has not yet been examined.

For deeper understanding of testicular 11KT biosynthesis induced by administration of hormones, the present study first investigated the steroidogenic pathway for 11KT production and identified 17KSR activity crucial for 11KT synthesis in the Japanese eel testis. Subsequently, cDNAs encoding candidate enzymes potentially exhibiting 17KSR activity were isolated and characterized. Furthermore, the effect of reGths on the 17KSR activity and the expression of the candidate genes was examined.

2. Materials and Methods

2.1 Animals, hormonal treatment, and tissue collection

Cultivated immature male Japanese eels (400-600 g body weight) raised from glass eels were kept in a flow-through freshwater tank at 15 °C, transferred into flow-through seawater tanks at 20°C prior to the experiments and used in this study.

In experiment 1, in order to obtain testes for analysis of the steroidogenic pathway for 11KT production, three fish were intraperitoneally injected with hCG (Asuka Pharmaceutical, Tokyo, Japan) at 625 ng (5IU)/g-BW and their testes were then collected at 1-day post-injection for the tissue-culture experiment.

In experiment 2, four fish were sampled before treatment as initial control and various tissues (pituitary, forebrain, midbrain, hindbrain, eye, gill, spleen, liver, head kidney, posterior kidney, heart, intestine, pancreas, muscle, skin, and testis) were collected from them. Twelve fish received a single intraperitoneal injection of hCG at a dose of 625 ng/g-BW and testes were collected from four fish at 1-, 3-, or 6-days post-injection. Another twelve fish served as the saline control group by injecting eel Ringer solution (150 mM NaCl, 3 mM KCl, 3.5 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM HEPES; pH 7.4). In addition, liver was collected from a fish at 3-day post hCG injection for isolation of *hsd17b3* cDNA.

In experiment 3, twelve fish were divided into four groups (n = 3/group): the saline control group, and the injection groups of hCG, recombinant single-chain Fsh (reFsh) (Kazeto et al., 2019), and Lh (reLh) (Kazeto et al., 2019) (625 ng/g-BW). Testes were collected at 1-day post-injection.

All the tissues collected were used for tissue-culture and/or RNA extraction. Samples for RNA extraction were stored in RNA later (Invitrogen, Carlsbad, CA) at -30 °C

until further use.

2.2 Culture of testicular fragments

To identify the steroid substrate of 17KSR activity for testicular 11KT production, eel testicular fragments at the first day after hCG administration (experiment 1) were distributed into 48-well plates (20 mg/well) and incubated in 400 μl of eel Ringer solution at 20 °C with 5×10^5 cpm [1,2,6,7-3H(N)]-androstenedione (Perkin-Elmer, Waltham, MA) at a final concentration of 100 nM. After 3, 6, and 12 hours incubation, media were collected and kept at -30 °C until used for thin-layer chromatography (TLC). Incubation was carried out for each time-point in three replicates.

To examine a time-dependent change in 17KSR activity, testicular fragments (experiment 2) at 0, 1, 3, or 6 days after either saline or hCG injection were incubated in eel Ringer with 11KA4 (100 ng/ml) as steroid substrate. After 24 hours of incubation, the media were collected and kept at -30 °C until used for time-resolved fluoroimmunoassay (TR-FIA) for 11KT. The 17KSR activity was expressed as a fold change in the enzymatic activity relative to the values in the corresponding control group.

Furthermore, testicular fragments (experiment 3) at 1 day after saline treatment, hCG, reFsh, or reLh treatment were also subjected to the tissue culture experiment described above to assess the differential effects of various gonadotropic reagents.

2.3 TLC

Steroid metabolites were extracted with 800 μl of dichloromethane twice after addition to the media of following non-radioactive standard steroid: A4 (Sigma, Poole, UK), T (Wako, Osaka, Japan), 11β-hydroxyandrostenedione (11OHA4; Steraloids, Newport, RI), 11β-

hydroxytestosterone (11OHT; Steraloids), 11-ketoandrostenedione (11KA4: TCI, Tokyo, Japan), and 11KT (Steraloids). The resultant extracts were concentrated in a water-bath at 40-50 °C under nitrogen gas and were subjected to TLC plates precoated with silica gel and separated in benzene-acetone (4:1 v/v) system. After separation, standard steroids were visualized under UV_{254}. Radioactive steroid metabolites on chromatograms were detected autoradiographically using an X-ray film (Eastman Kodak, Rochester, NY) and quantified using ImageJ (http://rsbweb.nih.gov/ij/).

2.4 TR-FIA

The concentration of 11KT in incubation media was measured by TR-FIA as described in chapter 2. In brief, steroids extracted with diethylether were incubated with an antiserum against 11KT (Cosmo Bio, Tokyo, Japan) in 96-well plates coated with BSA-conjugated 11KT prepared using the method described previously by Asahina *et al* (1995). After incubation and the washes, europium (Eu)-labeled goat anti-rabbit IgG (Perkin-Elmer, Waltham, MA) was added, followed by the addition of an enhancement solution (Perkin-Elmer, Waltham, MA). The intensity of fluorescent signals from dissociated Eu was measured by Infinite F200 (Tecan, Grodig, Austria).

2.5 RNA preparation and cDNA synthesis

Total RNA was extracted from testicular fragments and various tissues using the guanidium thiocyanate-phenol-chloroform extraction method using ISOGEN (Nippongene, Tokyo, Japan), according to manufacturer's instructions. Polyadenylated RNA was subsequently isolated from the total RNA extracted from a fish at 3 days-post hCG injection, using Oligotex-dT30 <Super> (Takara, Shiga, Japan). The cDNA was

synthesized from the total and polyadenylated RNA after priming with random hexamer using the Omniscript Reverse Transcription kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions.

2.6 Cloning of hsd17b3 cDNAs

To amplify *hsd17b3* cDNA fragment, PCR was conducted using the liver cDNA as template with a set of degenerate primers (forward 1 and reverse 1) designed from the consensus sequence of *hsd17b3* (Table 2). Subsequently, a nested PCR was carried out using the first step PCR product as template and another set of degenerate primers (forward 1 and reverse 2). The resultant PCR product was subcloned into pGEM-T Easy vector (Promega, Madison, WI) and subjected to sequence analysis using BigDye Terminator v3.1 Cycle Sequencing kit with 3130XL DNA sequencer (Applied Biosystems, Carlsbad, CA). In order to obtain the cDNA encoding *hsd17b3*, both 5'-and 3'-RACE were performed using SMARTer RACE cDNA Amplification Kit (Takara), following the manufacturer's protocol, using gene-specific primers, RACE forward 1 and RACE reverse 1 (Table 2). To confirm the validity of the nucleotide sequence of *hsd17b3* cDNA, cDNA fragments containing the open reading frame (ORF) were amplified using specific primers (Table 2) and Prime STAR HS DNA Polymerase (Takara) with proofreading ability and bi-directionally sequenced after subcloning into the vector as described above.

2.7 Cloning of hsd17b12a and hsd20b2 cDNAs

Putative nucleotide sequences encoding *hsd17b12a* and *hsd20b2* of Japanese eel were retrieved from the database of ovarian Expressed sequence tags (ESTs) (Ijiri et al., 2011b). The sets of primers to amplify the entire ORFs of the *hsd17b12a* and *hsd20b2* were

designed (Table 2) and used. PCR-based cloning of ORF sequences of these genes was carried out using cDNA prepared from non-treated testis using the same experimental protocols as described above.

2.8 Sequence alignments and phylogenetic analysis

The alignment analysis of sequences was performed using Clustal W. The neighbor-joining phylogenetic tree was constructed using Clustal W and displayed using MEGA version X (Kumer et al., 2018). The GenBank accession number or the Ensemble translation ID of Hsd17b3, Hsd17b12, Hsd20b2 (designated as Hsd17b12-like in masu salmon) and, Hsd17b1 sequences used in this study are as follow: Hsd17b3 - Zebrafish (AAS58451.1), Human (NP_000188.1), Mouse (AAB06793.1), Rat (NP_446459.1), Atlantic salmon a (ACI66194.1), Atlantic salmon b (ACI66672.1); Hsd17b12 - Zebrafish a (NP_957175.1), Zebrafish b (NP_955907.1), Medaka a (ENSORLP00000001758.1), Medaka b (ENSORLP00000040712.1), Stickleback a (ENSGACP00000009205.1), Stickleback b (ENSGACP00000022633.1), Catfish (AFA36443.2), Human (AAP36605.1), Mouse (NP_062631.1), Rat (Q6P7R8.1); Hsd20b2 - Zebrafish (XP_694907.2); Hsd17b12 - like - Masu salmon type1 (BAV89211.1), Masu salmon type2 (BAV89212.1); Hsd17b1 - Japanese eel (AAR88433.1), Zebrafish (NP_991147.2), Human (AAI11936.1), Mouse (NP_034605.1), Rat (NP_036983.1).

2.9 Functional assays of recombinant Hsd17b3, Hsd17b3 long form (Hsd17b3lf), Hsd17b12a, and Hsd20b2

For activity measurement, *hsd17b3*, *hsd17b3lf*, *hsd17b12a*, and *hsd20b2* cDNAs containing the entire open reading frames were inserted into a eukaryotic expression

vector, pCAGGS (Tokui et al., 1997), using In-fusion® HD Cloning Kit (Takara), according to the manufacturer's instructions and designated as Hsd17b3-pCAGGS, Hsd17b3lf-pCAGGS, Hsd17b12a-pCAGGS, and Hsd20b2-pCAGGS, respectively.

Human embryonic kidney 293 T (HEK293T) cells were maintained and propagated in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) at 37 °C. Thereafter, HEK293T cells were seeded into wells of six-well plates and transfected with either the expression vectors or empty-pCAGGS using FuGENE HD Transfection Reagent (Promega, Fitchburg, WI), according to the manufacturer's protocol. Transfected HEK293T cells were preincubated for 48 hours, and thereafter, incubated with 11KA4 (100 ng/ml) for 24 hours. After incubation, media were collected, and the 11KT concentration was measured by TR-FIA as described above. Three replicate incubations were done for each experimental group. Another set of experiments using eel hepatocyte-derived (Hepa-E1) cells was also conducted under the culture condition with E-RDF medium (Kyokuto, Tokyo, Japan) with 5% FBS at 28 °C.

Moreover, for substrate specificity analysis, Hepa-E1 cells transfected with Hsd17b3-pCAGGS, Hsd17b3lf-pCAGGS, and empty-pCAGGS were incubated with with $5x10^5$ cpm [1,2,6,7-3H(N)]-A4 (Perkin-Elmer), [1,2,6,7-3H(N)]-T (Perkin-Elmer), [2,4,6,7-3H(N)]- E1 (Perkin-Elmer), or [2,4,6,7-3H(N)]-estradiol-17β (E2) (Perkin-Elmer) at a final concentration of 100 nM. After 24 h of incubation, the media were collected and stored at -30 °C. The steroid metabolites in the media were analyzed by TLC. Similar experiments were also performed using HEK293T cells transfected with Hsd17b12a-pCAGGS, Hsd20b2-pCAGGS, and empty-pCAGGS.

Both cell-lines of HEK293T and Hepa-E1 were obtained from the Institute of Physical and Chemical Research (RIKEN) cell bank (Tsukuba, Japan).

2.10 Quantitative real-time PCR (qPCR)

The transcript abundances of genes encoding *hsd17b3*, *hsd17b12a*, or *hsd20b2* in various tissue were determined by qPCR using TaqMan Universal Master Mix2 with UNG (Applied Biosystems, Carlsbad, CA) or Quantifast SYBR Green PCR Kit (QIAGEN) on the Applied Biosystems 7500 Real-Time PCR System, according to the manufacturer's instructions. The primers and probes used for each gene are listed in Table 3. The detailed procedure to design the primer/probe sets, and to validate qPCR assays were based the handbook ABI PRISM Sequence User Guide and a previous report (Matsubara et al., 2003). Data were represented as relative mRNA levels to mRNA levels of an internal standard gene, *bactin* (*actb*) (Kazeto et al., 2008). The primers and probe for *β-actin* (*actb)* were shown in Table 2.

2.11 Statistical analysis

All data are expressed as mean ± SEM. Data analyses were carried out using one-way ANOVA followed by Tukey-Kramer test or unpair *t* test.

Table 2. Sequence of primers used for cloning.

Target	Primer	Nucleotide sequences (5’ → 3’)
hsd17b3	forward 1	AACAATGT(C/G/T)GG(A/G/T)AT(C/G/T)CT(A/C/G/T)CC
	reverse 1	TG(A/G/T)GA(A/G)AA(C/T)C(G/T)CTC(A/C)ACAAA
	reverse 2 (nested)	TC(A/C)ACAAA(A/C/T)AC(C/T)TT(G/T)GA(A/G/T)GC
	RACE forward 1	GGACCTAGAGCAGAGGATCACGAGCG
	RACE reverse 1	GCCGTACATGGTGTAGAGTGGGCAC
	ORF forward 1	GGTGTGGATCCGGATCCGAAGC
	ORF reverse 1	TGAATCAGTTACCGTTGACACTCTTG
hsd17b12a	ORF forward 1	CGCGAAAAGCCATTGCCGTGCAG
	ORF reverse 1	CGTTCCTCCTGGCGGGATGGC
hsd20b2	ORF forward 1	GACCATCGCAACCGCTCGG
	ORF reverse 1	CTGAACGCAACCTAATCACAAGGCAG

Table 3. Sequence of primers and probe used for qPCR.

Target	Primer	Nucleotide sequences (5' → 3')	Product size (bp)	Reference
hsd17b3	forward	CAGTGCCCAAATCCTTCTTCTC	186	
	reverse	CTTCTTTCCCTGTTGTTTCTCCTATC		
hsd17b12a	forward	AACGGGCAGCTGATTTCAAC	118	
	reverse	TCAGCATCACGGCAAATCC		
hsd20b2	forward	GGGATCGGCAGAGCTTACG	120	
	reverse	TCTCCCATGAAGCCTCTCAATC		
actb	forward	ACGGGCAGGTCATCACCAT	111	Kazeto et al. 2008
	probe	CCTTCCTTCCTGGGTATGGAGTCCTGC		
	reverse	AGTTGAAGGTGGTCTCGTGGATT		

3. Results

3.1 Metabolic pathway synthesizing 11KT

Based on identical mobility with non-radioactive standard steroid, 11OHA4, 11KA4, and 11KT were detected as major metabolites of radioactive A4 in the eel testis, although T and 11OHT were not found (Fig. 8A). Densitometrical analysis of the autoradiograph showed that the substrate (A4) decreased in a time-dependent manner, whereas 11OHA4 was constantly detected (19-25%) at all time-points (Fig. 8B). Meanwhile, 11KA4 and 11KT levels increased from 19% to 45% and from 5% to 19%, respectively (Fig. 8B).

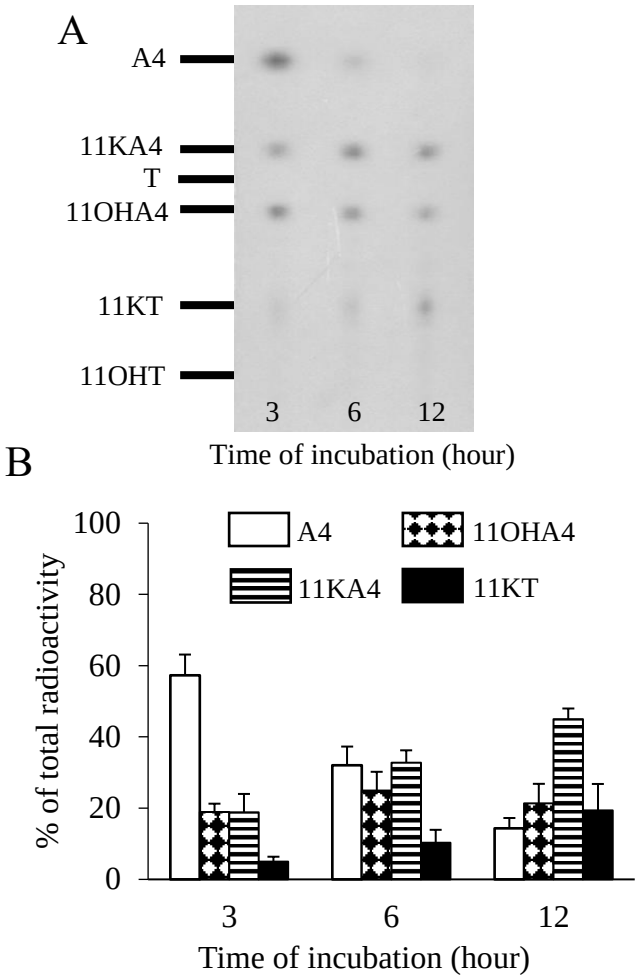

Figure 8. Metabolism of A4 in Japanese eel testis. (A) Autoradiogram of a thin-layer chromatogram of the metabolites converted from A4. (B) The conversion rates from A4 to major metabolites. The yield of steroid metabolites converted from A4 was calculated as a percentage against the total radioactivity. The bars represent the mean ± SEM of three replicates. A4: androstenedione, 11OHA4: 11β-hydroxyandrostenedione, 11KA4: 11-ketoandrostenedione, T: testosterone, 11OHT: 11β -hydroxytestosterone, 11KT: 11-ketotestosterone.

3.2 Sequence alignments and phylogenetic analyses of Hsd17b3, Hsd17b12a, and Hsd20b2

We obtained cDNAs of three enzymes, containing the open reading frame (ORF), using PCR-based strategy and EST-database information. The *hsd17b3* and *hsd17b3lf* cDNAs are composed of a 19 bp long 5'-untranslated region (UTR), a 7 bp long 3'-UTR, and an ORF of 924 and 948 bp encoding peptides of 308 and 316 amino acid residues long, respectively. An alignment of the amino acid sequence of Japanese eel Hsd17b3 and with its counterparts in other species demonstrated that the eel Hsd17b3 possessed 60% and 50% identity with Hsd17b3 of zebrafish and mammals, respectively. In Hsd17b3lf, eight amino acids were inserted between 127aa and 128aa in the Hsd17b3 (Fig. 9).

The *hsd17b12a* cDNA consists of a 9 bp long 5'-UTR, an 8 bp long 3'-UTR, and a 942 bp long ORF encoding 314 amino acids. Amino acid sequence of Hsd17b12a of the Japanese eel showed the highest identity with zebrafish Hsd17b12a (79%), followed by medaka Hsd17b12a (76%), stickleback Hsd17b12a (65%), Hsd17b12bs of other fish species (70-74%) and mammalian Hsd17b12s (59%) (Fig. 10).

The *hsd20b2* is composed of an 82 bp long 5'-UTR, a 74 bp long 3'-UTR, and an ORF of 981 bp encoding 327 amino acids long polypeptide. An alignment of amino acid sequence of eel and zebrafish or masu salmon Hsd20b2 (reported as Hsd17b12-like in masu salmon) demonstrated 61% and 66% identity, respectively (Fig. 11).

Japanese eel Hsd17b3 shows 36 and 39% homology to the Hsd17b12a and Hsd20b2, respectively. Hsd17b12a shares 40% sequence identity to the Hsd20b2. Hsd17b3lf shows identities to the other Hsds as well as the Hsd17b3 (Table 4). In addition, the deduced amino acid sequences of these HSDs contained the conserved signatures of short-chain dehydrogenase/reductase (SDR) family members, namely the cofactor binding (TGXXXGXG), the catalytic mechanism (YXXXK), and the structural integrity (NVG).

Phylogenetic analysis showed that isolated eel Hsd17b3, Hsd17b3lf, Hsd17b12a, and Hsd20b2 were included in the corresponding clusters of each HSD of vertebrates respectively (Fig. 12).

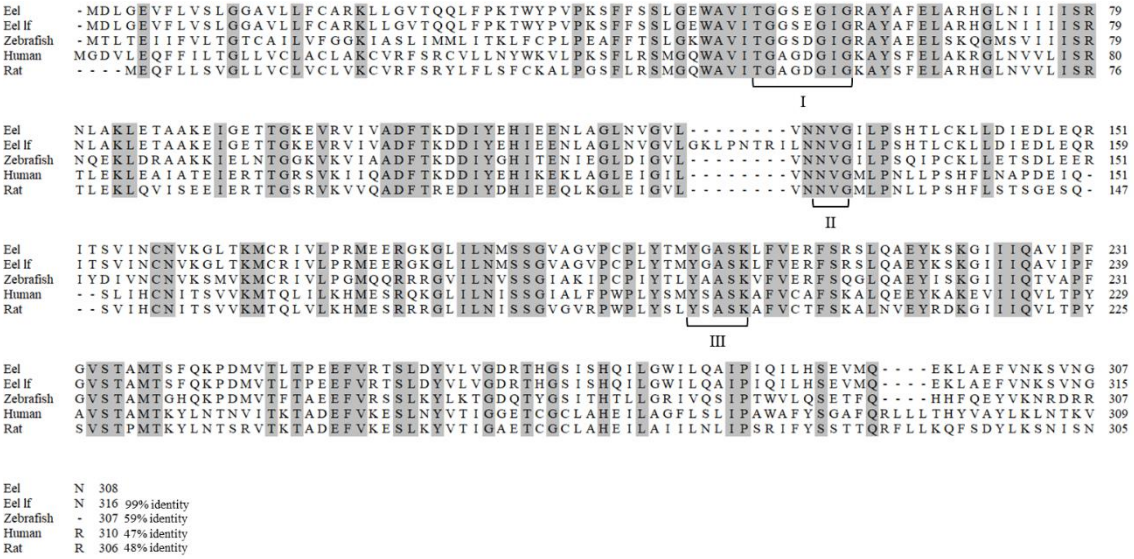

Figure 9. Alignment of the putative amino acid sequence of eel Hsd17b3 with those of Hsd17b3 of other animal species. I: cofactor binding site; II: the structural integrity site; III: catalytic mechanism site. Comparisons were made with sequence obtained from GenBank: Zebrafish (AAS58451.1), Human (NP_000188.1), Mouse (AAB06793.1), Rat (NP_446459.1).

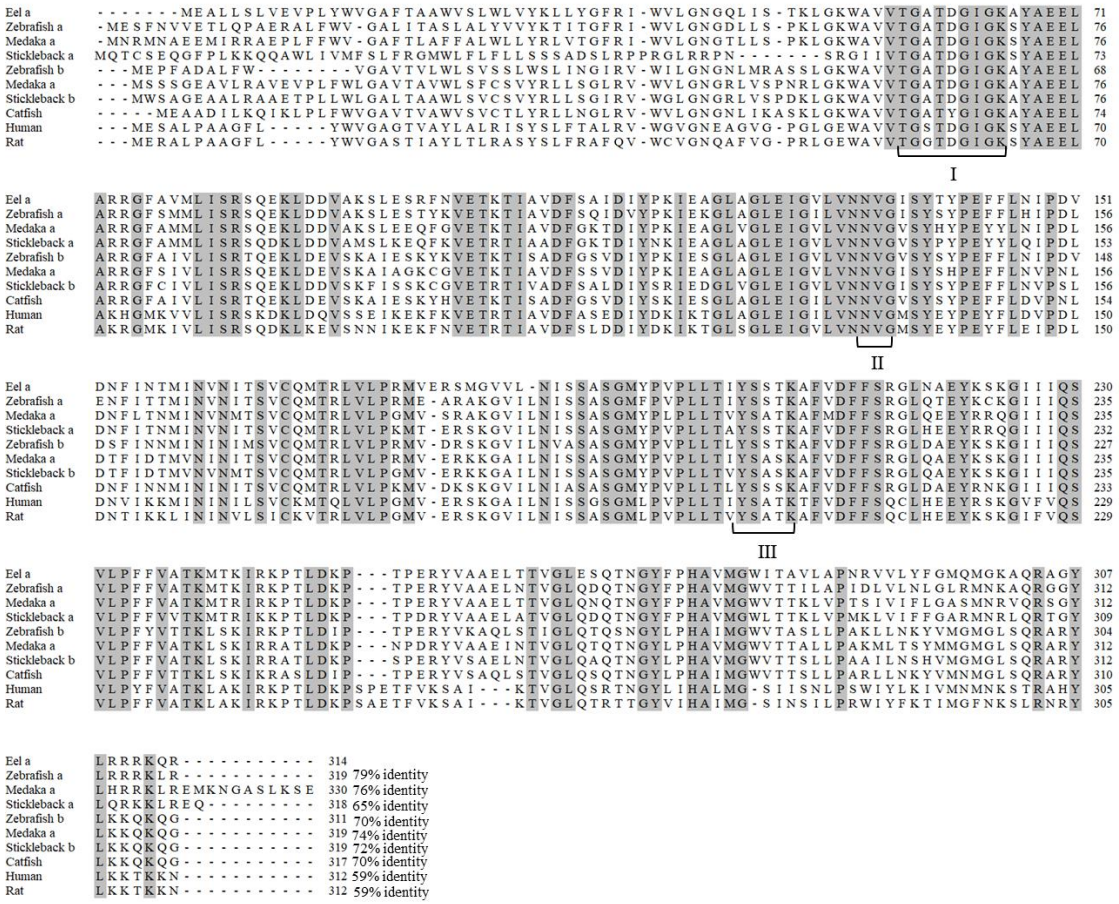

Figure 10. Alignment of the putative amino acid sequence of eel Hsd17b12a with those of Hsd17b12s of various organisms. I: cofactor binding site; II: the structural integrity site; III: catalytic mechanism site. Comparisons were made with sequence obtained from GenBank and Ensembl database (http://asia.ensembl.org/index.html) : Zebrafish a (NP_957175.1), Zebrafish b (NP_955907.1), Medaka a (ENSORLP00000001758.1), Medaka b (ENSORLP00000040712.1), Stickleback a (ENSGACP00000009205.1), Stickleback b (ENSGACP00000022633.1), Catfish (AFA36443.2), Human (AAP36605.1), Rat (Q6P7R8.1).

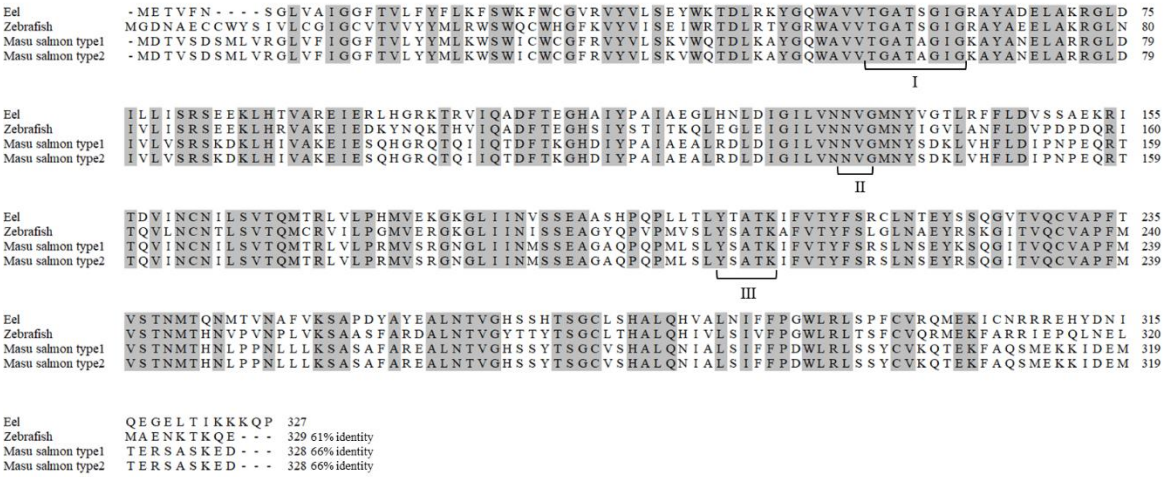

Figure 11. Alignment of the putative amino acid sequence of eel Hsd20b2 with those of Hsd20b2 of other animal species. I: cofactor binding site; II: the structural integrity site; III: catalytic mechanism site. Comparisons were made with sequence obtained from GenBank: Zebrafish (XP_694907.2), Masu salmon type1 (BAV89211.1), Masu salmon type2 (BAV89212.1).

Table 4. Amino acid sequence percentage identity between the candidate enzymes.

	Hsd17b3	Hsd17b3lf	Hsd17b12a	Hsd20b2
Hsd17b3	100			
Hsd17b3lf	99	100		
Hsd17b12a	36	35	100	
Hsd20b2	39	38	40	100

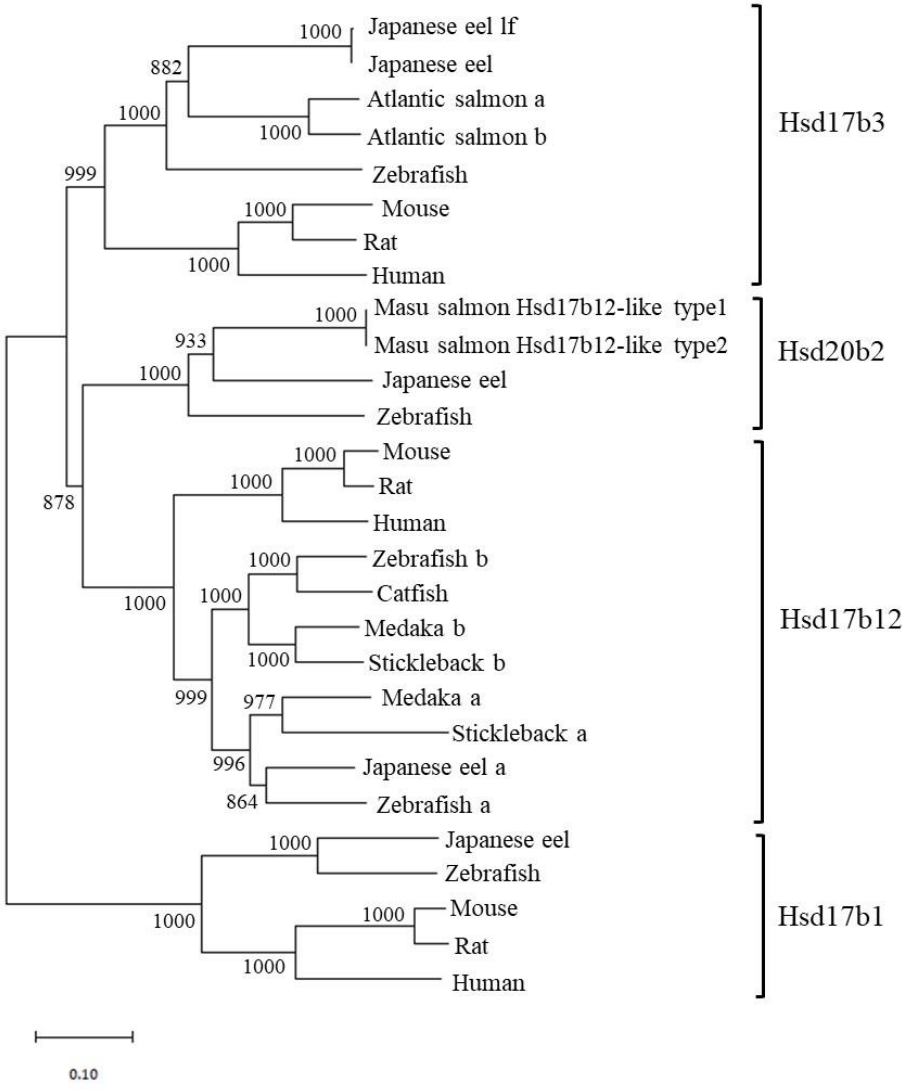

Figure 12. Phylogenetic analysis of Hsd17b3, Hsd17b12a, Hsd20b2, and Hsd17b1 proteins. The analysis was performed using Clustal W by the neighbor-joining method. The numbers beside the branches indicate bootstrap values from 1000 replicates.

3.3 The 17KSR activity of recombinant Hsd17b3, Hsd17b3lf, Hsd17b12a, and Hsd20b2

First, functional assays of these enzymes were performed using the recombinant proteins expressed in HEK293T cells. Hsd17b12a and Hsd20b2 catalyzed the conversion of 11KA4 into 11KT (approximately 75% and 26%, respectively), which was significantly higher than the 11% conversion observed in HEK293T cells transfected with an empty vector (mock). However, Hsd17b3 and Hsd17b3lf in HEK293T cells did not show any significant conversion of 11KA4 into 11-KT (Fig. 13A).

In addition, the Hepa-E1 cells transfected with the expression vector for Hsd17b3, Hsd17b12a, or Hsd20b2 significantly transformed 11KA4 to 11KT, with yields of 11KT of about 79%, 17% or 77%, respectively (Fig. 13B). The conversion by the recombinant Hsd17b3lf was similar to those observed in mock.

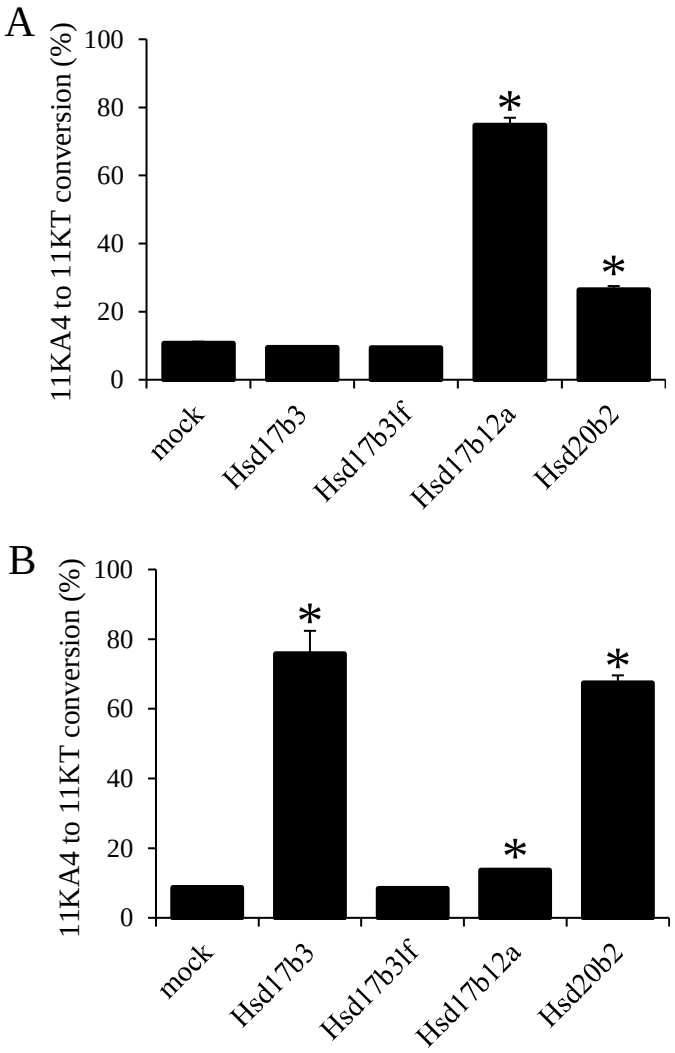

Figure 13. The 17KSR activity of recombinant eel Hsd17b3, Hsd17b3lf, Hsd17b12a, or Hsd20b2. The conversion of 11KA4 to 11KT by HEK293T (A) or Hepa-E1 (B) cells transfected with either Hsd17b3-pCAGGS, Hsd17b3lf-pCAGGS, Hsd17b12a-pCAGGS, Hsd20b2-pCAGGS, or empty-pCAGGS. Bars represent the means ± SEM of three replicates. The asterisk indicates significant differences from mock group ($P < 0.05$).

3.4. Substrate specificity of recombinant Hsd17b3, Hsd17b3lf, Hsd17b12a, and Hsd20b2

We further tested substrate specificity of Hsd17b3, Hsd17b3lf, Hsd17b12a, and Hsd20b2 by TLC. Reduction of A4 to T was catalyzed by Hsd17b3 expressed in Hepa-E1 with high efficiency when compared with Hepa-E1 cells transfected with an empty vector, while no oxidizations of T to A4 and E2 to E1 were confirmed by Hepa-E1 cells transfected with Hsd17b3-pCAGGS (Fig. 14A). Hsd17b3lf expressed in Hepa-E1 cells did not show any significant conversion of A4 into T, T into A4, and E2 and E1 (Fig. 14B). The reducing reactions of Hsd17b3 and Hsd17b3lf against E1 were not determined because Hepa-E1 cells possessed the endogenous enzymatic activity involved in the conversion of E1 toE2 (Fig. 14A and B).

Catalysis of the conversion of A4 to T by Hsd17b12a and Hsd20b2 expressed in HEK293T cells was significantly higher than that in controls. E1 was reduced to E2 by the Hsd17b12a but not the Hsd20b2 (Fig. 14C and D). On the other hand, the Hsd17b12a and Hsd20b2 did not show significant activity in the conversion of T to A4 or E2 to E1 compared to the mock (Fig. 14C and D).

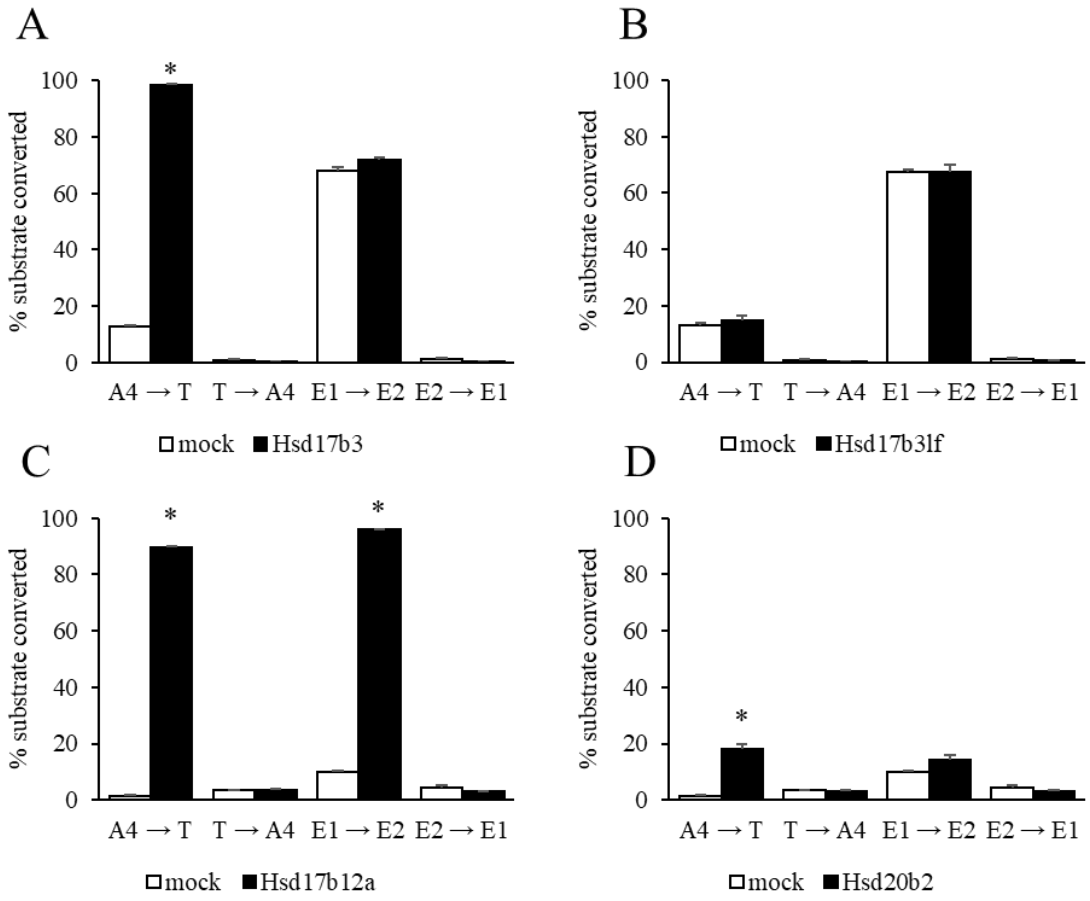

Figure 14. Substrate specificity of Hsd17b3 (A) and Hsd17b3lf (B) expressed in HepaE1 and Hsd17b12a (C) and Hsd20b2 (D) expressed in HEK293T. Conversion of A4 to T, T to A4, E1 to E2, and E2 to E1 was detected by thin-layer chromatography followed by autoradiography. Bars represent the mean ± SEM of triplicate measurements. Asterisks indicate significant differences from the mock group ($P < 0.05$).

3.5 Tissue-distribution of hsd17b3, hsd17b12a, and hsd20b2 mRNA

Genes of these Hsds were ubiquitously expressed in various tissues (Fig. 15). The *hsd17b3* mRNA was mainly detected in pituitary, forebrain, midbrain, eye, and muscle, while the transcripts were not detectable in the testis. The *hsd17b12a* expression was prominent in pituitary, forebrain, midbrain, hindbrain, eye, gill, and testis. In addition, the gene expression of *hsd20b2* was dominantly found in pituitary, liver, and testis.

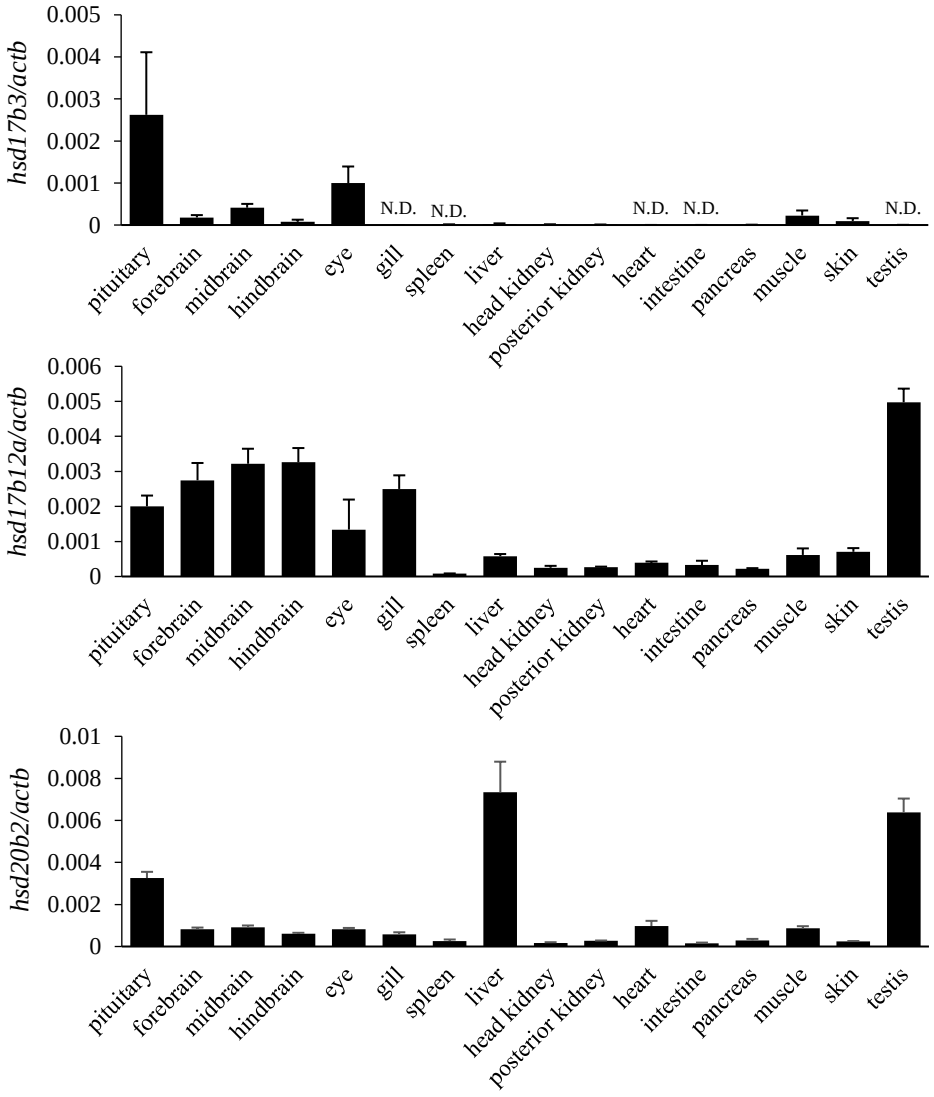

Figure 15. Tissue-specific expression of eel *hsd17b3*, *hsd17b12a*, and *hsd20b2*. The transcript abundance of each gene was determined by qPCR. Relative mRNA levels were normalized with *bactin* (*actb*). Bars represent the means ± SEM of four replicates. N.D., not detectable, values were below a limit of detection for qPCR analysis.

3.6 Effects of Gths on 17KSR activity responsible for 11KT synthesis in the testis

After hCG administration, the testicular 17KSR activity against 11KA4 forming 11KT increased significantly, reaching a maximum after 1 day, decreased gradually after 3 days, and returned to the basal level after 6 days (Fig. 16A). In contrast, the vehicle control group showed no significant change in the 17KSR activity throughout the experimental period (Fig. 16A).

Recombinant single-chain Lh displayed a significant upregulation (2.9-fold) of the 17KSR activity as well as hCG (3.5-fold) 1 day after the treatment, although the treatment by reFsh elicited an approximate 2-fold increase in the 17KSR activity with no statistical significance (Fig. 16B).

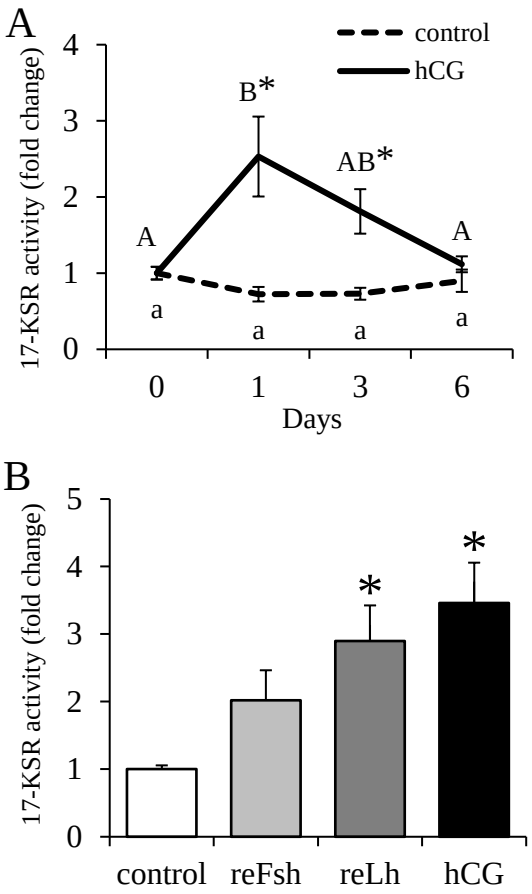

Figure 16. *In vivo* effects of Gths on 17KSR activity in testicular fragments. (A) The time-dependent change in 17-KSR activity forming 11KT from 11KA4 in hCG-treated group or in the vehicle control. (B) Effects of reFsh, reLh, or hCG on 17KSR activity. The values in the hormones-injected groups are expressed as fold change in the enzymatic activity relative to the values in the control group. Bars represent the means ± SEM of three (A) or four (B) replicates. The asterisk indicates significant differences from control group ($P < 0.05$). The different letters indicate significant differences in each group ($P < 0.05$).

3.7 Effects of reFsh, reLh, or hCG on the expression of hsd17b3, hsd17b12a, and hsd20b2

Transcript abundance of *hsd17b12a* gene increased significantly at 1 day after the hCG injection and decreased at 3 days at the basal level (Fig. 17A). In contrast, gene expression of *hsd20b2* was downregulated significantly at 1, 3, and 6 day after the treatment (Fig. 17A).

An injection of reFsh, reLh, or hCG significantly upregulated the expression level of *hsd17b12a* by about 2.9-, 4.4-, and 3.4-fold at 1 day after the treatment, respectively, compared with the control group (Fig. 17B). However, the expression level of *hsd20b2* tended to be reduced by reFsh, reLh, and hCG treatment with no statistical significance (Fig. 17B). In both experiments, abundance of *hsd17b3* mRNA was below the detection limit of qPCR (data not shown).

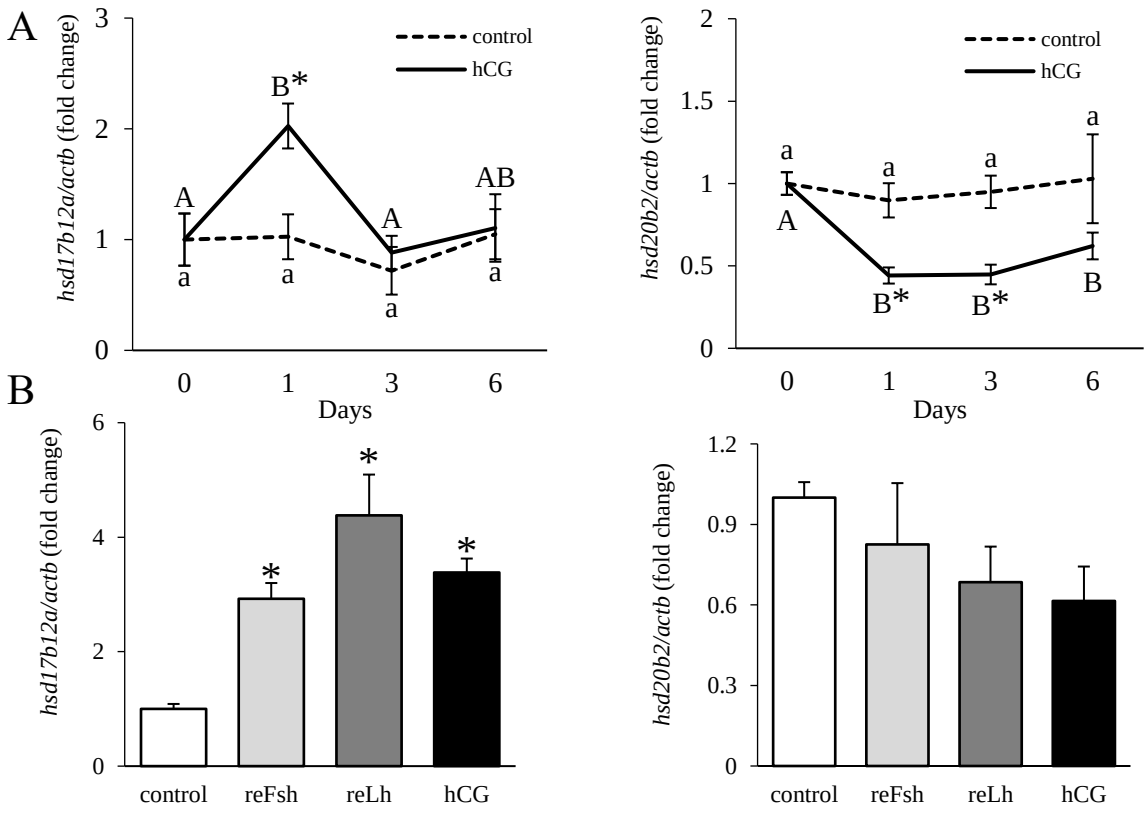

Figure 17. The changes in relative transcript levels of *hsd17b12a* and *hsd20b2* after the treatment with Gths. Relative mRNA levels were normalized with *bactin*. (A) The time-dependent change in the expression of *hsd17b12a* and *hsd20b2* genes in hCG-treated group or in the vehicle control. (B) Effect of reFsh, reLh, or hCG on gene expression of HSDs with 17-KSR activity. The values in the hormone-injected groups are expressed as fold changes in the transcript abundances relative to the values in the control group. Bars represent the means ± SEM of three (A) or four (B) replicates. The asterisk indicates significant differences from the control group ($P < 0.05$). The different letters indicate significant differences in each group ($P < 0.05$).

4. Discussion

11KT plays important roles in several physiological processes, including development of secondary sexual features, sexual behavior, and spermatogenesis, and is produced in testis under the regulation of Gths (Swanson et al., 2003). However, the Hsd17b enzyme with the 17KSR activity critical for testicular 11KT synthesis remains to be elucidated in teleost. The present study investigated the change in eel testicular 17KSR activity induced by Gths treatment. Furthermore, the isolation and characterization of candidate genes with such activity were thoroughly carried out.

To identify the steroid substrate of the 17KSR activity responsible for 11KT production in the Japanese eel testis, the metabolism of A4 in testicular fragments was first analyzed by TLC. 11KT was synthesized from A4 via 11OHA4 and 11KA4, which indicated that the Hsd17b with the 17KSR activity, catalyzing the conversion of 11KA4 to 11KT, is an indispensable mechanism for 11KT synthesis. In zebrafish, a similar steroidogenic pathway was shown by de Waal *et al*. (2008). On the other hand, in rainbow trout, A4 is converted to T and subsequently metabolized to 11OHT and 11KT (Arai and Tamaoki, 1967). In African catfish, 11KT was transformed via A4 → 11OHA4 → 11OHT (Cavaco et al., 1997). These findings suggested that the biosynthetic pathway for 11KT vary among fish species.

In mammals, Hsd17b3 plays a key role in regulating testicular production of the predominant potent androgen, testosterone. It has been previously demonstrated that HSD17B3 is essential for testosterone biosynthesis and is identified as the causative gene of hermaphroditism (Geissler at al., 1994). In addition, Hsd17b12 and Hsd20b2 were characterized as genes with sequences closely related to that of Hsd17b3 (Mindnich et al.,

2005; Tokarz et al., 2012). Therefore, in the present study, Japanese eel *hsd17b3*, *hsd17b12*, and *hsd20b2* cDNAs were isolated by PCR-based strategy and characterized. The amino acid sequences of Hsds share high identity with their counterparts in other animals and contain motifs common to the SDR family (Mindnich et al., 2004b), such as cofactor binding region, the catalytic mechanism region, and the structural integrity region. As expected in the phylogenetic analysis, Japanese eel Hsd17b3, Hsd17b3lf, Hsd17b12a, and Hsd20b2 clearly segregated into three branches with other Hsd17b3, Hsd17b12, and Hsd20b2 forms. In zebrafish, two variants of Hsd17b12, named Hsd17b12a and Hsd17b12b, were identified (Mindnich et al., 2004a) although Hsd17b12 was found as a single variant in mammal (Luu-The et al., 2006). In addition, the presence of two putative *hsd17b12* genes in medaka (*Oryzias latipes*), Hsd17b12a (ENSORLP00000001758.1) and Hsd17b12b (ENSORLP00000040712.1), and stickleback (*Gasterosteus aculeatus*), Hsd17b12a (ENSGACP00000009205.1) and Hsd17b12b (ENSGACP00000022633.1) could be confirmed in Ensembl database (http://asia.ensembl.org/index.html). The differences in the number of *hsd17b12* might be due to the whole genome duplication event (Zhou et al., 2005). However, in this study, only one form of Hsd17b12, clustered with zebrafish Hsd17b12a, was isolated from Japanese eel. Thus, it remains to be elucidated whether the Hsd17b12b exists in Japanese eel or not. The differences in function and gene regulation of Hsd17b12s need to be further examined if the isoforms exist.

Japanese eel Hsd17b3, Hsd17b12a, and Hsd20b2 expressed in HEK293T and/or Hepa-E1 cells proved to convert 11KA4 to 11KT, suggesting that these are functional enzymes with the 17KSR activity in Japanese eel. Mindnich *et al.* (2005) reported that human and zebrafish Hsd17b3 showed conversion of 11KA4 to 11KT. Hence, the reducing

activity of 11KA4 to 11KT by Hsd17b3 seems to be conserved across vertebrates.

Further analysis of the substrate specificity of the recombinant proteins was performed using Hepa-E1 cells expressing Hsd17b3 and Hsd17blf and HEK293T cells expressing Hsd17b12a and Hsd20b2. The Hsd17b3, Hsd17b12a, and Hsd20b2 preferably catalyzed the conversion of A4 to T and/or E1 to E2, suggesting that Hsd17b3, Hsd17b12a, and Hsd20b2 possess the reductase activity, but not oxidase activity, of androgens and/or estrogen. However, the expression of Japanese eel Hsd17b3lf in both cells did not lead to 11KT synthesis. In this form, eight amino acid residues were inserted in a region close to the functional domain of structural integrity, which might affect the 3-D structure, followed by subsequent loss of enzymatic activity. In this study, the reductase activity catalyzing the conversion E1 to E2 by Hsd17b3 and Hsd17b3lf could not be confirmed due to endogenous enzymatic activity in Hepa-E1 cells. Therefore, further experiments are required to investigate the reductase activity involved in the synthesis of C17-rduced estrogen of Hsd17b3 and Hsd17b3lf using other cell lines with no enzymatic activity involved in the estrogen production.

Previous reports in human and monkey showed that Hsd17b12 catalyzed the transformation of E1 into E2 selectively (Liu et al., 2007; Luu-The et al., 2006). In mouse, Hsd17b12 has been shown to convert E1 to E2 as efficiently as A4 to T (Blanchard et al., 2007). Japanese eel Hsd17b12a also catalyzed the transformation of 11KT from 11KA4 and E2 from E1. It should be noted that this is the first report demonstrating that this enzyme is responsible for 11KT formation to our best knowledge. These data indicated that the catalytic property depends on animal species, which suggests that this enzyme plays species-specific role in the synthesis of androgens and/or estrogens.

Tokarz *et al.* (2012) reported that zebrafish Hsd20b2, expressed in mammalian

cells, showed no 17KSR activity. Here, we indicated, for the first time, that the eel enzyme was able to convert 11KA4 to 11KT. In masu salmon, the enzyme catalyzed the conversion of a progestin, 17α-hydroxyprogesterone (17OHP), to 17α,20β-dihydroxy-4-pregnen-3-one (DHP) (Ijiri et al., 2017). Zebrafish Hsd20b2 displayed catalytic activity, converting a corticosteroid, cortisone, to 20β-hydroxycortisone (Tokarz et al., 2012). Therefore, the enzymatic activity of Hsd20b2 seems to be species-specific; however, it can be considered a necessity to further investigate its substrate-specificity due to different steroid substrates and experimental methodologies in this study and the previous studies.

Interestingly, three steroidogenic enzymes showed variable 17KSR activity depending on the cells where these enzymes were expressed. In this study, Japanese eel Hsd17b3 showed enzyme activity only when expressed in Hepa-E1 cells. In contrast, 17KSR activity of Hsd17b12a was much higher in HEK293T cells than in Hepa-E1 cells. A similar result has been reported that COS-1 cells expressing rat Hsd17b3 converted dehydroepiandrosterone to delta5-androstenediol with high efficiency, whereas HEK293 cells transfected with the same expression vector exhibited extremely low activity (Tsai-Morris et al., 1999). Considering these results, the varying activity of the recombinant steroidogenic enzyme is likely to be caused by difference of cells used in the experiments. Thus, it seems that the functional analysis of recombinant steroidogenic enzyme needs to comprehensively be evaluated with results obtained by experiments using multiple cell lines. In addition, the varying activity of recombinant steroidogenic enzymes expressed in HEK293T or Hepa-E1 cells might be caused by different levels of expression of the proteins, which was not tested by us. Hence, further experiments are required to generate specific antibody against steroidogenic enzymes and analyze expression levels of the enzymes in the hosts.

The analysis on the tissue-distribution of the transcript showed that *hsd17b3*, *hsd17b12a*, and *hsd20b2* genes are broadly expressed in Japanese eel. The ubiquitous expression of the three enzyme genes has also been reported in zebrafish (Mindnich et al., 2004a, 2005; Tokarz et al., 2012). 17KSR activity has also been detected in various tissues, such as liver, seminiferous tubes of African catfish (Cavaco et al., 1997), and liver, brain, spleen, intestine, kidney, and skin of rainbow trout (Schulz and Blüm, 1991). Taken together, not only testis but also extragonadal tissues are possibly associated with the final step of the biosynthesis of 11KT in teleost including Japanese eel.

The *in vivo* experiment revealed that the 17KSR activity was upregulated one day after reFsh, reLh or hCG administration. In chapter 2, it was reported in immature eel testis that 11KT production was regulated by Fsh and Lh at both transcriptional and translational levels *in vitro*. These findings strongly suggested that the gene expression of Hsd17b with the 17KSR activity responsible for 11KT production was regulated by Gths in male eel. Furthermore, the regulation of the 17KSR activity by Gth has also been demonstrated in rat (Tsai-Morris et al., 1999), and therefore, this gonadotropic regulation might be conserved across vertebrates.

The present study was performed to analyze the differential regulation of gene expression of these enzymes in the testis by Gths, i.e., reFsh, reLh, and hCG. Japanese eel *hsd17b3* mRNA could not be detected by qPCR in immature and Ghts-treated testis. In contrast, the expression of *hsd17b12a* gene was upregulated by Gths, especially reLh and hCG that are Gths the activating the receptor of Lh, which was identical to the regulation of 17KSR activity responsible for 11KT synthesis. It was demonstrated in chapter 2 that the Lh was more potent than Fsh in inducing androgen production in the testis of Japanese eel, which coincides with the finding in this study. In catfish, the changes in gene

expression of *hsd17b12* have been observed to be correlated with serum T levels during the reproductive cycle (Rajakumar et al., 2014). These findings suggested that Hsd17b12 is involved in production of C17-reduced androgens in teleost. The abundances of *hsd20b2* transcript decreased slightly by treatment of Gths in eel testis, which indicated that regulation of this gene is not consistent with that of the 17KSR activity in the Japanese eel testis. In masu salmon, a previous report demonstrated that Hsd20b2 plays an important role in synthesis of the maturation-inducing steroid, 17α, 20β-dihydroxy-4-pregnene-3-one, in the ovary, and that the expression was induced by Gth (Ijiri et al., 2017). Gene regulation of *hsd20b2* may vary in species-specific and/or tissue-specific manner although ovarian gene regulation of *hsd20b2* remains to be elucidated in Japanese eel. Taken together, only Hsd17b12a among the three Hsds would be one of the enzymes responsible for 17KSR activity involved in 11KT synthesis in immature Japanese eel testes.

In summary, the present study showed that the steroidogenic enzyme responsible for 17KSR activity, preferring 11KA4 as substrate in Japanese eel testis, was crucial for 11KT production. Subsequently, the cDNAs encoding Hsd17b3, Hsd17b12a, and Hsd20b2, as the candidate genes exhibiting the 17KSR activity, were cloned from Japanese eel and characterized using transient expression system of mammalian and piscine cells. The Hsd17b3, Hsd17b12a, and Hsd20b2 expressed either in HEK293T or in Hepa-E1 possessed 17KSR activity responsible for 11KT synthesis. Among these enzymes, only *hsd17b12a* mRNA was upregulated in the testis by Gths as well as the 17KSR activity forming 11KT from 11KA4. Thus, Hsd17b12a represents the steroidogenic enzyme mediating the final step in the testicular biosynthesis of 11KT in Japanese eel.

Chapter 4

Japanese eel retinol dehydrogenases 11/12-like are 17-ketosteroid reductases involved in sex steroid synthesis

1. Introduction

Androgens play important roles in the regulation of a variety of physiological processes, including gametogenesis in vertebrates (Hanukoglu, 1992; Tokarz et al., 2015). In particular, 11-ketotestosterone (11KT) has been recognized in teleosts as the most potent androgen (Borg, 1994; Kazeto et al., 2011; Tokarz et al., 2015). Recent studies in humans have also provided evidence for the production of 11KT in the adrenal gland and gonads (Imamichi et al., 2016; Pretorius et al., 2017). In male fish, 11KT controls sex determination, secondary sexual characteristics, and spermatogenesis (Borg, 1994). The biosynthesis of 11KT from cholesterol involves a complex cascade of several steroidogenic enzymes, which are controlled by the gonadotropins (Gths), follicle-stimulating hormone (Fsh), and luteinizing hormone (Lh) (Payne et al., 1995). Among these enzymes, 17β-hydroxysteroid dehydrogenases (Hsd17bs) are important enzymes for 11KT synthesis (de Waal et al., 2008; Pretorius et al., 2017).

Multiple types of Hsd17bs, which belong to short-chain dehydrogenase/reductase (SDR) or aldo-keto reductase families, are oxidoreductive steroidogenic enzymes that catalyze the oxidation of 17β-hydroxysteroids and the reduction of 17-ketosteroids (Luu-The et al., 2008; Peltoketo et al., 1999; Prehn et al., 2009). It is well known that the sequence identities between these enzymes are considerably low, and outside the SDR family-conserved regions similarity might be as low as 20% (Mindnich et al., 2004b). In addition, several types of Hsd17bs have been known as multifunctional proteins, which possess retinol dehydrogenase (RDH) activity, 3-ketoacyl-CoA reductase activity, 3-keto-reductase activity, and 17β-hydroxysteroid dehydrogenase/17-ketosteroid reductase activity (17HSD/17KSR activity) (Luu-The, 2001; Mindnich and Adamski, 2009). The

substrate specificity and oxidoreductive activity of the Hsd17bs are highly dependent on the type of enzyme, particularly Hsd17bs with 17KSR activity are essential for the biosynthesis of sex steroid hormones in gonads and other tissues in mammals, as well as in teleost (Labrie et al., 1997). In humans, HSD17B type 3 (HSD17B3) and type 5 (HSD17B5) are key enzymes catalyzing the synthesis of testosterone (T) as a precursor of dihydrotestosterone (DHT: a potent androgen in mammals) (Luu-The, 2013). In teleosts, Hsd17b3 catalyze the transformation of androstenedione (A4) and 11-ketoandrostenedione (11KA4) into T and 11KT, respectively (Mindnich and Adamski, 2009). The previous chapter (chapter 3) demonstrated that Hsd17b12 converted A4 to T and 11KA4 to 11KT.

A decade ago, a novel tentative HSD17B, HSD17B15, was first described in humans in a review (Luu-The et al., 2008), showing that the tentative HSD17B15 catalyzed the transformation of C17-reduced androgens, such as T, DHT, and androstanediol. However, the details, including the methods for the analysis, the nucleotide/protein sequences, and the gene expression levels, were not described. Since then, HSD17B15 was described as a prostate short-chain dehydrogenase reductase 1 (PSDR1) (Bertin et al., 2014). Kedishvili et al. (2002) reported that PSDR1 expressed in the *Spodoptera frugiperda*-derived cell line exhibited retinal reductase activity. Therefore, PSDR1 is also known as retinal reductase 1 (RalR1) or retinol dehydrogenase 11 (RDH11) (Lhor and Salesse, 2014). Thus, HSD17B15, PSDR1, and RDH11 are synonyms.

RDH11 has been reported to be involved in the regulation of retinoid metabolism/homeostasis in prostate and eye in humans (Kedishvili et al., 2002; Lhor and Salesse et al., 2014). It is also known as a causative gene for the eye disease retinitis pigmentosa (Xie et al., 2014). In mouse, Rdh11 is essential for retinol homeostasis in the liver and testis and is involved in dark adaptation (Belyaeva et al., 2018; Kasus-Jacobi et

al., 2005). Moreover, dark adaptation of *rdh11* knockout mice showed to delay compared with that of wild-type mice, which were analyzed by electroretinography (Kasus-Jacobi et al., 2005). However, it remains to be elucidated whether the murine enzyme possesses 17KSR activity for androgen synthesis. Thus, a detailed assessment of Rdhs and the 17KSR activity they might possess in vertebrates has been lacking.

In the present study, to reveal whether Japanese eel Rdh11 belongs to a group of Hsd17bs and is related with the 11KT synthesis, three cDNA encoding candidate Rdh11s with 17KSR activity involved in the synthesis of sex steroid hormones were isolated and characterized. In addition, gene expression analyses were conducted using quantitative real-time PCR (qPCR).

2. Materials and Methods

2.1 Animals

Male cultivated Japanese eels (400–600 g body weight) raised from glass eels were kept in a flow-through freshwater tank at 15 °C. Prior to the experiments, immature eels were acclimated to seawater (20 °C) and used in the present study. Various tissues (pituitary, forebrain, midbrain, hindbrain, eye, gill, spleen, liver, head kidney, posterior kidney, heart, intestine, pancreas, muscle, skin, and testis) were collected for RNA extraction and testicular culture. Before the collection of tissues, eels were anesthetized with 0.05 % 2-phenoxyethanol (Wako, Osaka, Japan) to limit distress and to sacrifice.

2.2 RNA preparation and cDNA synthesis

Total RNA was extracted from various tissues by the guanidium thiocyanate-phenol-chloroform extraction method using ISOGEN (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. The cDNA was synthesized from total RNA after priming with random hexamers using the Omniscript Reverse Transcription Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

2.3 Isolation of cDNAs

Primer pairs were designed based on three partial sequences obtained from a transcriptome database of Japanese eel (http://molas.iis.sinica.edu.tw/jpeel/) by interrogating the database using the coding region of human *rdh11* cDNA (GenBank accession numbers NP_016026.4). The 5'- and 3' ends of three cDNAs were amplified by RACE using testicular cDNA as template with the SMARTer^{TM} RACE cDNA Amplification Kit

(Takara, Shiga, Japan) and specific primers (table 4), according to manufacturer's instructions. The resultant PCR product was subcloned into a pGEM-T Easy vector (Promega, Madison, WI) and sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit on 3130XL DNA sequencer (Applied Biosystems, Carlsbad, CA). The fragments containing the open reading frame (ORF) were amplified to confirm the validity of the nucleotide sequence using specific primers (table 4) and Prime STAR HS DNA Polymerase (Takara) with proofreading ability and bi-directionally sequenced after subcloning into the vector as described above.

2.4 Sequence alignments and phylogenetic analysis

The homology search of the deduced amino acid sequences of the isolated cDNAs was carried out using BLAST in the NCBI (Altschul et al., 1990). Alignments of deduced amino acid sequences were performed using Clustal W (Thompson et al., 2002). The phylogenetic analyses were conducted with MEGA version X (Kumar et al., 2018) using the neighbor-joining method and method of maximum likelihood and 1000 bootstrap replicates. The GenBank accession numbers of Rdh sequences used in this study are as follows: RDH11-human, NP_057110.3; Rdh11-mouse, NP_067532.2; Rdh11-mummichog, XP_012727744.1; Rdh11-channel catfish, XP_017319503.1; Rdh11 isoform X1-European eel, XP_035270419.1; Rdh11 isoform X2-European eel, XP_035270421.1; RDH12-human, NP_689656.2; Rdh12-mouse, NP_084293.1; Rdh12-tilapia, XP_003445520.1; Rdh12-zebrafish, NP_001002325.1; Rdh12-European eel, XP_035270418.1, XP_035251138, XP_035251898, XP_035248107; Rdh12 like-European eel, XP_035283990.1; RDH13-human, NP_001139443.1; Rdh13-mouse, NP_780581.1; RDH14-human, NP_065956.1; Rdh14-mouse, NP_076186.1; RDH5 (HSD17B9) -human,

NP_001186700.1; HSD17B6-human, NP_003716.2; Hsd17b6-mouse, NP_001346306.1.

2.5 Functional assay of recombinant proteins

cDNAs were ligated into a eukaryotic expression vector, pCAGGS (Tokui et al., 1997), using an In-fusion® HD Cloning Kit (Takara), according to the manufacturer's protocol. Transient expression of the protein was performed using human embryonic kidney 293 T (HEK293T) cells as described in chapter 2. Briefly, HEK293T cells were seeded into six-well plates and transfected using X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions.

Estrone (E1), A4 and 11KA4 treatments were applied in cell culture (100ng/mL) for 24 h, after which the media were collected and kept at -30 °C until use.

For substrate specificity analysis, transfected HEK293T cells were incubated with $5x10^5$ cpm [1,2,6,7-3H(N)]-A4 (Perkin-Elmer, Waltham, MA), [1,2,6,7-3H(N)]-T (Perkin-Elmer), [2,4,6,7-3H(N)]- E1 (Perkin-Elmer), or [2,4,6,7-3H(N)]-estradiol-17β (E2) (Perkin-Elmer) at a final concentration of 100 nM. After 24 h of incubation, the media were collected and stored at -30 °C.

The HEK293T cell line was obtained from the Institute of Physical and Chemical Research (RIKEN) cell bank (Tsukuba, Japan).

2.6 TR-FIA

The concentrations of 11KT, T, and E2 in the media were determined by TR-FIA as described in chapter 2. Briefly, steroids extracted with diethyl ether or serially diluted 11KT, T, and E2 (0.0039–8 ng/mL) as standards, and antiserum against 11KT (Cosmo Bio, Tokyo, Japan), T (Cosmo Bio), and E2 (Cosmo Bio) were added to 96-well plates coated

with bovine serum albumin-conjugated 11KT, T, and E2. After incubation and washes, each well was incubated with europium (Eu)-labeled goat anti-rabbit IgG (Perkin-Elmer), followed by the addition of enhancement solution (Perkin-Elmer). The fluorescence intensity from dissociated Eu was measured using an Infinite F200 fluorescence microplate reader (Tecan, Grodig, Austria).

2.7 TLC

Identification and detection of radioactive steroid metabolites were performed as described previously (in chapter 3) with minor modifications. In brief, steroid metabolites were extracted with dichloromethane after the following non-radioactive carrier steroids were added as standard: A4 (Sigma, Poole, UK), T (Sigma), E1 (Sigma), and E2 (Sigma). Subsequently, the resultant steroid metabolites were subjected to TLC in a benzene-acetone (4:1 v/v) system. Substrates and metabolites were identified by the comparison of mobility with standard steroids. Standard steroids were observed under UV_{254} or detected by baking the TLC plate after it was sprayed with ethanol-sulfuric acid (4:1, v/v). Radioactive spots on chromatograms were revealed by autoradiography using an X-ray film (Eastman Kodak, Rochester, NY) and quantified using ImageJ (http://rsbweb.nih.gov/ij/).

2.8 Isolation of Leydig cells, Sertoli cells, and germ cells

Fractions including enriched Leydig cells, Sertoli cells, and germ cells were prepared according to the method of chapter 2. In brief, the testicular fragments were digested using collagenase (Gibco, Gaithersburg, MD), dispase (Gibco), and TryPLE express (Gibco). The digested fragments were filtered successively through nylon meshes with pore sizes of 40 μm and 20 μm. After washing with Dulbecco's phosphate-buffered saline (DPBS), the

dispersed cells were placed in a Percoll gradient of 10, 30, 35, and 40 % and then centrifuged at 3000 rpm for 30 min. The formed cell layer was collected and washed with DPBS. The fraction including enriched Leydig cells and germ cells was obtained from the region between Percoll gradient layers 35-% and 40-%. The cells obtained were harvested in 0.2 % gelatin-coated dishes containing DMEM/F-12 GlutaMAX™ (Gibco) with 50 mM NaCl, antibiotics, and 10 % KnockOut Serum Replacement (Gibco). After 1 d, non-adhering germ cells were collected and transferred to newly prepared 0.2 % gelatin-coated dishes. After incubation for a further day, attached cells (i.e., Leydig cells) and suspended cells (i.e., germ cells) were collected and kept at -80 ºC until total RNA isolation and cDNA synthesis. Sertoli cell fractions that migrated to the interface between the 10 % and 30 % Percoll layers were harvested, transferred into 0.2 % gelatin-coated dishes, and cultured for 30 min. After discarding suspended cells, the adhering cells were cultured as described above. After 2 d, the adhering cells were collected and kept at -80 ºC until further processing.

2.9 Culture of testicular fragments

Japanese eel testicular fragments were distributed into wells of a 48-well plates (30 mg/well) containing 300 μL of L-15 medium (Gibco) supplemented with 0.5 % bovine serum albumin and 10 mM of HEPES buffering agent (pH 7.4) and incubated with different doses (0, 30, 100, 300, and 1000 ng/mL) of recombinant single-chain Fsh (reFsh), Lh (reLh) (Kazeto et al., 2019), or 11KT (10 and 100ng/mL; Sigma) for 72 h at 20 ℃. Vehicle (DPBS) only was used as the zero-dose control. This culture experiment was repeated four times using samples from different animals. After incubation, the testicular fragments were collected and kept at -80 ºC until further analysis.

2.10 qPCR

The expression of three mRNAs in various tissues, testicular cell fractions, and cultured testicular fragments was determined by qPCR using Gene Ace Probe qPCR Mix α Low ROX (Nippongene) on an Applied Biosystems 7500 Real-Time PCR System, according to the manufacturer's instructions. Specific primers and probes were designed based on previous literature (Matsubara et al., 2003) and Primer Express Software Ver. 3.0 (Applied Biosystems) (table 5). The detailed conditions and the validation of the qPCR assay were performed according to ABI PRISM Sequence User Guide and a previous report (Matsubara et al., 2003). Relative mRNA levels of the target mRNAs were normalized to those of β-actin (*actb*; Kazeto et al., 2008) or *rps15* levels as internal controls.

2.11 Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). The unpaired t-tests were carried out for data obtained from TR-FIA and TLC. Results of localization and expression change of Japanese eel rdh11/12-like mRNAs in the testis were analyzed by one-way-ANOVA followed by Tukey's and Dunnett's test. EZR version 1.41 (Kanda, 2013) was used for all statistical analyses.

Table 4. Sequence of primers used for cloning.

Target	Primer	Nucleotide sequence (5’ → 3’)
rdh11/12-like 1	RACE forward	CATCAGTAACGCTGGCGTGATG
	RACE reverse	CCAGGCCGCTGTTGTAGCTG
	ORF forward	GTTGCGAGGGTAAACCCTGCAG
	ORF reverse	CGTCTTGGCACATTTGGAATGGCTG
rdh11/12-like 2	RACE forward	CCCAGCCAGAATTGTCATCGTG
	RACE reverse	CAATACGCCTTGGCTGTGTCATAGC
	ORF forward	GAACTCAAGGAAGAAGGGACCTG
	ORF reverse	GCATTCATTCCCATGTAATTCCAAGCATC
rdh11/12-like 3	RACE forward	TGGGCCACTTCCTGCTTACTCAC
	RACE reverse	CCGTACGCCTTCTTGTCGCTG
	ORF forward	GCTAGGCACGAAAGCGTAGTCGTAG
	ORF reverse	GCAACTGAGGTCGCCTCTGCGACG

Table 5. Sequence of primers used for qPCR.

Target	Primer/Probe	Nucleotide sequence (5’ → 3’)	Reference
rdh11/12-like 1	forward	GCAGACACCAGCTCCATAC	
	probe	CCTCAGAGAGGTGAACCATCTGCAT	
	reverse	CAGGAAGTGACCCAGGTGAT	
rdh11/12-like 2	forward	AGTGAGAAGAGCTATGACACAG	
	reverse	CACAGATGTCTTGGTGAAAGGC	
rdh11/12-like 3	forward	GAGAAGGGGTACAGCGACAAG	
	reverse	CGGTCTTGGTGAAGGGCTTC	
actb	forward	ACGGGCAGGTCATCACCAT	Kazeto et al., 2012
	probe	CCTTCCTTCCTGGGTATGGAGTCCTGC	
	reverse	AGTTGAAGGTGGTCTCGTGGATT	
rps15	forward	ACCAGGTGGAAATCAAGCC	Suzuki et al., 2020a
	reverse	GGAATGAAGCGGGAAGAATG	

3. Results

3.1 Characterization and phylogeny of Japanese eel Rdh11 related sequences

Three cDNAs containing Rdh11-related ORFs were obtained from Japanese eel testis. The BLAST searches using the deduced amino acid sequences indicated that one sequence had high scores with both Rdh11s and Rdh12s of teleost fish, while two other sequences showed high scores with only Rdh12s of teleost. Comparison of the amino acid sequences of the putative Japanese eel Rdh sequences, putative European eel Rdh11s and 12s, and human RDH11 and RDH12, indicated that sequences from the two eels were almost identical (97-99%), and 54-65% identical to human Rdh11 and Rdh12 (Table 6). Therefore, none of the three Japanese eel gene candidates for Rdh11 could be identified as Rdh11 or Rdh12, and, therefore, they were designated as Rdh11/12-like: Rdh11/12-like 1 (LC 536524), Rdh11/12-like 2 (LC 589956), and Rdh11/12-like 3 (LC 589957).

The deduced amino acid sequences (Fig. 18) of all three proteins (Rdh11/12-like 1–3) included conserved signatures of the SDR family, such as cofactor binding (TGXXXGXG), the catalytic mechanism (YXXXK), and the structural integrity (NVG or NAG) patterns, similar to those found in human RDH11 and RDH12. The nucleotide sequence of *rdh11/12-like* 1 is composed of a 5′-untranslated region (UTR) of 2 bp, a 3′-UTR of 45 bp, and an ORF of 951 bp encoding a protein of 317 amino acids. The nucleotide sequence of *rdh11/12-like* 2 consists of a 5′-untranslated region (UTR) of 99 bp, a 3′-UTR of 14 bp, and an ORF of 894 bp encoding a protein of 298 amino acids; that of *rdh11/12-like* 3 is composed of a 5′-untranslated region (UTR) of 59 bp, a 3′-UTR of 31 bp, and an ORF of 894 bp encoding a protein of 298 amino acids. The amino acid sequence of Japanese eel Rdh11/12-like 1 showed the highest similarity to piscine Rdh11 and Rdh12

(82–84%), followed by mammalian Rdh11 and Rdh12 (61–66%), and mammalian Rdh13 and Rdh14 (52–54 %). The Japanese eel Rdh11/12-like 2 and Rdh11/12-like 3 sequences have higher homology with Rdh11 and Rdh12 of teleosts and mammals (54–60%) compared to those with Rdh13 and Rdh14 (47–53%).

In the phylogenetic analyses using neighbor-joining and maximum likelihood methods (Fig. 19), human RDH11 and RDH12 clustered with the corresponding mouse Rdhs, while piscine Rdh11s and Rdh12s did not form clades with the corresponding Rdhs. Japanese eel Rdh11/12-like 1 clustered with piscine Rdh11 and Rdh12, however the cluster is formed outside that of mammalian Rdh11 and Rdh12. In contrast, Rdh11/12-like 2 and Rdh11/12-like 3 formed a clade with putative European eel Rdh11s and Rdh12s outside that of mammalian and piscine Rdh11/Rdh12.

Table 6. Amino acid sequence percentage identity comparison of Japanese eel Rdh11/12-like proteins, European eel Rdh11s and Rdh12s, and human Rdh11 and Rdh12.

			Japanese eel			European eel							human	
			Rdh11/12-like 1	Rdh11/12-like 2	Rdh11/12-like 3	Rdh11_isoform_X1	Rdh11_isoform_X2	Rdh12	Rdh12	Rdh12	Rdh12	Rdh12like	RDH11	RDH12
		accession number				XP_035270419.1	XP_035270421.1	XP_035251138.1	XP_035270418.1	XP_035251898.1	XP_035248107.1	XP_035283990.1	NP_057110.3	NP_689656.2
Japanese eel	Rdh11/12-like 1		100											
	Rdh11/12-like 2		60	100										
	Rdh11/12-like 3		55	77	100									
European eel	Rdh11_isoform_X1	XP_035270419.1	52	59	57	100								
	Rdh11_isoform_X2	XP_035270421.1	53	61	57	100	100							
	Rdh12	XP_035251138.1	99	59	55	52	52	100						
	Rdh12	XP_035270418.1	60	99	77	59	61	60	100					
	Rdh12	XP_035251898.1	53	58	58	51	51	53	58	100				
	Rdh12	XP_035248107.1	48	48	46	45	47	48	48	53	100			
	Rdh12like	XP_035283990.1	56	77	97	57	57	56	77	58	46	100		
human	RDH11	NP_057110.3	65	56	54	55	55	65	55	51	47	55	100	
	RDH12	NP_689656.2	66	59	59	55	55	65	59	50	50	58	73	100

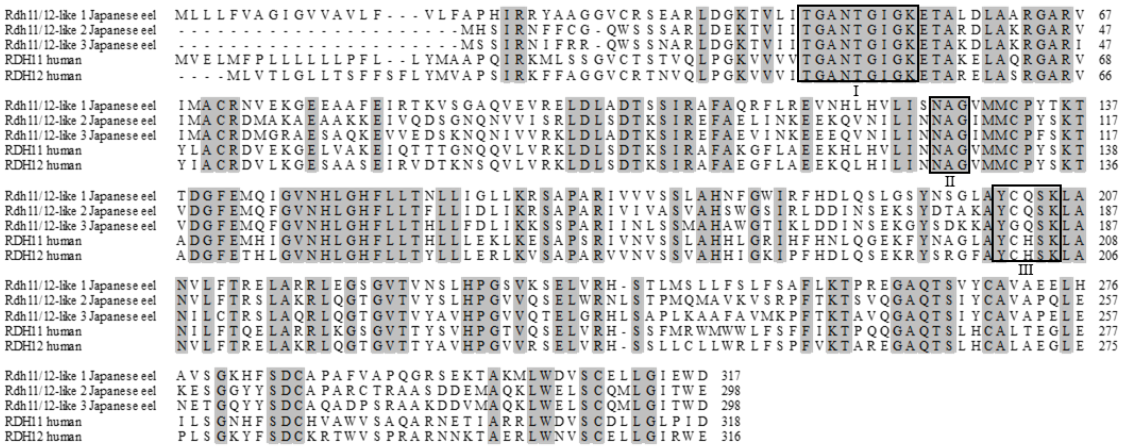

Figure 18. Alignment of the deduced amino acid sequences of three Japanese eel Rdh11/12-like genes, human RDH11 (NP_057110.3), and RDH12 (NP_689656.2). I: cofactor-binding site; II: structural integrity site; III: catalytic mechanism site.

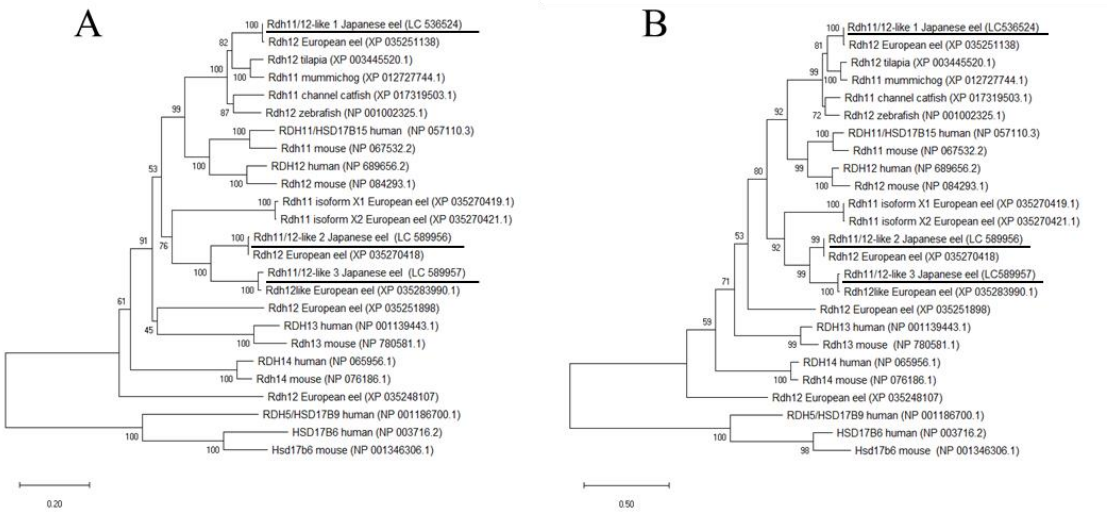

Figure 19. Phylogenetic analysis of Rdh proteins. The analysis was performed using Clustal W by the neighbor-joining (A) and Maximum likelihood (B) method. Numbers beside the branches indicate percentage of bootstrap values from 1000 replicates.

3.2 17-ketosteroid reductase activity of Japanese eel Rdh11/12-like 1–3

Functional assays for reductase activity of Rdh11/12-like 1–3 against 11KA4, A4, and E1 were performed using the recombinant proteins expressed in HEK293T cells (Fig. 20). HEK293T cells expressing Rdh11/12-like 1 more efficiently converted 11KA4 to 11KT while HEK293T cells transfected with an empty vector (mock control) also showed low enzymatic activity. No significant was detected for recombinant Rdh11/12-like 2 or Rdh11/12-like 3. In contrast, all three recombinant Rdh11/12-like proteins exhibited significant catalytic activity in transformation of T from A4, and E2 from E1, compared with controls.

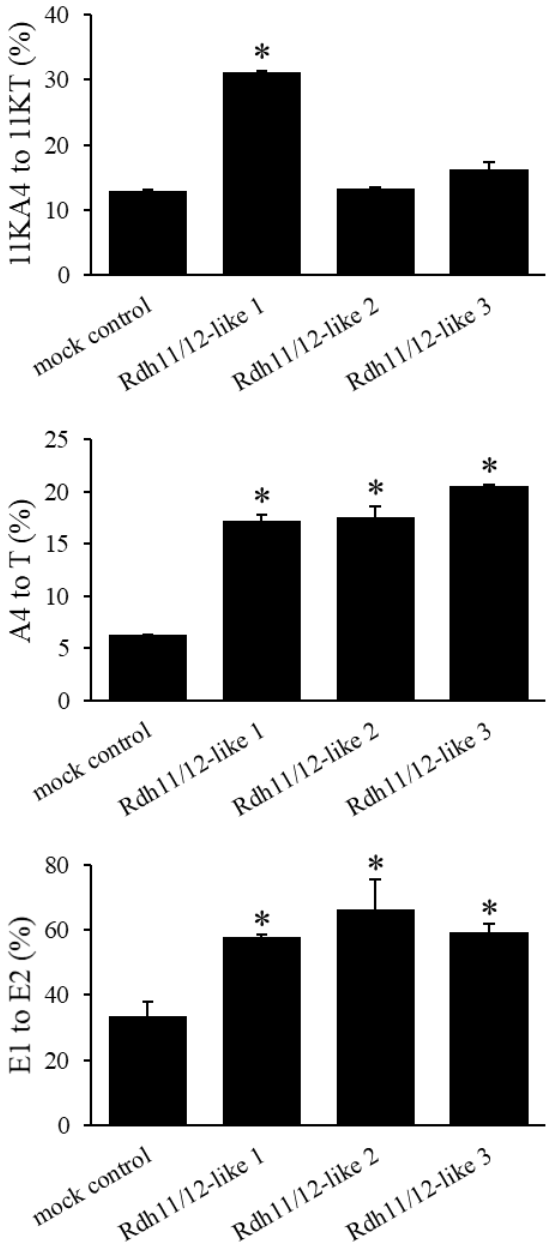

Figure 20. Enzymatic activity of HEK293T cells transfected with empty-pCAGGS (mock control), Rdh11/12-like 1-pCAGGS, Rdh11/12-like 2-pCAGGS, or Rdh11/12-like 3-pCAGGS. Conversion of 11KA4 to 11-KT, A4 to T, and E1 to E2 was examined by TR-FIA. Bars represent the mean ± SEM of triplicate measurements. Asterisks indicate significant differences from the mock control group ($P < 0.05$).

3.3 Substrate specificity of Japanese eel Rdh11/12-like 1

Substrate specificity of Rdh11/12-like 1 was analyzed using TLC. Catalysis of the conversion of A4 to T and E1 to E2 by recombinant Rdh11/12-like 1 was significantly higher than that in the mock controls. On the other hand, the Rdh11/12-like 1 expressed in HEK293T cells did not show significant activity in conversion of T to A4 or E2 to E1 compared to the mock control (Fig. 21).

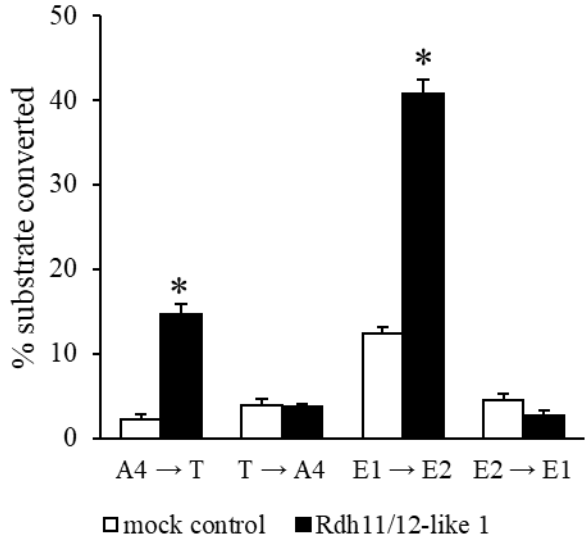

Figure 21. Substrate specificity of Rdh11/12-like 1 expressed in HEK293T. Conversion of A4 to T, T to A4, E1 to E2, and E2 to E1 was detected by thin-layer chromatography followed by autoradiography. Bars represent the mean ± SEM of triplicate measurements. Asterisks indicate significant differences from the mock control group ($P < 0.05$).

3.4 Distribution of Japanese eel rdh11/12-like 1–3 mRNA

The pituitary and parts of brain and testis were the predominant sources of *rdh11/12-like 1* transcript, while *rdh11/12-like 2* mRNA was mostly detected in pituitary, parts of the brain, eye, and testis (Fig. 22). Expression of *rdh11/12-like 3,* on the other hand, was ubiquitous across the tissues analyzed (Fig. 22).

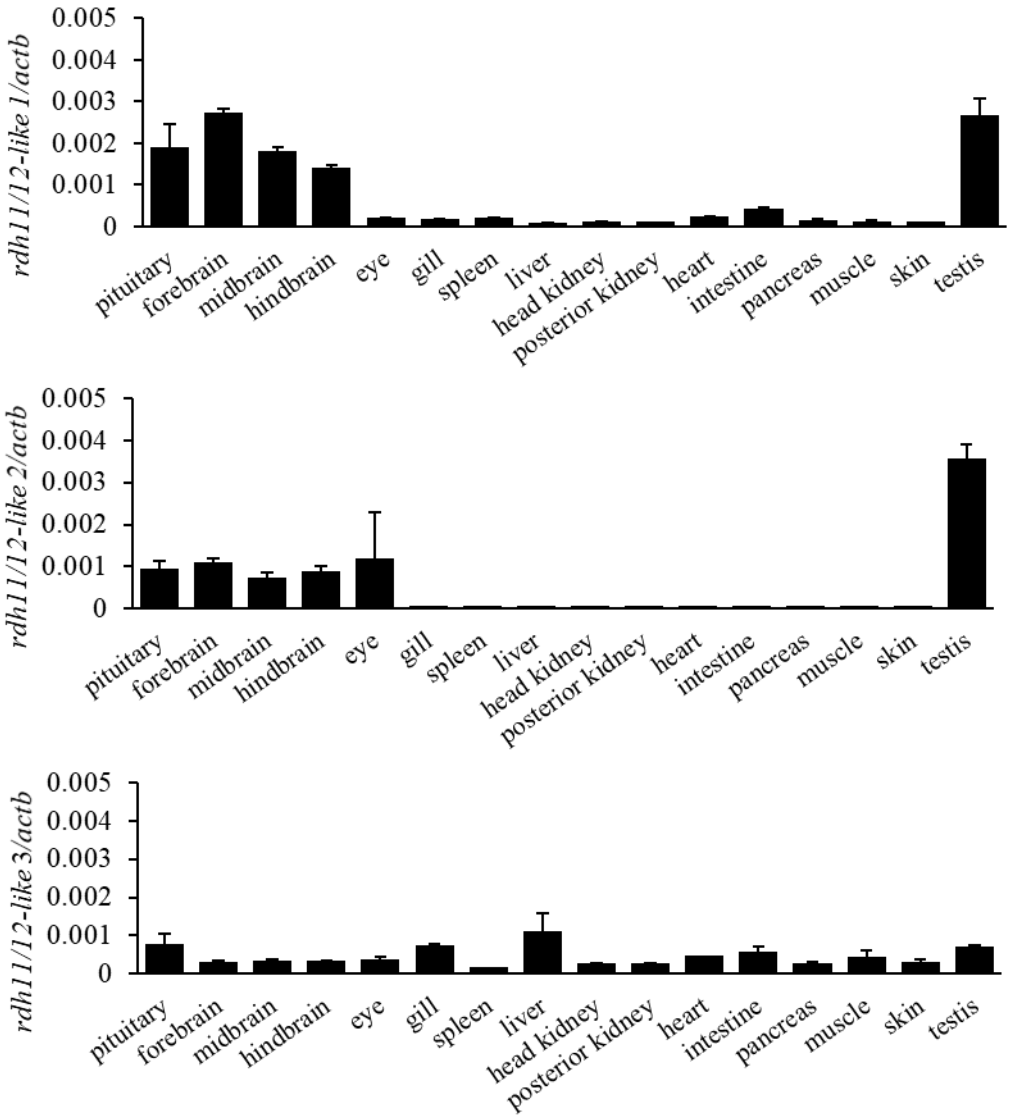

Figure 5. Tissue mRNA expression levels of Japanese eel *rdh11/12-like* genes as measured by qPCR. The transcript levels of *rdh11/12-like 1, rdh11/12-like 2, and rdh11/12-like 3* were determined by qPCR. Relative mRNA levels were normalized to those of *actb*. Various tissues were collected from four Japanese eels. Bars represent mean ± SEM of data obtained in four experiments using different Japanese eels.

3.5 Localization of the Japanese eel rdh11/12-likes mRNA in the testis

The cellular localization of *rdh11/12-like 1–3* mRNAs in the testis was analyzed by qPCR using three cell fractions including enriched Leydig cells, Sertoli cells, and germ cells. The *rdh11/12-like 1* and *rdh11/12-like 3* transcripts were detected in all three fractions with no significant differences in expression level among the different fractions (Fig. 23). In contrast, *rdh11/12-like 2* mRNA was detected only in the germ cell fraction (Fig. 23).

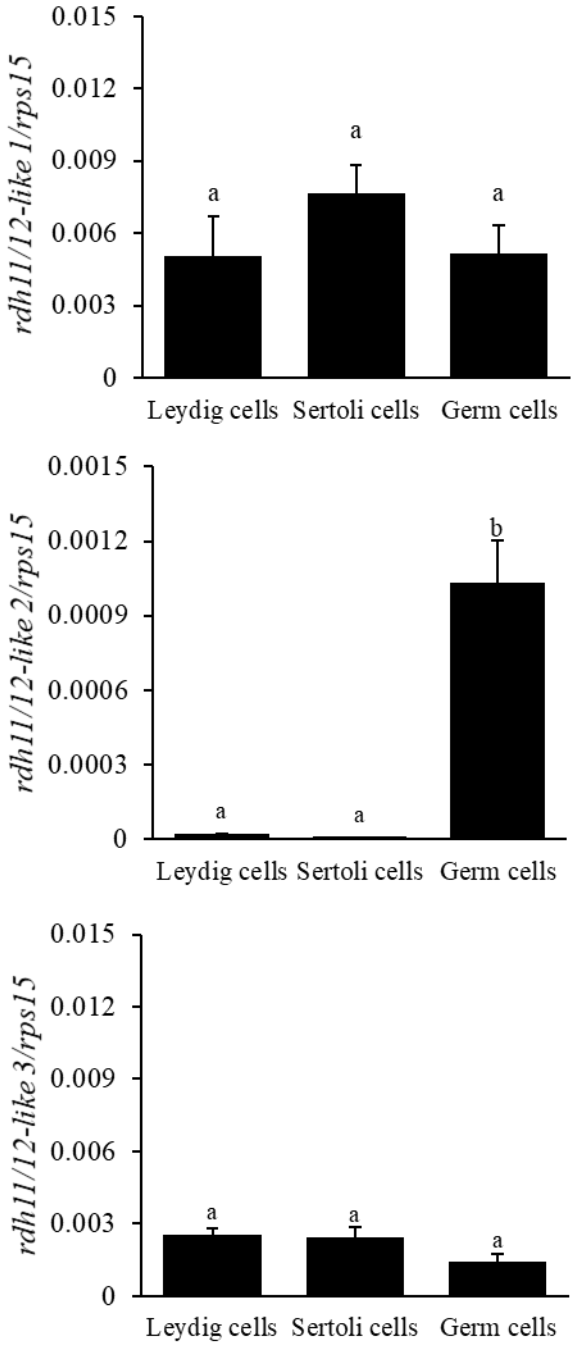

Figure 23. Expression patterns of *rdh11/12-like* genes mRNA in Japanese eel testes. The relative mRNA levels were normalized to those of *rps15*. The cell preparations were independently performed four times using testis tissues from different Japanese eel specimens. Bars represent mean ± SEM calculated from data obtained in four sets of experiments. Different letters indicate significant differences in each group ($P < 0.05$).

3.6 Effects of reFsh, reLh and 11KT on the expression of rdh11/12-like transcripts

Testicular fragments were cultured with various concentrations of reFsh, reLh, or 11KT to investigate the regulation of three *rdh11/12-like* genes, and the changes in the transcript abundance were examined by qPCR. There was no statistically significant effect of any of the treatments on abundance of any of the transcripts (Fig. 24).

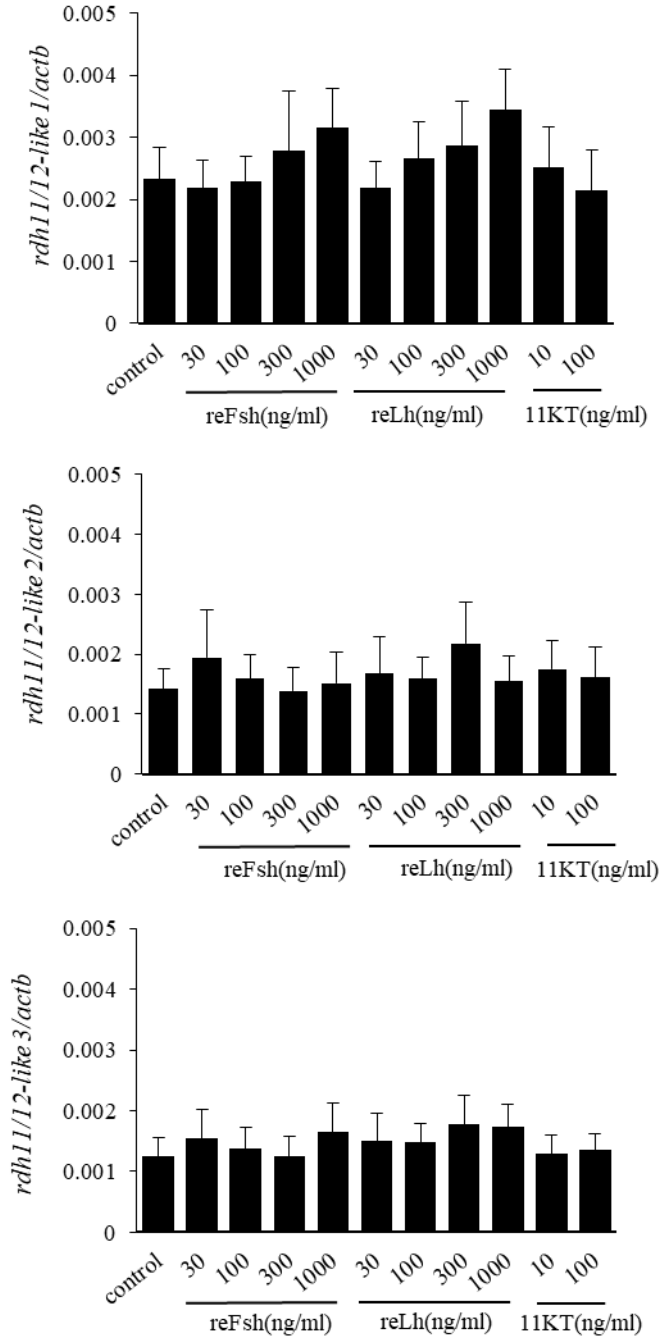

Figure 24. Effects of Gths and 11-KT on the expression of *rdh11/12-like1, rdh11/12-like 2, and rdh11/12-like 3* genes. The transcript levels of *rdh11/12-like* genes were determined by qPCR. Relative mRNA levels were normalized to *actb*. Bars represent mean ± SEM calculated from data obtained in four sets of experiments using different Japanese eels.

4. Discussion

In this study, cDNAs encoding three candidate Rdh11/12 proteins were isolated from Japanese eel testes using a PCR-based strategy. The deduced amino acid sequences included three conserved regions of the SDR family related to cofactor binding, catalytic activity, and structural integrity (Mindnich et al., 2004),which was confirmed by their capacity to reduce endogenous steroids such as 11KA4, androstenedione and estrone. The Japanese eel deduced Rdh proteins exhibited a high similarity to Rdh11s and Rdh12s from teleost fishes, and among human RDHs, RDH11 and RDH12 were the closest. A similar result was obtained in the comparison of tentative European eel Rdh11s and Rdh12s to human RDH11 and RDH12. In mammals, it is well known that Rdh11 and Rdh12 are closely related and share 73% identity (Albalat et al., 2011; Lhor and Salesse et al., 2014). In two phylogenetic analyses, each human RDH protein clusters together with the corresponding mouse Rdh protein. However, putative teleost Rdh proteins from different species not always fall in the mammalian clades. Further, the candidate of the Japanese eel showed a closer relationship to mammalian Rdh11 and Rdh12 than to Rdh13 and Rdh14. For these reasons, we concluded that the candidates are either Rdh11 or Rdh12. However, the phylogenetic analyses could not unambiguously place the Japanese eel Rdh proteins into either Rdh11 or Rdh12, thus they were designated as Rdh11/12-like proteins (Rdh11/12-like 1, Rdh11/12-like 2, and Rdh11/12-like 3).

It is well known that teleost fishes have undergone extra genome duplication, unlike mammals (Meyer and Schartl, 1999). A number of Rdh11/12-like may be teleost fishes-specific. To reveal the evolutionary background of the rdh11 and rdh12 in teleosts, it would be necessary to isolate and characterize these genes in various fish species and analysis

whether some of teleost rdh sequences correspond to different genes or at least some are different transcripts from the same gene, utilizing genome information of various teleost fishes including Japanese eel.

In the present study, we performed phylogenetic analyses using teleost Rdh11 and Rdh12 sequences registered in the database. However, the sequences formed several clades with a mixture of Rdh11 and Rdh12 for both, which is probably due to the incorrect annotations.

All three Rdh11/12-like proteins were able to catalyze the conversion from A4 to T and E1 to E2; only Rdh11/12-like 1 could convert 11KA into 11KT. These results suggest that Rdh11/12-like 1–3 are functional enzymes with 17KSR activity and involved in sex steroid hormone production and belong to a group of Hsd17bs, unlike the findings of Kedishvili et al. (2002) suggesting human RDH11 does not play roles on steroid metabolism. This may be due to the difference in species studied and/or in experimental design, including the type of host cells used for expression of recombinant proteins and sample preparation for enzymatic assays. However, the most likely reason is the differences in steroids used in functional assays. DHT, androsterone, dehydroepiandrosterone, and 5a-androstanediol were employed as steroid substrates in functional assays involving human RDH11 (Kedishvili et al. 2002), while A4, T, E1, E2, and 11KA4 were used in the present study. However, we did not profess that Japanese eel Rdh11/12-like proteins and human RDH11 were orthologues. Therefore, Japanese Rdh11/12-like proteins may be teleost fishes-specific proteins with the 17KSR activity.

Further investigation of the substrate specificity of Rdh11/12-like 1 was conducted because only this protein, among the three, catalyzed the formation of 11KT from 11KA4, suggesting that Rdh11/12-like 1 may be more important for testicular function. Rdh11/12-

like 1 preferably catalyzed the conversion from A4 to T and E1 to E2, suggesting that Rdh11/12-like 1 plays a role in reduction, but not oxidation, of androgens and estrogens. To the best of our knowledge, this is the first report, demonstrating that genes closely related to Rdh11/12 are involved in the synthesis of C17-reduced androgen and estrogen. A previous study in mice reported that Hsd17b12 efficiently catalyzed the conversion of E1 to E2 as well as A4 to T (Blanchard et al., 2007). Conversely, human and monkey Hsd17b12s have been shown to transform selectively E1 into E2 (Liu et al., 2007; Luu-The et al., 2006). Moreover, human Hsd17b3, but not zebrafish Hsd17b3, has been reported to catalyze the transformation androstanedione and androsterone into dihydrotestosterone and androstanediol, respectively (Mindnich et al., 2005). The data indicated that the catalytic properties of Hsd17bs, including Rdh11/12-likes, varies in the species, which suggests that the production of androgens and/or estrogens by Rdh11/12-likes may be species-specific.

The expressions of *rdh11/12-like 1* and *rdh11/12-like 2* transcripts was observed predominantly in testis, while gene expression of *rdh11/12-like 3* was ubiquitous. Moreover, the transcripts of *rdh11/12-like 1 and rdh11/12-like 3* were detected in Leydig, Sertoli, and germ cells, while *rdh11/12-like 2* was specifically expressed in the germ cells. It is well established that Leydig cells express several steroidogenic enzymes that catalyze the transformation of cholesterol into sex steroid hormones (Payne and Youngblood, 1995). These findings support the concept that Rdh11/12-like proteins, especially Rdh11/12-like 1 and Rdh11/12-like 3, participate in the production of sex steroid hormones in testis. In humans, *psdr1* (*rdh11)* is more highly expressed in prostate (Lin et al., 2001). Additionally, mouse *psdr1* (*rdh11*) shows the highest expression levels in testis (Moore et al., 2002). Hence, expression of *rdh11*-related genes in tissues involved in the production of potent androgens seems to be conserved across vertebrates.

In males of teleost fishes, E2 plays an important role in early spermatogenesis. For example, in Japanese eels, *in vivo* and *in vitro* experiments have revealed that E2 induces the renewal of spermatogonial stem cells (Miura et al., 1999). Additionally, in three-spot wrasse (*Halichoeres trimaculatus*) and in three-spot wrasse (Halichoeres trimaculatus), intraperitoneal injection of E2 promoted the renewal and proliferation of spermatogonia in the testis (Kobayashi et al., 2011). Therefore, Rdh11/12-like proteins could be involved in E2 synthesis during early spermatogenesis because they show 17KSR activity and are expressed in immature testes.

In addition, the *rdh11/12-like* genes mRNAs were also expressed in the pituitary, forebrain, midbrain, and hindbrain, as well as testis. Previous *in vitro* studies demonstrated that 17KSR activity was detected in several tissues of teleosts (Cavaco et al., 1997; Schulz et al., 1991), which suggests that the Japanese eel Rdh11/12-like proteins might be involved in the production of androgen and estrogen in extragonadal tissues. In particular, it is known that steroids locally-synthesized in brain, called neurosteroids, play important roles in the central nervous system, such as neuronal excitability and function, brain plasticity, and behavior (Diotel et al., 2018). In adult zebrafish, in vitro incubation of brain indicated that DHT and E2 were synthesized from pregnenolone, indicating the brain has several steroidogenic enzymes, such as cytochrome P450 side-chain cleavage enzyme, 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase, cytochrome P450 17α-hydroxylase/C17-21 lyase, Hsd17bs, 5α-reductase, and aromatase (Diotel et al., 2011). Lorenzi et al. (2012) indicated that 11KT and T amount are higher in brain than in muscle and gonad, suggesting that the brain is a tissue involved in androgens production. Moreover, brain homogenates converted 11KA4 to 11KT (Schulz and Blüm, 1991). Hence, the Japanese eel Rdh11/12-likes may be involved in neurosteroidogenesis in the brain.

RDH11 is known to possess retinaldehyde reductase activity responsible for retinoid metabolism (Albalat et al., 2011; Belyaeva et al., 2018; Kedishvili et al., 2002). In general, retinoid metabolism plays important roles in fertility, embryogenesis, immunity, and vision (Lhor and Salesse et al., 2014). In particular, retinoic acid (RA) controls spermatogonial differentiation and meiosis and is synthesized from retinol by Rdh10 and retinaldehyde dehydrogenases (Busada and Geyer, 2016; Griswold, 2016). In the testes of mice, Rdh10 and retinaldehyde dehydrogenases are observed in both Sertoli cells and germ cells (Arnold et al., 2015; Tong et al., 2013). In the present study, Sertoli cells fraction expressed *rdh11/12-like 1* and *rdh11/12-like 3,* while germ cells fraction expressed three *rdh11/12-like* genes. Hence, the Japanese eel Rdh11/12-likes might associate homeostasis of the retinoid in Sertoli cells and germ cells. Moreover, previous reports indicated that RA signaling is necessary during the development of brain in embryonic stage (Grandel et al., 2002; Samarut et al., 2015). Since, *rdh11/12-like* transcripts were detected in the brain, they could be involved in the modulation of RA level in the brain of Japanese eel. For a deeper understanding the roles of Rdh11/12-likes as retinol dehydrogenase, further experiments are required to investigate the gene expression during embryogenesis, the enzymatic activity of the eel Rdh11/12-likes against retinal and conduct kinetic analysis using several substrates, such as steroids and retinoids.

The present study was analyzed the regulation of transcription of *rdh11/12-like 1*, *rdh11/12-like 2*, and *rdh11/12-like 3* genes in Japanese eel testes by reFsh, reLh, and 11KT. The results of qPCR demonstrated that the expression levels of *rdh11/12-like 1*, *rdh11/12-like 2*, and *rdh11/12-like 3* did not change by treatment of reFsh, reLh, and 11KT. In the testes of juvenile rhesus monkeys, *rdh11* mRNA expression was not significantly changed by administration of Fsh, Lh, or a combination of both (Nourashrafeddin and Hosseini

Rashidi, 2018). Therefore, gonadotropins might not regulate the expression of *rdh11* and *rdh11/12-likes* in vertebrates. In mammals, *rdh11* was initially identified as a gene regulated by androgen in the prostate, named *psdr1* (Lin et al., 2001). The expression of human *psdr1* in LNCaP cells was upregulated by androgen (Lin et al., 2001). Moore et al. (2002) demonstrated that the *psdr1* expression level in the prostate was lower in castrated mice than in control mice, suggesting that the gene was regulated by androgen in the mouse prostate. However, in our in vitro study, 11KT did not affect testicular *rdh11/12-like* expression.

As shown in chapter 2, in male Japanese eels, it was demonstrated that the enhancement of 11KT synthesis by Gths was mediated by the increased expression of several steroidogenic enzymes (Suzuki et al., 2020b). In addition, Suzuki et al. (2020a) reported 17KSR activity responsible for conversion of 11KA4 to 11KT and expression of hsd17b12a (encoding an enzyme catalyzing the transformation of 11KA4 into 11KT) were both upregulated by Gths in Japanese eel testis, unlike our lack of Gth effects on rdh11/12-like expression. This suggests that Rdh11/12-like 1 could be involved in maintaining a basal-level of 11KT production in Japanese eel immature testis but does not function in Gths-induced 11KT production.

In summary, three cDNAs encoding Rdh11/12-like proteins were cloned from Japanese eel and characterized. The Japanese eel Rdh11/12-likes expressed in HEK293T cells possessed 17KSR activity involved in androgen and estrogen production. Moreover, only Japanese eel Rdh11/12-like 1 catalyzed the conversion of 11KT from 11KA4. Expression analyses of the rdh11/12-like genes by qPCR revealed that the transcripts were detected predominantly in the testis, although no significant expression changes by Gths or 11KT was detected. These findings suggest that Japanese eel Rdh11/12-like proteins are

involved in sex steroid hormone synthesis in Japanese eel testes.

Chapter 5

Summary and conclusion

Testicular steroid hormones play important roles in spermatogenesis. In particular, 11KT is identified as one of the major inducers of spermatogenesis in teleost and synthesized by successive enzymatic reaction of steroidogenic enzymes (Fig. 25). It is known that the 11KT synthesis is regulated by Gths, but the physiological roles of Fsh and Lh remain to be elucidated in many fish species because it is difficult to purify Gths from pituitaries. Recently, advancements in biotechnology have enabled the production of bioactive recombinant Gths in teleost. In Japanese eel, Kazeto et al (2019) established FreeStyle 293-F cell lines producing reFsh and reLh. However, effects of reFsh and reLh on the production of 11KT in male Japanese eel have not yet been clarified. Therefore, the functional analyses of reGths were conducted focusing on the binding specificities to their receptors and effects on testicular steroidogenesis *in vitro*.

Assays with gonadotropin receptors-expressing COS-7 cells indicated reFsh stimulated its cognate receptor, meanwhile reLh activated both receptors (Fig. 25). Although results of *in vitro* incubations showed that reFsh and reLh induced testicular 11KT production in a dose and time-dependent manner by upregulating expression of steroidogenic enzymes, the effective doses of reLh were apparently lower and the effects of reLh emerged faster in comparison with reFsh. Results of quantitative real-time PCR using testicular cell fractions showed that *fshr* and *lhcgr1* mRNA were detected in both Sertoli and Leydig cells. These analyses revealed that reFsh and reLh were biologically active and hence will be useful for future studies. Moreover, the data showed that both eel Fsh and Lh acted as steroidogenic hormones through their receptors in testicular somatic cells; however, Lh was more potent on androgen production, implying their differential functions on spermatogenesis.

The expression of steroidogenic enzymes was regulated by the treatment of reGths

as described above. However, Hsd17b with 17KSR activity involved in the synthesis of 11KT has not yet been well clarified in Japanese eel. Therefore, for the deeper understanding of testicular 11KT biosynthesis regulated by Gths, the steroidogenic pathway of 11KT synthesis and Hsd17bs with the 17KSR activity were characterized.

In vitro incubation of the testis with androstenedione (A4) and the subsequent analysis of the metabolites by thin-layer chromatography indicated that 11KT was synthesized from A4 via 11β-hydroxyandrostenedione (11OHA4) and 11-ketoandrostenedione (11KA4), which indicated that the steroidogenic enzyme exhibiting the 17KSR activity responsible for converting 11KA4 to 11KT is crucial for 11KT production. Subsequently, cDNAs encoding three candidate enzymes, Hsd17b type3 (Hsd17b3), Hsd17b type12a (Hsd17b12a), and 20β-hydroxysteroid dehydrogenase type2 (Hsd20b2), potentially with the 17KSR activity were isolated and characterized in the Japanese eel. The isolated *hsd17b3*, *hsd17b12a*, and *hsd20b2* cDNAs putatively encoded 308, 314, and 327 amino acid residues with high homology to those of other vertebrate counterparts, respectively. The Hsd17b3, Hsd17b12a, and Hsd20b2 expressed either in HEK293T or in Hepa-E1 converted 11KA4 to 11KT. Tissue-distribution analysis by quantitative real time PCR revealed that *hsd17b12a* and *hsd20b2* mRNAs were detected in the testis, while *hsd17b3* mRNA was not detectable. Furthermore, the effects of Gths on the 17KSR activity and the expression of the candidate genes in the immature testis were examined. The 17KSR activity was upregulated by the administration of Gths. Furthermore, only expression of *hsd17b12a* among three candidates was upregulated by Gths as well as the 17KSR activity. These findings strongly suggested that Hsd17b12a is one of the enzymes with 17KSR activity responsible for 11KT synthesis in the testis of Japanese eel (Fig. 25).

A group of Hsd17bs, including Hsd17b12a, has been known as multifunctional proteins, which possess retinol dehydrogenase (RDH) activity, 3-ketoacyl-CoA reductase activity, 3-keto-reductase activity, and 17β-hydroxysteroid dehydrogenase/17-ketosteroid reductase activity (17HSD/17KSR activity). RDH11 has been suggested to be a novel tentative HSD17B (HSD17B15) in humans for a decade, however no definitive proof has been provided yet. Moreover, it has not been reported whether the protein is involved in the synthesis of 11KT in vertebrate including teleost. Hence to identify the new hsd17b with 17KSR activity involved in testicular 11KT synthesis, three genes as candidate Rdh11s were isolated and characterized.

Based on their structural features revealed by the analyses of sequence identity and a phylogenetic analysis, these isolated genes are found to be comparably related to human *rdh11* and *rdh12* genes. Therefore, these genes were designated as *rdh11/12-like 1*, *rdh11/12-like 2*, and *rdh11/12-like 3*. Three recombinant Rdh11/12-like proteins expressed in HEK293T cells catalyzed the transformation of estrone into E2 and androstenedione into testosterone. Only Rdh11/12-like 1 catalyzed the conversion of 11-ketoandrostenedione into 11KT. Tissue-distribution analysis by quantitative real-time PCR revealed, in immature male Japanese eel, that *rdh11/12-like 1* and r*dh11/12-like 2* are predominantly expressed in testis and brain, while *rdh11/12-like 3* is expressed ubiquitously. Moreover, the effects of Gths and 11KT on the expression of the three *rdh11/12-like* genes were analyzed in the immature testis. *In vitro* incubation of immature testes with various doses of recombinant Fsh, Lh, and 11KT indicated that the expression levels of *rdh11/12-like 1*, *rdh11/12-like 2,* and *rdh11/12-like 3* did not change. These findings suggest that the three Rdh11/12-like proteins play roles in sex steroid metabolism and belong to a group of Hsd17bs. In particular, Rdh11/12-like 1 may be one of the enzymes with 17KSR activity

involved in the production of 11KT in the testis (Fig. 25).

In conclusion, reGths were strongly suggested to be reliable hormones for the artificial induction of sexual maturation in Japanese eel. The effective doses of reLh were apparently lower and the effects of reLh emerged faster in comparison with reFsh, suggested their differential roles in spermatogenesis. In addition, it was revealed that a final step of 11KT synthesis was catalyzed by Hsd17b12a and Rdh11/12-like 1; in particular, the expression of *hsd17b12a* gene was regulated by reGth. Moreover, it would be important for understanding gametogenesis to define the differential effects of Fsh and Lh in Japanese eel.

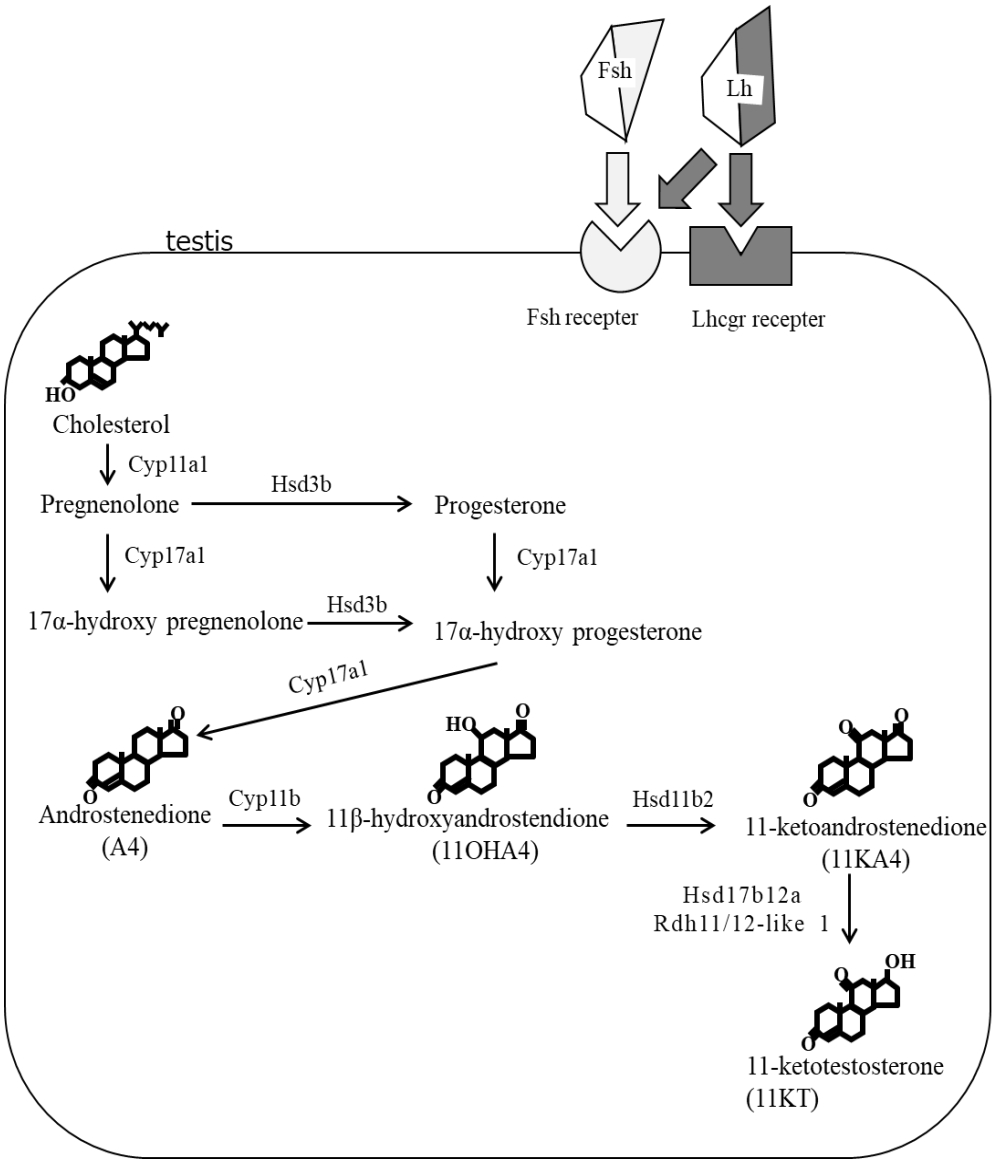

Figure 25. Schematic illustration of testicular steroidogenesis in Japanese eel.

References

Albalat, R., Brunet, F., Laudet, V., Schubert, M., 2011. Evolution of retinoid and steroid signaling: vertebrate diversification from an amphioxus perspective. Genome Biol. Evol. 3, 985-1005.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403-410.

Arai, R., Tamaoki, B.I., 1967. Steroid biosynthesis in vitro by testes of rainbow trout, *Salmo gairdneri*. Gen. Comp. Endocrinol. 8, 305-313.

Arnold, S.L., Kent, T., Hogarth, C.A., Schlatt, S., Prasad, B., Haenisch, M., Walsh, T., Muller, C.H., Griswold, M.D., Amory, J.K., Isoherranen, N., 2015. Importance of ALDH1A enzymes in determining human testicular retinoic acid concentrations. J. Lipid. Res. 56, 342-357.

Asahina, K., Kanbegawa, A., Higashi, T., 1995. Development of a microtiter plate enzyme-linked immunosorbent assay for 17α, 20β, 21-trihydroxy-4-pregnen-3-one, a teleost gonadal steroid. Fish. Sci. 61, 491–494.

Baenziger, JU., Kumar, S., Brodbeck, RM., Smith, PL., Beranek, MC., 1992. Circulatory half-life but not interaction with the lutropin/chorionic gonadotropin receptor is modulated by sulfation of bovine lutropin oligosaccharides. Proc. Natl. Acad. Sci.

USA. 89, 334-338.

Belyaeva, O.V., Wu, L., Shmarakov, I., Nelson, P.S., Kedishvili, N.Y., 2018. Retinol dehydrogenase 11 is essential for the maintenance of retinol homeostasis in liver and testis in mice. J. Biol. Chem. 293, 6996-7007.

Bertin, J., Dury, A.Y., Ouellet, J., Pelletier, G., Labrie, F., 2014. Localization of the androgen-synthesizing enzymes, androgen receptor, and sex steroids in the vagina: possible implications for the treatment of postmenopausal sexual dysfunction. J. Sex Med. 11, 1949-1961.

Blanchard, P.G., Luu-The, V., 2007. Differential androgen and estrogen substrates specificity in the mouse and primates type 12 17beta-hydroxysteroid dehydrogenase. J. Endocrinol. 194, 449-455.

Boime, I., Ben-Menahem, D., 1999. Glycoprotein hormone structure-function and analog design. Rec. Prog. Hormone Res. 54,271-289.

Borg, B., 1994. Androgens in teleost fishes. Comp. Biochem. Physiol. 109, 219-245.

Busada, J.T., Geyer, C.B., 2016. The Role of Retinoic Acid (RA) in Spermatogonial Differentiation. Biol. Reprod. 94, 1-10.

Campbell, B., Dickey, JT., Swanson, P., 2003. Endocrine changes during onset of puberty

in male spring Chinook salmon, Oncorhynchus tshawytscha. Biol Reprod. 69(6):2109-17.

Cavaco, J.E.B., Lambert, J.G.D., Schulz, R.W., Goos, H.J.Th., 1996. Pubertal development of male African catfish, *Clarias gariepinus*. In vitro steroidogenesis by testis and interrenal tissue and plasma levels of sexual steroids. Fish Physiol. Biochem. 16, 129-138.

Cavaco, J.E.B., Vischer, H.F., Lambert, J.G.D., Goos, H.J.Th., Schulz, R.W., 1997. Mismatch between patterns of circulating and testicular androgens in African catfish, *Clarias gariepinus*. Fish Physiol. Biochem. 17, 155–162.

Chang, Y.S., Huang, F.L., 1982. The mode of action of carp gonadotropin on the stimulation of androgen production by carp testis in vitro. Gen. Comp. Endocrinol. 48, 147-53.

Chauvigné, F., Tingaud-Sequeira, A., Agulleiro, M.J., Calusinska, M., Gómez, A., Finn, R.N., Cerdà, J. 2010. Functional and evolutionary analysis of flatfish gonadotropin receptors reveals cladal- and lineage-level divergence of the teleost glycoprotein receptor family. Biol. Reprod. 82, 1088–1102.

Chauvigné, F., Verdura, S., Mazón, M.J., Duncan, N., Zanuy, S., Gómez, A., Cerdà, J., 2012. Follicle-stimulating hormone and luteinizing hormone mediate the androgenic pathway in Leydig cells of an evolutionaly advanced teleost. Biol. Reprod. 87, 1-11.

Chauvigné, F., Zapater, C., Crespo, D., Planas, JV., Cerdà, J., 2014a. Fsh and Lh direct conserved and specific pathways during flatfish semicystic spermatogenesis. J. Mol. Endocrinol. 53, 175-90.

Chauvigné, F., Zapater, C., Gasol, J.M., Cerdà, J., 2014b. Germ-line activation of the luteinizing hormone receptor directly drives spermiogenesis in a nonmammalian vertebrate. Proc. Natl. Acad. Sci. USA. 111, 1427-1432.

Chauvigné, F., Ollé, J., González, W., Duncan, N., Giménez, I., Cerdà, J., 2017. Toward developing recombinant gonadotropin-based hormone therapies for increasing fertility in the flatfish Senegalese sole. PLoS One. 12, e0174387

Chen, J., Zhang, Y., Tang, Z., Mao, J., Kuang, Z., Qin, C., Li, W., 2012. Producyion of recombinant orange-spotted grouper (*Epinephelus coioides*) follicle-stimulating hormone (FSH) in single-chain form and dimer form by *Pichia pastoris* and their biological activities. Gen. Comp. Endocrinol. 178, 237-249.

Chu, L., Li, J., Liu, Y., Hu, W., Cheng, CH., 2014. Targeted gene disruption in zebrafish reveals noncanonical functions of LH signaling in reproduction. Mol. Endocrinol. 28, 1785-95.

Chu, L., Li, J., Liu, Y., Cheng, CH., 2015. Gonadotropin Signaling in Zebrafish Ovary and Testis Development: Insights From Gene Knockout Study. Mol. Endocrinol. 29, 1743-58.

Combarnous, Y., 1992. Molecular basis of the specificityof binding of glycoprotein hormones to their receptors. Endocr. Rev. 13, 670-691.

de Waal, P.P., Wang, D.S., Nijenhuis, W.A., Schulz, R.W., Bogerd, J., 2008. Functional characterization and expression analysis of the androgen receptor in zebrafish (*Danio rerio*) testis. Reproduction 136, 225-234.

Diotel, N., Do Rego, J. L., Anglade, I., Vaillant, C., Pellegrini, E., Gueguen, M. M., Mironov, S., Vaudry, H., and Kah, O. 2011. Activity and expression of steroidogenic enzymes in the brain of adult zebrafish. Eur. J. Neurosci. 34, 45–56.

Diotel, N., Charlier, T. D., Lefebvre d'Hellencourt, C., Couret, D., Trudeau, V. L., Nicolau, J. C., Meilhac, O., Kah, O., Pellegrini, E., 2018. Steroid Transport, Local Synthesis, and Signaling within the Brain: Roles in Neurogenesis, Neuroprotection, and Sexual Behaviors. Front. Neurosci. 12, 84.

Garcia-Campayo, V., Boime, L., 2001. Novel recombinant gonadotropins. Trends Endocrino. Metab. 12, 72-77.

García-López, A., Fernández-Pasquier, V., Couto, E., Canario, A.V., Sarasquete, C., Martínez-Rodríguez, G., 2006. Testicular development and plasma sex steroid levels in cultured male Senegalese sole *Solea senegalensis* Kaup. Gen. Comp. Endocrinol. 147, 343-351.

García-López, Á., Bogerd, J., Granneman, J.C.M., van Dijk, W., Trant, J.M., Taranger, G.L., Schulz, R.W., 2009. Leydig cells express follicle-stimulating hormone receptors in African catfish. Endocrinology 150, 357-365.

García-López, Á., de Jonge, H., Nóbrega, R.H., de Waal, P.P., van Dijk, W., Hemrika, W., Taranger, G.L., Bogerd, J., Schulz, R.W., 2010. Studies in zebrafish reveal unusual cellular expression patterns of gonadotropin receptor messenger ribonucleic acids in the testis and unexpected functional differentiation of the gonadotropins. Endocrinology 151, 2349-2360.

Geissler, W.M., Davis, D.L., Wu, L., Bradshaw, K.D., Patel, S., Mendonca, B.B., Elliston, K.O., Wilson, J.D., Russell, D.W., Andersson, S., 1994. Male pseudohermaphroditism caused by mutations of testicular 17 beta-hydroxysteroid dehydrogenase 3. Nat. Genet. 7, 34-39.

Grandel, H., Lun, K., Rauch, G. J., Rhinn, M., Piotrowski, T., Houart, C., Sordino, P., Küchler, A. M., Schulte-Merker, S., Geisler, R., Holder, N., Wilson, S. W., Brand, M., 2002. Retinoic acid signalling in the zebrafish embryo is necessary during pre-segmentation stages to pattern the anterior-posterior axis of the CNS and to induce a pectoral fin bud. Development 129, 2851–2865.

Griswold, M.D., 2016. Spermatogenesis: The Commitment to Meiosis. Physiol. Rev. 96, 1-17.

Han, Y.S., Sun, Y.L., Liao, Y.F., Liao, I.C., Shen, K.N., Tzeng, W.N., 2008. Temporal analysis of population geneticcomposition in the overexploited Japanese eel Anguillajaponica. Marine Biology 155, 613–621.

Hanukoglu, I., 1992. Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone biosynthesis. J. Steroid. Biochem. Mol. Biol. 43, 779-804.

Henkel, CV., Dirks, RP., de Wijze, DL., Minegishi, Y., Aoyama, J., Jansen, HJ., Turner, B., Knudsen, B., Bundgaard, M., Hvam, KL., Boetzer, M., Pirovano, W., Weltzien, FA., Dufour, S., Tsukamoto, K., Spaink, HP., van den Thillart, GE., 2012. First draft genome sequence of the Japanese eel, *Anguilla japonica*. Gene 511, 195-201

Higuchi, T., Watanabe, S., Manabe, R., Kaku, T., Okamura, A., Yamada, Y., Miller, M.J., Tsukamoto K., 2018. Tracking *Anguilla japonica* silver eels along the West Marina Ridge using pop-up archival transmitting tags. Zool. Stud. 57, 24-37.

Higuchi, T., Yamada, Y., Watanabe, S., Kojima, T., Tsukamoto, K., 2020. Estimation of the spawning time of Japanese eels in the open ocean. Scientific reports 10, 4172.

Huhtaniemi, I.T., Themmen, A.P., 2005. Mutations in human gonadotropin and gonadotropin receptor genes Endocrine 26, 2702-2711.

Ijiri, S., Takei, N., Kazeto, Y., Todo, T., Adachi, S., Yamauchi, K., 2006. Changes in localization of cytochrome P450 cholesterol side-chain cleavage (P450scc) in

Japanese eel testis and ovary during gonadal development. Gen. Comp. Endocrinol. 145, 75-83.

Ijiri, S., Tsukamoto, K. Chow, S., Kurogi, H., Adachi, S., Tanaka, H., 2011a. Controlled reproduction in the Japanese eel (Anguilla japonica), past and present. Aquacult. Europe. 36, 13–17.

Ijiri, S., Tsukamoto, K., Chow, S., Kurogi, H., Gen, K., Tanaka, H., Adachi, S., 2011b. Construction of EST database from ovaries of wild maturing eels. Indian J. Sci. Technol., 9th ISRPF Issue, 4(S8), 295-296.

Ijiri, S., Shibata, Y., Takezawa, N., Kazeto, Y., Takatsuka, N., Kato, E., Hagihara, S., Ozaki, Y., Adachi, S., Yamauchi, K., Nagahama, Y., 2017. 17β-HSD Type 12-Like Is Responsible for Maturation-Inducing Hormone Synthesis During Oocyte Maturation in Masu Salmon. Endocrinology 158, 627-639.

Imamichi, Y., Yuhki, K.I., Orisaka, M., Kitano, T., Mukai, K., Ushikubi, F., Taniguchi, T., Umezawa, A., Miyamoto, K., Yazawa, T., 2016. 11-Ketotestosterone Is a Major Androgen Produced in Human Gonads. J. Clin. Endocrinol. Metab. 101, 3582-3591.

Jacoby, D., Gollock, M., 2014. Anguilla japonica. The IUCN Red List of Threatened Species. Version 2014.3.

Jiang, J.Q., Kobayashi, T., Ge, W., Kobayashi, H., Tanaka, M., Okamoto, M., Nonaka, Y.,

Nagahama, Y., 1996. Fish testicular 11β-hydroxylase: cDNA cloning and mRNA expression during spermatogenesis. FEBS Lett. 397, 250-252.

Jiang, J.Q., Young, G., Kobayashi, T., Nagahama, Y., 1998. Eel (*Anguilla japonica*) testis 11beta-hydroxylase gene is expressed in interrenal tissue and its product lacks aldosterone synthesizing activity. Mol. Cell. Endocrinol. 146, 207-211.

Kagawa, H., Ohta, H., Tanaka, H., 2001. Recent progress of research on larvae production of Japanese eel, *Anguilla japonica*. Suisan Zoshoku 49, 127-132.

Kagawa, H., Tanaka, H., Ohta, H., Unuma, T., Nomura, K., 2005. The first success of glass eel production in the world: basic biology on fish reproduction advances new applied technology in aquaculture. Fish Physiol. Biochem. 31, 193-199.

Kanda Y., 2013. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant. 48, 452-458.

Kasus-Jacobi, A., Ou, J., Birch, D. G., Locke, K. G., Shelton, J. M., Richardson, J. A., Murphy, A. J., Valenzuela, D. M., Yancopoulos, G. D., & Edwards, A. O., 2005. Functional characterization of mouse RDH11 as a retinol dehydrogenase involved in dark adaptation in vivo. J. Biol. Chem. 280, 20413–20420.

Kazeto, Y., Ijiri, S., Todo, T., Adachi, S., Yamauchi, K., 2000. Molecular cloning and characterization of Japanese eel ovarian P450c17 (CYP17) cDNA. Gen. Comp.

Endocrinol. 118, 123-133.

Kazeto, Y., Ijiri, S., Matsubara, H., Adachi, S., Yamauchi, K., 2003. Molecular cloning and characterization of 3β-hydroxysteroid dehydrogenase/Δ4-Δ5 isomerase cDNAs from Japanese eel ovary. J. Steroid Biochem. Mol. Biol. 85, 49-56.

Kazeto, Y., Ijiri, S., Adachi, S., Yamauchi, K., 2006. Cloning and characterization of a cDNA encoding cholesterol side-chain cleavage cytochrome P450 (CYP11A1): tissue-distribution and changes in the transcript abundance in ovarian tissue of Japanese eel, *Anguilla japonica*, during artificially induced sexual development. J. Steroid Biochem. Mol. Biol. 99, 121-128.

Kazeto, Y., Kohara, M., Miura, T., Miura, C., Yamaguchi, S., Trant, J.M., Adachi, S., Yamauchi, K., 2008. Japanese eel follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh): production of biologically active recombinant Fsh and Lh by Drosophila S2 cells and their differential actions on the reproductive biology. Biol. Reprod. 79, 938-946.

Kazeto, Y., Tosaka, R., Matsubara, H., Ijiri, S., Adachi, S., 2011. Ovarian steroidogenesis and the role of sex steroid hormones on ovarian growth and maturation of the Japanese eel. J. Steroid. Biochem. Mol. Biol. 127, 149-154.

Kazeto, Y., Kohara, M., Tosaka, R., Gen, K., Yokoyama, M., Miura, C., Miura, T., Adachi, S., Yamauchi, K., 2012. Molecular characterization and gene expression of Japanese

eel (*Anguilla japonica*) gonadotropin receptors. Zool. Sci. 29, 204-211

Kazeto, Y., Tanaka, T., Suzuki, H., Ozaki, Y., Fukada, H., Gen, K., 2019. Development and validation of specific enzyme-linked immunosorbent assays for follicle-stimulating hormone and luteinizing hormone of Japanese eel. Fish. Sci. 85, 829–837.

Keay, S.D., Vatish, M., Karteris, E., Hillhouse, E.W., Randeva, H.S., 2004. The role of hCG in reproductive medicine. BJOG. 111, 1218-1228.

Kedishvili, N.Y., Chumakova, O.V., Chetyrkin, S.V., Belyaeva, O.V., Lapshina, E.A., Lin, D.W., Matsumura, M., Nelson, P.S., 2002. Evidence that the human gene for prostate short-chain dehydrogenase/reductase (PSDR1) encodes a novel retinal reductase (RalR1). J. Biol. Chem. 277, 28909-28915.

Kobayashi, M., Hayakawa, Y., Park, W., Banba, A., Yoshizaki, G., Kumamaru, K., Kagawa, H., Kaki, H., Nagaya, H., Sohn, Y.C., 2010. Production of recombinant Japanese eel gonadotropins by baculovirus in silkworm larvae. Gen. Comp. Endocrinol. 167, 379-386.

Kobayashi, Y., Nozu, R., Nakamura, M., 2011. Role of estrogen in spermatogenesis in initial phase males of the three-spot wrasse (*Halichoeres trimaculatus*): effect of aromatase inhibitor on the testis. Dev. Dyn. 240, 116-121.

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K., 2018. MEGA X: Molecular

Evolutionary Genetics Analysis across computing platforms. Mol. Biol. Evol. 35, 1547-1549.

Kusakabe, M., Nakamura, I., Evans, J., Swanson, P., Young, G., 2006. Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins during spermatogenesis in rainbow trout testes. J. Endocrinol. 189, 541-544.

Labrie, F., Luu-The, V., Lin, S.X., Labrie, C., Simard, J., Breton, R., Bélanger, A., 1997. The key role of 17 beta-hydroxysteroid dehydrogenases in sex steroid biology. Steroids 62, 148-158.

Legardinier, S., Duonor-Cérutti, M., Devauchelle, G., Combarnous, Y., Cahoreau, C., 2005. Biological activities of recombinant equine luteinizing hormone/chorionic gonadotropin (eLH/CG) expressed in Sf9 and Mimic insect cell lines. J. Mol. Endocrinol. 34, 47-60.

Levavi-Sivan, B., Bogerd, J., Mañanós, E.L., Gómez, A., Lareyre, J.J., 2010. Perspectives on fish gonadotropins and their receptors. Gen. Comp. Endocrinol. 165, 412-437.

Lhor, M., Salesse, C., 2014. Retinol dehydrogenases: membrane-bound enzymes for the visual function. Biochem. Cell Biol. 92, 510-523.

Li, J., Cheng, CHK., 2018. Evolution of gonadotropin signaling on gonad development:

insights from gene knockout studies in zebrafish. Biol. Reprod. 99, 686-694.

Li, Y.Y., Inoue, K., Takei, Y., 2003. Steroidogenic acute regulatory protein in eels: cDNA cloning and effects of ACTH and seawater transfer on its mRNA expression. Zool. Sci. 20, 211-219.

Lin, B., White, J.T., Ferguson, C., Wang, S., Vessella, R., Bumgarner, R., True, L.D., Hood, L., Nelson, P.S., 2001. Prostate short-chain dehydrogenase reductase 1 (PSDR1): a new member of the short-chain steroid dehydrogenase/reductase family highly expressed in normal and neoplastic prostate epithelium. Cancer Res. 61, 1611-1618.

Liu, H., Zheng, S., Bellemare, V., Pelletier, G., Labrie, F., Luu-The, V., 2007. Expression and localization of estrogenic type 12 17beta-hydroxysteroid dehydrogenase in the cynomolgus monkey. BMC Biochem. 8, 2.

Lorenzi, V., Earley, R. L., Grober, M. S., 2012. Differential Responses of Brain, Gonad and Muscle Steroid Levels to Changes in Social Status and Sex in a Sequential and Bidirectional Hermaphroditic Fish. PLoS ONE 7, e51158.

Luu-The, V., 2001. Analysis and characteristics of multiple types of human 17beta-hydroxysteroid dehydrogenase. J. Steroid. Biochem. Mol. Biol. 76, 143-151.

Luu-The, V., Tremblay, P., Labrie, F., 2006. Characterization of type 12 17beta-hydroxysteroid dehydrogenase, an isoform of type 3 17beta-hydroxysteroid

dehydrogenase responsible for estradiol formation in women. Mol. Endocrinol. 20, 437-443.

Luu-The, V., Bélanger, A., Labrie, F., 2008. Androgen biosynthetic pathways in the human prostate. Best Pract. Res. Clin. Endocrinol. Metab. 22, 207-221.

Luu-The, V., 2013. Assessment of steroidogenesis and steroidogenic enzyme functions. J. Steroid. Biochem. Mol. Biol. 137, 176-182.

Matsubara, H., Kazeto, Y., Ijiri, S., Hirai, T., Adachi, S., Yamauchi, K., 2003. Changes in mRNA levels of ovarian steroidogenic enzymes during artificial maturation of Japanese eel *Anguilla japonica*. Fish. Sci. 69, 979-988.

Maugars, G., Dufour, S., 2015. Demonstration of the Coexistence of Duplicated LH Receptors in Teleosts, and Their Origin in Ancestral Actinopterygians. PLoS One. 10, e0135184.

Maugars, G., Schmitz, M., 2008. Gene expression profiling during spermatogenesis in early maturating male Atlantic salmon parr testes. Gen.Comp. Endocrinol. 159, 178-187.

Mazón, MJ., Molés, G., Rocha, A., Crespo, B., Lan-Chow-Wing, O., Espigares, F., Muñoz, I., Felip, A., Carrillo, M., Zanuy, S., Gómez, A., 2015. Gonadotropins in European sea bass: Endocrine roles and biotechnological applications. Gen. Comp. Endocrinol. 221, 31-41.

Menzer, C., Schams, D., 1979. Radioimmunoassay for PMSG and its application to in-vivo studies. J. Reprod. Fertil. 55, 339-345.

Meyer, A., Schartl, M., 1999. Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. Curr. Opin. Cell Biol. 11, 699-704.

Mikawa, N., Yamada, Y., Horie, N.,Okamura, A. Utoh, T., Tanaka, A., Tsukamoto, K., 2019. A preliminary experiment regarding the natural induction of gonadal development in female Japanese eels without hormone treatment. Aquac. Res. 50, 3749–3754.

Mindnich, R., Deluca, D., Adamski, J., 2004a. Identification and characterization of 17 beta-hydroxysteroid dehydrogenases in the zebrafish, *Danio rerio*. Mol. Cell. Endocrinol. 215, 19-30.

Mindnich, R., Möller, G., Adamski, J., 2004b. The role of 17 beta-hydroxysteroid dehydrogenases. Mol. Cell. Endocrinol. 218, 7-20.

Mindnich, R., Haller, F., Halbach, F., Moeller, G., Hrabé de Angelis, M., Adamski, J., 2005. Androgen metabolism via 17beta-hydroxysteroid dehydrogenase type 3 in mammalian and non-mammalian vertebrates: comparison of the human and the zebrafish enzyme. J. Mol. Endocrinol. 35, 305-316.

Mindnich, R., Adamski, J., 2009. Zebrafish 17beta-hydroxysteroid dehydrogenases: an evolutionary perspective. Mol. Cell. Endocrinol. 301, 20-26.

Miura, T., Yamauchi, K., Nagahama, Y., Takahashi, H., 1991. Induction of spermatogenesis in male Japanese eel, *Anguilla japonica*, by a single injection of human chorionic gonadotropin. Zool. Sci. 8, 63-73.

Miura, C., Miura, T., Yamashita, M., Yamauchi, K., Nagahama, Y., 1996. Hormonal induction of all stages of spermatogenesis in germ-somatic cell coculture from immature Japanese eel testis. Dev. Growth Differ. 38, 257-262.

Miura, T., Miura, C., Ohta, T., Nader, M.R., Todo, T., Yamauchi, K., 1999. Estradiol-17beta stimulates the renewal of spermatogonial stem cells in males. Biochem. Biophys. Res. Commun. 264, 230-234.

Molés, G., Gómez, A., Rocha, A., Carrillo, M., Zanuy, S., 2008. Purification and characterization of follicle-stimulating hormone from pituitary glands of sea bass (*Dicentrarchus labrax*). Gen. Comp. Endocrinol. 158, 68-76.

Molés, G., Zanuy, S., Muñoz, I., Crespo, B., Martínez, I., Mañanós, E., Gómez, A., 2011. Receptor specificity and functional comparison of recombinant sea bass (*Dicentrarchus labrax*) gonadotropins (Fsh and Lh) produced in different host systems. Biol. Reprod. 84, 1171-1181.

Molés, G., Hausken, K., Carrillo, M., Zanuy, S., Levavi-Sivan, B., Gómez, A., 2020. Generation and use of recombinant gonadotropins in fish [published online ahead of print, 2020 Jul 17]. Gen. Comp. Endocrinol. 113555.

Moore, S., Pritchard, C., Lin, B., Ferguson, C., Nelson, P.S., 2002. Isolation and characterization of the murine prostate short-chain dehydrogenase/reductase 1 (Psdr1) gene, a new member of the short-chain steroid dehydrogenase/reductase family. Gene. 293, 149-160.

Murozumi, N., Nakashima, R., Hirai, T., Kamei, Y., Ishikawa-Fujiwara, T., Todo, T., Kitano, T., 2014. Loss of follicle-stimulating hormone receptor function causes masculinization and suppression of ovarian development in genetically female medaka. Endocrinology 155, 3136-3145.

Nourashrafeddin, S., Hosseini Rashidi, B., 2018. Gonadotropin Regulation of Retinoic Acid Activity in the Testis. Acta. Med. Iran. 56, 34-42.

Nyuji, M., Kazeto, Y., Izumida, D., Tani, K., Suzuki, H., Hamada, K., Mekuchi, M., Gen, K., Soyano, K., Okuzawa, K., 2016. Greater amberjack Fsh, Lh, and their receptors: plasma and mRNA profiles during ovarian development. Gen. Comp. Endocrinol. 225, 224–234

Ohkubo, M., Yabu, T, Yamashita, M., Shimizu, A., 2013. Molecular cloning of two gonadotropin receptors in mummichog Fundulus heteroclitus and their gene

expression during follicular development and maturation. Gen Comp Endocrinol. 184:75–86.

Ohta, T., Miyake, H., Miura, C., Kamei, H., Aida, K., Miura, T., 2007. Follicle-stimulating hormone induces spermatogenesis mediated by androgen production in Japanese eel, *Anguilla japonica*. Biol. Reprod. 77,970-977.

Ohta, T., Sato, Y., Imaizumi, H., Kazeto, Y., 2017. Changes in milt volume and sperm quality with time after an injection of recombinant Japanese eel luteinizing hormone in male Japanese eels. Aquaculture 479, 150-154.

Ozaki, Y., Higuchi, M., Miura, C., Yamaguchi, S., Tozawa, Y., Miura, T., 2006. Roles of 11β-hydroxysteroid dehydrogenase in fish spermatogenesis. Endocrinology 147, 5139-46.

Ozaki, Y., Damsteegt, EL., Setiawan, AN., Miura, T., Lokman, PM., 2019. Expressional regulation of gonadotropin receptor genes and androgen receptor genes in the eel testis. Gen. Comp. Endocrinol. 280,123-133.

Payne, A.H., Youngblood, G.L., 1995. Regulation of expression of steroidogenic enzymes in Leydig cells. Biol. Reprod. 52, 217-25.

Peltoketo, H., Luu-The, V., Simard, J., Adamski, J., 1999. 17beta-hydroxysteroid dehydrogenase (HSD)/17-ketosteroid reductase (KSR) family; nomenclature and main

characteristics of the 17HSD/KSR enzymes. J. Mol. Endocrinol. 23, 1-11.

Peñaranda, DS., Gallego, V., Rozenfeld, C., Herranz-Jusdado, JG., Pérez, L., Gómez, A., Giménez, 3., Asturiano, JF., 2018. Using specific recombinant gonadotropins to induce spermatogenesis and spermiation in the European eel (Anguilla anguilla). Theriogenology 107, 6-20.

Pierce, J.G., Parsons, T.F., 1981. Glycoprotein hormones: structure and function. Annu. Rev. Biochem. 50, 465-495.

Prehn, C., Möller, G., Adamski, J., 2009. Recent advances in 17beta-hydroxysteroid dehydrogenases. J. Steroid Biochem. Mol. Biol. 114, 72-77.

Pretorius, E., Arlt, W., Storbeck, K.H., 2017. A new dawn for androgens: Novel lessons from 11-oxygenated C19 steroids. Mol. Cell. Endocrinol. 441, 76-85.

Rajakumar, A., Senthilkumaran, B., 2014. Molecular cloning and expression analysis of 17b-hydroxysteroid dehydrogenase 1 and 12 during gonadal development, recrudescence and after in vivo hCG induction in catfish, *Clarias batrachus*. Steroids 92, 81-89.

Rocha, A., Zanuy, S., Carrillo, M., Gómez, A., 2009. Seasonal changes in gonadal expression of gonadotropin receptors, steroidogenic acute regulatory protein and steroidogenic enzymes in the European sea bass. Gen. Comp. Endocrinol.162, 265–

275.

Rolland, AD., Lareyre, JJ., Goupil, AS., Montfort, J., Ricordel, MJ., Esquerré, D., Hugot, K., Houlgatte, R., Chalmel, F., Le Gac, F., 2009. Expression profiling of rainbow trout testis development identifies evolutionary conserved genes involved in spermatogenesis. BMC Genomics. 10, 546.

Saito, K., Chida, Y., Adachi, S., Yamauchi, K., 2003. *In vitro* induction of luteinizing hormone synthesis by estrogen in organ-cultured pituitary glands of the Japanese eel, *Anguilla japonica*. Zool. Sci. 20, 69-73.

Sambroni, E., Rolland, AD., Lareyre, JJ., Le Gac F.,2012. FSH and LH have common and distinct effects on gene expression in rainbow trout testis. J Mol Endocrinol. 50, 1-18.

Sambroni, E., Lareyre, JJ., Le Gac F., 2013 Fsh controls gene expression in fish both independently of and through steroid mediation. PLoS One. 8, e76684.

Samarut, E., Fraher, D., Laudet, V., Gibert, Y., 2015. ZebRA: An overview of retinoic acid signaling during zebrafish development. Biochim. Biophys. Acta. 1849, 73–83.

Schulz, R., Blüm, V., 1991. Extragonadal 17 beta-hydroxysteroid dehydrogenase activity in rainbow trout. Gen. Comp. Endocrinol. 82, 197-205.

Schulz, R.W., de França, L.R., Lareyre, J.J. LeGac, F., Chiarini-Garcia, H., Nobrega, R.H.,

Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390-411.

Sha, J.A., Dudley, K., Rajapaksha, W.R., O'Shaughnessy, P.J., 1997. Sequence of mouse 17beta-hydroxysteroid dehydrogenase type 3 cDNA and tissue distribution of the type 1 and type 3 isoform mRNAs. J. Steroid. Biochem. Mol. Biol. 60, 19-24.

Smith, PL., Baenziger, JU., 1992. Molecular basis of recognition by the glycoprotein hormone-specific N-acetylgalactosamine-transferase. Proc. Natl. Acad. Sci. USA. 89, 329-333.

Stocco, D.M., 2000. The role of StAR protein in steroidogenesis: challemges for the furture. J. Endocrinol., 164, 247-253.

Swanson, P., Suzuki, K., Kawauchi, H., Dickhoff, W.W., 1991. Isolation and characterization of two coho salmon gonadotropins, GtH-I and GtH-II. Biol. Reprod. 44, 29-38.

Swanson, P., Dickey, J.T., Cambell, B., 2003. Biochemistry and physiology of fish gonadotropins. Fish Physiol. Biochem. 28, 53-59.

Tanaka, H. 2015. Progression in artificial seedling production of Japanese eel *Anguilla japonica*. Fish. Sci. 81, 11–19.

Tokarz, J., Mindnich, R., Norton, W., Möller, G., Hrabé de Angelis, M., Adamski, J., 2012.

Discovery of a novel enzyme mediating glucocorticoid catabolism in fish: 20beta-hydroxysteroid dehydrogenase type 2. Mol. Cell. Endocrinol. 349, 202-213.

Tokarz, J., Möller, G., Hrabě de Angelis, M., Adamski, J., 2015. Steroids in teleost fishes: A functional point of view. Steroids. 103, 123-144.

Tokui, M., Takei, I., Tashiro, F., Shimada, A., Kasuga, A., Ishii, M., Ishii, T., Takatsu, K., Saruta, T., Miyazaki, J., 1997. Intramuscular injection of expression plasmid DNA is an effective means of long-term systemic delivery of interleukin-5. J. Biochem. Biophys. Res. Commun. 233, 527-531.

Thompson, J.D., Gibson, T.J. & Higgins, D.G., 2002. Multiple Sequence Alignment Using ClustalW and ClustalX. Curr. Protoc. Bioinformatics. 2.3.1–2.3.22.

Tong, M.H., Yang, Q.E., Davis, J.C., Griswold, M.D., 2013. Retinol dehydrogenase 10 is indispensible for spermatogenesis in juvenile males. Proc. Natl. Acad. Sci. USA., 110, 543-548.

Tsai-Morris, C.H., Khanum, A., Tang, P.Z., Dufau, M.L., 1999. The rat 17beta-hydroxysteroid dehydrogenase type III: molecular cloning and gonadotropin regulation. Endocrinology 140, 3534-3542.

Tsukamoto, K., Chow, S., Otake, T., Kurogi, H., Mochioka, N., Miller, M.J., Aoyama, J., Kimura, S., Watanabe, S., Yoshinaga, T., Shinoda, A., Kuroki, M., Oya, M., Watanabe,

T., Hata, K., Ijiri, S., Kazeto, Y., Nomura, K., Tanaka, H., 2011. Oceanic spawning ecology of freshwater eels in the western North Pacific. Nat. Commun. 2, 179.

Vischer, HF., Granneman, JC., Linskens, MH., Schulz, RW., Bogerd, J., 2003 Both recombinant African catfish LH and FSH are able to activate the African catfish FSH receptor. J. Mol. Endocrinol. 31:133–140

Von Hofsten, J., Karlsson, J., Jone, I., Olsson, P.E., 2002. Expression and regulation of *fushi tarazu* factor-1 and steroidogenic genes during reproduction in Arctic char (*Salvelinus alpines*). Biol.Reprod. 67, 1297-1304.

Wu, GC., Jeng, SR., Pan, YT., Li, HW., Ku, WL., Lin, CJ., Chang, CF., 2019. The germline-specific expression of Foxl3a and its paralogous Foxl3b are associated with male gonadal differentiation in the Japanese eel, *Anguilla japonica*. Gen. Comp. Endocrinol. 277:56-65.

Xie, Y. A., Lee, W., Cai, C., Gambin, T., Nõupuu, K., Sujirakul, T., Ayuso, C., Jhangiani, S., Muzny, D., Boerwinkle, E., Gibbs, R., Greenstein, V. C., Lupski, J. R., Tsang, S. H., Allikmets, R., 2014. New syndrome with retinitis pigmentosa is caused by nonsense mutations in retinol dehydrogenase RDH11. Human molecular genetics, 23, 5774–5780.

Xie, Y., Chu, L., Liu, Y., Sham, KWY., Li, J., Cheng, CHK., 2017. The highly overlapping actions of Lh signaling and Fsh signaling on zebrafish spermatogenesis. J. Endocrinol.

234, 233-246.

Yamada, H., Satoh, R., Yamashita, T., Kambegawa, A., Iwata, M., 1997. Development of a time-resolved fluoroimmunoassay (TR-FIA) for testosterone: measurement of serum testosterone concentrations after testosterone treatment in the rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 106, 181-188.

Zhang, Z., Lau, SW., Zhang, L., Ge, W., 2015a. Disruption of Zebrafish Follicle-Stimulating Hormone Receptor (*fshr*) But Not Luteinizing Hormone Receptor (*lhcgr*) Gene by TALEN Leads to Failed Follicle Activation in Females Followed by Sexual Reversal to Males. Endocrinology. 156, 3747-62.

Zhang, Z., Zhu, B., Ge, W., 2015b. Genetic analysis of zebrafish gonadotropin (FSH and LH) functions by TALEN-mediated gene disruption. Mol. Endocrinol. 29, 76-98.

Zhou, L.Y., Wang, D.S., Senthilkumaran, B., Yoshikuni, M., Shibata, Y., Kobayashi, T., Sudhakumari, C.C., Nagahama, Y., 2005. Cloning, expression and characterization of three types of 17beta-hydroxysteroid dehydrogenases from the Nile tilapia, *Oreochromis niloticus*. J. Mol. Endocrinol. 35, 103-116.

Zmora, N., Kazeto, Y., Kumar, R.S., Schulz, R.W., Trant, J.M., 2007. Production of recombinant channel catfish (*Ictalurus punctatus*) Fsh and LH in S2 *Drosophila* cell line and induction of their different actions. J. Endocrinol. 194, 407- 416.