BIOLOGY OF REPRODUCTION **67,** 1297–1304 (2002)
DOI 10.1095/biolreprod.102.005181

Expression and Regulation of *Fushi Tarazu* Factor-1 and Steroidogenic Genes During Reproduction in Arctic Char (*Salvelinus alpinus*)[1]

Jonas von Hofsten,[4] Johnny Karlsson,[4] Iwan Jones,[3,4] and Per-Erik Olsson[2,4,5]

Department of Molecular Biology,[4] Umeå University, SE-901 87 Umeå, Sweden
Department of Natural Sciences,[5] Örebro University, SE-701 82 Örebro, Sweden

ABSTRACT

Teleost *fushi tarazu* factor-1 (FTZ-F1) is a potential regulator of steroidogenesis. The present study shows sex-specific regulation of Arctic char *fushi tarazu* factor-1 (acFF1) and steroidogenic genes during reproductive maturation and in response to hormone treatment. A link between gonadal expression of acFF1, steroidogenic acute regulatory protein (StAR), and cytochrome P450-11A (CYP11A), was observed in the reproductive maturation process, as elevated acFF1 mRNA and protein levels preceded increased StAR and CYP11A transcription. Sex-specific differences were observed as estrogen treatment resulted in down-regulated levels of acFF1 mRNA in testis and male head kidney, whereas no significant effect was observed in females. 11-Ketotestosterone (11-KT) down-regulated CYP11A and 3β-hydroxysteroid dehydrogenase (3βHSD) in head kidney and up-regulated CYP11A in testis. StAR remained unaffected by hormone treatment. This suggests that acFF1 is controlled by 17β-estradiol, whereas the effects on CYP11A and 3βHSD are mediated by 11-KT. Coexpression of acFF1, StAR, and CYP11A was observed in head kidney, in addition to gonads, indicating correlation between these steroidogenic genes. StAR and acFF1 were also coexpressed in liver, suggesting a potential role in cholesterol metabolism. Although these results indicate conserved steroidogenic functions for FTZ-F1 among vertebrates, they also raise the question of additional roles for FTZ-F1 in teleosts.

gene regulation, seasonal reproduction, steroid hormones

INTRODUCTION

Fushi tarazu factor-1 (FTZ-F1) is an orphan nuclear receptor that was initially described as an activator of the *Drosophila* segmentation gene *fushi tarazu* (ftz) [1, 2]. FTZ-F1 homologues have since been recognized in numerous species, and FTZ-F1 now constitutes a distinct family (NR5A) in the superfamily of nuclear receptors [3]. The FTZ-F1 family includes two major subgroups of related genes with separate functions and expression patterns among higher vertebrates. The NR5A2 subgroup contains the liver receptor hormone-1 (LRH-1) or α-feto protein transcription factor (FTF), which regulates the expression of the α-feto protein [4] and is involved in cholesterol metabolism [5] in mammals. The NR5A1 subgroup contains the steroidogenic factor-1 (SF-1) or adrenal 4-binding protein (Ad4BP) genes. SF-1 is an important regulator of steroidogenesis in mammals because it controls the transcription of several cytochrome P450 (CYP) enzymes involved in steroidogenic pathways [6, 7]. The mechanism of FTZ-F1 function in lower vertebrates such as amphibians and fish have not been completely elucidated. The expression patterns of teleost FTZ-F1 homologues partly correlate to mammalian patterns [8, 9], but their roles in reproductive mechanisms have not been determined.

SF-1 knockout mice develop without steroidogenic tissues and lack expression of a number of components involved in steroidogenesis [10, 11], of which one is the steroidogenic acute regulatory protein (StAR) [12]. StAR is a mitochondrial phosphoprotein that delivers cholesterol to the inner mitochondrial membrane, where steroid hormone biosynthesis is initiated. Mammalian expression of StAR is restricted to steroidogenic tissues and is rapidly induced by trophic hormones and cAMP [13]. A targeted disruption of the mouse gene that codes for StAR resulted in lower steroid levels, confirming its essential role in steroidogenesis [12]. Regulation of murine StAR gene expression is dependent on SF-1 [14]. In humans, SF-1 has been shown to interact with Sp-1 and cAMP in the regulation of StAR gene expression [15]. A recently isolated zebrafish StAR cDNA revealed high sequence similarity to other vertebrate StAR homologues [16]. However, a potential conservation of StAR expression and function between mammals and teleosts has not yet been determined.

Once StAR has delivered cholesterol to the inner membrane of the mitochondria, the initial step in steroid hormone biosynthesis is the conversion of cholesterol to pregnenolone, which is carried out by cholesterol side-chain cleavage P450 (CYP11A). Mammalian CYP11A genes are transcriptionally regulated by SF-1 in vitro [17–19], and SF-1 expression precedes CYP11A expression during gonadal development [20]. However, SF-1 knockout mice still express CYP11A [11]. In lower vertebrates, CYP11A has been identified only in rainbow trout (*Oncorhynchus mykiss*) [21], southern stingray (*Dasyatis americana*) [22], and channel catfish (*Ictalurus punctatus*; GenBank accession number AF063836), and function and expression patterns have been poorly investigated. Following conversion, pregnenolone is further modified into steroid hormones in the cytosol. One of the key enzymes in the early conversion steps is 3β-hydroxysteroid dehydrogenase (3β-HSD). SF-1 is a crucial component in steroidogenesis and regulates a majority of the enzymes that govern steroid biosynthesis in gonads and adrenal glands. 17β-Estradiol (E_2) treatment has been implicated in repressing SF-1 expression in mammals [23] and reptiles [24], and if the functions of FTZ-F1

[1]This work was funded by the Swedish Agriculture and Forestry Research Council and the Kempe Foundation, Sweden.
[2]Correspondence: Per-Erik Olsson, Department of Natural Sciences, Örebro University, SE-701 82, Sweden. FAX: 46 19 303566; e-mail: per-erik.olsson@nat.uru.se
[3]Current address: Department of Human Genetics, Mount Sinai School of Medicine, 1425 Madison Avenue, New York, NY 10029.

Received: 4 March 2002.
First decision: 31 March 2002.
Accepted: 8 May 2002.
© 2002 by the Society for the Study of Reproduction, Inc.
ISSN: 0006-3363. http://www.biolreprod.org

Downloaded from https://academic.oup.com/biolreprod/article/67/4/1297/2683675 by guest on 19 April 2024

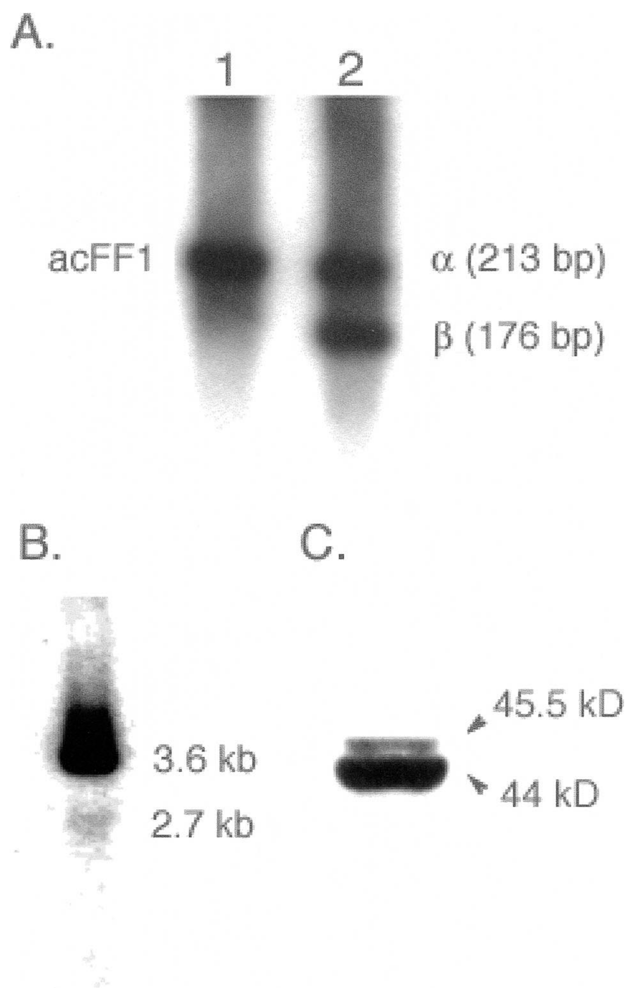

FIG. 1. Arctic char possess two FTZ-F1 homologues. **A**) Digestion with *Bst*ZI (C↓GGCCG) distinguishes between FTZ-F1α and FTZ-F1β. Total RNA extracted from Arctic char testis was reverse transcribed and amplified by PCR using oligonucleotides designed against vertebrate FTZ-F1 sequences. The amplified product was fractionated by electrophoresis through a 2% (w/v) agarose gel before (1) and after (2) *Bst*ZI digestion. **B**) Two acFF1 transcripts (3.6 and 2.7 kb) detected by Northern hybridization with [α^{32}P]dCTP-radiolabeled cDNA. **C**) The acFF1 proteins (44 and 45.5 kDa) were detected using an FF1 primary antibody (rabbit), and a horseradish-coupled anti-rabbit secondary antibody, and were visualized with enhanced chemiluminescence.

homologues are conserved among vertebrates, such a repression may be involved in the feminization observed in salmonid fish following E_2 treatment [25, 26]. Modulation of sex ratios and steroidogenic activity by hormone treatments have been studied previously in salmonid fish, and E_2 has been proven to be a powerful feminizing agent in these studies [25, 26]. The mechanism underlying this effect has, however, not been elucidated.

Because the mechanisms of teleost FTZ-F1 during reproduction and in steroidogenesis are poorly understood, our aim was to determine whether teleost FTZ-F1 is linked to reproductive maturation and whether it is under hormonal regulation. In order to study the expression of components involved in the initial steps of steroidogenesis we isolated partial FTZ-F1, StAR, CYP11A, and 3βHSD cDNA homologues from Arctic char (*Salvelinus alpinus*). Using these, we determined the tissue expression patterns of the FTZ-F1 genes and compared them with the expression of the putative downstream genes. Thereafter, we determined their expression during reproductive maturation. Finally, in order to achieve a better understanding of the role of these genes, we analyzed their regulation in response to steroid-hormone treatment.

MATERIALS AND METHODS

Arctic Char Maintenance

Adult Arctic char were kept in 1600-L aquaria supplied with a continuous flow of aerated water at a temperature and a photoperiod that followed natural conditions. The fish had ad libitum access to commercial trout food. The Ethical Review Committee in Umeå, Sweden, approved the use of animals for all studies described in this report (approvals A83-98 and A75-01).

Isolation of Arctic Char FF1 homologues, StAR, CYP11A, and 3βHSD

Total RNA was extracted from Arctic char testes using TRI reagent (Sigma-Aldrich, Tyresö, Sweden) and treated with 1 unit of DNAse I (Promega, Madison, WI) for 1 h at 37°C. Thereafter, cDNA was synthesized from 1 μg of total RNA using 500 ng of random hexamer primer (Promega) and the SuperScript reverse transcriptase kit (Gibco BRL, Täby, Sweden). Amplification was performed using forward and reverse oligonucleotides based on conserved regions of vertebrate FTZ-F1 sequences. For FF1 the primers were 5′-TGTAAGGGCTTCTTCAAGCGC-3′ (forward), and 5′-GGAGAACAGTGTCTGGTCAGCC-3′ (reverse 1). Amplification was performed at 95°C for 30 sec, 54°C for 30 sec, and 72°C for 1 min for a total of 40 cycles. Independent detection of Arctic char FF1α (acFF1α) and acFF1β was made possible due to the presence of a *Bst*ZI restriction site in the acFF1β sequence. For CYP11A, the primers were 5′-GACTGCATCTCTCTGATG-3′ (forward), and 5′-CTCCACCT-GACGCTGTTT-3′ (reverse 1). Amplification was performed at 95°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min for a total of 40 cycles. For 3βHSD, the primers were 5′-GTGTGTGTGGTRACAGGAGC-3′ (forward) and 5′-RTGTAGTARAAGTTYCCWCC-3′ (reverse). Amplification was performed at 95°C for 30 sec, 52°C for 45 sec, and 72°C for 1 min for a total of 40 cycles. All amplified products were ligated into pGEM-T (Promega) and recombinant plasmids were isolated using the Wizard *Plus* SV Miniprep System (Promega). Cycle sequencing was performed using the DYEnamic ET Terminator Cycle Sequencing Premix Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The reactions were resolved on an ABI Prism 377 DNA Sequencer (Perkin Elmer, Upplands Väsby, Sweden) and the data obtained were analyzed using EditView version 1.0.1 (Perkin Elmer). These clones were employed for generating probes for all subsequent analyses. The sequence data have been deposited in the GenBank database under the following accession numbers: acFF1α, AF468978; acFF1β, AF468977; StAR, AF468973; CYP11A, AF468974; 3βHSD, AF468975; and 18S rRNA, AF469620.

Northern Blot Analysis

Northern analyses of poly(A)$^+$ RNA (3 μg) extracted from Arctic char testes by the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech) were performed according to the method described by Sambrook et al. [27]. Membranes were probed using random primed [α-^{32}P]dCTP-radiolabeled cDNAs encoding Arctic char FF1β, which was isolated and sequenced as described above. Hybridizations were performed at 50°C O/N (6× SSC, 0.1% SDS [w/v], 100 μg ml^{-1} tRNA, and 5× Denhardt solution). The membranes were washed twice for 30 min at 37°C and at 50°C in 1× SSC/0.1% SDS (w/v), and hybridizing signals were exposed to Hyperfilm-MP film (Amersham Pharmacia Biotech) at −70°C. The films were visualized using a Curix 60 Film Developer (AGFA-Gevaert AB, Kista, Sweden).

Tissue Distribution of Messenger RNA

Total RNA was extracted from male (n = 4) and female (n = 4) Arctic char tissues using TRI reagent and processed for polymerase chain reaction (PCR) amplification. For FF1α and FF1β, the primers were 5′-TGTAAG-GGCTTCTTCAAGCGC-3′ (forward 1), and 5′-CCGGTCTCGCTTGTA-CATGGG-3′ (reverse 2). Amplification was performed at 95°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec for a total of 40 cycles, followed by digestion with *Bst*ZI at 50°C for 2 h (Fig. 1A). StAR and CYP11A were amplified as described above. 18S rRNA was amplified using Classic Internal Standard Primers (Ambion Inc., Austin, TX) according to the man-

Downloaded from https://academic.oup.com/biolreprod/article/67/4/1297/2683675 by guest on 19 April 2024

ufacturer's instructions. All PCR products were fractionated by electorphoresis on 2% (w/v) agarose gels in 1× TAE buffer and transferred onto Hybond-Nfp membranes (Amersham Pharmacia Biotech) by capillary transfer in 20× SSC according to the method described by Sambrook et al. [27]. Hybridizations were performed using random primed [α-^{32}P]dCTP-labeled cDNA at 65°C O/N (6× SSC, 0.1% SDS [w/v], 100 μg/ml^{-1} tRNA, and 5× Denhardt solution). The membranes were washed twice for 30 min at 42°C and 65°C in 0.1× SSC/0.1% SDS (w/v) and exposed to Hyperfilm-MP film (Amersham Pharmacia Biotech) at −70°C. The films were visualized using a Curix 60 Film Developer (AGFA-Gevaert AB). Polymerase chain reactions from the most representative male and female were transferred onto membranes, and were probed and detected as described above. Tissues were sampled in April.

Antibody Production

A peptide sequence (MYKRDRALKQQKKAL) located in the FTZ-F1 box of the acFF1 protein was synthesized, linked to a BSA carrier, and used to create an immune response in rabbit (Agrisera, Vännäs, Sweden). The rabbit was immunized three times, and serum was collected and stored at −80°C until used further. The antibody was evaluated for specificity and sensitivity using Western blot and ELISA techniques. An antibody dilution of 1:1000 was used to detect peptide quantities as little as 3 ng.

Western Blot Analysis

Protein preparations from various tissues were obtained by homogenization of 50 mg of tissue in 500 μl of sample buffer containing 63 mM Tris pH 6.8, 10% glycerol, 17.5% SDS, and 5% β-mercaptoethanol. Samples were boiled for 1 min and centrifuged at 3000 × g for 5 min to separate protein and debris. The supernatants were collected, and protein concentrations were decided by the Lowry method. Ten micrograms of total protein from each tissue, male and female respectively, were run on 12% SDS-PAGE gels and transferred to a polyvinylidene difluoride membrane. The membranes were blocked in 3% powdered milk for 1 h before adding primary antibody 1:1000 in blocking solution for 5 h at 4°C. After primary antibody incubation, the membranes were washed three times for 15 min in Tris-buffered saline-Tween 20 (TBST) before adding secondary horseradish peroxidase-coupled anti-rabbit antibody (Amersham Pharmacia Biotech), diluted 1:2500. After three 15-min subsequent washes in TBST the Western blots were developed using enhanced chemiluminescence Western blotting detection reagent (Amersham Pharmacia Biotech) and exposed to Hyperfilm-MP film (Amersham Pharmacia Biotech). The films were visualized using a Curix 60 Film Developer (AGFA-Gevaert AB).

Semiquantitative Reverse Transcription-PCR Analyses on Hormone Treatment and Reproductive Maturation

Juvenile Arctic char (20–50 g) were injected i.p. with 11-ketotestosterone (11-KT), testosterone (T), and E_2 dissolved in peanut oil (1.0 mg/kg). Control fish remained uninjected or were injected with peanut oil. After 4 days (96 h), the fish were killed and dissected. The tissues removed were kept at −70°C for RNA preparation. The sex of each fish was determined by examining their excised gonads with a microscope before freezing. Each test group contained at least four animals of each sex. Total RNA was extracted, cDNA was synthesized, and reverse transcription (RT)-PCR was conducted as described above. The semiquantitative analyses were carried out as previously described [9] and the number of cycles was determined to be 18 for 18S, 26 for FF1α/β, 40 for CYP11A, 40 for StAR, and 38 for 3βHSD for logarithmic amplifications. The PhosphorImager quantifications were made using a Storm 860 (Molecular Dynamics, Sunnyvale, CA) and analyzed with Mulitquant software (Molecular Dynamics). For semiquantitative mRNA measurements during reproductive maturation, four males and four females were sampled at five time points (n = 4) from March until November. and analyzed as described above.

Enzyme-Linked Immunosorbent Assay

Levels of acFF1 in gonads were determined by ELISA during reproductive maturation. The protein was prepared and quantified as described above. An excess of total protein (5 μg) in Na_2CO_3 buffer (pH 9.6) was used to coat the 96 wells of a MaxiSorp plate (Nalge Nunc International, Roskilde, Denmark). The wells were blocked for 1 h with 3% milk powder in PBS before adding the FF1 antibody (1:1000) in PBS. Incubations of primary antibody were conducted at 4°C for 4 h. Horseradish peroxidase secondary antibodies (Amersham Pharmacia Biotech) were added (1:2000) in PBS, followed by a color reaction with 1-Step Turbo TMB-ELISA (Pierce, Rockford, IL) and analysis in a spectrophotometrical plate reader at 450 nm. Four males and four females were used for each of four sampling points, and each sample was loaded in triplicate. The molar quantification was accomplished by comparing absorbance to a peptide standard curve. A triplicate negative control (muscle tissue) was used to exclude background cross-reaction.

Hormone Assays

Plasma levels of E_2 were determined by radioimmunoassay (Coat-a-Count, DPC, Los Angeles, CA). Triplicate measurements were made for each group, except for the first group, for which single measurements were made. Plasma levels of 11-KT were determined by ELISA using an 11-KT Kit (Biosense, Bergen, Norway).

Statistics

Data from all quantitative analyses are shown as means ± SEM. Analysis of variance was applied to each series of data followed by the Student t-test determination of significance. In the figures, significance is illustrated as *$P < 0.1$ or **$P < 0.01$.

RESULTS

Arctic char FTZ-F1, StAR, CYP11A, and 3βHSD mRNA sequences were amplified by RT-PCR from testicular tissue. Sequence analyses of several independent clones demonstrated that Arctic char possess two FTZ-F1 variants (designated acFF1α and acFF1β; Fig. 1A). The partial cDNAs were both 783 base pairs (bp) in length and exhibited a 95% identity to each other at both the nucleotide and amino acid levels. Analysis of testicular poly(A)+ RNA indicated that acFF1 mRNA consists of multiple transcripts. Gene products of 3.6 kilobases (kb) and 2.7 kb were detected (Fig. 1B). The approximate sizes of the two proteins detected with the acFF1 antibody were 44 and 45.5 kDa (Fig. 1C), which is similar to FTZ-F1 proteins in other species.

A single gene transcript of StAR was detected both by sequencing clones and by Northern blot analysis. The partial cDNA obtained by RT-PCR was 754 bp, and the mRNA detected in Northern analyses indicated that the full-length message was 1.8 kb (data not shown). A single type of CYP11A was also generated by RT-PCR. This cDNA fragment was 769 bp. Northern blot analysis of CYP11A showed two mRNAs of 1.4 kb and 1.2 kb (data not shown).

Tissue Distribution of acFF1, StAR, and CYP11A

Complementary DNA from gonads, liver, and head kidney were examined by PCR to determine the tissue distribution of acFF1α, acFF1β, StAR, and CYP11A. Expression of acFF1α and acFF1β was detected in all tissues examined, in both sexes (Fig. 2A). Individual differences between fish were observed; however, the most representative fish of each sex was used to illustrate tissue distribution. Expression of StAR was detected in gonads, head kidney, and liver in both sexes (Fig. 2A). The tissue distribution of CYP11A was restricted to gonads and head kidney in male and female fish alike (Fig. 2A).

The Arctic char FF1 antibody detected strong signals in ovary, testis, head kidney, and liver (Fig. 2B). The preserum incubated control did not show any immunoreactivity.

Gonadal Expression of FF1α/β, StAR, and CYP11A During Reproductive Maturation

Arctic char ovaries and testes were sampled at several time points during the reproductive maturation cycle. The

Downloaded from https://academic.oup.com/biolreprod/article/67/4/1297/2683675 by guest on 19 April 2024

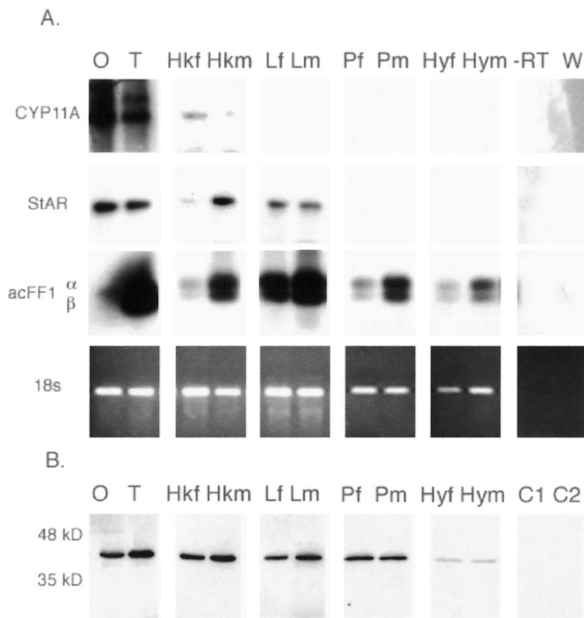

FIG. 2. Tissue distribution of acFF1α, acFF1β, StAR, and CYP11A mRNA and acFF1 protein. Tissues that were examined included O, ovary; T, testis; Hk, head kidney; L, liver; P, pituitary; Hy, hypothalamus; and Br, brain. A male is indicated by "m" and a female by "f" after the tissue abbreviation. **A**) Messenger RNA detected by RT-PCR after hybridization with [α^{32}P]dCTP-radiolabeled cDNAs. **B**) The acFF1 proteins (44 and 45.5 kDa) were detected using an FF1 primary antibody (rabbit) and a horseradish-coupled anti-rabbit secondary antibody. and were visualized with enhanced chemiluminescence.

relative levels of FF1α/β, CYP11A, and StAR mRNA were measured by semiquantitative RT-PCR. Gonadal expressions of acFF1α and acFF1β mRNA were higher in July, in parallel with a higher gonad-somatic index (GSI; Table 1) at the onset of reproductive maturation (Fig. 3, A and B). Although testicular levels of both acFF1α and acFF1β remained elevated during September and November, ovarian acFF1 levels declined during the later part of maturation (Fig. 3, A and B). The gonadal transcription of StAR mRNA was higher in both sexes during reproductive maturation (Fig. 3C). However, although a small increase in StAR levels was observed in males in July and it peaked in November, levels in females increased drastically in September and remained high in November. CYP11A levels increased in July and decreased in November (Fig. 3D). Although higher ovarian levels of CYP11A were observed in July and September, only testicular levels showed a statistically significant difference. This was due to high individual differences in ovarian samplings.

The relative levels of acFF1 protein during the reproductive maturation period were determined by ELISA. To achieve a higher resolution during the prespawning period, an additional sampling, conducted in August, was used in this analysis. The acFF1 content per microgram of total protein was high during the early part of the reproductive maturation cycle in both sexes (Table 1). During the later part of the reproductive cycle, the increase in protein content in gonads was mostly due to larger oocyte size and higher sperm number, which also accounted for a large part of the increase in the GSI. Taking GSI into consideration, significant increases in acFF1 levels were observed in testicular tissue in August (Fig. 3E). In females, acFF1 levels were higher in July, they peaked in August, and then declined during the later part of reproductive maturation. The general pattern was similar in both sexes, although levels were higher in females than in males. This pattern was similar for both mRNA and protein levels, as is seen in Figure 3, A, B, and E.

Hormone Levels and Treatment

Plasma levels of E_2 in females and 11-KT in males were measured and compared with the GSI and liver-somatic index (LSI) (Table 1). In females, E_2 levels were slightly elevated between April and May, and significantly elevated between May and July. The high level persisted during September but it decreased in November. The pattern was congruent with the LSI pattern. GSI increased during the entire period and reached its highest level in November. In males, 11-KT levels increased in July, peaked in September, and declined in November. The increase in 11-KT coincided with the increase in testicular size and the decrease coincided with the end of spermatogenesis. Levels of E_2 in females and 11-KT in males were both up-regulated during early reproductive maturation, in parallel to the onset of an increasing GSI, and they were down-regulated at the end of reproductive maturation, when vitellogenesis and spermatogenesis were complete.

Gonads and head kidneys from juvenile fish treated with 11-KT, T, and E_2 were analyzed for alterations in mRNA expression levels of acFF1α, acFF1β, CYP11A, StAR, and 3βHSD by semiquantitative RT-PCR. No change in gonadal expression of acFF1α or acFF1β was observed in fish treated with 11-KT or T, whereas in males, both testicular

TABLE 1. Liver-somatic index, gonad-somatic index, plasma levels of 11-KT and E_2, and gonadal acFF1 levels during reproductive maturation.

Month	Sex	Liver-somatic index	Gonad-somatic index	11KT ng/ml	E2 pg/ml	acFF1 fmol/μg
April	M	1.03 (0.26)*	0.29 (0.10)	0.77 (0.58)	—†	28.3 (19.1)
May	M	1.57 (0.65)	0.26 (0.20)	1.15 (0.28)	—	52.7 (14.6)
July	M	1.06 (0.39)	2.66 ().51)	12.8 (1.3)	—	34.7 (21.0)
August	M	1.23 (0.48)	2.90 (0.90)	—	—	121.2 (22.9)
September	M	1.08 (0.36)	2.66 (0.37)	45.2 (10.8)	—	34.0 (34.0)
November	M	0.76 (0.21)	2.98 (0.18)	18.4 (5.4)	—	12.4 (7.2)
April	F	1.27 (0.61)	1.21 (0.46)	—	94.6 (12.7)	217.9 (45.0)
May	F	1.13 (0.22)	1.65 (0.21)	—	275.3 (66.5)	21.1 (14.5)
July	F	1.46 (0.89)	8.79 (4.82)	—	913.8 (288.0)	109.2 (18.6)
August	F	1.94 (0.38)	13.19 (3.44)	—	—	115.5 (23.6)
September	F	1.14 (0.77)	11.98 (5.28)	—	778.4 (412.1)	102.3 (28.8)
November	F	0.85 (0.24)	15.32 (2.90)	—	86.6 (14.1)	52.9 (14.6)

* Standard deviations are indicated in parentheses.
† — indicates no value was measured.

Downloaded from https://academic.oup.com/biolreprod/article/67/4/1297/2683675 by guest on 19 April 2024

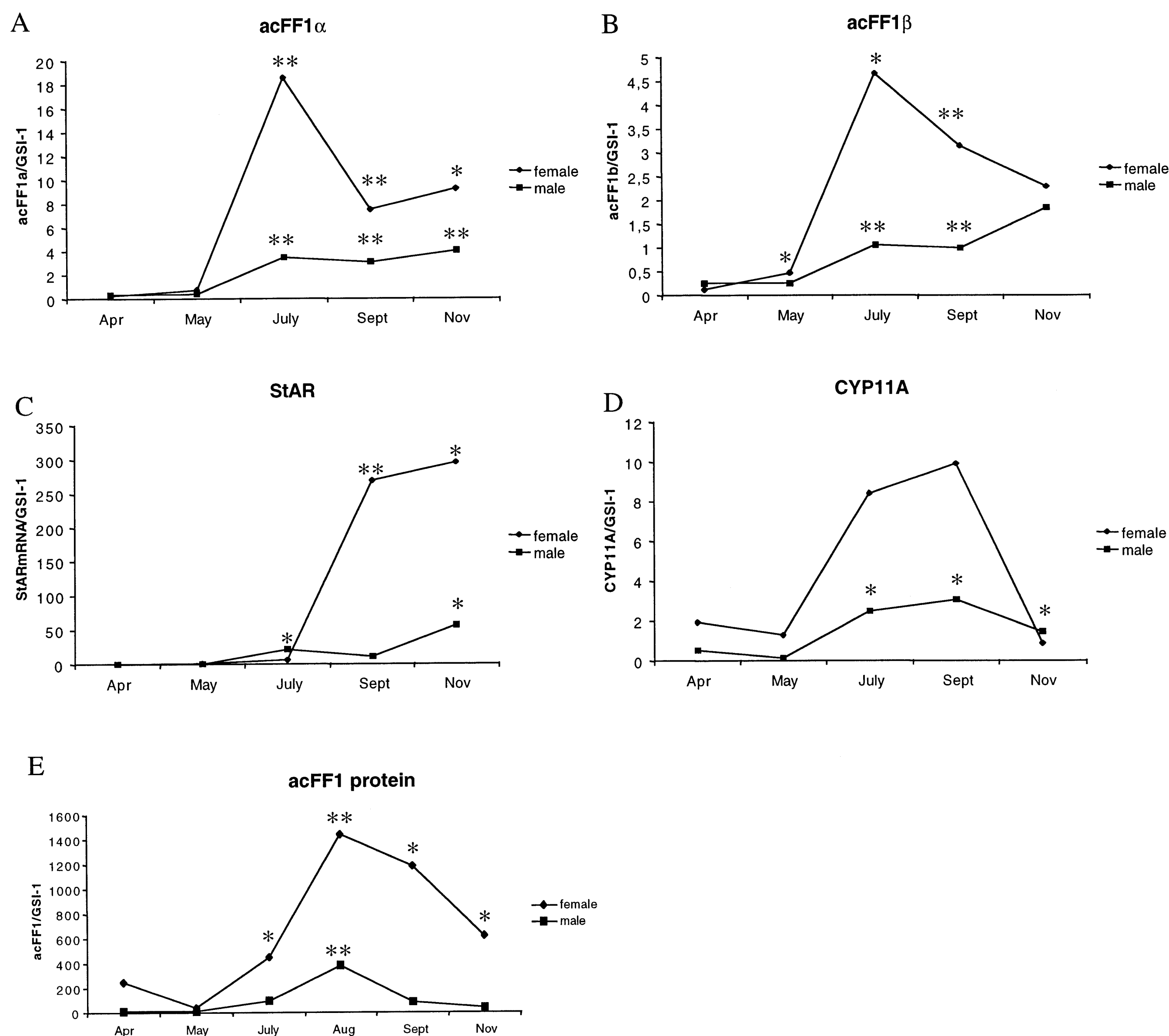

FIG. 3. Gonadal expression of acFF1α, acFF1β, StAR, and CYP11A during the reproductive cycle. Semiquantitative RT-PCR illustrates the mRNA expression of acFF1α (**A**), acFF1β (**B**), StAR (**C**), and CYP11A (**D**) relative to 18S expression and the GSI between April and November. **E**) Arctic char FF1 protein content in 1.0 μg of gonadal total protein correlated to the GSI during reproductive maturation. Statistically significant deviations from expression levels in April (May for CYP11A) are indicated by $*P < 0.1$ and $**P < 0.01$.

and head kidney acFF1α and acFF1β levels dropped following E_2 treatment (Fig. 4, A and B). In ovaries, none of the hormone treatments resulted in altered levels, although in females, levels in head kidney showed a lower trend during E_2 treatments.

Testicular CYP11A levels displayed a significant increase following 11-KT treatment (Fig. 4C), whereas ovarian expression remained unaffected. Treatment with 11-KT resulted in lower CYP11A levels in head kidney in both sexes (Fig. 4D). However, the decrease in males was not supported by statistical analysis due to the considerable individual differences in the male control group.

Similar to those of CYP11A, 3βHSD mRNA levels rose in testicular tissue after 11-KT treatment (Fig. 4E). The induction was not, however, statistically significant. Expression in head kidney also resembled that of CYP11A after 11-KT treatment, with lower levels in testis (Fig. 4F).

Gonadal StAR expression was low and undetectable in most fish in this experiment and did not show any altered expression following hormone treatments (data not shown). Levels in head kidney were detectable, but they did not appear to be influenced by the hormone treatments (Fig. 4G).

DISCUSSION

The present study demonstrates that expression levels of acFF1α and acFF1β mRNA and protein increase in the gonads of both sexes at the onset of reproductive maturation (Fig. 3). Up-regulation of acFF1 preceded the up-regulation of both StAR and CYP11A. All these genes showed more pronounced up-regulation in females than in males. Sex differences in the expression of enzymes involved in steroidogenesis have previously been observed during sex differentiation for aromatase (CYP19) and CYP11β, two enzymes involved in the final conversion of cholesterol to E_2

Downloaded from https://academic.oup.com/biolreprod/article/67/4/1297/2683675 by guest on 19 April 2024

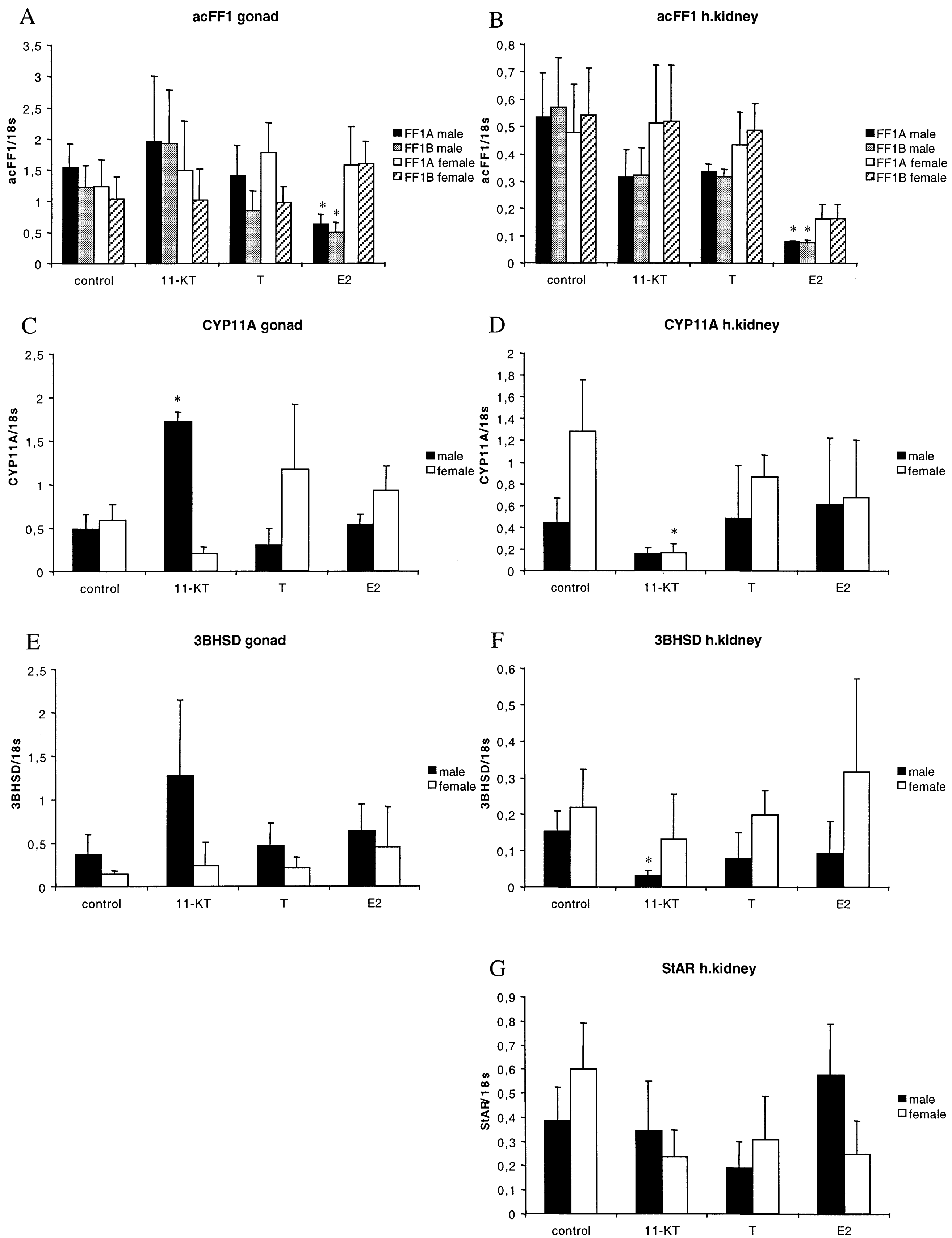

FIG. 4. Expression of acFF1α, acFF1β, StAR, CYP11A, and 3βHSD in response to hormone treatment. Juvenile fish were injected with 1.0 mg/kg 11-KT, testosterone, or 17β-estradiol, and examined for acFF1α, acFF1β, StAR, CYP11A, and 3βHSD mRNA expression by semiquantitative RT-PCR. **A**) Expression of acFF1α and acFF1β in gonads. **B**) Expression of acFF1α and acFF1β in head kidney. **C**) CYP11A expression in gonads. **D**) CYP11A expression in head kidney. **E**) 3βHSD expression in gonads. **F**) 3βHSD expression in head kidney. **G**) StAR expression in head kidney. Significant differences from control are indicated by $^*P < 0.1$.

Downloaded from https://academic.oup.com/biolreprod/article/67/4/1297/2683675 by guest on 19 April 2024

and 11-KT, respectively [28, 29]. This indicates that sex-specific regulatory pathways are involved in the steroidogenic process. However, during sex differentiation in rainbow trout, no significant differences in CYP11A, 3β-HSD, or CYP17 levels were observed [26], suggesting that sex differences in steroidogenesis are more prominent in adult fish.

The expression of both acFF1 mRNA and protein coincided with the highest plasma E_2 and 11-KT levels during the reproductive cycle (Table 1). Higher FTZ-F1 levels have been observed in the pituitaries of maturing male and female salmonids in response to GnRH [30, 31], and the correlation between elevated 11-KT plasma levels and acFF1 levels observed in the present study is in agreement with an earlier observation on sockeye salmon [30]. This indicates a link between acFF1, 11-KT, and E_2 in males and females, respectively, during the reproductive maturation process.

Zebrafish FF1a has been shown to regulate expression of the LHβ subunit in synergism with E_2 by binding to its gene promoter [32], indicating a mechanistic relation between teleost FTZ-F1 and E_2. Teleost FTZ-F1 has been put forward as a likely candidate that works upstream of several genes involved in steroidogenesis, and it has been suggested that teleost FTZ-F1 may be involved in feminization of developing testis in response to estrogen exposure [26]. The present study indicates that acFF1α and acFF1β levels are repressed in the testis, but not in the ovaries, following exposure to E_2. This has been previously observed in rats [23] and turtles [24], and could therefore be a conserved vertebrate FTZ-F1 function. Although E_2 resulted in down-regulation of acFF1, E_2 was observed to have no effect on CYP11A, 3βHSD, or StAR. Expression of testicular 3βHSD has been previously shown to be down-regulated by E_2 in an all male populations of rainbow trout during the period of sex differentiation [26]. However, in the same study, the researchers observed down-regulation of CYP11B and CYP17, whereas E_2 had no effect on CYP11A. Taken together, the differences observed in our study and the previous studies indicate that both sex and stage of life may affect the regulation of steroidogenic genes in teleosts.

Following 11-KT treatment, testicular CYP11A was up-regulated, whereas ovarian levels remained unaffected (Fig. 4C). A similar trend was also observed for 3βHSD, although it was not supported by the statistical analysis. Contrary to the effect seen in testis, the expression of CYP11A and 3βHSD was down-regulated by 11-KT in head kidney. Previous studies have shown that 11-KT suppresses cortisol levels in the interrenal cells in the head kidney of rainbow trout [33, 34]. These results suggest that higher 11-KT levels result in a general decrease in steroidogenic activity in head kidney. Although SF-1 has been indicated to regulate StAR in mammals, both ovarian CYP11A and StAR were down-regulated by E_2 in neonatal rats [35] without affecting SF-1 levels, suggesting that SF-1 is not directly involved in this down-regulation. The mechanism underlying this down-regulation remains to be determined. However, plasma E_2 and 11-KT levels may affect secretion of gonadotropins, leading to up- or down-regulation of StAR, CYP11A, or 3βHSD due to secondary effects after negative feedback on the hypothalamic-pituitary axis. In the present study, we did not observe a correlation between the down-regulation of acFF1 and downstream genes after hormone treatment. It is possible that this was due to the relatively short-term hormone exposure. Hence, the up-regulation of CYP11A expression in testis following 11-KT exposure may be independent of acFF1, or at least not due to greater transcription of acFF1 mRNA. Thus, the acFF1 isoforms, CYP11A and StAR, are all up-regulated during reproductive maturation, and acFF1, CYP11A, and 3βHSD are susceptible to hormonal treatment, indicating reproductive involvement.

The tissue distributions of CYP11A, StAR, and acFF1α and acFF1β were determined, and observed to coexpress in gonads and head kidney (Fig. 2A). Because these tissues are the main sites for conversion of cholesterol into steroid hormones, this colocalization supports a role for these enzymes in steroidogenesis. However, StAR transcripts were also detected in liver (Fig. 2A). No function for StAR action in liver has previously been suggested. Because the liver is involved in cholesterol metabolism and StAR is an intracellular transporter of cholesterol, the presence of StAR in the liver may indicate that StAR is involved in hepatic cholesterol metabolism. Although acFF1α and acFF1β were coexpressed with StAR in liver, they were also detected in the brain tissues both as mRNA and proteins. Studies with mammals have shown that SF-1 regulates high-density lipoprotein (HDL) receptor scavenger receptor class B type I [36, 37]. HDL plays a critical role in cholesterol metabolism and has been suggested to deliver cholesteryl esters to the liver and steroidogenic tissues. The expression of acFF1 and StAR in liver may indicate that these functions of SF-1 are conserved in teleosts.

In summary, the present study shows sex-specific regulation of teleost FTZ-F1 and steroidogenic genes in response to hormone treatment. A link between acFF1, StAR, and CYP11A expression in the reproductive maturation process is also suggested, as elevated acFF1 mRNA and protein levels preceded greater transcription of both StAR and CYP11A. Studies of mammals have shown that both CYP11A and StAR are regulated by SF-1 [13–15, 38, 39]. They are also expressed to a greater degree during follicle maturation [40], similar to the observations made in the present study. Promoter studies are needed to establish whether the up-regulation of StAR and CYP11A are due to interactions with acFF1, because both genes may be regulated by gonadotropins as well. Other researchers have shown down-regulation of several steroidogenic enzymes in male rainbow trout testis following E_2 treatment [26]. Although we did not observe that effect in this study, α and β forms of acFF1 were down-regulated and may account for downstream effects, either through indirect actions on the hypothalamus and pituitary, or directly through promoter binding. Several in vivo and in vitro studies are needed in order to understand the delicate regulation of teleost steroidogenesis.

Downloaded from https://academic.oup.com/biolreprod/article/67/4/1297/2683675 by guest on 19 April 2024

ACKNOWLEDGMENTS

We acknowledge Dr. Liselotte Westerlund for her advise on oligo construction and Håkan Berg for E_2 measurements.

REFERENCES

1. Ueda H, Sonoda S, Brown JL, Scott MP, Wu C. A sequence-specific DNA-binding protein that activates *fushi tarazu* segmentation gene expression. Genes Dev 1990; 4:624–635.
2. Lavorgna G, Ueda H, Clos J, Wu C. FTZ-F1, a steroid hormone receptor-like protein implicated in the activation of *fushi tarazu*. Science 1991; 252:848–851.
3. Nuclear Receptors Committee. A unified nomenclature system for the nuclear receptor superfamily. Cell 1999; 97:161–163.
4. Galarneau L, Paré JF, Allard D, Hamel D, Levesque L, Tugwood JD,

Green S, Belanger L. The alpha$_1$-fetoprotein is activated by a nuclear receptor of the *Drosophila* FTZ-F1 family. Mol Cell Biol 1996; 16: 3853–3865.

5. Nitta M, Ku S, Brown C, Okamoto AY, Shan B. CPF: an orphan nuclear receptor that regulates liver-specific expression of the human cholesterol 7α-hydroxylase gene. Proc Natl Acad Sci U S A 1999; 96:6660–6665.
6. Lala DS, Rice DA, Parker KL. Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of *fushi tarazu*-factor I. Mol Endocrinol 1992; 6:1249–1258.
7. Ikeda Y, Lala DS, Luo X, Kim E, Moisan MP, Parker KL. Characterization of the mouse FTZ-F1 gene, which encodes a key regulator of steroid hydroxylase gene expression. Mol Endocrinol 1993; 7:852–860.
8. Lin WW, Wang HH, Sum C, Liu D, Hew C, Chung B. Zebrafish ftz-f1 gene has two promoters, is alternatively spliced, and is expressed in digestive organs. Biochem J 2000; 348:439–446.
9. von Hofsten J, Jones I, Karlsson J, Olsson PE. Developmental expression patterns of FTZ-F1 homologues in Zebrafish (*Danio rerio*). Gen Comp Endocrinol 2001; 121:146–155.
10. Luo X, Ikeda Y, Parker KL. A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell 1994; 77:481–490.
11. Sadovsky Y, Crawford PA, Woodson KG, Polish JA, Clements MA, Tourtellotte LM, Simburger K, Milbrandt J. Mice deficient in the orphan receptor steroidogenic factor 1 lack adrenal glands and gonads but express P450 side-chain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids. Proc Natl Acad Sci U S A 1995; 92:10939–10943.
12. Caron KM, Clark BJ, Ikeda Y, Parker KL. Steroidogenic factor 1 acts at all levels of the reproductive axis. Steroids 1997; 62:53–56.
13. Christenson LK, Strauss III JF. Steroidogenic acute regulatory protein (StAR) and the intramitochondrial translocation of cholesterol. Biochim Biophys Acta 2000; 1529:175–187.
14. Caron KM, Ikeda Y, Soo SC, Stocco DM, Parker KL, Clark BJ. Characterization of the promoter region of the mouse gene encoding for the steroidogenic acute regulatory protein. Mol Endocrinol 1997; 11: 138–147.
15. Sugawara T, Saito M, Fujimoto S. Sp1 and SF-1 interact and cooperate in the regulation of human steroidogenic acute regulatory protein gene expression. Endocrinology 2000; 141:2895–2903.
16. Bauer MP, Bridgham JT, Langenau DM, Johnson AL, Goetz FW. Conservation of steroidogenic acute regulatory (StAR) protein structure and expression in vertebrates. Mol Cell Endocrinol 2000; 168:119–125.
17. Rice DA, Kirkman MS, Aitken LD, Mouw AR, Schimmer BP, Parker KL. Analysis of the promoter region of the gene encoding mouse cholesterol side-chain cleavage enzyme. J Biol Chem 1990; 265: 11713–11720.
18. Clemens JW, Lala DS, Parker KL, Richards JS. Steroidogenic factor-1 binding and transcriptional activity of the cholesterol side-chain cleavage promoter in rat granulosa cells. Endocrinology 1994; 134: 1499–1508.
19. Takayama K, Morohashi K, Honda S, Hara N, Omura T. Contribution of Ad4BP, a steroidogenic cell-specific transcription factor, to regulation of the human CYP11A and bovine CYP11B genes through their distal promoters. J Biochem 1994; 116:193–203.
20. Hatano O, Takayama K, Imai T, Waterman MR, Takakusu A, Omura T, Morohashi K. Sex-dependent expression of a transcription factor, Ad4BP, regulating steroidogenic P-450 genes in the gonads during prenatal and postnatal rat development. Development 1994; 120: 2787–2797.
21. Takahashi M, Tanaka M, Sakai N, Adachi S, Miller WL, Nagahama Y. Rainbow trout ovarian cholesterol side-chain cleavage cytochrome P450 (P450scc). cDNA cloning and mRNA expression during oogenesis. FEBS Lett 1993; 319:45–48.
22. Nunez S, Trant JM. Isolation of the putative cDNA encoding cholesterol side chain cleavage cytochrome P450 (CYP11A) of the southern stingray (*Dasyatis americana*). Gene 1997; 187:123–129.
23. Majdic G, Sharpe RM, Saunders PTK. Maternal oestrogen/xenoestrogen exposure alters expression of steroidogenic factor-1 (SF-1/Ad4BP) in the fetal rat testis. Mol Cell Endocrinol 1997; 127:91–98.
24. Fleming A, Crews D. Estradiol and incubation temperature modulate regulation of steroidogenic factor 1 in the developing gonad of the red-eared slider turtle. Endocrinology 2001; 142:1403–1411.
25. Guiguen Y, Barollier JF, Ricordel MJ, Iseki K, McMeel OM, Martin SA, Fostier A. Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (*Oncorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*). Mol Reprod Dev 1999; 54:154–162.
26. Govoroun M, McMeel OM, Mecherouki H, Smith TJ, Guiguen Y. 17β-Estradiol treatment decreases steroid enzyme messenger ribonucleic acid levels in the rainbow trout testis. Endocrinology 2001; 142: 1841–1847.
27. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual, 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.
28. Govoroun M, McMeel OM, D'Cotta H, Ricordel MJ, Smith T, Fostier A, Guiguen Y. Steroid enzyme gene expressions during natural and androgen-induced gonadal differentiation in the rainbow trout, *Oncorhynchus mykiss*. J Exp Zool 2001; 290:558–566.
29. Trant JM, Gavasso S, Ackers J, Chung BC, Place AR. Developmental expression of cytochrome P450 aromatase genes (CYP19a and CYP19b) in zebrafish fry (*Danio rerio*). J Exp Zool 2001; 290:475–483.
30. Higa M, Ando H, Urano A. Expression of *fushi tarazu* factor 1 homolog and pit-1 genes in the pituitaries of pre-spawning chum and sockeye salmon. Comp Biochem Physiol B 2001; 129:503–509.
31. Higa M, Kanda H, Kitahashi T, Ito M, Shiba T, Ando H. Quantitative analysis of *fushi tarazu* factor 1 homolog messenger ribonucleic acids in the pituitary of salmon at different prespawning stages. Biol Reprod 2000; 63:1756–1763.
32. Liu D, Le Drean Y, Ekker M, Xiong F, Hew CL. Teleost FTZ-F1 homolog and its splicing variant determine the expression of the salmon gonadotropin IIβ subunit gene. Mol Endocrinol 1997; 11:877–890.
33. Young G, Thorarensen H, Davie PS. 11-Ketotestosterone suppresses interrenal activity in rainbow trout (*Oncorhynchus mykiss*). Gen Comp Endocrinol 1996; 103:301–307.
34. Pottinger TG, Carrick TR, Huges SE, Balm PHM. Testosterone, 11-ketotestosterone, and estradiol-17β modify baseline and stress-induced interrenal and corticotropic activity in trout. Gen Comp Endocrinol 1996; 104:284–295.
35. Ikeda Y, Nagai A, Ikeda MA, Hayashi S. Neonatal estrogen exposure inhibits steroidogenesis in the developing rat ovary. Dev Dyn 2001; 221:443–453.
36. Cao G, Garcia CK, Wyne KL, Schultz RA, Parker KL, Hobbs HH. Structure and localization of the human gene encoding SR-BI/CLA-1. Evidence for transcriptional control by steroidogenic factor 1. J Biol Chem. 1997; 272:33068–33076.
37. Rigotti A, Trigatti BL, Penman M, Rayburn H, Herz J, Krieger M. A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. Proc Natl Acad Sci U S A 1997; 94: 12610–12615.
38. Hu MC, Chiang EF, Tong SK, Lai W, Hsu NC, Wang LC, Chung BC. Regulation of steroidogenesis in transgenic mice and zebrafish. Mol Cell Endocrinol 2001; 171:9–14.
39. Hu MC, Hsu NC, Pai CI, Wang CK, Chung BC. Functions of the upstream and proximal steroidogenic factor-1 (SF-1)-binding sites in the CYP11A1 promoter in basal transcription and hormonal response. Mol Endocrinol 2001; 15:812–818.
40. Garmey JC, Guthrie HD, Garrett WM, Stoler MH, Veldhuis JD. Localization and expression of low-density lipoprotein receptor, steroidogenic acute regulatory protein, cytochrome P450 side-chain cleavage and P450 17-α-hydroxylase/C17–20 lyase in developing swine follicles: in situ molecular hybridisation and immunocytochemical studies. Mol Cell Endocrinol 2000; 170:57–65.

Downloaded from https://academic.oup.com/biolreprod/article/67/4/1297/2683675 by guest on 19 April 2024