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 $\alpha$ and LH $\beta$ 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 $\alpha$ and LH $\beta$ 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$\beta$ (E2) and 17$\alpha$,20$\beta$-dihydroxy-4-pregnen-3-one (17$\alpha$,20$\beta$-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 $\beta$, whereas E2 decreased steady state mRNA levels of FSH $\beta$ 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$\alpha$,20$\beta$-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$\alpha$,20$\beta$-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.

Journal of Molecular Endocrinology (1998) 21, 291–306

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. 1988b,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 $\alpha$ and a unique $\beta$ subunit. In salmon, the GTH I heterodimer is composed of a I$\beta$ subunit and either an $\alpha$1 or an $\alpha$2 subunit. GTH II, on the other hand, is composed of an $\alpha$2–II$\beta$ 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 pelamis) (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-Bernsten 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-17β (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, Schreibman 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) × 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 guanidium 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-GGAATTCTAGAT(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+

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Primer</th>
<th>Direction</th>
<th>Number of bases</th>
<th>Sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH β</td>
<td>FSH β-5′</td>
<td>Forward</td>
<td>31</td>
<td>CGCGAAATTCCTTCTGGAGCATTATGACTGT</td>
</tr>
<tr>
<td></td>
<td>Adaptor</td>
<td>Reverse</td>
<td>37</td>
<td>GACTGGATCCGAATTCTAGAT(17)</td>
</tr>
<tr>
<td></td>
<td>FSH β-BS</td>
<td>Reverse</td>
<td>30</td>
<td>CGCGAAATTCAGGTCGGTTTGCACAGGC</td>
</tr>
<tr>
<td>LH β</td>
<td>LH β-5′</td>
<td>Forward</td>
<td>26</td>
<td>CGCGAAATTCAGGATTTAGTTCTTCA</td>
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<tr>
<td></td>
<td>Adaptor</td>
<td>Reverse</td>
<td>37</td>
<td>CGCGAAATTCAGGATTTCTAGAT(17)</td>
</tr>
<tr>
<td></td>
<td>LH β-BS</td>
<td>Reverse</td>
<td>30</td>
<td>CGCGAAATTCACCGCTCCTTGTTGCACGC</td>
</tr>
</tbody>
</table>

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. tschawytscha) (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 (same concentrations for each PCR) and 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 µg) 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 fragment 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 µg) 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 fragment 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 µg) 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
overnight with 2 Gilbert 1984) for 0.5–2 h and then hybridized using Church hybridization solution (Church & Indianapolis, IN, USA). Each filter was prehybridized using Quick Spin columns (Boehringer Mannheim, Amersham, Cleveland, OH, USA) and purified using a Maxiscript kit (Ambion, Austin, TX, USA) and [α-32P]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 x 10^7 c.p.m. of probe with fresh Church solution at 60 °C. The blots were subsequently rinsed twice in 0.3 M NaCl, 0.03 M sodium citrate (2 x SSC), 0.1% SDS at room temperature for 5 min and twice in 0.2 x 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 x SSC, 0.1% SDS containing 1.0 μg/ml RNase A (Boehringer Mannheim) at room temperature and washed for an additional 20 min, with 0.2 x 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 spermatoïds 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 synthesis at the transcriptional level, we also measured LHβ transcript levels as positive controls. The effects of exogenous steroids (testosterone, E2 and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P)) on plasma and pituitary FSH and LH levels and steady state levels of LHβ and FSHβ 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 ± 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 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); 17α,20β-P-treated females 0·86 ± 0·03 (n=12); 17α,20β-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 αβ 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 (ED₈₀) ranging from 11·2 to 20·0 ng/ml and ED₈₀ ranging from 2·2 to 3·5 ng/ml. The LH RIA ED₈₀ ranged from 2·9 to 3·0 ng/ml and ED₈₀ 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/µl. Antisense RNA probes were synthesized using [α-32P]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 µl) were hybridized to antisense RNA probes (3 x 10^5 c.p.m./3 µl) in 20 µl hybridization solution overnight at 42 °C. Subsequently, 100 µl 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 µl) of all reactions were then added to 5 µl yeast tRNA (5 mg/ml) and precipitated with 55 µl 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 µl 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/µg 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.

### 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 µg total RNA from pituitaries of spermatizing male and vitellogenic female coho salmon and antisense RNA probes for FSH β and LH β (Fig. 4). As a negative control, 10 µg 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 µg) 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...
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.

**FSH β cDNA**

```
  TGA ATT CCT TCT GGA AGC ATT ATG TAC TGC ACC CAC TTA AAG AGC CTT CAG CGC TGC GTC GTC
-24
  M Y C T H L K T L Q L V V
-12
  ATG GCA AGC CTT TGG GTG ACA CCA GTC AGG GCC GGA ACA GAC TGC AGG AGT GGC TGC CTA
-11
  M A T L W V T P V R A A T G T D C R Y G C R
  121
  CTA AAC AAC ATG ACC ATC ACC GTG GAG AGA GAG GAC TGT CAC GGA AGC ATC ACC ATC
  L N N M T I T V E R E D C H O S I T I T
  30
  ACC TGC GCC GGC CTU TGC GAA AGC AGC CAG CTG AAC TAT CAG ACC ACA TGG CTG CCA CCG
  T C A G L C E T T D L N Y Q S T W L P R
  181
  TCC CAG GGG GTG TGT TAC TCC AGA GAG TGG TAC TAC GAG AAG GTC TGG TGC CAA
  S Q G V C N F K E W S Y E K V Y L E G C
  241
  CCA GCC GGC GTC GAC CCC TTC TCC ATG CTA CCC GTT GCC AAG AGC TGC GTG ATC ATC
  71
  P S G V D P F F I P V A K S C D C I K C
  301
  AAG AGC GAC AAC ACC GAC TGG TGT CAC GCA ACG ATG AGT GCA ACA CCC ACC TGC GTT GTA
  420
  C T A C G A A G T A A T A C T A G A GTG AGG CAG TCG TCT CAG CCA CTA TGT TTA GCA ACA
  M A T P S C I N V
  480
  111
  P L E M *
  481
  GTT TCA CTA TTA GTG CTT TAA ACC GTG GCC ARA TAA AAC ACT ACT
  540
  TGT AAA AAA AAA AAA AAA
```

**LH β cDNA**

```
  GAA TTC AGG TTA GCT CAT GTA GCC ACC CTG ATC ACC CTG TTT CTC TCT CTC TCT A
-23
  M L G L L T V G T L I S L F L C I L
  -7
  61
  CTG GAC AAC ATG GAG GGG TCT CTC ATG CAG CCC TGT CAG CCC ATC AAC CAG ACT GTC TCT
  L E P M E G A S L M Q P C Q P I N Q T V S
  -121
  CTG GAG AAG AGC GGC TGC CCA AGG TGC TTA GTG CTA CAC ACC CTC ATC GTC ATG GCC CAC
  L E K E G C P T C L V I Q T P I C S G H
  181
  TOC GTC ACC AGC GAG CGG GTT TCC AAG AGC CCA TTT TOC ACC GTG TAC CAG CAT GTG TGC
  35
  C V T K E P F V K S P F S T V Y Q H V C
  241
  ACC TAC CGG GAC GTC CCC TAT GAA AGC ATC CCG CTA CTA GAC TCT CTC GTT CCC CGG TGT GAC
  55
  T Y R D V R Y E T I R L P D C P P W V D
  301
  CCT CAT GTC ACC TAC CTT GCT GTG ATG ACG ATC TGC TGT AAC ATG GAC ACT
  75
  P H V T Y P V A L S C D C S L C N M D T
  361
  TCT GAC TGG ACC ATC AGG CTC CAG CCA GAC TCC TCC TCT ACC CAA AGA GTA CTA ACG
  95
  S D C T I E S L Q P D F C I T Q R V L T
  421
  GAT GGT GAC ATG TGG TGA CAT GTC AGC CAA CAA GCC TCC GTA CTG TAA GAT GAC TTA TAT
  105
  D G D M W *
  480
  109
  481
  TAC ACC CCG CTC GTT AAT GAC ATG TAG GGC CAA TGT ATG GTC ACC TAC ACA TAA AGC TAA
  540
  541
  GGG TAC GAA ATC ACG TGC CTG CTA CCC TCA GCA AAA AAA AAA AAA AA A
  601
```

**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.

**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.
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 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.

**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 µg) 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 µg (D) and 5·7 µg (E) total RNA from the pituitary of a vitellogenic female coho salmon.

**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.

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

Experiment 2

In the second experiment, conducted during February 1996, the effects of testosterone, E2 and 17α,20β-P on plasma and pituitary FSH and LH levels, as well as pituitary FSHβ and LHβ 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 17α,20β-P-treated males. In females, E2 treatment caused a significant reduction in FSH plasma levels, while testosterone and 17α,20β-P were not significantly different from those in controls. Plasma FSH levels prior to treatment were 1·97 ± 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 expressed as pg mRNA/µg DNA. Steady state transcript levels for FSHβ were unaltered by the steroid treatment in females; however, in males E2 caused a significant reduction in FSHβ mRNA levels. As in the first experiment, both testosterone and E2 caused a significant increase in LHβ 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 17α,20β-P groups, only females were analyzed for pituitary gonadotropin content.
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. 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...
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 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 (FSHβ and LHβ) from coho salmon were cloned and sequenced. The cDNAs were used to synthesize RNA probes for development of RPAs to quantify levels of FSHβ and LHβ 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 FSHβ and LHβ 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
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 FSHβ or LHβ gonadotropin subunits. This was expected since the cDNA comparisons among FSHβ and LHβ showed no greater than 33% sequence identity.

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

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**Figure 12.** Effects of acute in vivo treatment with testosterone, E2 and 17α,20β-P on steady state levels of mRNA for FSHβ and LHβ 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 ≤ 0.05.

**Figure 13.** Effects of acute in vivo treatment with testosterone, E2 and 17α,20β-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 ≤ 0.05.
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 17α,20β-P. Since these experiments were conducted when plasma levels of 17α,20β-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, Bommel aer et al. 1981, Van Putten et al. 1981). Larsen & Swanson (1997) also found a post-castration rise in plasma LH in spermatizing 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 (Bom mel aer 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 11β-hydroxyandrostosterone 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.

ACKNOWLEDGEMENTS

We thank Dr Walton W Dickhoff for a critical review of the manuscript. Financial Support for this project was supplied by the United States Department of Agriculture, Grant #93-327203-9409, and is gratefully acknowledged. We would also like to express gratitude to one reviewer whose thoughtful comments significantly improved the manuscript.

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RECEIVED 9 January 1998

Journal of Molecular Endocrinology (1998) 21, 291–306