

Effects of sex steroids on gonadotropin (FSH and LH) regulation in coho salmon (*Oncorhynchus kisutch*)

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ABSTRACT

The effect of steroid hormone treatment on coho salmon (*Oncorhynchus kisutch*) was examined. The cDNAs for coho salmon FSH β and LH β subunits were cloned and sequenced using reverse transcriptase PCR. Northern blot analysis revealed that a single transcript of 1 kb for each of these subunits was present in the pituitaries of vitellogenic and spermiating coho salmon. RNase protection assays (RPAs) were developed to quantify FSH β and LH β subunit transcript levels. For the RPAs, antisense RNA probes and sense RNA standards were prepared from a region of the cDNAs which spanned the signal peptide and a portion of the mature protein. These RPAs were used to examine the effects of exogenous steroids including testosterone, estradiol-17 β (E2) and 17 α ,20 β -dihydroxy-4-pregnen-3-one (17 α ,20 β -P) *in vivo*, in coho salmon at three time points during the spring period of gonadal growth when plasma levels of FSH are increasing. Both testosterone and E2 increased steady state mRNA levels of LH β , whereas E2

decreased steady state mRNA levels of FSH β in one experiment. Thus, the RPAs were able to detect changes in steady state mRNA levels in response to exogenous steroid treatment. Plasma and pituitary levels of FSH and LH were also measured using RIA. Throughout the experimental series, FSH plasma levels decreased in response to exogenous testosterone and E2 administration, while 17 α ,20 β -P had no effect on FSH plasma levels. Plasma LH levels were not detected throughout the course of the experiment. Pituitary LH increased in response to testosterone and E2, while pituitary FSH levels did not change. 17 α ,20 β -P had no effect on pituitary FSH or LH content during the experiment. Thus, regulation of the gonadotropins in coho salmon occurs at both the transcriptional as well as the translational level. Testosterone and E2 appear to have negative feedback effects on FSH, but positive feedback on LH.

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INTRODUCTION

The vertebrate pituitary produces a family of chemically related glycoproteins including follicle-stimulating hormone (FSH), luteinizing hormone (LH) and thyroid-stimulating hormone (TSH) (Pierce & Parsons 1981). In salmonids, two types of gonadotropins, GTH I and GTH II have been purified (Itoh *et al.* 1988, Suzuki *et al.* 1988*b,c*, Kawauchi *et al.* 1989, Sekine *et al.* 1989, Swanson *et al.* 1991, Govoroun *et al.* 1997) and their cDNA structures have been determined (Trinh *et al.* 1986, Sekine *et al.* 1989, Gen *et al.* 1993, Kato *et al.* 1993). Salmon gonadotropins are structurally similar to tetrapod FSH and LH, and are heterodimers composed of an

α and a unique β subunit. In salmon, the GTH I heterodimer is composed of a I β subunit and either an α 1 or an α 2 subunit. GTH II, on the other hand, is composed of an α 2–II β heterodimer. GTH I and GTH II have been identified in other teleost species such as carp (*Cyprinus carpio*) (Van der Kraak *et al.* 1992), red seabream (*Pagrus major*) (Tanaka *et al.* 1993), bonito (*Katsuwonus plelamis*) (Koide *et al.* 1993) and killifish (*Fundulus heteroclitus*) (Lin *et al.* 1992). In other fish, such as African catfish (*Clarias gariepinus*), European eel (*Anguilla anguilla*) and some cyprinids, only GTH II has been identified (reviewed by Schulz *et al.* 1995). Because of growing evidence of structural homology of GTH I to FSH and GTH II to LH (Querat 1994, 1995),

this nomenclature will be adopted and used in this report.

Many studies in salmonid fish have indicated that FSH and LH are regulated independently of one another. Pituitary content (Suzuki *et al.* 1988a, Nozaki *et al.* 1990a,b, Naito *et al.* 1991) and plasma levels of FSH and LH (Suzuki *et al.* 1988a, Swanson *et al.* 1989, Swanson 1991, Oppen-Berntsen *et al.* 1994, Slater *et al.* 1994, Prat *et al.* 1996) vary differently during gametogenesis with FSH being elevated during vitellogenesis and spermatogenesis and LH increasing during final maturation. Subunit mRNA levels for FSH and LH also vary throughout gametogenesis (Weil *et al.* 1995, Yoshiura *et al.* 1997). However, the mechanisms involved in regulating the differential synthesis and release of the two salmon GTHs have not been determined.

It has been well established in mammals that gonadotropin synthesis and secretion are regulated primarily by gonadotropin-releasing hormone (GnRH) from the hypothalamus and feedback by gonadal sex steroids and peptides such as inhibins and activins (for a review see Gharib *et al.* 1990). In fish, considerable information is available on the regulation of LH synthesis and secretion (Schulz *et al.* 1995). However, due to the relatively recent discovery of FSH in salmon, very little is known about the mechanisms for regulation of FSH synthesis and secretion. Numerous studies in teleosts have demonstrated that aromatizable androgens and estradiol-17 β (E2) increase pituitary levels of immunoreactive LH (Crim & Evans 1979, Crim *et al.* 1981, Gielen *et al.* 1982, Dufour *et al.* 1983, Borg *et al.* 1985, Magri *et al.* 1985, Schreiberman *et al.* 1986) and increase levels of LH β subunit mRNA (Trinh *et al.* 1986, Querat *et al.* 1991a,b). Recent studies (Xiong *et al.* 1994a,b,c) have shown that the promoter for the LH β subunit contains estrogen response elements. A single study by Larsen & Swanson (1997) revealed that gonadectomy of mature salmon resulted in an increase in plasma FSH levels, suggesting a factor present in the gonads was responsible for regulating plasma FSH levels in a negative feedback manner. However, investigations of the effects of sex steroids on FSH synthesis and secretion are lacking.

The goals of this study were threefold: to clone and sequence coho salmon (*Oncorhynchus kisutch*) FSH β and LH β subunits; to develop RNase protection assays (RPAs) to quantify levels of transcripts for these subunits; and to determine if sex steroids play a role in regulating FSH levels in salmonids. We focused our studies of steroid feedback regulation of FSH during the period of gonadal growth in the spring when plasma and

pituitary levels of FSH are increasing (Swanson 1991).

MATERIALS AND METHODS

Animals

Two-year-old coho salmon, from two different brood years, were obtained from Domsea Broodstock Inc. (Rochester, WA, USA) and reared in fresh water at the Northwest Fisheries Science Center in Seattle in circular fiberglass tanks (1.6 m diameter) under natural photoperiod. Water temperature ranged from a minimum of 8 °C (December) to 14 °C (August). Fish were fed to satiation once daily with BioDiet Grower Pellet (BioProducts, Warrenton, OR, USA). In coho salmon, the gonads develop synchronously and fish of this stock spawn under these rearing conditions in November, at 3 years of age. Experiments were conducted on maturing salmon. The term maturing is used to refer to fish that have initiated secondary oocyte growth or are in later stages of spermatogenesis, but do not yet produce viable gametes. For all fish that were killed except spermiating males, whole body and gonad weights were determined to calculate a gonadosomatic index (GSI=(gonad weight/body weight) \times 100).

Total RNA isolation and first strand cDNA synthesis

Pituitaries were removed from female coho salmon in early October, prior to spawning in November, and immediately frozen in liquid nitrogen. This stage of reproductive development was selected because transcripts for the two gonadotropin subunits (FSH β , LH β) would likely be present. Total RNA was extracted from a single pituitary using a guanidinium isothiocyanate procedure modified from Chomczynski & Sacchi (1987). First strand cDNA synthesis was performed using a reverse transcriptase PCR (RT-PCR) kit (Stratagene, La Jolla, CA, USA). A poly(t) primer (GACTGGATC-CGAATTCTAGAT(17)) containing a restriction site for EcoRI was used for the reaction (Frohman 1993).

Cloning and sequencing of coho salmon FSH and LH subunits

The cDNAs for coho salmon FSH β and LH β subunits were generated by RT-PCR and subsequently cloned and sequenced. Products of two independent PCRs were cloned and three clones of

TABLE 1. Oligonucleotide primers used in PCR and sequencing. Commercial primers used in sequencing were T7 and PUC/M13 Forward for clones in pT7 Blue, and T7 and T3 for subclones in pBluescript SK+

	Primer	Direction	Number of bases	Sequence (5'–3')
Subunit				
	FSH β			
	FSH β -5'	Forward	31	CGCGAATTCCCTTCTGGAAGCATTATGTACTG
	Adaptor	Reverse	37	GACTGGATCCGAATTCTAGAT(17)
	FSH β -BS	Reverse	30	CGCGAATTCAGGTCCGTCGTTTCGCACAGGC
LH β	LH β -5'	Forward	26	CGCGAATTCAGGATGTTAGGTCTTCA
	Adaptor	Reverse	37	GACTGGATCCGAATTCTAGAT(17)
	LH β -BS	Reverse	30	CGCGAATTCAAACCGGCTCCTTGGTGACGC

each PCR were sequenced to determine a consensus sequence since *Taq* errors were likely to occur. Where base pair differences occurred, a single clone would contain one base while all other clones would contain another common base at this position. Sequence data for chum (*O. keta*) (Sekine *et al.* 1989), masu (*O. masou*) (Gen *et al.* 1993, Kato *et al.* 1993) and chinook salmon (*O. tshawytscha*) (Trinh *et al.* 1986) were compared using MacDNASIS Pro (Hitachi, San Bruno, CA, USA) and used to design a forward primer in conjunction with the poly(t) reverse primer. Primers for each subunit are specified in Table 1. Each forward primer spanned a portion of the 5' untranslated region and the initial portion of the signal peptide. An *EcoRI* restriction site was added to aid in cloning. Initially ten cycles of PCR were carried out using the forward primer only. Conditions were as follows: one cycle for 2 min at 94 °C followed by ten cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 2 min. The reaction mixture consisted of the following: 10 μ l first strand cDNA reaction mixture; the forward primer (0.24 μ M FSH β or 0.29 μ M LH β) in double distilled water (0.5 μ l); 0.25 mM each of dATP, dCTP, dGTP and dTTP (2.0 μ l each); 50 mM KCl; 10 mM Tris-HCl, pH 9.0; 0.1% Triton X-100 (4.0 μ l); and 2.5 mM MgCl₂ (2.0 μ l) in a final volume of 40 μ l (rest as water). *Taq* polymerase (2 units, Promega, Madison, WI, USA) was added just prior to the first extension (72 °C). Following the completion of this reaction, a 20 μ l portion was removed and subjected to 30 cycles of PCR using the forward primer (same concentrations as above) and the poly(t) primer (0.22 μ M). The reaction conditions and mixture remained the same with a final volume of 80 μ l. All PCRs were carried out on a Perkin Elmer Gene Amp Thermal Cycler (Perkin Elmer Cetus, Foster City, CA, USA). The same conditions were followed for both subunits, although the 5' forward primer differed.

The PCR products were run on a 1.2% Nusieve agarose gel (FMC Bioproducts, Rockland, ME, USA). A 1 kb ladder (Gibco BRL, Gaithersburg,

MD, USA) was used as a size reference. The gel was stained with ethidium bromide and a single band for each of the subunits (FSH β and LH β) was cut from the gel and purified using the Sephaglass BandPrep kit (Pharmacia-LKB, Piscataway, NJ, USA). The purified cDNAs were then ligated into a pT7 Blue Vector (Novagen, Madison, WI, USA) using a DNA ligation kit (Novagen). NovaBlue competent cells (Novagen) were then used in the transformation reactions. Positive clones were subjected to large-scale plasmid amplification followed by purification using a plasmid purification kit (Qiagen, Chatsworth, CA, USA).

All clones were sequenced using the Dye Terminator Cycle Sequencing Ready Reaction DNA Sequencing kit (Perkin Elmer Cetus) and run on an ABI Prism 310 Genetic Analyzer (Perkin Elmer Cetus). Purified plasmid (0.37 μ g) was used for each reaction. Primers (PUC/M13 Primer Forward and T7 Promoter Primer; Promega) were used to sequence the clones starting in both 5' and 3' directions. Sequences were analyzed using MacDNASIS.

Template of RNA probes and standards

To generate templates for production of RNA probes and standards, a 197–204 bp fragment of each subunit (Fig. 1) was cloned into pBluescript S/K+ vector (Stratagene) at the *EcoRI* site. These fragments were generated by PCR using the original forward primer and a new reverse primer with an *EcoRI* site present (Table 1). PCR and conditions were the same as those described above with one of the original cDNA clones (0.5 μ g) as template for each specific subunit. The original clone chosen was identical to the consensus sequence in the region cloned. PCR products were restriction digested with *EcoRI*, and purification followed by ligation and transformation were identical to initial cloning, except for the vector. Positive clones were selected and subjected to large-scale amplification. Each

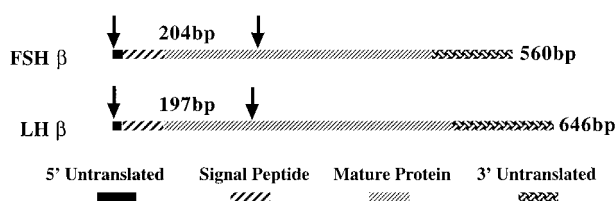


FIGURE 1. Diagrammatic representation of cDNAs for FSH β and LH β subunit mRNAs. Length in base pairs is shown on the far right for each subunit. Arrows signify location and length of each subunit that was subcloned into pBluescript vector and used for development of RPAs.

clone was sequenced in both directions to confirm sequence identity.

Northern blot analysis

Total RNA was isolated and pooled from pituitaries of spermiating male coho salmon ($n=8$). GSIs of spermiating males were not measured, but all were releasing milt at the time of killing. Three vitellogenic female ($GSI=1.98 \pm 0.26$, mean \pm s.e.) coho salmon were also killed and total RNA was isolated from the pituitaries and pooled. As a negative control, total RNA was also extracted from ovarian theca-interstitial cells isolated from a female coho salmon in the preovulatory stage. Ten micrograms from each sample were loaded on a 1% agarose gel equilibrated with 0.2 M formaldehyde, 0.4 M (3-(N-morpholino)propane sulfonic acid), 0.1 M sodium acetate, 0.01 M EDTA and electrophoresed for 2.5 h at 100 V using a VGS 1600 Vertical Gel System (Stratagene). The RNA ladder (0.24–9.5 kb, Gibco BRL) was cut from the gel and stained with ethidium bromide. RNA was capillary transferred overnight to a flash nylon membrane (Stratagene), crosslinked to the membrane using a UV Stratalinker 1800 (Stratagene) and stained with 0.02% methylene blue in 0.3 M sodium acetate, pH 5.5 to detect 28S and 18S ribosomal bands. The blot was stored at -20°C until hybridization with RNA probes.

Antisense RNA probes for each subunit were generated using the pBluescript subclones. The probes were labeled using a Maxiscript kit (Ambion, Austin, TX, USA) and [α - ^{32}P]CTP (Amersham, Cleveland, OH, USA) and purified using Quick Spin columns (Boehringer Mannheim, Indianapolis, IN, USA). Each filter was prehybridized with Church hybridization solution (Church & Gilbert 1984) for 0.5–2 h and then hybridized overnight with 2×10^7 c.p.m. of probe with fresh Church solution at 60°C . The blots were subse-

quently rinsed twice in 0.3 M NaCl, 0.03 M sodium citrate ($2 \times \text{SSC}$), 0.1% SDS at room temperature for 5 min and twice in $0.2 \times \text{SSC}$ containing 0.1% SDS at 65°C for 20 min. Blots were exposed to Kodak BioMax film with an intensifying screen for 2–3 h. The blot was then washed in $0.2 \times \text{SSC}$, 0.1% SDS containing 1.0 $\mu\text{g/ml}$ RNase A (Boehringer Mannheim) at room temperature and washed for an additional 20 min, with $0.2 \times \text{SSC}$, 0.1% SDS at 65°C . The filter was then repacked with Kodak BioMax film and an intensifying screen for 4–6 h.

Experiments

Three experiments were conducted to determine possible feedback effects of sex steroids on FSH levels in coho salmon during a period when plasma FSH levels are increasing and plasma LH levels are not detected. These experiments were therefore conducted during February, April and June when FSH levels increase in coho salmon that will spawn in the following November (Swanson 1991). During this period of gonadal growth, GSIs of males and females that were used in the experiments increased 10- and 2-fold respectively (see data below) and initial (day 0) plasma levels of FSH in males and females increased 6- and 11-fold respectively. Although histology was not performed on gonad tissue collected during these experiments, other studies of coho salmon have shown that during this time of year oocytes are in the early yolk globule stage (Chestnut 1970, Peterschmidt 1991) and the testes are in stage III of spermatogenesis (Chestnut 1970, Planas & Swanson 1993). Stage III of spermatogenesis refers to testes with spermatogonia, primary and secondary spermatocytes but no spermatids or mature sperm. We have referred to fish used in these experiments as maturing since they did not yet have fully mature gametes, but were destined to complete maturation in the subsequent autumn. Because previous studies indicated that treatment of gonad-intact juvenile or maturing (but not fully mature) rainbow trout with E2 or testosterone stimulated LH β synthesis at the transcriptional level, we also measured LH β transcript levels as positive controls. The effects of exogenous steroids (testosterone, E2 and $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ($17\alpha,20\beta$ -P)) on plasma and pituitary FSH and LH levels and steady state levels of LH β and FSH β subunit transcripts were determined in all three experiments to determine whether the feedback effects varied during this period of gonadal growth. Data are expressed as mean \pm s.e. when presented graphically or in text.

Experiment 1

In June 1995, 2-year-old maturing coho salmon (638.9 ± 26.7 g) were anesthetized in buffered tricaine methanesulfonate (MS-222, 0.05%) and divided into three groups of ten fish. Each fish was implanted with a passive integrated transponder (PIT) tag. Control fish received an i.p. injection of molten cocoa butter (1.0 ml/kg body weight). Steroid hormone treatments (testosterone or E2) were injected i.p. at a dose of 0.1 mg/ml per kg body weight. Blood was taken prior to (day 0) and 7 days after steroid injection and plasma samples were frozen at -80°C until analyzed for FSH and LH by RIA (Swanson *et al.* 1989). On day 7, the fish were killed by decapitation and body as well as gonad weights were recorded. Pituitaries were quickly removed, placed individually in sterile 1.5 ml microfuge tubes, frozen in liquid nitrogen and placed at -80°C until analyzed for gonadotropin subunit transcript levels by RPAs, or FSH and LH levels using RIAs. GSIs of the fish at the termination of the experiment were: control females 0.92 ± 0.12 ($n=6$); control males 0.37 ± 0.12 ($n=4$); testosterone-treated females 1.24 ± 0.16 ($n=7$); testosterone-treated males 0.17 ± 0.13 ($n=2$); E2-treated females 1.07 ± 0.08 ($n=6$); E2-treated males 0.07 ± 0.02 ($n=4$).

Experiment 2

In February 1996, 2-year-old maturing coho salmon (290.4 ± 3.6 g) were divided into four groups of 15 fish, each fish being PIT tagged. Control fish received an i.p. injection of cocoa butter (1.0 ml/kg body weight). Steroid treatments (testosterone, E2 or $17\alpha,20\beta\text{-P}$) were injected i.p. at a dose of 0.1 mg/ml per kg body weight. Blood was taken prior to (day 0) and 4 days after treatment and plasma samples were frozen at -80°C until assayed for FSH and LH by RIA. Total body weight as well as gonad weights were recorded at the termination of the experiment on day 4. Pituitaries were removed, placed individually in sterile microfuge tubes and frozen in liquid nitrogen until assayed for gonadotropin subunit transcript levels by RPAs or FSH and LH content by RIA. At the end of the experiment the GSIs of experimental fish were as follows: control females 0.52 ± 0.04 ($n=7$); control males 0.04 ± 0.006 ($n=7$); testosterone-treated females 0.61 ± 0.030 ($n=8$); testosterone-treated males 0.05 ± 0.007 ($n=6$); E2-treated females 0.63 ± 0.04 ($n=10$); E2-treated males 0.05 ± 0.007 ($n=5$); $17\alpha,20\beta\text{-P}$ -treated females 0.67 ± 0.04 ($n=10$); $17\alpha,20\beta\text{-P}$ -treated males 0.04 ± 0.006 ($n=5$).

Experiment 3

In April 1996, 2-year-old maturing coho salmon (387.6 ± 6.5 g) were divided into four groups of 20

fish, each fish receiving a PIT tag. Control fish received an i.p. injection of cocoa butter (1.0 ml/kg body weight). Steroid treatments (testosterone, E2 or $17\alpha,20\beta\text{-P}$) were injected i.p. at a dose of 0.1 mg/ml per kg body weight. Blood was taken prior to (day 0) and 4 days after steroid injection and plasma samples were frozen at -80°C until assayed for FSH and LH by RIA. On day 4 the fish were killed by decapitation and body and gonad weights were recorded. Pituitary glands were removed and assayed for gonadotropin subunit transcripts or FSH and LH content as described above. At the termination of the experiment, the GSIs were as follows: control females 0.813 ± 0.05 ($n=11$); control males 0.18 ± 0.06 ($n=9$); testosterone-treated females 0.91 ± 0.06 ($n=10$); testosterone-treated males 0.09 ± 0.008 ($n=10$); E2-treated females 0.832 ± 0.06 ($n=11$); E2-treated males 0.07 ± 0.008 ($n=9$); $17\alpha,20\beta\text{-P}$ -treated females 0.86 ± 0.03 ($n=12$); $17\alpha,20\beta\text{-P}$ -treated males 0.09 ± 0.007 ($n=8$).

Gonadotropin measurement

Plasma hormone levels were determined using homologous RIAs for FSH and LH (Swanson *et al.* 1989), except that a new, more specific antiserum against LH β was employed. Both of these assays detect the β subunits as well as the $\alpha\beta$ dimer. The cross-reactivity of FSH in the LH assay was $<0.01\%$ and that of LH in the FSH assay was 6.0% . Cross-reactions of growth hormone, prolactin, somatolactin and TSH in both assays were $<0.01\%$. The sensitivity of the FSH assay varied as the label decayed over the 4-week period of assays, with effective dose (ED_{50} s) ranging from 11.2 to 20.0 ng/ml and ED_{80} s ranging from 2.2 to 3.5 ng/ml. The LH RIA ED_{50} s ranged from 2.9 to 3.0 ng/ml and ED_{80} s from 0.16 to 0.28 ng/ml. Pituitaries were sonicated in 0.2 M barbital buffer, pH 8.6 with 1.0 mM phenylmethylsulfonyl fluoride and centrifuged for 5 min at 7700 g at 4°C . The supernatant was then collected and assayed by RIAs after dilution in 1.0% BSA in barbital buffer (assay buffer).

RPAs

Total nucleic acid (TNA) was prepared from pituitaries by digestion of homogenized tissue with proteinase K, followed by phenol/chloroform extraction (Durnam & Palmiter 1983). The DNA content in each sample was determined using Hoechst 33258 dye and a TKO 102 fluorometer (Hoefer Scientific Instruments, San Francisco, CA, USA).

Sense RNA standards for each subunit were generated by transcription of linearized vector with the subunit cDNA clone. For both subunits, linearization with XbaI (New England Biolabs, Beverly, MA, USA) and transcription with T7 RNA polymerase produced sense RNA using the Maxiscript kit (Ambion). The amount of sense RNA standard generated was determined by spectrophotometry (absorbance at 260 nm). The standard was diluted using RNase-free water to prepare stock solutions of 160 pg RNA/ μ l. Antisense RNA probes were synthesized using [α - 32 P]CTP after linearizing each pBluescript clone with AccI (New England Biolabs). Probes were purified by affinity chromatography using Sephaglass columns.

The RPAs were performed using the RPA II kit (Ambion). Samples of pituitary TNA or sense RNA standards (total volume 12 μ l) were hybridized to antisense RNA probes (3×10^4 c.p.m./3 μ l) in 20 μ l hybridization solution overnight at 42 °C. Subsequently, 100 μ l RNase mix (5 U/ml RNase A, 200 U/ml RNase T1) were added to the samples as well as the standard curve and digested for 2 h at 36 °C. Aliquots (50 μ l) of all reactions were then added to 5 μ l yeast tRNA (5 mg/ml) and precipitated with 55 μ l 20% trichloroacetic acid (TCA) for 30 min on ice. The reaction mixture was then transferred to a Whatmann GF/C membrane (2.5 mm) using a vacuum manifold (Millipore Corporation, Bedford, MA, USA). Each filter was washed five times with 500 μ l 5% TCA in 0.02 M sodium pyrophosphate followed by a single wash in 70% ethanol (1.0 ml). Filters were then allowed to dry for at least 30 min at room temperature before counting on a Packard Tri-Carb 300 scintillation counter. Sample values were then calculated from the standard curve after linear regression and expressed as pg RNA/ μ g DNA. The sizes of the RNA/RNA hybrid samples and standards were analyzed by electrophoresis (Duan *et al.* 1993). Cross-reactivity among the subunits was checked by cross-hybridization, followed by electrophoresis of the protected RNA fragments.

Statistical analysis

Results of the experiments were analyzed using one-way ANOVA followed by the Fisher protected least significant difference test (Dowdy & Weardon 1991) using the Statview 512+ program (Abacus Concepts, Inc., Berkeley, CA, USA). Differences between groups were considered to be significant at $P < 0.05$.

RESULTS

Sequence analysis of coho salmon gonadotropins (FSH β and LH β)

The consensus coho salmon FSH β subunit cDNA (FSH β) is 560 bp long and consists of a portion of the 5' untranslated region (16 bp), signal peptide (72 bp, 24 amino acids), mature protein (339 bp, 113 amino acids) and a 3' untranslated region following the stop codon of approximately 128 bp (Fig. 2). A polyadenylation signal (AATAAA) was located in the 3' untranslated region. The FSH β -5' forward primer (Table 1) was incorporated into the clone. Since this primer was designed comparing sequence data from chum and masu salmon, we cannot be sure that this is the actual coho salmon FSH β cDNA sequence in that region.

The consensus coho salmon LH β subunit cDNA (LH β) is 646 bp long (including the 5' EcoRI site), consisting of 6 bp of 5' untranslated region, signal peptide (69 bp, 23 amino acids), mature protein (327 bp, 109 amino acids) and a 3' untranslated region of approximately 210 bp with a polyadenylation signal (AATAAA) (Fig. 3). The LH β -5' forward primer was part of the cDNA clone; thus, we cannot be sure that this portion of the cDNA clone actually represents the coho salmon LH β cDNA sequence.

Northern blot analysis

Northern blots were performed using 10 μ g total RNA from pituitaries of spermiating male and vitellogenic female coho salmon and antisense RNA probes for FSH β and LH β (Fig. 4). As a negative control, 10 μ g total RNA from coho salmon ovarian thecal cell layers were run along with the pituitary RNA. In each hybridization (FSH β and LH β), a single message of approximately 1 kb was observed from both pituitary samples. No bands were detected in the thecal cell RNA. Cross-reactivity for the FSH β and LH β probes was tested by dot blot hybridization using cDNA probes of one subunit hybridized to the cDNA of the other subunit. These results (data not shown) revealed no cross-reactivity among the β subunits probes.

RPA validation

The RPAs for FSH β and LH β were validated in a similar fashion. For each RPA, either sense RNA standards (400 and 800 pg) or total pituitary RNA (0.57 and 5.7 μ g) were hybridized to antisense RNA probe overnight at 42–43 °C. Antisense RNA probe alone was also allowed to incubate overnight. The reactions were then treated with RNase (except

FSH β cDNA

1	TGA ATT CCT TCT GGA AGC ATT ATG TAC TGC ACC CAC TTA AAG ACG CTG CAG CTG GTC GTC	60
-24	M Y C T H L K T L Q L V V	-12
61	ATG GCA ACG CTG TGG GTG ACA CCA GTG AGG GCG GGG ACA GAC TGC AGG TAT GGC TGC CGA	120
-11	M A T L W V T P V R A G T D C R Y G C R	9
121	CTA AAC AAC ATG ACC ATC ACC GTG GAG AGA GAG GAC TGT CAC GGA AGC ATC ACC ATC ACC	180
10	L N N M T I T V E R E D C H G S I T I T	30
181	ACC TGC GCC GGC CTG TGC GAA ACG ACG GAC CTG AAC TAT CAG AGC ACA TGG CTG CCA CGC	240
31	T C A G L C E T T D L N Y Q S T W L P R	50
241	TCC CAG GGG GTG TGT AAC TTC AAG GAG TGG TCC TAC GAG AAG GTC TAC CTG GAA GGC TGT	300
51	S Q G V C N F K E W S Y E K V Y L E G C	70
301	CCA TCC GGG GTC GAC CCC TTC TTC ATA CCC GTT GCC AAG AGC TGC GAT TGC ATC AAA TGC	360
71	P S G V D P F F I P V A K S C D C I K C	90
361	AAG ACG GAC AAC ACC GAC TGT GAT CGC ATA AGC ATG GCA ACA CCC AGC TGC ATT GTA AAC	420
91	K T D N T D C D R I S M A T P S C I V N	110
421	CCA CTA GAA ATG TAA TAC TGT AGA GTT AGG CTG TTG CAG CAG CCA GTT TGT TTA GCA ACA	480
111	P L E M *	114
481	GTT TGA CTA TTC TAT GGT ACC GTT TCC ATT GTT GAT GTG CTA ACG <u>CAA TAA AAC</u> ACT ACT	540
541	TTG AAA AAA AAA AAA AA	560

FIGURE 2. Nucleotide sequence for coho salmon FSH β cDNA (upper line) and deduced amino acid sequence (lower line). Numbers represent nucleotide sequence (bold) and amino acid sequence (negative numbers indicate signal peptide). The arrow indicates the cleavage site between the signal peptide and the mature protein. Stop codon is indicated with an asterisk. The polyadenylation signal is underlined.

LH β cDNA

1	GAA TTC AGG ATG TTA GGT CTT CAT GTA GGC ACC CTG ATC TCC CTG TTT CTG TGC ATT CTC	60
-23	M L G L H V G T L I S L F L C I L	-7
61	CTG GAA CCC ATG GAG GGG TCT CTC ATG CAG CCC TGT CAG CCC ATC AAC CAG ACT GTG TCT	120
-6	L E P M E G S L M Q P C Q P I N Q T V S	14
121	CTG GAG AAG GAA GGC TGC CCA ACG TGC TTA GTC ATT CAA ACC CCT ATC TGC AGT GGC CAC	180
15	L E K E G C P T C L V I Q T P I C S G H	34
181	TGC GTC ACC AAG GAG CCG GTT TTC AAG AGC CCA TTT TCC ACC GTG TAC CAG CAT GTG TGC	240
35	C V T K E P V F K S P F S T V Y Q H V C	54
241	ACC TAC CGG GAC GTC CGC TAT GAA ACG ATC CGC CTA CCT GAC TGT CCC CCG TGG GTG GAC	300
55	T Y R D V R Y E T I R L P D C P P W V D	74
301	CCT CAT GTC ACC TAC CCT GTG GCT CTG AGC TGT GAC TGC AGC CTC TGT AAC ATG GAC ACT	360
75	P H V T Y P V A L S C D C S L C N M D T	94
361	TCT GAC TGT ACC ATC GAG AGC CTG CAG CCA GAC TTC TGC ATT ACC CAA AGA GTA CTA ACG	420
95	S D C T I E S L Q P D F C I T Q R V L T	104
421	GAT GGT GAC ATG TGG TGA CAT GTC AGC CAA CAT GCC TGC CTA TGT AAT GAC ATG TTA TAT	480
105	D G D M W *	109
481	TAC ACC CGC CCA TGT AAT GAC ATG TAG GCC CAA TGT ATG GTG AAC TCA AAC TGA ACT CAA	540
541	ATT TCA TAG GTA CTA GAC ATG GGC TAG TAG GTT GTA TGT GGA TGT ACT TGT ACT CAT ATT	600
601	GGG ATC TGA <u>ATA AAC</u> TGA CTC CAA CTC TGA AAA AAA AAA AAA AA	646

FIGURE 3. Nucleotide sequence for coho salmon LH β cDNA (upper line) and deduced amino acid sequence (lower line). Numbers represent nucleotide sequence (bold) and amino acid sequence (negative numbers indicate signal peptide). The arrow indicates the cleavage site between the signal peptide and the mature protein. An asterisk indicates the stop codon. The polyadenylation signal is underlined.

for probe alone), inactivated, precipitated and resuspended. The reactions were then run on an acrylamide gel to observe size and relative amounts of protected probe, which represented the amount of mRNA present in the reaction (Fig. 5).

The size of the probes (approximately 305 bp) is larger than the protected RNA because part of the polylinker is present in the probes during the labeling procedure. The protected RNA fragments from the standard reactions are slightly smaller

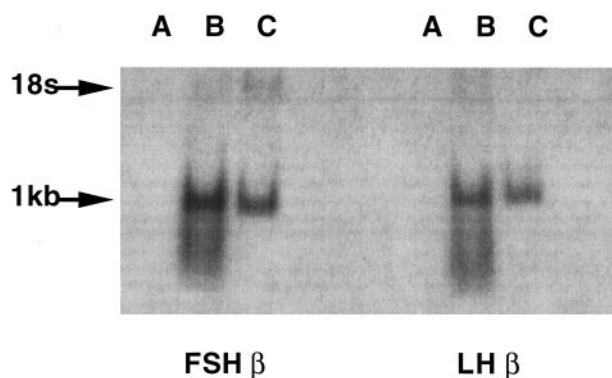


FIGURE 4. Northern blot depicting size and number of FSH β and LH β subunit transcripts from ovarian theca cell layer (control tissue) (A), pituitary RNA from spermiating male (B), and vitellogenic female (C) coho salmon. A single message of approximately 1 kb was present for each subunit in maturing females and mature males. No transcripts for FSH β or LH β subunits were detected in RNA from ovarian thecal cells. Total RNA (10 μ g) from each sample was hybridized with antisense RNA probes which were also used for the RPAs.

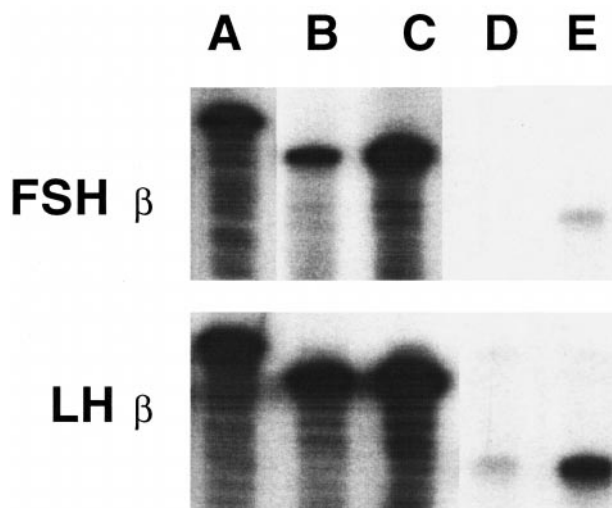


FIGURE 5. RPAs for FSH β and LH β using acrylamide gels and autoradiography. Antisense RNA probe (A); 400 pg (B) and 800 pg (C) sense RNA standard; 0.57 μ g (D) and 5.7 μ g (E) total RNA from the pituitary of a vitellogenic female coho salmon.

(approximately 250 bp) than the probe because the sense RNA standards have less complementary sequence with the probe in the polylinker region, thus hybridization in this region is poor and the resulting RNA is digested by the RNase. Finally, the protected RNA fragments from the pituitary total RNA samples are smaller (approximately

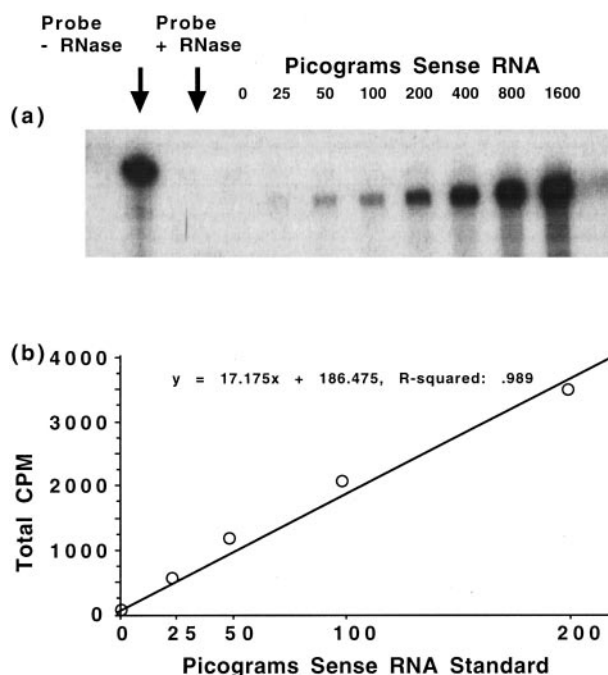


FIGURE 6. The autoradiograph (a) shows a standard curve for the RNase protection assay developed for the LH β subunit. Probe without RNase is larger in size due to the presence of some polylinker sequence, whereas the bands for the sense standards are smaller due to less complementarity of the polylinker between the antisense probe and sense standard. TCA precipitations of standard curve reactions were spotted on GF/C filters and counted on a scintillation counter (b). The linear range of the curve falls within 0–200 pg.

205 bp) because the sample RNA does not contain polylinker, thus there is no possibility to hybridize with the polylinker in the probe. These size differences were incorporated into the assays to ensure that RNase digestion conditions were stringent enough to remove any excess probe that was not hybridized to standard RNA or mRNA. A concentration-related protection was found for the protected RNA fragment from sense RNA standard reactions (Fig. 5, lanes B and C) and sample total RNA reactions (Fig. 5, lanes D and E).

The standard curves used in the assay were also scrutinized after acrylamide gel electrophoresis and found to protect the antisense probe in a concentration dependent fashion (Fig. 6, data shown are for the LH β RPA only). Although the sense RNA standards were serially diluted in the range of 1600 pg to 25 pg per tube, the curve was linear only from 0.0 to 200 pg. Standard curves for FSH β are not shown, but had a similar range of sensitivity to that of the LH β RPA.

Experiment 1

In the first experiment, conducted in June 1995, the effects of testosterone and E2 on plasma FSH and LH levels, as well as pituitary FSH β and LH β subunit mRNA levels were investigated. Data from females only were analyzed in this experiment since there were few males (2–4/group) and some of these males appeared not to be maturing based on visual examination of their gonads and GSIs.

By 7 days post-treatment there was a significant reduction in plasma FSH levels in both testosterone- and E2-treated fish compared with the control group (Fig. 7a). Plasma FSH levels prior to treatment were 22.73 ± 1.78 ng/ml. LH levels were not detected (<0.2 ng/ml) at this time for any group. Plasma steroid levels were not measured. However, preliminary experiments indicated that treatment with testosterone in cocoa butter resulted in elevated plasma testosterone levels for 3–28 days after treatment.

Steady state mRNA levels for FSH β and LH β subunits (Fig. 7b) were measured in pituitaries from female fish. No significant differences were seen among the three treatment groups with respect to the FSH β subunit mRNA levels. However, treatments with testosterone and E2 significantly elevated the steady state mRNA levels for the LH β subunit compared with the control group.

Experiment 2

In the second experiment, conducted during February 1996, the effects of testosterone, E2 and $17\alpha,20\beta$ -P on plasma and pituitary FSH and LH levels, as well as pituitary FSH β and LH β subunit mRNA levels were examined. Because initial levels of FSH were variable, the effects of the treatments on FSH levels are expressed as a percentage of the levels at day 0 (Fig. 8). Four days after the treatment there was a significant reduction in plasma FSH levels in males treated with testosterone or E2, but not in control or $17\alpha,20\beta$ -P-treated males. In females, E2 treatment caused a significant reduction in FSH plasma levels, while testosterone and $17\alpha,20\beta$ -P were not significantly different from those in controls. Plasma FSH levels prior to treatment were 1.974 ± 0.33 ng/ml for males and 1.76 ± 0.16 ng/ml for females. In all fish, irrespective of treatment, plasma LH levels were not detected (<0.2 ng/ml).

Pituitaries from five males and five females from each treatment were analyzed for steady state mRNA levels for the gonadotropin subunits (Fig. 9a and b). Total nucleic acid extractions were performed on individual pituitaries and results are

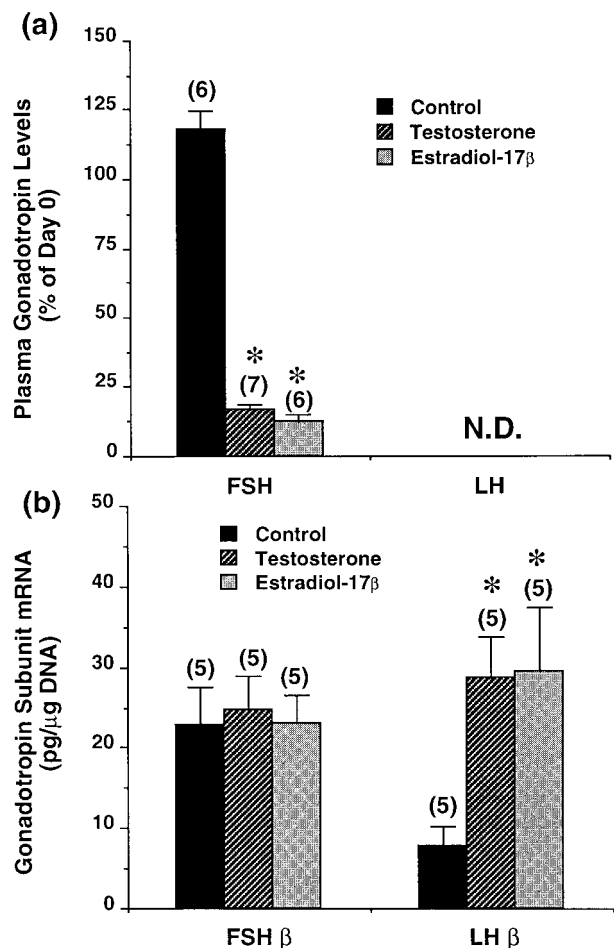


FIGURE 7. Effects of acute *in vivo* treatment of testosterone and E2 on plasma gonadotropin levels (a) and pituitary gonadotropin subunit transcript levels (b). Fish were treated in June 1995, 5 months prior to spawning, with steroid (0.1 mg/kg body weight) in molten cocoa butter. Samples were collected 7 days post-treatment. GSI of females was 1.03 ± 0.06 . Error bars indicate s.e. Number of replicates per treatment is shown in parentheses above columns. *Significantly different from controls of the same sex, $P \leq 0.05$. N.D., non-detectable.

expressed as pg mRNA/ μ g DNA. Steady state transcript levels for FSH β were unaltered by the steroid treatment in females; however, in males E2 caused a significant reduction in FSH β mRNA levels. As in the first experiment, both testosterone and E2 caused a significant increase in LH β subunit mRNA levels in both sexes.

The remaining pituitaries were analyzed for FSH and LH content by RIA. Since there were no extra males in the E2 or $17\alpha,20\beta$ -P groups, only females were analyzed for pituitary gonadotropin content

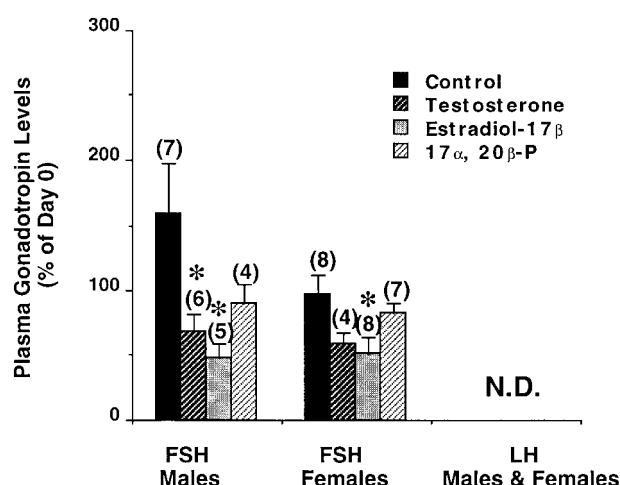


FIGURE 8. Effects of acute *in vivo* treatment with testosterone, E2 and 17α,20β-P on plasma levels of gonadotropin in coho salmon treated in February 1996, 10 months prior to spawning. Fish were treated with steroid (0.1 mg/kg body weight) in molten cocoa butter and samples were collected 4 days after treatment. GSIs of males and females were 0.047 ± 0.003 and 0.613 ± 0.021 respectively. Error bars indicate S.E. Number of replicates per treatment is shown in parentheses above columns. *Significantly different from controls of the same sex, $P \leq 0.05$. N.D., non-detectable.

and results are expressed as µg/pituitary (LH or FSH). There were no significant differences among the groups with regard to pituitary FSH levels, but a significant increase in LH content was seen in those fish treated with testosterone or E2 (Fig. 10a and b).

Experiment 3

In the third experiment, which was conducted in April 1996, the effects of testosterone, E2 and 17α,20β-P on plasma and pituitary FSH and LH levels, as well as pituitary FSH β and LH β subunit mRNA levels were examined. By 4 days post-treatment, FSH levels were significantly reduced in those groups treated with testosterone or E2, but significantly increased by 17α,20β-P (Fig. 11). These results were the same in both sexes. Plasma FSH levels prior to treatment were 8.22 ± 0.70 ng/ml for males and 11.49 ± 1.10 ng/ml for females. Plasma LH levels were not detected in any fish (<0.2 ng/ml).

Pituitaries from five males and five females were analyzed for steady state levels of gonadotropin subunit mRNAs (Fig. 12a and b). No significant differences in FSH β transcripts were observed among the treatments in either males or females.

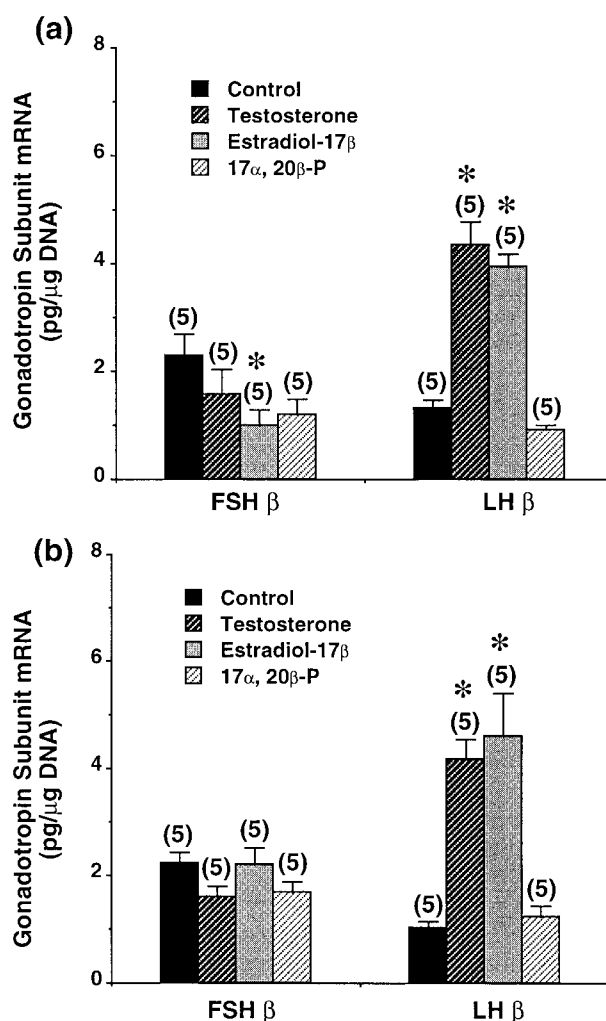


FIGURE 9. Effects of acute *in vivo* treatment with testosterone, E2 and 17α,20β-P on steady state levels of FSH β and LH β subunit transcripts in male (a) and female (b) coho salmon treated in February 1996, 10 months prior to spawning. Fish were treated with steroid (0.1 mg/kg body weight) in molten cocoa butter and samples were collected 4 days after treatment. GSIs of males and females were 0.047 ± 0.003 and 0.613 ± 0.021 respectively. Error bars indicate S.E. Number of replicates per treatment is shown in parentheses above columns. *Significantly different from controls of the same sex, $P \leq 0.05$.

Both males and females treated with either testosterone or E2 had significantly higher levels of LH β subunit transcripts than controls.

The remaining pituitaries were analyzed for FSH and LH content (Fig. 13a and b). No significant difference was seen with regard to pituitary FSH content in males or females for any treatment group. However, pituitary LH levels were significantly

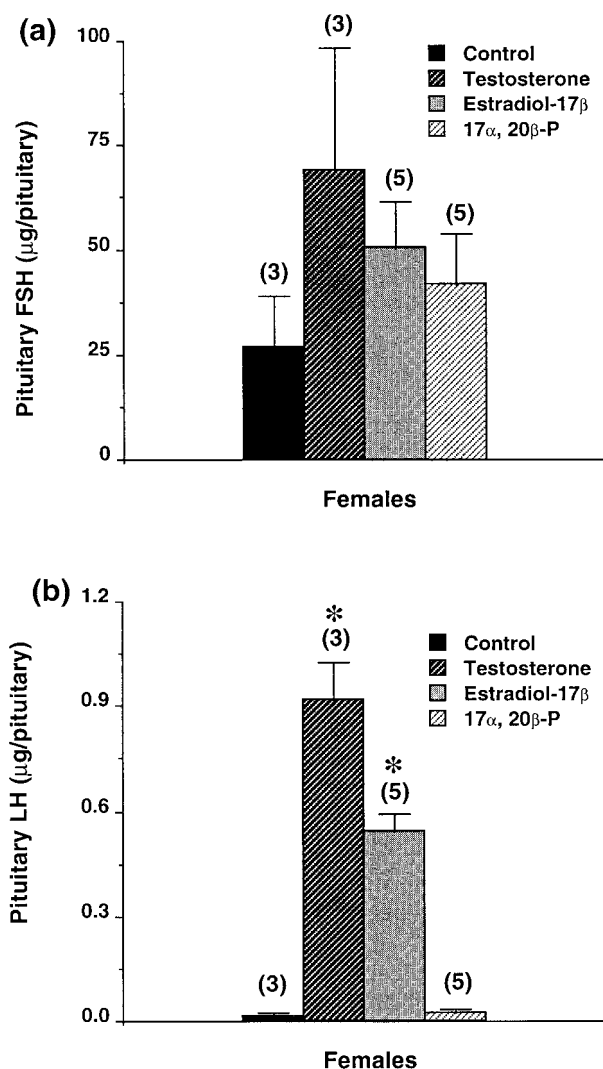


FIGURE 10. Effects of acute *in vivo* treatment with testosterone, E2 and 17 α ,20 β -P on pituitary FSH (a) and LH (b) in female coho salmon treated in February 1996, 10 months prior to spawning. Fish were treated with steroid (0.1 mg/kg body weight) in molten cocoa butter and samples were collected 4 days after treatment. GSIs indices of males and females were 0.047 ± 0.003 and 0.613 ± 0.021 respectively. Error bars indicate s.e. Number of replicates per treatment is shown in parentheses above columns. *Significantly different from controls of the same sex, $P \leq 0.05$.

higher in fish treated with E2 (males and females) and testosterone (females only).

DISCUSSION

Sex steroids have both positive and negative feedback effects on gonadotropins, depending on

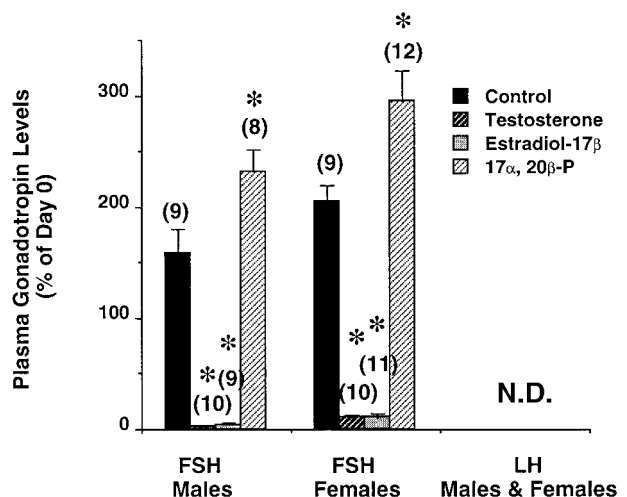


FIGURE 11. Effects of acute *in vivo* treatment with testosterone, E2 and 17 α ,20 β -P on plasma gonadotropin levels. Fish were treated in April, 8 months prior to spawning, and samples were collected 4 days after injection of steroid (0.1 mg/kg body weight) in molten cocoa butter. GSIs of males and females were 0.108 ± 0.017 and 0.855 ± 0.026 respectively. Error bars indicate s.e. Number of replicates per treatment is shown in parentheses above columns. *Significantly different from controls of the same sex, $P \leq 0.05$. N.D., non-detectable.

the stage of maturation and level – transcription, translation, or secretion. Many studies have been undertaken to determine sex steroid effects on LH synthesis or secretion in fish, but few data are available for sex steroid feedback regulation of FSH. In this study cDNAs for the gonadotropin subunits (FSH β and LH β) from coho salmon were cloned and sequenced. The cDNAs were used to synthesize RNA probes for development of RPAs to quantify levels of FSH β and LH β subunit transcripts. The RPAs along with RIAs for FSH and LH were then used to determine effects of sex steroids on FSH and LH in coho salmon during gonadal growth, a period when plasma FSH levels increase in the spring prior to spawning in the fall.

The amino acid sequences deduced from cDNAs for coho salmon FSH β , and LH β subunits show greater than 96% sequence identity to their respective subunits in other salmonids (Sekine *et al.* 1989). Northern blot analysis of coho salmon pituitary RNA revealed a single mRNA transcript, approximately 1 kb in size, for each subunit, in agreement with the transcript size and number found in masu salmon (Kato *et al.* 1993).

The coho salmon gonadotropin subunit cDNAs were then used to develop RPAs for quantification of steady state mRNA levels of the subunits. We

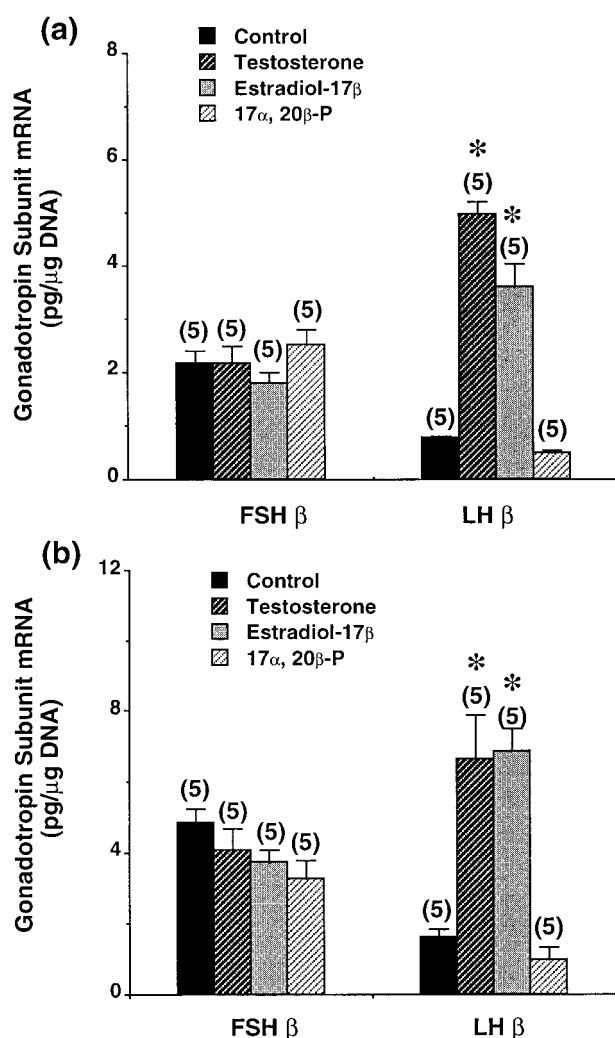


FIGURE 12. Effects of acute *in vivo* treatment with testosterone, E2 and 17 α ,20 β -P on steady state levels of mRNA for FSH β and LH β in male (a) and female (b) coho salmon. Fish were treated in April, 8 months prior to spawning, and samples were collected 4 days after injection of steroid (0.1 mg/kg body weight) in molten cocoa butter. GSIs of males and females were 0.108 ± 0.017 and 0.855 ± 0.026 respectively. Error bars indicate s.e. Number of replicates per treatment is shown in parentheses above columns. *Significantly different from controls of the same sex, $P \leq 0.05$.

selected RPAs because of their ability to quantitatively measure large numbers of samples in a single assay and their high degree of specificity over dot blot analysis and Northern blot. The probes used in the RPAs had less than 1% cross-reactivity, based upon comparisons of hybridizations to sense standard RNAs of FSH β or LH β gonadotropin subunits. This was expected since the cDNA

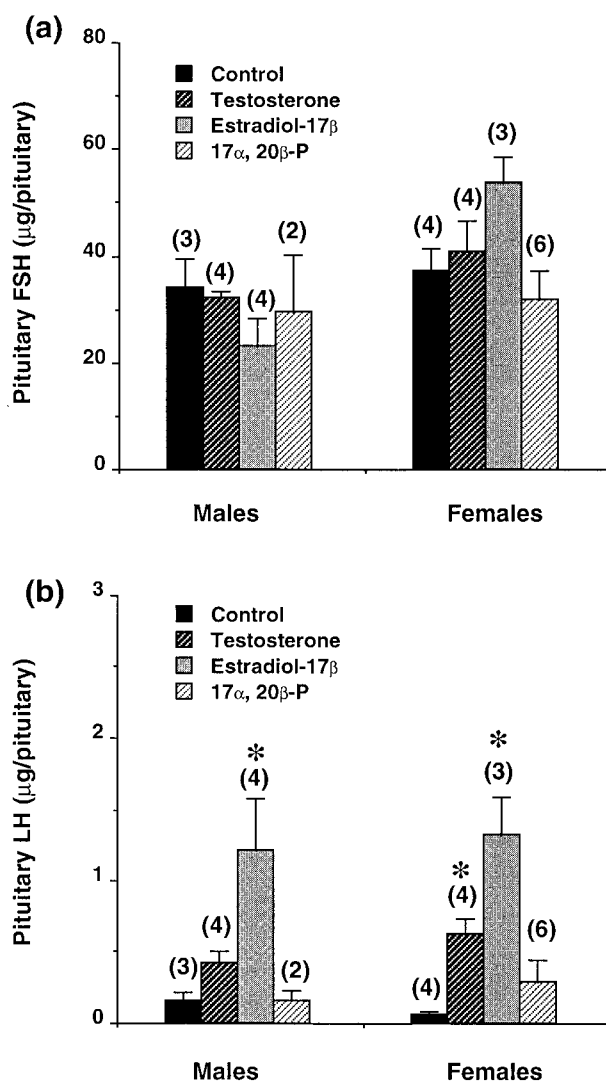


FIGURE 13. Effects of acute *in vivo* treatment with testosterone, E2 and 17 α ,20 β -P on pituitary FSH (a) and LH (b) levels in coho salmon. Fish were treated in April, 8 months prior to spawning, and samples were collected 4 days after injection of steroid (0.1 mg/kg body weight) in molten cocoa butter. GSIs of males and females were 0.108 ± 0.017 and 0.855 ± 0.026 respectively. Error bars indicate s.e. Number of replicates per treatment is shown in parentheses above columns. *Significantly different from controls of the same sex, $P \leq 0.05$.

comparisons among FSH β and LH β showed no greater than 33% sequence identity.

In this study the effects of acute sex steroid treatment *in vivo* (testosterone, E2 and 17 α ,20 β -P) on plasma and pituitary FSH and LH, as well as steady state levels of FSH β and LH β subunit transcripts were investigated in coho salmon at

three different times during the spring period of gonadal growth. This period was selected because FSH levels increase at this time and LH levels are not detected (Swanson 1991). Treatment with either testosterone or E2 significantly lowered plasma FSH levels compared with control fish by 4–7 days after treatment suggesting a negative feedback effect of these steroids on FSH in salmon. In only one of the three experiments, plasma FSH levels increased after treatment with 17 α ,20 β -P. Since these experiments were conducted when plasma levels of 17 α ,20 β -P are not detected, the physiological significance of this effect is uncertain. Similar negative feedback effects of testosterone on plasma FSH levels were found in maturing male Atlantic salmon parr (Borg *et al.* 1998) when sampled in the summer (July), but positive effects of testosterone were observed when fish were sampled in the fall (November). The negative feedback effects of gonadal sex steroids on FSH in salmon are consistent with previous studies demonstrating that surgical removal of the ovary or testis in coho salmon in late stages of vitellogenesis and spermatogenesis causes an acute (3–14 days post-surgery) increase in plasma FSH levels in fish (Larsen & Swanson 1997).

The mechanism whereby testosterone or E2 causes a decline in plasma FSH levels is not known. The decline in plasma FSH levels in response to testosterone or E2 could be due to decreased synthesis, secretion and/or increased clearance. In one experiment (February), E2 caused a significant decline in FSH β mRNA levels, but only in males. However, pituitary levels of immunoreactive FSH for all groups were not significantly different from controls. Thus, it appears that the decrease in FSH plasma levels seen throughout the experimental series may be in part due to changes in steady state levels of FSH β mRNA; however, changes in clearance, secretion, or mRNA processing cannot be ruled out.

Although we did not observe negative feedback effects of sex steroids on LH in coho salmon, there is evidence for negative feedback regulation of LH in teleosts. Surgical removal of the ovary or testis causes an increase in plasma LH (maturational GTH or GTH II) levels in mature rainbow trout, suggesting factors from the gonad exert negative feedback effects on GTH (Billard *et al.* 1976, 1977, Billard 1978, Bommelaer *et al.* 1981, Van Putten *et al.* 1981). Larsen & Swanson (1997) also found a post-castrational rise in plasma LH in spermiating male coho salmon. The post-ovariectomy rise in LH in female rainbow trout at the conclusion of vitellogenesis could be depressed by injections of E2 (Bommelaer *et al.* 1981). Kobayashi & Stacey (1990)

found a post-ovariectomy rise in plasma GTH levels (LH) in female goldfish. In goldfish and trout, E2 increases dopaminergic inhibition of LH release (Sokolowska *et al.* 1985, Peter *et al.* 1986, Trudeau *et al.* 1993b, Linard *et al.* 1995). Recent studies in rainbow trout by Saligaut and colleagues (1992) have suggested that E2 increases dopamine biosynthesis in early to late stages of vitellogenesis, thus maintaining a strong inhibition of LH release. In the present study, we found no evidence for negative feedback regulation of LH, although the fish used in our study were relatively immature compared with fish used in the gonadectomy studies described above.

In all three experiments, positive feedback effects of E2 or testosterone on LH were found. Both steroids increased pituitary LH content and steady state levels of LH β subunit mRNA. These results are consistent with results of studies in other salmonids which have shown that E2 or aromatizable androgens increase pituitary LH (maturational GTH) in intact juvenile trout (Crim & Peter 1978, Crim & Evans 1979, Gielen & Goos 1983, Magri *et al.* 1985) and LH β transcripts in trout and eel (Trinh *et al.* 1986, Querat *et al.* 1991a,b). The effects of E2 are likely through increases in transcription as has been found in rats (Shupnik *et al.* 1989, Shupnik & Rosenzweig 1991) since the upstream regulatory region of the salmon LH β gene contains functional estrogen response elements (Xiong *et al.* 1994b).

In all cases where measured, LH pituitary content increased in response to testosterone or E2 treatment. Plasma LH levels were not detected in all three of the experiments, which was expected since these fish were at a relatively immature stage of reproduction, when plasma LH levels are not detected (Swanson 1991). Earlier studies in rainbow trout indicated E2 or aromatizable androgens exert positive feedback on LH synthesis. GTH (LH) levels in the pituitary of immature rainbow trout increased in response to *in vivo* injections of estrogen and aromatizable androgens that were administered using a cocoa butter vehicle (Crim *et al.* 1981). Elevation of pituitary LH in intact juvenile trout by exogenous testosterone treatment has also been previously demonstrated (Crim & Peter 1978, Crim & Evans 1979, Gielen & Goos 1983, Magri *et al.* 1985, Borg *et al.* 1998).

Sex steroids may be affecting gonadotropin synthesis and/or secretion indirectly at the level of the hypothalamus or directly at the level of the pituitary. Huggard and colleagues (1996), using goldfish pituitary cell culture and Northern blots, showed that testosterone and 11 β -hydroxyandrosterone stimulated LH β mRNA production in goldfish.

Preliminary results in our laboratory looking at E2 effects *in vitro* have shown negative feedback on FSH β mRNA levels and positive feedback on LH β mRNA levels in both male and female coho salmon at late stages of gametogenesis (2 months prior to spawning). In addition, sex steroids have been shown to enhance LH secretion in response to GnRH *in vitro* in rainbow trout (Weil & Marcuzzi 1990a,b) and *in vivo* in goldfish (Trudeau *et al.* 1991, 1993a).

In conclusion, we have confirmed the positive effects of E2 and testosterone on LH β subunit mRNA levels as well as pituitary content in coho salmon. In contrast, we have shown that E2 and testosterone have negative feedback effects on FSH by decreasing plasma FSH levels in fish. These negative feedback effects of E2 or testosterone may involve alterations in transcription since these steroids decreased FSH β subunit mRNA levels in male coho salmon, but only during February. Future experiments involving different doses as well as length of exposure, in conjunction with varying stages of sexual maturation are required to clarify the role of sex steroid feedback in the regulation of FSH and LH synthesis.

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