Quantification and Regulation of Thyroid Stimulating Hormone (TSH) and TSH messenger RNA in salmon

by

Donald A. Larsen

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Abstract

Quantification and Regulation of Thyroid Stimulating Hormone (TSH) and TSH messenger RNA in salmon

by Donald A. Larsen

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School of Fisheries

Thyroid activity is regulated by the hypothalamic-pituitary-thyroid axis. Using a recently developed radioimmunoassay (RIA) for coho salmon (*Oncorhynchus kisutch*) thyroid stimulating hormone (TSH), an *in vitro* pituitary cell culture technique was employed to examine regulation of TSH secretion by corticotropin-releasing hormone (CRH) family peptides (ovine CRH, carp urotensin I, and frog sauvagine) as well as thyrotropin-releasing hormone, salmon growth hormone-releasing hormone, and salmon gonadotropin-releasing hormone. The results of these experiments demonstrated the potency of a CRH-like peptide in the hypothalamic regulation of TSH in fish similar to that which has been observed by others in premetamorphic amphibians.

In order to study salmon TSH transcription, an RNase protection assay (RPA) was designed for quantification of steady state levels of salmon TSH β subunit mRNA expression. This assay, along with a similar RPA previously designed for coho salmon total α-subunit mRNA, was used to examine the effects of feeding T3 and methimazole (a thyroid inhibitor) on TSH subunit

gene expression. These experiments confirmed that, as in mammals, TSH α and β subunit expression in teleosts may be differentially regulated by negative feedback from the thyroid hormones.

Thyroid activity increases dramatically during salmonid smoltification. We quantified, and examined feedback relations between, pituitary TSH β mRNA (by RPA) and pituitary and plasma TSH and plasma thyroid hormone levels (by RIA) during smoltification of coho salmon. Pituitary TSH β mRNA changed dramatically, from high levels in winter to low levels in the spring, while pituitary and plasma TSH levels showed a small, but statistically non-significant change. In contrast with TSH protein levels, large increases in plasma T4 and modest increases in plasma T3 were observed. Regression analyses showed a significant positive relationship between plasma T4 and T3, and a negative relationship between plasma T3 and pituitary TSH β mRNA. All other relations were not significant. These data are supportive of a peripheral (i.e. change in T4-T3 conversion, tissue sensitivity and clearance) rather than central regulatory pathway for thyroid activity in fish.

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Chapter 1

INTRODUCTION

Thyroid stimulating hormone (TSH), otherwise known as thyrotropin, is produced in the thyrotropes of the anterior pituitary of all vertebrate classes. Its function is to regulate synthesis and secretion of the thyroid hormones (Magner, 1990). The thyroid hormones, in turn, control various metabolic and developmental processes including protein synthesis, carbohydrate metabolism, thermogenesis (mammals), and cell growth (Oppenheimer, 1979, 1985; Morley, 1981; Shupnik *et al.*, 1989).
The first investigators to demonstrate that the pituitary contained a thyrotropic substance were Smith (1916) and Allen (1916) through pituitary extirpation experiments in frog larvae. Reviews of the discovery of TSH have been prepared by numerous scholars including the work by Sawin (1988).

TSH is a member of the vertebrate pituitary glycoprotein hormone family. Included within this family are the gonadotropins: follicle-stimulating hormone (FSH), luteinizing hormone (LH), and placental chorionic gonadotropic hormone (CG) (Pierce and Parsons, 1981). In teleost fish the gonadotropic hormones, which are homologous to tetrapod FSH and LH, have historically been called gonadotropin I (GTH I) and gonadotropin II (GTH II), respectively (Suzuki *et al.*, 1988a,b; Itoh *et al.*, 1988, 1990; Sekine *et al.*, 1989; Kawauchi *et al.*, 1989; Swanson *et al.*, 1991; Gen *et al.*, 1993; Kato *et al.*,

1993). More recently, this nomenclature for the fish gonadotropins has been changed to that of tetrapods (Querat, 1993, 1995).

The pituitary glycoproteins consist of two non-identical, non-covalently linked, glycosylated subunits termed α and β. In general, within a species, the α subunits are identical among the gonadotropins and TSH while the β subunits confer hormonal specificity. Fish are unique in that two types of α subunit are present in some species (Chang *et al.*, 1988; Suzuki *et al.*, 1988c; Gen *et al.*, 1993; Koide *et al.*, 1993); however, the functional significance of these two forms is, to date, unknown. The β subunit is essential for receptor recognition while the α subunit is involved in receptor activation. Furthermore, it has been shown that after the initial association of the two subunits, a conformational change affecting both subunits leads to an active heterodimer (Combarnous, 1992).

As is the case with most hormones, the most significant strides in our understanding of TSH regulation and function have come with the ability to quantify the hormone. Several investigators have developed radioimmunoassays for quantification of pituitary and plasma TSH in mammalian species (Magner, 1990). However, until now, investigation of pituitary control of the fish thyroid has been hampered by the lack of sufficient quantities of highly purified fish TSH for studies of its biological effects and for development of sensitive homologous TSH immunoassays.

Studies of the brain-pituitary-thyroid axis of fish have been limited to either histology or measurement of thyroxine levels as an index of TSH activity (Gorbman, 1946, 1969; Pickford and Atz, 1956; Grau and Stetson, 1977; Ng *et al.*, 1982; Kuhn *et al.*, 1986; Swanson *et al.*, 1987). The first published attempts to isolate TSH were conducted by Fontaine and Condliffe (1963) and Fontaine (1969) in the European eel (*Anguilla anguilla*) and common carp (*Cyprinus carpio*). Ng *et al.* (1982) isolated two thyrotropic fractions from American plaice (*Hippoglossoides platessoides*), winter flounder (*Pseudopleuronectes americanus*), chum salmon (*O. keta*) and, common carp. More recently, isolation of TSH has been reported for a variety of fish species: the common carp (Kuhn *et al.* 1986), European eel (Marchelidon *et al.*, 1991), coho salmon, (*O. kisutch*) (Swanson *et al.*, 1987), tilapia (*Oreochromis nilotica*), (Byamungu *et al.*, 1991), Indian major carp (*Cirrhinus mrigala*) (Bandyopadhyay and Bhattacharya, 1993), and black silver carp (*Aristichthys nobilis*) (Banerjee *et al.*, 1994). However, due to the lack of significant quantities of hormone in the pituitary and the difficulties associated with separating TSH from the closely related gonadotropins, no TSH immunoassays had been developed as a result of these studies.

Isolation, characterization, and development of a radioimmunoassay for coho salmon TSH represented nearly a 20 year odyssey. In the initial doctoral work of Dr. Penny Swanson, a specific *in vivo* bioassay for TSH was developed (Swanson and Dickhoff, 1987; Swanson *et al.*, 1987), and coho

salmon thyrotropic activity was separated from gonadotropic activity (Swanson *et al.*, 1987, 1991). Although this TSH fraction induced a significant and dose-dependent increase in thyroxine levels of coho salmon and lacked gonadotropic activity, the preparation was later found to be insufficiently pure for amino acid sequence analysis or antisera production. After analysis by reverse-phase high-performance liquid chromatography (rpHPLC), the major protein in the TSH fraction was identified as α subunit, and the amount of TSH β subunit was too low for subsequent analysis.

Through the continuing efforts of Moriyama *et al.* (1997), highly purified coho salmon TSH was eventually isolated from pituitary glands by sequential gel filtration chromatography, ion-exchange chromatography, and rpHPLC. A relatively high yield of protein, compared with previous efforts, allowed for subsequent screening of the rpHPLC fractions by immunoblot analysis and homologous bioassay (Swanson and Dickhoff, 1987; Swanson *et al.*, 1987), subunit size determination by SDS-PAGE, and N-terminal sequence analysis. Finally, TSH β antiserum was generated, allowing for immunocytochemical analysis and development of the first homologous TSH RIA for fish.

An alternative, but complementary, approach to measuring TSH activity is to use molecular techniques to examine steady state levels of the pituitary messenger RNA (mRNA) coding for the TSH subunits. The RNase

protection assay (RPA or solution hybridization assay), first described by Durnam and Palmiter (1983), was used by Carr *et al.* (1985) to examine regulation of pituitary α and TSH β mRNA expression in the rat. In non-mammalian vertebrates, the only study to quantify total α and TSH β mRNA expression was conducted by Buckbinder and Brown (1993) in the African clawed frog, *Xenopus laevis,* using Northern blot analysis followed by scanning laser densitometry. No such studies had previously been conducted in fish. Chapter 3 of this dissertation describes the development of an RPA for quantification of coho salmon pituitary TSH β mRNA subunit levels.

In mammals, thyroid activity is regulated through the hypothalamic-pituitary-thyroid (H-P-T) axis: the hypothalamus regulates pituitary TSH which, in turn regulates thyroid activity. Furthermore, negative feedback of the thyroid hormones at the level of the hypothalamus and pituitary plays a significant regulatory role as well (Oppenheimer, 1979, 1985). Prior to the development of direct quantification techniques for fish TSH, regulation of this axis in fish was poorly understood. Chapter 2 describes the use of a *in vitro* pituitary cell culture technique to examine hypothalamic regulation of TSH secretion. Chapter 3, in addition to describing the development of an RPA for TSH β mRNA, examines negative feedback of the thyroid hormones on TSH subunit transcription. Finally, anadromous salmonids undergo a period of heightened thyroid activity during the physiological process of

smoltification (freshwater to seawater transformation) (Hoar, 1988). While it is known that TSH stimulates thyroid activity, the lack of a reliable assay for quantifying fish TSH has impeded efforts to understand the role of endogenous TSH in this process. Chapter 4 examines the smoltification-associated changes in pituitary TSH β mRNA, pituitary and plasma TSH and plasma thyroid hormones during this important developmental period.

Chapter 2

Hypothalamic regulation of the pituitary-thyroid axis in coho salmon, *Oncorhynchus kisutch*.

A. INTRODUCTION

Investigation of hypothalamic regulation of thyrotropin (TSH) secretion in teleost fish has been impeded by the lack of a sensitive immunoassay for TSH. Most studies have relied on indirect measures of TSH release, such as histological and immunohistological assessment of thyroid activity or quantification of plasma thyroid hormone levels (Eales, 1979; Eales and Brown, 1993). While thyrotropin-releasing hormone (TRH, *p*Glu-His-Pro-NH_2) appears to be a principal positive regulator of the thyroid axis in mammals (Morley, 1981) and birds (Sharp and Klandorf, 1985), it is not clear what role TRH plays in regulation of thyrotropic activity in ectothermic vertebrates including reptiles, amphibians (for review see Licht and Denver, 1990) and fish (for review see Crim *et al.*, 1978). In studies of reptiles and postmetamorphic amphibians, regulation of TSH seems less specific than in mammals; being stimulated by various neurohormones including TRH, gonadotropin-releasing hormone (GnRH), growth hormone-releasing hormone (GHRH), vasoactive intestinal peptide (VIP) and corticotropin-releasing hormone (CRH)-like peptides (Jacobs *et al.*, 1988a,b; Jacobs and Kuhn, 1989; Denver, 1988; Denver and Licht, 1989a; Licht and Denver, 1990). However, in premetamorphic amphibians, stimulation of the pituitary-

thyroid axis appears to be more specific; only being responsive to CRH-like peptides and unresponsive to all other neurohormones tested, including TRH (Jacobs *et al.*, 1988a; Denver and Licht, 1989b; Licht and Denver, 1990; Denver, 1993, 1996, 1997a). In fish, the effects of TRH on the thyroid axis range from stimulatory (Kaul and Vollrath, 1974; Deering and Jones, 1975; Tsuneki and Fernholm, 1975; Eales and Himick, 1988) to no effect (Wildmeister and Horster, 1971; Peter, 1973; Peter and McKeown, 1975; Deering, 1975; Crim *et al.*, 1978; Tsuneki and Fernholm, 1975; Dickhoff *et al.*, 1978a; Gorbman and Hyder, 1973) to inhibitory (Bromage, 1975; Bromage *et al.*, 1976). Finally, it has been proposed by some investigators that thyrotropin secretion in fish is principally regulated via inhibitory hypothalamic control (Ball *et al.*, 1972; Crim *et al.*, 1978; Peter, 1973; Peter and Fryer, 1983; MacKenzie *et al.*, 1987; Sukumar *et al.*, 1997). Both somatostatin and dopamine are potential candidates for this inhibitory control based on their actions in mammals (Morley, 1981).

The objective of the present study was to investigate hypothalamic regulation of TSH in fish. Using a *in vitro* pituitary cell culture technique, we employed a recently developed radioimmunoassay (RIA) for coho salmon TSH (Moriyama *et al.*, 1997) to examine directly, for the first time in fish, regulation of TSH secretion by TRH as well as salmon (s)GHRH, sGnRH and CRH family peptides (ovine CRH, carp urotensin I (UI), and frog sauvagine

(SV)). We also examined the effect of the CRH antagonist, α-helical CRF $_{(9-41)}$ on oCRH-stimulated TSH release by salmon pituitary cells.

B. MATERIALS AND METHODS

Animals

All experiments were conducted using 2-year-old immature coho salmon, *Oncorhynchus kisutch* (body weight ranged from approx. 200 to 600 g) obtained from either Domsea Farms Inc. (Rochester, WA) or the offspring from the stock that returns yearly to the University of Washington, School of Fisheries hatchery (Seattle, WA). The fish were reared in fresh water at the Northwest Fisheries Science Center (Seattle, WA) in 1.3-or 2.6-m-diameter cylindrical fiberglass tanks with flow-through dechlorinated municipal water, and fed standard rations of Biodiet Grower Pellets (Bioproducts, Warrenton, OR). They were maintained under natural photoperiod; temperature ranged from 8°C (January) to 15°C (August).

Hormones

Thyrotropin releasing hormone (TRH) was purchased from Sigma (St. Louis, MO). Salmon GnRH was purchased from Peninsula Laboratories (Belmont, CA). Salmon GHRH, ovine CRH, SV, UI and α-helical CRF $_{(9-41)}$ where generously provided by Jean Rivier (Salk Institute, La Jolla, CA). All peptides were dissolved directly in Hank's balanced salt solution (HBSS)

(136.9 mM NaCl, 5.4 mM KCl, 0.8 mM $MgS0_4$, 0.3 mM Na_2HPO_4, 8.0 mM $NaHCO_3$, 1.26 mM $CaCl_2$, 2.0 mM glucose, 10 mM HEPES, and phenol red) except sGHRH which was dissolved in HBSS containing ascorbic acid at a final concentration of 0.5 mM.

Pituitary Cell Culture

The pituitary cell culture technique was modified from Weil *et al.* (1986). For each experiment, pituitary glands were removed from 10-20 fish and placed in ice-cold modified HBSS supplemented with gentamycin sulfate (10 µg/ml) (Gibco, 15750-011) and antibiotic-antimycotic (100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate and 0.25 µg/ml amphotericin B as Fungizone in 0.85% saline) (Gibco, 15240-039). Pooled glands were rinsed three times in HBSS and then minced to approximately 0.1 mm^3 with a sterile razor blade in several drops of 0.1% trypsin 1:250 (Gibco, 840-7072IC) in HBSS containing 0.3% bovine serum albumin (BSA) (Fraction V, Sigma). All subsequent steps were carried out in a 15°C incubator under atmospheric air. Minced tissue was transferred to a trypsinizing flask containing 10 ml of the trypsin solution and stirred for 30 min at 1 rev/sec. Every 10 min, throughout the trypsinization process, the cells were suspended via gentle suction with a plastic Pasteur pipette. The suspension was centrifuged (Model CL with swinging buckets; International Equipment Co., Needham, MA) at 200 g for 10 min to collect the cell pellet. The pellet was then suspended in 10

ml trypsin inhibitor (25 mg/10 ml HBSS-0.3% BSA) (Sigma, Type I-S 3T-9003) and incubated under gentle shaking (Orbit Shaker, Lab-Line Instruments Inc., Melrose Park, IL) for 5 min. The pellet was centrifuged as above; then gently shaken for 5 min in 10 ml DNase II (100 µg/10 ml HBSS-0.3% BSA) (Sigma, D-8764) and reconcentrated via centrifugation as above. To reduce cell clumping, the cell pellet was subjected to three washes (followed by centrifugation as above): first, 10 ml Ca^{++}-free HBSS containing 2 mM EGTA; then 10 ml, Ca^{++}-free HBSS containing 1 mM EGTA; finally, 10 ml Ca^{++}-free HBSS. The cell suspension was filtered through a 100-mesh stainless steel screen, centrifuged as above, and suspended in 10 ml Ca^{++}-free HBSS.

The number of cells was determined by diluting 0.2 ml of the cell suspension in 0.8 ml of Ca^{++}-free HBSS and counting using a Thoma hemocytometer. The viability of the cells was determined by trypan blue staining and ranged from 82 to 95%. Following cell number determination, the cells were centrifuged and resuspended in Waymouth's medium (Gibco, 51400-026) supplemented with gentamycin sulfate and antibiotic-antimycotic (same concentrations as above) and plated at a density of 2 X 10^5 cells/ml in either 48- (500 µl/well) or 96- (250 µl/well) well sterile cell culture plates (Costar; Cambridge, MA).

Cells were allowed to settle for 3 days, at 15°C under atmospheric air prior to exposure to treatments. After 3 days, the cells were washed once with HBSS then incubated for 6 hr with HBSS-0.1% BSA with or without various

concentrations of peptide from 0.01 to 100 nM. All experiments were conducted with 6 replicate wells/concentration. For the experiment with the CRH antagonist, the cells were first preincubated for 4 hr with or without 1 μM α-helical CRF $_{(9\text{-}41)}$, then incubated for 24 hr (as opposed to 6 hrs for all other experiments) with or without oCRH. The longer incubation time for oCRH in the antagonist experiment was used because a similar protocol had been used successfully in other GnRH antagonist experiments in our laboratory (unpublished data). Furthermore, in a preliminary experiment (data not shown), antagonist-treated cells were less responsive to CRH treatment under shorter incubation times. Following incubation, the medium was collected and stored at -80°C until assay.

Radioimmunoassay and Data Analysis

Pituitary medium was assayed in 100 μl duplicates for TSH using a homologous salmon TSH radioimmunoassay (RIA) described previously by Moriyama *et al.* (1997). The range (average ± SEM) of the TSH RIA (n = 8; assays run over 6-wk period) was from 0.4 ± 0.4 ng/tube (ED 80) to 5.1± 0.7 ng/tube (ED 20), and the intra- and inter-assay coefficients of variation were 11.2 and 18.1%, respectively. For the experiment examining sGnRH, medium was also assayed for GTH I (Swanson *et al.*, 1989). Differences between the amount of hormone secreted by control cells and cells receiving different concentrations of neurohormones were statistically analyzed by analysis of

variance (ANOVA) followed by the Fisher Protected Least Significant Difference Test (Dowdy and Weardon, 1991) using the Statview 4.0 program (Abacus Concepts, Inc.). Due to variation in the level of basal secretion from cells in different experiments, the data are expressed graphically as a percentage of control values. In each figure the average % SEM for the control levels is indicated by the shaded region around the 100% line.

C. RESULTS

Six separate cell culture experiments were conducted; the results from four experiments are presented in Figure 1. The first two examined the TSH concentration response of pituitary cells to increasing concentrations of TRH or oCRH at two different times of year (May and November) (Fig. 1A and B). This experiment was conducted during two separate seasons to demonstrate that the results were repeatable and to examine whether the sensitivity of the cells might differ between spring and autumn. In both May and November, TRH did not elicit a concentration-dependent change in TSH secretion (Fig. 1A). However, at a single concentration of 1.0 nM TRH, there was a significant increase above control levels in TSH secretion in November. In contrast to the results found with TRH, oCRH caused a significant concentration dependent increase in TSH secretion during both May and November (Fig. 1B). In both oCRH concentration-response curves, there was

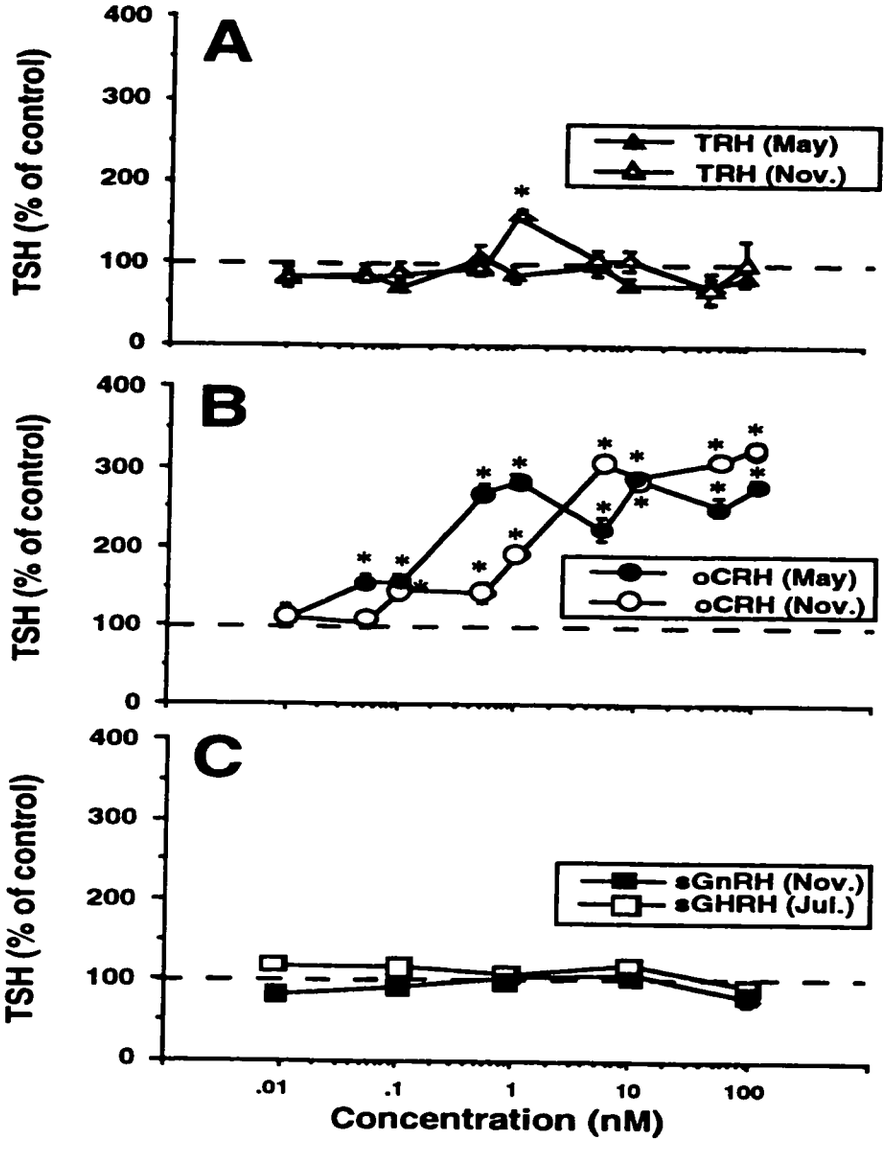


FIGURE 1. Release of TSH from cultured pituitary cells, isolated from 2-yr-old coho salmon, treated for 6 hr with various concentrations of (A) thyrotropin-releasing hormone (TRH) during May and November, (B) ovine corticotropin-releasing hormone (oCRH) during May and November, (C) gonadotropic-releasing hormone (sGnRH) during November and salmon growth hormone-releasing hormone (sGHRH) during July. Data are expressed as a mean percent of control ± percent SEM; n = 6 replicate wells/treatment. Shaded region shows SEM for control wells. Asterisks indicate significant differences from control ($P < 0.05$).

a plateau which varied seasonally. In May, peak secretion was attained with a concentration of 0.5 nM oCRH, whereas in November peak secretion required a tenfold higher concentration of oCRH.

The concentration response of sGnRH and sGHRH was examined in two separate experiments conducted in November and July, respectively (Fig. 1C). Similar to TRH, neither sGnRH nor sGHRH stimulated secretion of TSH from salmon pituitary cells. Some additional wells from the sGHRH experiment were treated with various doses of oCRH and found to secrete TSH in a concentration-dependent manner (data not shown). Thus, the cells were responsive to hypophysiotropic stimulation by CRH, but not sGHRH. The medium from the sGnRH treated cells was also assayed for GTH I content and showed elevated GTH I indicating that gonadotropes in this culture system were responsive (Fig. 2).

Following the observation that oCRH was a potent stimulator of TSH secretion from salmon pituitary cells, we compared the effects of oCRH to two CRH related peptides; frog skin sauvagine (SV) and carp Urotensin I (UI) (Fig. 3). Similar to the first experiment (Fig. 1B), oCRH-treated cells secreted significantly more TSH than control cells. However, the concentration-response curve was irregular in that cells were significantly stimulated at the lowest concentration tested of 0.01 nM, as well as at the higher concentrations of 1.0, 10 and 100 nM. They were not, however, significantly stimulated at the intermediate concentration of 0.1 nM. SV caused a significant, concentration-

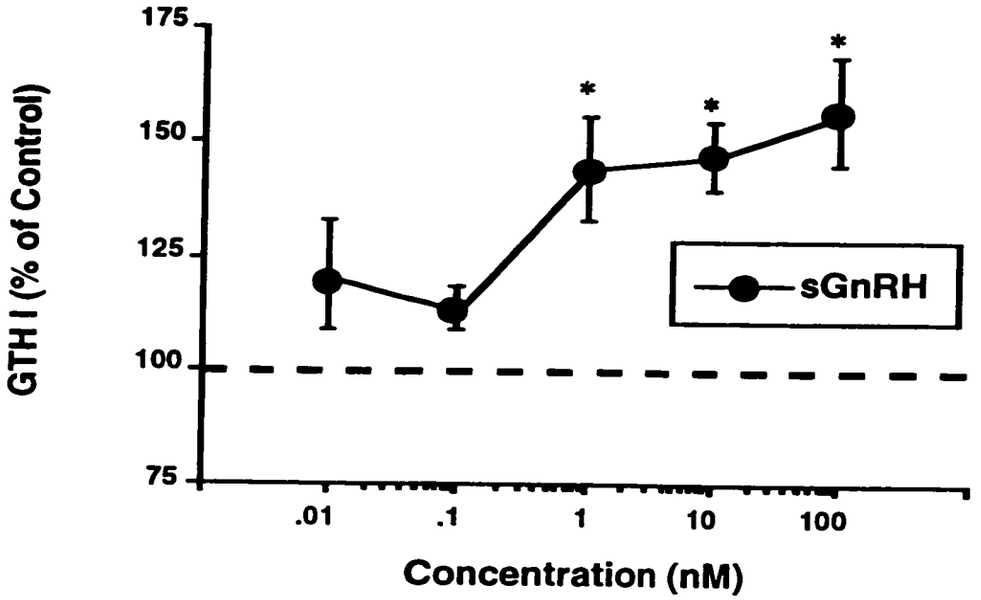


FIGURE 2. Release of GTH I from cultured pituitary cells, isolated from 2-yr-old coho salmon, treated for 6 hr with sGnRH during November. Data are expressed as a mean percent of control ± percent SEM; n = 6 replicate wells/treatment. Shaded region shows SEM for control wells. Asterisks indicate significant differences from control ($P < 0.05$).

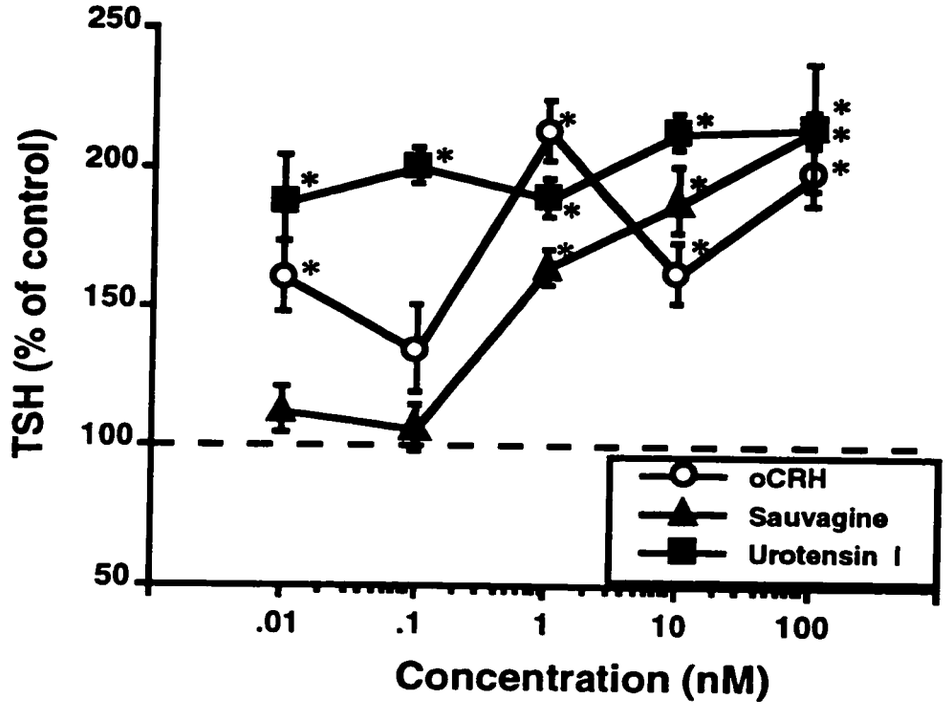


FIGURE 3. Release of TSH from cultured pituitary cells, isolated from 2-yr-old coho salmon, treated for 6 hr with oCRH, sauvagine (SV) or urotensin I (UI) in June. Data are expressed as a mean percent of control ± percent SEM; n = 6 replicate wells/treatment. Shaded region shows SEM for control wells. Asterisks indicate significant differences from control ($P < 0.05$).

dependent increase in TSH secretion as well. There was no significant difference between treated and control cells at the lowest concentrations of 0.01 and 0.1 nM SV, but higher concentrations (1 to 100 nM) stimulated TSH release. Finally, UI stimulated TSH secretion at all concentrations tested, with maximum secretion of TSH being achieved at the lowest concentration tested of 0.01 nM and maintenance of that level at all higher concentrations (Fig. 3).

The final experiment was conducted to examine whether oCRH-dependent TSH secretion in salmon could be blocked by a known antagonist of CRH-dependent ACTH secretion in goldfish (Weld *et al.*, 1987). Cells were either preincubated with or without 1 μM α-helical CRF $_{(9\text{-}41)}$ for 4 hr then treated with or without increasing concentrations of oCRH for 24 hr. Once again oCRH caused a significant concentration-dependent increase in TSH secretion above control levels at the concentrations from 1 to 1000 nM oCRH (Fig. 4). However, cells preincubated with 1 μM α-helical CRF $_{(9\text{-}41)}$ secreted significantly less TSH than the cells receiving no antagonist at the intermediate concentrations of 1 and 10 nM oCRH (Fig. 4). At the higher concentrations of oCRH (100 and 1000 nM), the pretreatment concentration of antagonist was insufficient to block oCRH action (Fig. 4).

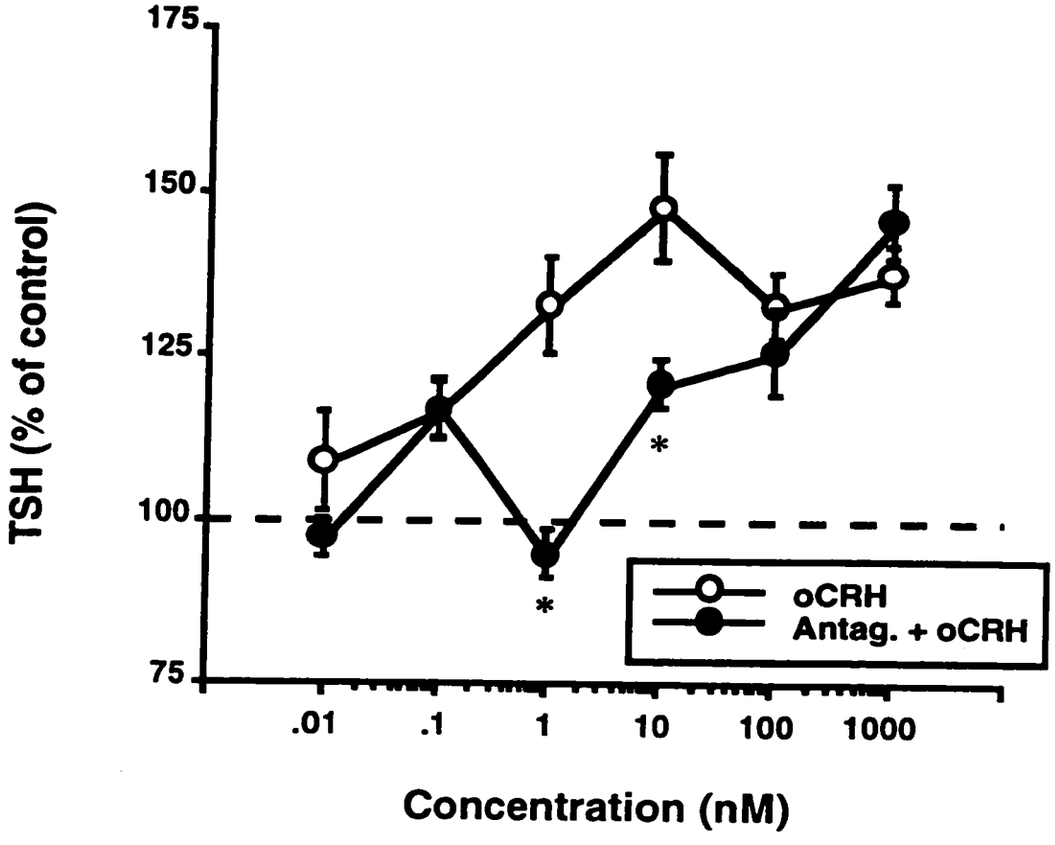


FIGURE 4. Release of TSH from cultured pituitary cells, isolated from 2-yr-old coho salmon, preincubated for 4 hr with or without 1 μM CRH antagonist, α-helical CRF $_{(9\text{-}41)}$ (Antag.), then incubated for 24 hr with various concentrations of oCRH in August. Data are expressed as a mean percent of control ± percent SEM; n = 6 replicate wells/treatment. Shaded region shows SEM for control wells. Asterisks indicate significant difference from cells not pretreated with antagonist ($P < 0.05$).

D. DISCUSSION

Historically, the lack of a reliable assay for TSH has impeded efforts to clarify neuroendorcrine regulation of the thyroid axis in fish. With the recent development of an RIA for salmon TSH (Moriyama *et al.*, 1997) we were able to directly quantify TSH secretion from pituitary cells treated with various hypophysiotropins. Results from these experiments demonstrate that the CRH-family peptides are potent stimulators of *in vitro* TSH secretion from salmon pituitary cells, while TRH, sGHRH and sGnRH are not. Furthermore, we successfully demonstrated that α-helical CRF $_{(9\text{-}41)}$ antagonizes CRH-stimulated TSH release similar to its effect on CRH stimulated ACTH release in goldfish (*Carassius aurata*) (Weld *et al.*, 1987).

It has been established that TSH is a central regulator of the thyroid axis in fish (see reviews by Leatherland, 1988; Eales, 1979; Eales and Brown, 1993). However, prior to the development of direct quantification techniques for TSH, the identities of the stimulatory as well as inhibitory factors for TSH regulation have been elusive. Studies, using hypothalamic lesions, *in vitro* pituitary cell culture, and pituitary transplantation have established that the hypothalamus also regulates activity of the thyrotrophs in various teleosts. TRH has received the greatest attention in studies designed to examine neuroendocrine control of the thyroid axis of fish, due to its proven stimulatory role in TSH synthesis and secretion in mammals (Morley, 1981), birds (Sharp and Klandorf, 1985), reptiles and adult amphibians (Darras and

Kuhn, 1982; Denver, 1988, 1993; Preece and Licht, 1987; Jacobs *et al.*, 1988a; Denver and Licht, 1989a; Licht and Denver, 1990). But, contrary to what is observed in other vertebrates, most studies in fish have indicated that the thyroid axis is regulated primarily through inhibitory hypothalamic control (Ball *et al.*, 1972; Crim *et al.*, 1978; Peter and Fryer, 1983; MacKenzie *et al.*, 1987; Sukumar *et al.*, 1997).

In the present investigation, TRH did not consistently affect secretion of TSH from salmon pituitary cells (Fig. 1A). These results are in agreement with earlier studies in fish (Wildmeister and Horster, 1971; Peter, 1973; Peter and McKeown, 1975; Deering, 1975; Crim *et al.*, 1978; Tsuneki and Fernholm, 1975; Dickhoff *et al.*, 1978a; Gorbman and Hyder, 1973) and larval amphibians (see Norris and Dent, 1989; Jacobs *et al.*, 1988a; Denver and Licht, 1989b; Denver, 1993) which suggested that TRH did not influence the thyroid system. In amphibians, the stimulatory action of TRH on TSH secretion appears to develop after metamorphosis (Denver, 1988, 1996, 1997a; Denver and Licht, 1989b; Jacobs *et al.*, 1988a). The results from this study are in disagreement with the stimulating effect of TRH in fish reported by Kaul and Vollrath (1974) and Tsuneki and Fernholm (1975), who used histological changes in thyrotroph cells, and Deering and Jones (1975), who measured pituitary adenyl cyclase activity. Furthermore, Eales and Himick (1988) demonstrated that TRH acutely elevates plasma T4, but not T3 in arctic charr (*Salvelinus alpinus*) and rainbow trout (*O. mykiss*). These data suggest that

TRH may act directly on the thyroid gland without pituitary involvement or there may be species differences in TSH responsiveness to TRH.

Similar to TRH, neither sGnRH nor sGHRH stimulated secretion of TSH from pituitary cells (Fig. 1C). The responsiveness of the gonadotrophs in the sGnRH experiment was demonstrated by the secretion of GTH I from these pituitary cells (Fig. 2). However, it is noteworthy that the thyrotrophs in this preparation were not tested for responsiveness to oCRH. In the sGHRH experiment, additional cells were found to be responsive to oCRH (data not shown). Furthermore, similar studies have shown that sGHRH weakly stimulates GH release from salmon pituitary cells (Parker *et al.*, 1997). Thus, assuming all cell types were responsive in both experiments, the thyrotrophs did not secrete TSH in response to sGnRH and sGHRH, but the gonadotrophs and somatotrophs were stimulated to secrete GTH I and GH, respectively, in response to these hypophysiotropins.

All of the fish used in our study had already gone through smoltification; a metamorphic-like process whereby a freshwater-adapted parr goes through a series of morphological, physiological, and behavioral changes to become a sea water-adapted smolt (Hoar, 1988). In light of the change in sensitivity of the pituitary to TRH observed in amphibians after metamorphosis, one might anticipate that the salmon pituitary would be responsive to TRH following smoltification. The lack of response to TRH in

salmon compared to post-metamorphic amphibians may simply represent a phylogenetic difference between the separate organisms.

Recently, several studies of nonmammalian vertebrates have shown that CRH-like peptides stimulate the thyroid axis (birds: Meeuwis *et al.*, 1989; Geris *et al.*, 1996; reptiles: Denver and Licht, 1989a; Licht and Denver, 1990; amphibians: Denver, 1988; Jacobs and Kuhn, 1989; Denver and Licht, 1989b; Gancedo *et al.*, 1992, Denver, 1993, 1996, 1997a; fish: De Pedro *et al.*, 1995). Furthermore, treatment of various frog species with CRH-family peptides (Gancedo *et al.*, 1992; Denver, 1993; 1997b) accelerated metamorphosis, while treatment with CRH antisera inhibited metamorphosis (Denver, 1993; 1997b). It is believed that the primary role of CRH in mammals is stimulation of ACTH activity (Plotsky, 1985). CRH has also been shown to stimulate ACTH secretion in amphibians (Tonon *et al.*, 1986) and fish (Fryer *et al.*, 1983, 1984). In fact, it has been proposed that during amphibian metamorphosis, a CRH-like molecule(s) may be involved in co-regulating both the thyroid and interrenal axes (Denver and Licht, 1989b; Licht and Denver, 1990; Denver, 1993; 1996; 1997a,b).

In the current investigation, the CRH-family peptides (oCRH, UI, and SV) proved to be potent stimulators of TSH secretion in salmon (Fig. 1B and Fig. 3). Ovine CRH consistently stimulated TSH secretion from pituitary cells in four experiments conducted at different times of the year. However, the concentration-response characteristics; maximal responses and effective

concentrations were different in each experiment. These differences may be inherent variation in different cell preparations. On the other hand, these differences may be due to seasonal and/or life history variation in the sensitivity of the hypothalamic-pituitary-thyroid axis to stimulation. In coho salmon, Specker and Schreck (1984) and Swanson and Dickhoff (1987) observed a developmental change in the sensitivity of the thyroid to TSH injection during smoltification; being low in January, then increasing throughout smoltification in May. Similar seasonal changes in sensitivity at the level of the pituitary in response to hypothalamic stimulation may exist as well.

UI, isolated from the urophysis of teleost fish (Ichikawa *et al.*, 1982; Lederis *et al.*, 1982), and SV, isolated from frog skin (Erspamer and Melchiorri, 1980), are CRH-like peptides that have been shown to stimulate TSH secretion in reptiles (Denver and Licht, 1989a) and amphibians (Denver and Licht, 1989b; Denver, 1993). In the current study, UI appeared to be more potent than oCRH and SV, although additional data are needed to clearly demonstrate this point. Nonetheless, these results are similar to those of Fryer *et al.* (1983), who found that *in vitro*, UI was 2-3 times more potent than oCRH or SV in stimulating ACTH release from superfused goldfish anterior pituitary cells. Thus, while inhibitory hypothalamic control (Ball *et al.*, 1972; Crim *et al.*, 1978; Peter and Fryer, 1983; MacKenzie *et al.*, 1987; Sukumar *et al.*, 1997) may predominate in regulating TSH in fish, the present findings

demonstrate potential for positive hypothalamic regulation through a CRH-like molecule.

The CRH antagonist, α-helical CRF $_{(9\text{-}41)}$, has been shown to antagonize the ACTH-releasing activity of CRH in mammals (Rivier *et al.*, 1984) and goldfish (Weld *et al.*, 1987) and to reduce the whole-body T_4 concentration and metamorphic rate in the Western spadefoot toad, *Scaphiopus hammondii* (Denver, 1997b). It is an analogue of CRH in which the first 8 NH_2-terminal amino acids have been deleted and the α-helix formation has been optimized. It is thought to act by specifically competing with CRH in binding to the CRH receptors without causing activation (Rivier *et al.*, 1984). Our results directly demonstrate an inhibitory affect of α-helical CRF $_{(9\text{-}41)}$ on oCRH-stimulated TSH release. Taken together, the results from these various studies suggest that CRH may act on similar receptors in both the pituitary thyrotophs and corticotrophs during activation of the thyroid and interrenal axes.

The results from this investigation suggest that fish are similar to larval amphibians with regard to neurohormonal specificity of TSH stimulation. Of the hypophysiotropins tested, only the CRH-like peptides stimulated TSH secretion from salmon pituitary cells while TRH (Fig. 1A), sGnRH, and sGHRH (Fig. 1 C) did not. *In vitro* and *in vivo* studies of larval amphibians have demonstrated a similar specificity in responsiveness to only CRH-like molecules, but not TRH or GHRH (Denver and Licht, 1989b). As

previously mentioned, in amphibians, the stimulatory action of TRH on TSH secretion appears to develop after metamorphosis (Denver, 1988; Denver and Licht, 1989b; Jacobs *et al.*, 1988a).

The thyroid and the interrenal axes are key endocrine pathways which interact to regulate amphibian metamorphosis (Dodd and Dodd, 1976; White and Nicoll, 1981; Norris and Dent, 1989; Kikuyama *et al.*, 1993; Hayes, 1995; Hayes and Wu, 1995). While less is known in fish, these pathways appear to interact similarly during larval marine fish development (Brown and Kim, 1995) and flatfish metamorphosis (De Jesus *et al.*, 1990; 1991), and both axes are activated during salmonid smoltification (see Hoar, 1988). In amphibians, all available evidence indicates that a CRH-like molecule functions as a co-regulator of both TSH and ACTH release (see Denver, 1997a,b). Although more research is necessary on a diversity of fish species, based on the results of this investigation, this co-regulatory action of CRH may be extended to include fish as well.

Chapter 3

Quantification of salmon α- and thyrotropin (TSH) β-subunit messenger RNA by an RNase protection assay: regulation by thyroid hormones.

A. INTRODUCTION

Thyrotropin (thyroid-stimulating hormone; TSH) is a member of the pituitary glycoprotein hormone family because of its similarity to follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (GTH I and GTH II in fish). Each of these hormones is a heterodimer composed of two non-covalently linked α and β subunits. The α subunit is identical in TSH, FSH and LH; whereas, the β subunit of each hormone is structurally distinct and confers hormonal specificity (Pierce and Parsons, 1981). The subunits are synthesized as separate peptides from distinct messenger RNA (mRNA) (Shupnik *et al.*, 1989).

TSH is the principal regulator of thyroid hormone (thyroxine: T4) synthesis and secretion. T4 is converted by monodeiodinase in peripheral tissues to the more biologically active form, triiodothyronine (T3). The thyroid hormones, in turn, control various metabolic and developmental processes including protein synthesis, carbohydrate metabolism, thermogenesis (mammals), and cell growth (Oppenheimer, 1979; 1985; Morley, 1981; Shupnik *et al.*, 1989).

In mammals, regulation of TSH gene expression, production and secretion is well established (Morley, 1981; Carr *et al.*, 1985; Shupnik *et al.*, 1989; Magner, 1990). Synthesis and secretion of TSH are under positive hypothalamic control by thyrotropin-releasing hormone (TRH) (Taylor and Weintraub, 1985) and negative control by somatostatin (Hirooka *et al.*, 1978) and dopamine (Morley, 1981). Other factors including serotonin, neurotensin, norepinephrine, cholecystokinin, estrogen, (Morley, 1981), glucagon-like peptide-1 (Beak *et al.*, 1996) and, in fish and amphibians corticotrophin-releasing hormone-like peptides (see Chapter 2) are believed to play roles in TSH regulation as well. Perhaps the most important negative regulators of TSH, however, are the thyroid hormones, via a classic negative feedback loop. Elevated circulating concentrations of thyroid hormone feed back at the level of the pituitary and the hypothalamus to suppress synthesis and secretion of TSH. Thus, hypothalamic, thyroid and other hormones interact to modulate TSH gene expression, production and secretion.

Regulation of the thyroid axis in non-mammalian vertebrates has not been completely described, principally due to the lack of a means to directly quantify TSH. In teleost fish, indirect measures of TSH activity and regulation have been used by various investigators (reviewed by Leatherland, 1988). The cDNA for TSH β has been cloned in the rainbow trout (*Oncorhynchus mykiss*) (Ito *et al.*, 1993) and the European eel (*Anguilla anguilla*) (Salmon *et al.*, 1993). In amphibians, Buckbinder and Brown (1993)

cloned the cDNAs encoding TSH α and β subunits in the African clawed frog, *Xenopus laevis*, and quantified mRNA expression during metamorphosis. To date, quantification and regulation of the TSH subunits have not been examined in teleost fish.

The objective of this investigation was to clone the cDNA for the coho salmon (*O. kisutch*) TSH β subunit and design a solution hybridization, or RNase protection assay (RPA), for quantification of steady state levels of TSH β mRNA. This assay was used along with a similar RPA for coho salmon α subunit mRNA, developed by Dickey and Swanson (1995), to examine the effects of T3 and methimazole (a thyroid inhibitor) on TSH gene expression.

B. MATERIALS AND METHODS

Animals

Two separate stocks of immature, two-year old coho salmon (*O. kisutch*) were used. For development of the RPA, broodstock originally obtained from Aquaseed Inc. (Rochester, WA) were used for extraction of total pituitary RNA. Validation of the RPA was conducted using the offspring from broodstock that returns yearly to the University of Washington, School of Fisheries hatchery (Seattle, WA). The fish were reared in fresh water at the Northwest Fisheries Science Center (Seattle, WA) in 2.6 or 1.3 m diameter cylindrical fiberglass tanks, and fed standard rations of Biodiet Grower Pellets

(Bioproducts, Warrenton, OR). They were maintained under natural photoperiod; temperature ranged from 8 °C (January) to 15 °C (August).

Isolation of coho salmon TSH β cDNA

Total RNA was extracted from a pool of pituitaries from five fish of mixed sex (approximate body weight 200-300 g) using a guanidinium isothiocyanate procedure modified from Chomczynski and Sacci (1987). First strand cDNA was synthesized using the reverse transcriptase polymerase chain reaction (RT-PCR) with MMLV reverse transcriptase (Promega, Madison, WI) and a GeneAmp RNA PCR Kit (Perkin Elmer Cetus, Norwalk, CT). A poly (t) primer (5'-GACTGGATCCGAATTCTAGAT$_{(17)}$-3') containing an Eco RI restriction site was employed for the reaction (Frohman, 1993).

The cDNA for coho salmon TSH β was generated by RT-PCR using a forward primer based on TSH β cDNA sequence data from rainbow trout, *O. mykiss* (Ito *et al.*, 1993) and the poly (t) reverse primer. The forward primer (5'-CGCGAATTCGCTGTTTGCCAAATGGAATTG-3'), which spanned part of the 5' untranslated region and the signal peptide, contained an Eco RI restriction site to aid in cloning. The PCR was initially performed with only the forward primer using a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT) under the following conditions: 1 cycle at 94 °C (2 min), followed by 10 cycles of the following sequence; 94 °C (1 min), 52 °C (1 min) and 72 °C (2 min). The reaction mixture contained 0.12 μM forward primer in

ddH_20; 10 µl of first strand cDNA template; 0.25 mM of each dNTP; 50 mM KCl; 10 mM Tris-HCl, pH 9.0; 0.1% Triton X-100 and 2.5 mM $MgCl_2$ at a final volume of 40 µl. Two units of *taq* polymerase (Promega, Madison, WI) were added prior to the first extension (72 °C). A 20 µl aliquot from the original PCR was then subjected to 30 cycles using the forward primer and the poly (t) reverse primer at a concentration of 0.6 µM each, under the same conditions outlined above, but at a final volume of 80 µl.

The PCR product was restriction digested with Eco RI and electrophoresed on a 2% Nusieve agarose gel (FMC Bioproducts, Rockland, ME) with a one kilobase (kb) ladder (Gibco BRL, Gaithersburg, MD) for size reference. Following staining with ethidium bromide, two bands of approximately 1.0 and 0.9 kb were found. The smaller 0.9 kb band, which was the expected size for TSH β, was cut from the gel and purified with a Sephaglass BandPrep kit (Pharmacia-LKB, Piscataway, NJ) and ligated into pUC19 (New England Biolabs, Inc., Beverly, MA) with a DNA ligation kit (Novagen, Madison, WI). Plasmid transformation and amplification were conducted with NovaBlue competent cells (Novagen) and positive clones were purified using a plasmid purification kit (Qiagen, Chatsworth, CA).

Two cloned fragments were partially sequenced approximately 273 bp from the 5′ end and 130 bp from the 3′ end using the Sanger dideoxy method (Sanger *et al.*, 1977) using PUC/M13 primers (Forward#1211, Reverse#1201;

New England Biolabs, Beverly, MA) with a Sequenase Version 2.0 kit (United States Biochemical, Cleveland, OH) on a Model S2 sequencing gel apparatus (Gibco, BRL). Sequence analysis was performed using the MacDNASIS program (Hitachi Software Engineering America, Ltd., San Bruno, CA). The 273 bp at the 5′ end and the 130 bp at the 3′ end showed 99% and 97% base pair identity with the cDNA sequence for rainbow trout (Ito *et al.*, 1993).

Preparation of antisense TSH β riboprobe and sense RNA standard

To develop an RNA probe as well as standard for the RPA, a 199 bp fragment of the TSH β subunit was cloned. The fragment was generated by the PCR using the forward primer previously mentioned and the following reverse primer, once again, containing an Eco RI restriction site: (5′- CGCGAATTCCGGCCAGCTCCTTCATCCTTA-3′) with 5.0 μg of the original cDNA clone as template. Except for the use of a different ligation vector; pBluescript S/K+ vector (Stratagene, La Jolla, CA), the PCR product was restriction digested, purified, ligated, transformed and amplified under identical conditions as described above for the TSH β cDNA. Two positive clones were sequenced in both directions, as above, using commercial primers (T3 and T7; Stratagene) to confer TSH β subunit identity.

A radioactively labeled anti-sense RNA probe for TSH β was generated by linearizing the pBluescript sub-clone with Xba I (New England Biolabs,

Beverly, MA) followed by transcription using a Maxiscript kit (Ambion, Austin, TX) with the T7 RNA polymerase and α-^{32}P CTP (Amersham, Cleveland, OH). The probe was purified using a Quick Spin column (Boehringer Mannheim, Indianapolis, IN).

Sense standard RNA for TSH β was generated by transcription of Hinc II (New England Biolabs, Beverly, MA) linearized plasmid with the Maxiscript kit using the T3 RNA polymerase. The amount of sense RNA standard generated was determined spectrophotometrically at an absorbance of 260 nm and diluted in RNase free water to 160 pg RNA/μl for use in the assay.

RNase Protection Assay (RPA)

Total nucleic acid (TNA) was prepared from salmon pituitary glands and muscle tissue (used for negative control) by digestion of homogenized tissue with proteinase K, followed by extraction with phenol/chloroform (Durnam and Palmiter, 1983). DNA quantification was then determined using Hoechst 33258 dye with a TKO 102 fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

The RPAs were conducted using a RPA II kit (Ambion, Austin, TX). The radioactive antisense RNA probe ($3x10^4$ cpm/3 μl) was combined with either sense RNA standard or nucleic acid samples (total volume 12 μl) in

20 µl of hybridization solution; denatured for 5 minutes at 90 °C, then hybridized overnight at 42 °C. Following hybridization, the unprotected fragments were digested by adding 100 µl of RNase mix (5 U/ml RNase A, 200 U/ml RNase T1) to the samples and the standards and incubating for 2 hrs at 36 °C. For validation, protected fragments from both tissue samples and graded amounts of synthetic sense TSH β standard were precipitated with 100 µl of Dx solution (Ambion, Austin, TX) and analyzed by electrophoresis on a 5% polyacrylamide/8 M urea gel. The gel was transferred to filter paper, covered with plastic wrap and exposed to X-ray film (Kodak XAR5) overnight at room temperature.

Quantification of protected sample fragments and standards was done by adding 50 µl aliquots of the digestion reactions to 5 µl of yeast tRNA carrier (5 mg/ml) and precipitating with 55 µl of 20% trichloroacetic acid (TCA) for 30 min on ice. The reaction mixture was subsequently transferred to a Whatman GF/C membrane (25 mm diameter) using a vacuum manifold (Millipore Corp., Bedford, MA). The filters were washed five times with 500 µl of 5% TCA in 0.02 M sodium pyrophosphate and once with 70% ethanol (1.0 ml) then dried under an incandescent lamp for 30 min and counted on a scintillation counter (Packard Tri-Carb 300). The hybridized signal was compared to that of a standard curve following simple linear regression and expressed as pg mRNA/µg DNA (modified from Duan *et al.*, 1993).

Thyroid hormone feedback on mRNA levels

Feedback of thyroid hormones on TSH subunit transcription was studied using two-year old immature coho salmon during August-September, 1995. Fish were divided into four treatments of 36 fish each (avg. body wt. 111.0 ± 1.8 g). They were fed Biodiet Grower Pellets (Bioproducts, Warrenton, OR) using automatic belt feeders (Babington Enterprises, Inc., Hagerman, ID). Treatment groups received a control diet or diets supplemented with triiodothyronine (T3) (10 or 100 μg/g) or the goitrogen, methimazole (2.5 mg/g). T3 and methimazole were incorporated into the diet by dissolving in 70% alkaline ethanol solution (33 ml 95% ethanol; 12 ml 0.1% NaOH) and spraying the solution onto the surface of the food with an airbrush (Badger 350 airbrush, Badger Air-Brush Co. Franklin Park, IL) using nitrogen as the vehicle. The food was sprayed using 5.0 ml of solution per kg of food. The control diet was prepared in identical fashion, but without T3 or methimazole. All treatment groups were fed at 2% body weight per day.

Treatment groups were reared under the various dietary regimes for four weeks. After two and four weeks, six fish from each treatment group were anesthetized in a buffered solution of 0.05% tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA.), measured for weight and fork length, examined for general appearance and sampled for pituitary glands and plasma. On each sampling date, all treatment groups were sampled at the same time of day (0900-1200); approximately 16 hours

after the last feeding. Pituitary glands were removed, frozen in liquid nitrogen, and stored at -80 °C until assayed by RPA for both TSH β-subunit mRNA as described above and total α subunit mRNA (α1 and α2; Gen *et al.*, 1993) as described by Dickey and Swanson (1995). Blood samples were collected from severed caudal vessels into heparinized Natelson tubes (VWR Scientific), centrifuged and stored frozen at -80 °C until assayed for plasma thyroxine (T4) and triiodothyronine (T3) (Dickhoff *et al.*, 1978b; 1982).

Statistical analysis

Results were analyzed by one-way analysis of variance followed by the Fisher Protected Least Significance Difference test (Dowdy and Weardon, 1991) using Statview 512+ (Brain Power, Inc.). Differences between treatments were considered significant at $P < 0.05$.

C. RESULTS

TSH β RPA standard curve and hybridization signal

The TSH β RPA standard curve was analyzed by autoradiography and scintillation counting of the hybridization reaction products (Fig. 5). Figure 5A shows an autoradiogram of the polyacrylamide gel displaying the following: a 314 bp probe (which includes 115 bp of labeled polylinker RNA), probe digested with RNase, probe hybridized to muscle tissue total nucleic

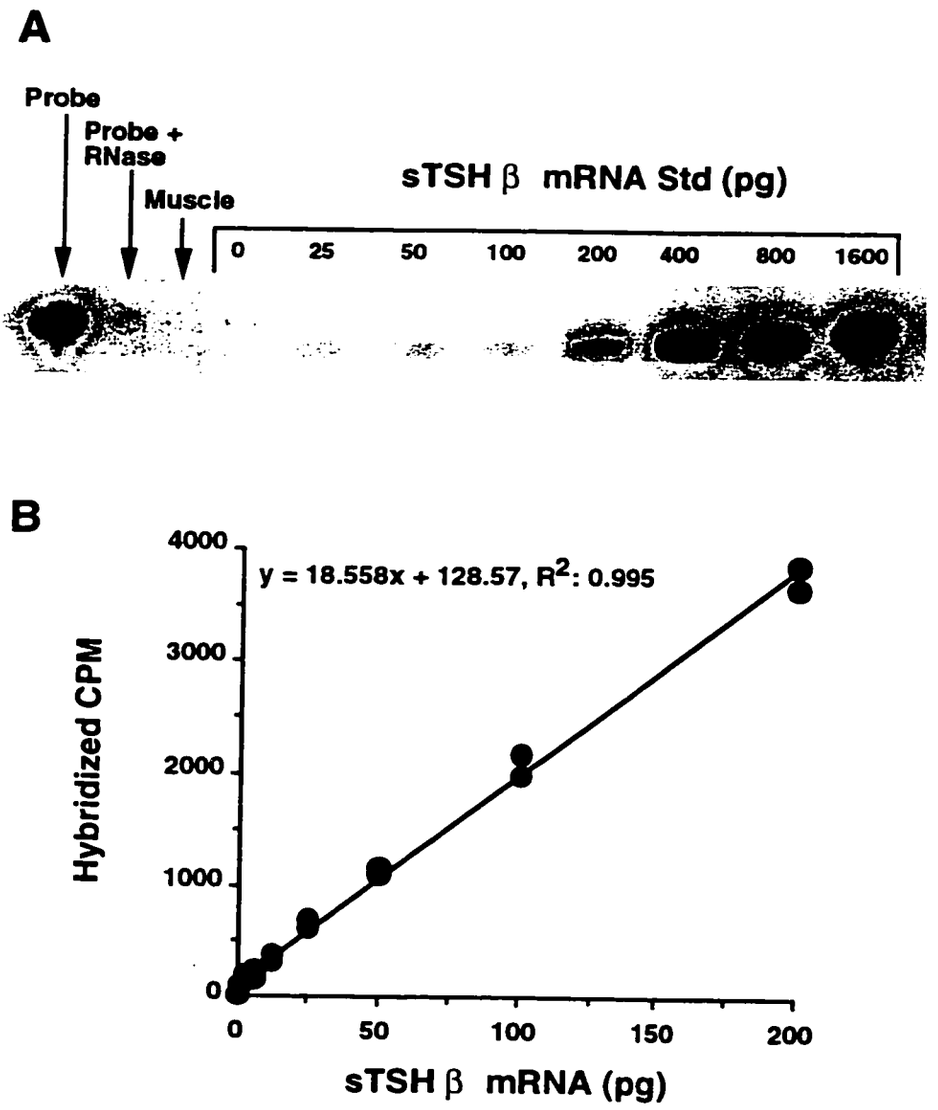
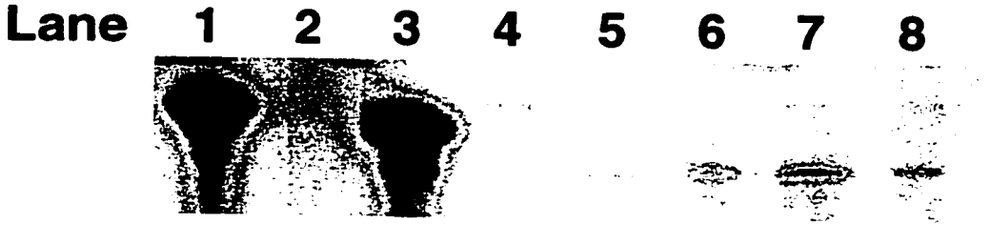


FIGURE 5. Autoradiograph of acrylamide gel and scintillation counting of hybridized probe for TSH β. (A) Autoradiograph shows, from left to right: activity of probe alone, probe treated with RNase, probe hybridized to muscle nucleic acid (negative control), and probe hybridized to TSH β mRNA standard from 0 to 1600 pg. (B) Standard curve from scintillation counting for 0 to 200 pg of TSH β mRNA.

acid (TNA) and graded amounts of synthetic sense TSH β mRNA standard. No visible band appeared when the probe was hybridized to 20 µg of coho muscle tissue TNA, which was the negative control. The protected fragments on the gel from the sense standard curve were slightly smaller than the single stranded probe alone due to the loss of unprotected polylinker DNA during RNase digestion. Although the sense RNA standards were serially diluted from 1600 pg to 0 pg, only that portion of the curve between 0 to 200 pg was linear.

Figure 5B shows a typical curve prepared from protected TCA-precipitated standards with hybridized radioactivity as a function of TSH β mRNA. Background cpm is typically less than 1% of the total cpm. The assay could detect values as low as 1.56 pg/tube. Typically, 5-20 µg of DNA per extracted TNA sample were used. Thus, as little as 0.1 pg/µg DNA can be reliably detected.

The size of hybridized mRNA from salmon tissues was analyzed by electrophoresis on polyacrylamide gels and autoradiography (Fig. 6). Lanes 1 through 4 (Fig. 6) display the following: TSH β probe, probe digested with RNase, an 800 pg protected synthetic sense standard (included as a positive control) and 20 µg of muscle tissue TNA (included as a negative control). Lanes 5 through 8 (Fig. 6) display the protected hybridization signal for either



1) TSH β Probe
2) TSH β Probe + RNAse
3) TSH β Standard (800 pg)
4) Muscle tissue - coho sub-adult (20 μg TNA)
5) Pituitary - coho parr (20 μg TNA)
6) Pituitary - chinook parr (20 μg TNA)
7) Pituitary - coho sub-adult (20 μg Total RNA)
8) Pituitary - spermiating coho (20 μg Total RNA)

FIGURE 6. Autoradiograph of acrylamide gel of radioactive TSH β probe hybridized to nucleic acids extracted from salmon muscle and pituitary tissues.

pituitary TNA from coho and chinook parr or pituitary total RNA from sub-adult and mature coho salmon. The signal from pituitary tissues showed a single band of approximately 199 bp, as predicted. Once again the variation in size between the probe, the 800 pg standard fragment and the pituitary sample fragments results from variation in the amount of protected, radioactively labeled polylinker present following RNase digestion.

Thyroid hormone feedback on mRNA levels

Thyroid hormone feedback on TSH transcription was investigated by comparing the amount of TSH subunit hybridization in pituitary glands from T3 and methimazole-treated versus control animals. The efficacy of the T3 feeding treatment was assessed by measuring plasma T3 and T4 levels. Plasma T3 and T4 levels in fish fed 0, 10 and 100 μg/g T3 for two and four weeks are shown in Figure 7. After two weeks of treatment, there were no significant differences in either plasma T3 or T4 in any of the treatment groups. After four weeks of treatment, fish treated with the low dose of T3 (10 μg/g) had a significant elevation in plasma T4, but no significant change in plasma T3, compared to control fish. However, the fish treated with the high dose of T3 (100 μg/g) showed an approximate twofold increase in plasma T3 and a significant decrease in plasma T4 compared to control fish. It is noteworthy that, after four weeks of treatment with T3, the fish on the low

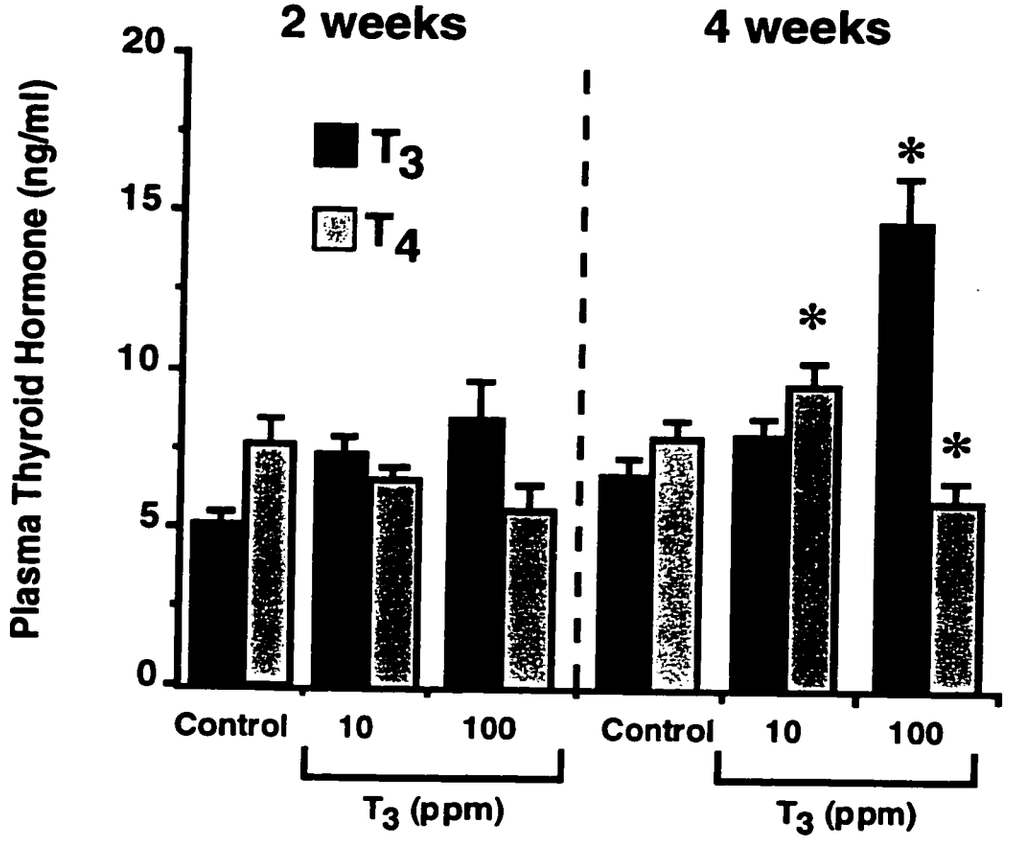


FIGURE 7. Plasma levels of thyroxine (T4) and triiodothyronine (T3) in fish fed food containing T3 at 0 (control), 10 or 100 μg/g after two and four weeks. Feed ration was 2% body weight per day. Asterisks indicate significant differences compared to control value ($P < 0.05$).

and high doses showed cranial hyperplasia, which is commonly seen in thyroid hormone treated fish.

Pituitary TSH β and total α mRNA levels in fish fed 0, 10 and 100 μg/g T3 for two and four weeks are shown in Figure 8. After two weeks, there were no significant differences in the amount of TSH β mRNA between any of the treatment groups. After four weeks, the fish fed the high dose of T3 showed significantly lower pituitary TSH β mRNA levels than control and low dose fish (Fig. 8A). After two weeks, the fish fed the low dose of T3 had significantly higher total α mRNA levels (approximately twofold) than control and high dose fish, while after four weeks there were no significant differences in total α mRNA between any of the treatments (Fig. 8B).

The efficacy of dietary methimazole treatment (2.5 mg/g) in reducing thyroid hormone production was assessed by measuring plasma T3 and T4 levels (Fig. 9). After two weeks, there were no significant differences in plasma T3 or T4 between control or methimazole treated fish. However, after four weeks of methimazole treatment, both plasma T3 and T4 were significantly depressed (approximately three- to fourfold) below that of control fish.

Pituitary TSH β and total α mRNA levels in fish fed methimazole treated food for two and four weeks are shown in Figure 10. After two weeks, there were no significant differences in the amount of either TSH β mRNA

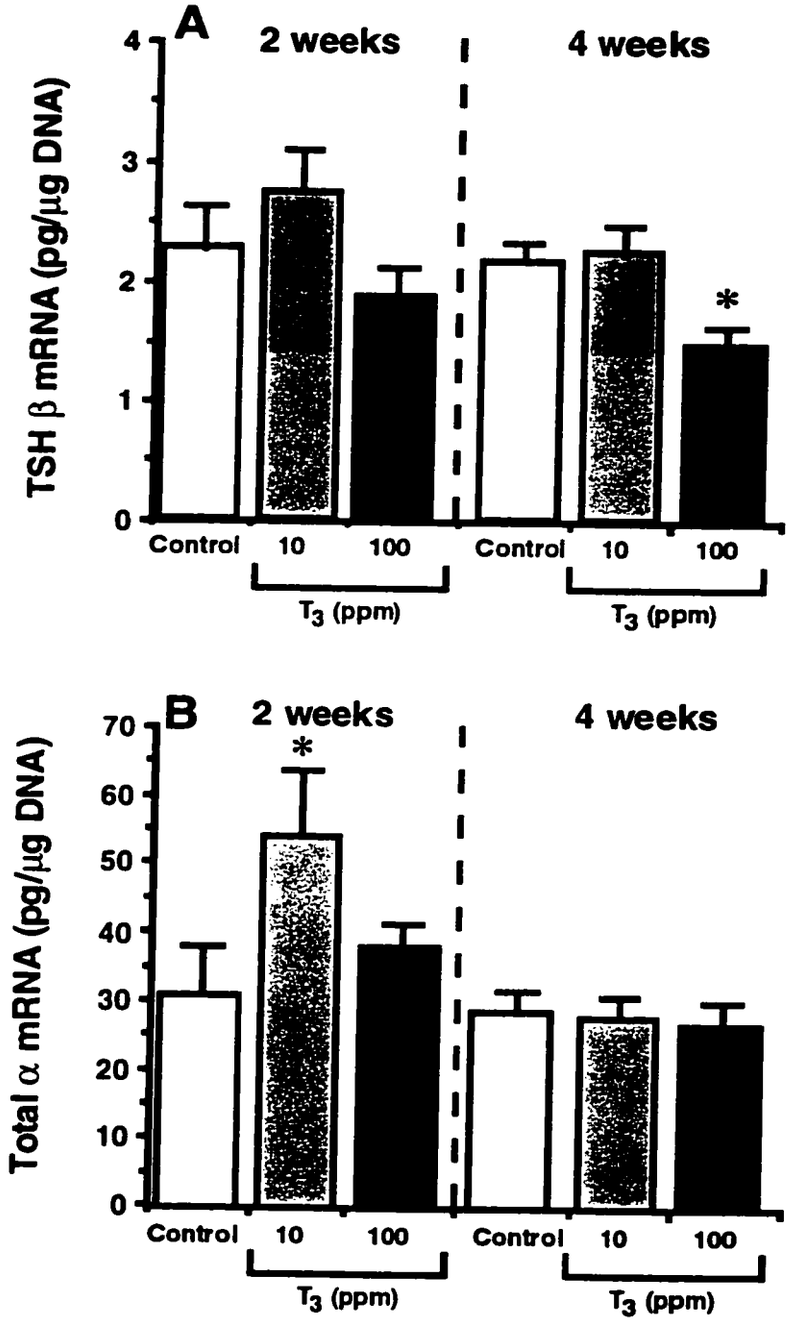


FIGURE 8. Pituitary TSH β-subunit mRNA (A) and total α-subunit mRNA (B) levels in coho salmon treated with dietary T3 and control fish after two and four weeks. Asterisks indicate a significant difference compared to control value ($P < 0.05$).

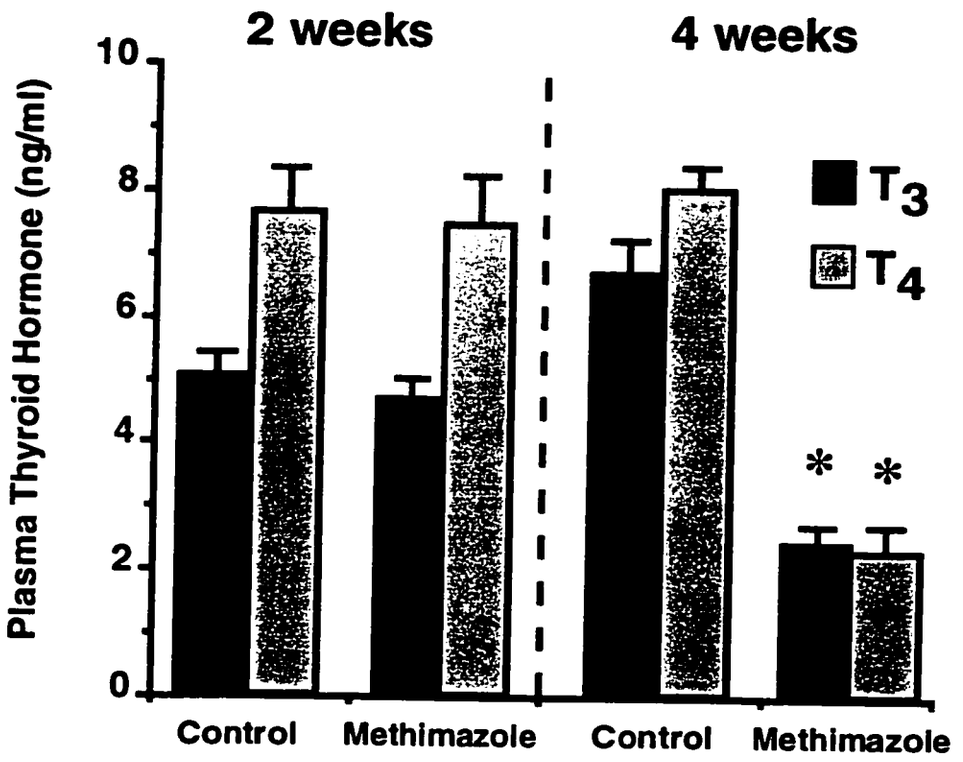


FIGURE 9. Plasma thyroxine and triiodothyronine levels in coho salmon treated with dietary methimazole after two and four weeks. Asterisks indicate a significant difference compared to control values ($P < 0.05$).

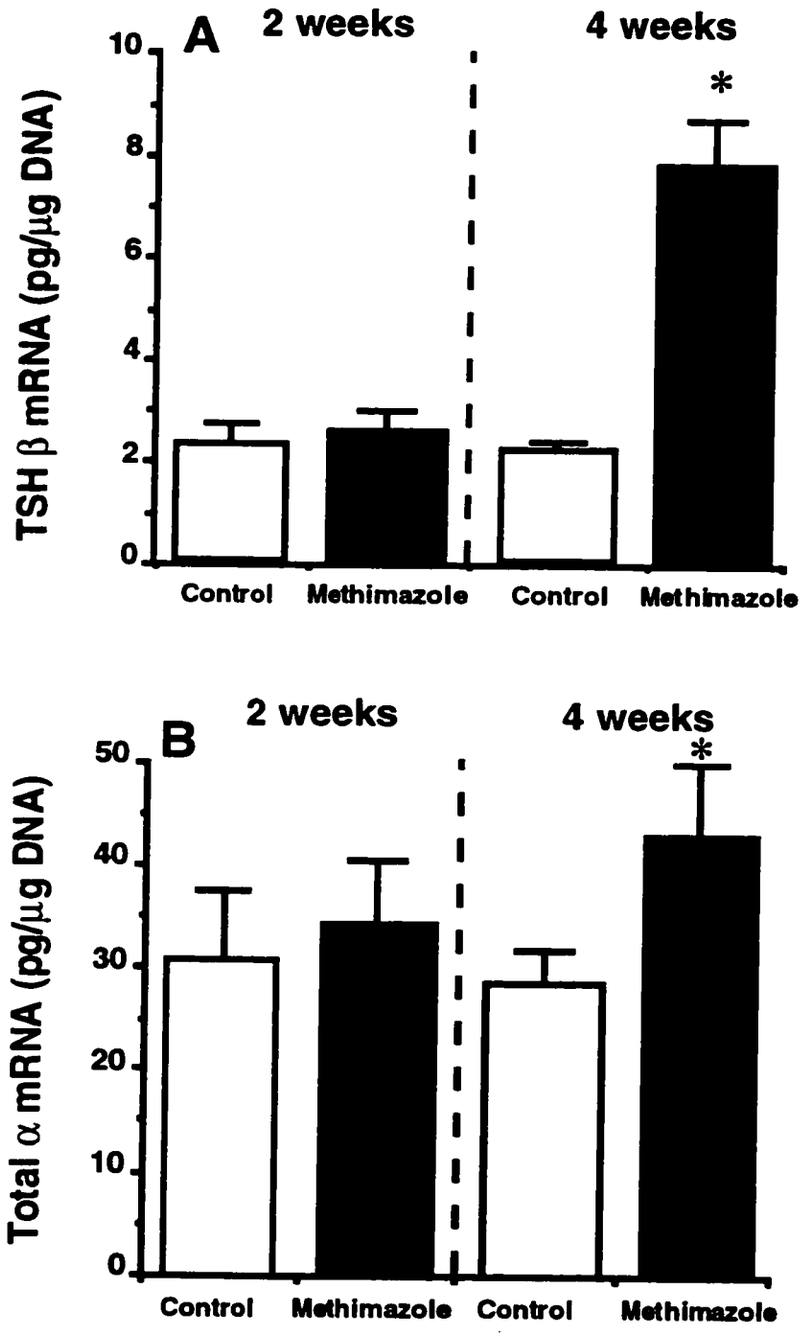


FIGURE 10. Pituitary TSH β-subunit mRNA (A) and total α-subunit mRNA (B) levels in coho salmon treated and untreated (control) with dietary methimazole after two and four weeks. Asterisks indicate a significant difference compared to control values ($P < 0.05$).

(Fig. 10A) or total α mRNA (Fig. 10B) between control or methimazole treated fish. However, after four weeks there was a significant increase in both pituitary TSH β mRNA (approximately fourfold) (Fig. 10A) and total α mRNA (Fig. 10B).

D. DISCUSSION

Thyroid hormones play important roles in the regulation of teleost growth, development (Leatherland, 1988; Eales, 1988; Specker, 1988; Dickhoff and Sullivan, 1987; Dickhoff and Darling, 1983) and reproduction (Cyr and Eales, 1996). TSH is a central positive modulator of thyroidal activity; injection of bovine or ovine TSH stimulates the thyroid of fish (reviewed by Leatherland, 1988). Prolactin and growth hormone may affect thyroid activity in some teleosts as well (Grau, 1988). Direct investigation of pituitary TSH in fish has been difficult because of the lack of purified fish TSH and the lack of sensitive and specific assays for it. The relatively small amount of TSH in the pituitary gland and the difficulty in separating TSH from the gonadotropic hormones have resulted in only limited quantities of purified hormone available for development of direct quantification techniques such as radioimmunoassays (Swanson *et al.*, 1987).

In this investigation, we have employed molecular techniques to develop a highly sensitive assay for measuring salmon TSH β mRNA in

pituitary RNA and TNA samples. When used in conjunction with an RPA for total pituitary α mRNA, we were able to accurately quantify steady-state levels of TSH subunit expression in a teleost for the first time. The term steady-state is used because, while mRNA is relatively stable, the levels that are measured are the result of a balance between synthesis and degradation.

The level of TSH β mRNA measured by this technique is an order of magnitude lower than the level of total α mRNA. Total α mRNA may be much higher than TSH β mRNA because the α subunit is also used for the gonadotropic hormones. Furthermore, the expression of coho salmon TSH β mRNA is also much lower than the mRNA expression for coho salmon GTH I β (approx. 25 pg/μg DNA) or GTH II β (approx. 10 pg/μg DNA) in fish of similar age and developmental stage (Dickey and Swanson, 1995). Thus, the levels of mRNA for the α and β subunits of glycoprotein hormones in the salmon pituitary gland have approximately the same relative abundance as the mature proteins (Swanson *et al.*, 1987). The low level of apparent expression of TSH β necessitates an assay with high sensitivity.

The RPA (solution hybridization assay) was used by Carr *et al.* (1985) to examine regulation of pituitary α and TSH β mRNA expression in the rat. In non-mammalian vertebrates, the only study to quantify total α and TSH β mRNA expression was conducted by Buckbinder and Brown (1993) in the

African clawed frog, *X. laevis*, using Northern blot analysis followed by scanning laser densitometry. The RPA has some advantages over Northern blot and dot blot analysis. Carr *et al.* (1985) found the RPA for pituitary α and TSH β mRNA to be tenfold more sensitive than Northern blot analysis. Furthermore, the RPA is more specific and requires less sample and fewer sample manipulations than the other techniques. Finally, the absolute amount of mRNA in a sample can be determined by the RPA whereas Northern blot and dot blot analysis only give a relative ratio.

The second objective of this investigation was to demonstrate directly, for the first time in a teleost, the negative feedback relationship between the thyroid hormones and TSH expression. Negative feedback of the thyroid hormones on the pituitary in teleosts has been well established (for review see Eales, 1979). However, due to the lack of a technique for direct quantification of TSH, all previous studies have relied on indirect methods. Histological studies have demonstrated reduced thyroid and pituitary thyrotroph activity following T4 treatment in the eel, *A. anguilla*, (Olivereau, 1962) and the rainbow trout, *O. mykiss*, (Baker, 1965; 1969) and increased pituitary thyrotroph activity following radiothyroidectomy in the eel (Olivereau, 1963). Peter (1971; 1972) demonstrated that T4 implantation resulted in reduced radioiodine uptake in the thyroid of the goldfish, *Carassius auratus*. Furthermore, Higgs *et al.* (1979) showed that feeding of T3 and T4 caused a decrease in thyroid follicular cell height in coho salmon,

O. kisutch. Finally, Brown and Stetson (1985) demonstrated that injection of thyroid hormones in the killifish, *Fundulus heteroclitus*, also caused a reduction in thyroid follicle cell height; however, the effect varied with photoperiod.

The efficacy of the various feeding treatments used in this investigation was assessed by measuring plasma thyroid hormone levels. The low T3 treatment (10 μg/g) did not cause a significant increase in the plasma T3 levels throughout the experimental period and only a small, but significant, elevation in plasma T4 after four weeks (Fig. 7). However, there were visible morphological changes (cranial chondroplasia) in these fish after just two weeks of treatment. Thus, although a physiological effect of the low T3 treatment was clearly evident, this effect was not reflected in the plasma thyroid hormone levels of these fish. In contrast, the plasma T3 levels of the high T3 treatment (100 μg/g) were significantly elevated (approx. twofold) above control levels after four weeks. Plasma T4 levels were significantly depressed by high T3 treatment after four weeks. This depression in plasma T4 corresponds well with the significant decrease in pituitary TSH β mRNA compared to control fish. Finally, the methimazole treatment did not cause a significant change in thyroid status after two weeks; however, after four weeks the fish were hypothyroid based on significant declines (approx. three- to fourfold) in both plasma T3 and T4 (Fig. 9). Thus, it can be concluded that

the thyroid hormone and methimazole feeding manipulations altered plasma thyroid hormone levels in the desired direction after four weeks.

This is the first study that directly demonstrates a negative feedback relationship between the thyroid hormones and TSH expression in a fish. Negative feedback of thyroid hormones on TSH subunit expression is well established in mammals. Cloned TSH cDNAs have been used as molecular hybridization probes in mammalian studies of changes in steady-state levels of total α and TSH β subunit mRNAs in response to thyroid hormones (Gurr *et al.*, 1983; Gurr and Kourides, 1984; Croyle and Maurer, 1984; Chin *et al.*, 1985a,b). In addition, Carr (1985) used an RPA to examine regulation of rat TSH subunit mRNAs by thyroid hormones as well. In mice, thyroid hormones reduced pituitary TSH subunit mRNA within one day of treatment. Hybridizable levels of TSH β mRNA were decreased after only one hour in TtT 97 tumor cells from T4 injected mice (Shupnik *et al.*, 1983; Chin *et al.*, 1985b; Shupnik *et al.*, 1989). Our results show a significant reduction in TSH β expression occurring between two and four weeks of the high T3 treatment. The large discrepancy in time required for thyroid hormone suppression of TSH β mRNA between mammals and teleosts may reflect differences in biochemical processes in endotherms and poikilotherms; the degradation rate of pituitary mRNA transcripts may be much lower in poikilotherms compared to endotherms. However, differences in responses

between the fish from this investigation and mammals may be related to thyroid hormone dosage. Furthermore, the fish used in this investigation were treated with T3 in their food, to reduce handling stress; whereas the mammalian studies involved relatively faster acting intraperitoneal injections into either intact animals, thyroid hormone sensitive radiothyroidectomized animals or hypothyroid animals injected with transplantable thyrotropic tumors (TtT97) (Chin *et al.*, 1985a; Carr *et al.*, 1985; Shupnik *et al.*, 1989).

Our findings suggest a basic similarity in regulation of α and TSH β mRNA in the salmon compared to mammals. The absolute amount of α mRNA was approximately fifteenfold greater than TSH β, although most of this excess α subunit mRNA may be present in the relatively more abundant gonadotropes. Numerous mammalian studies have shown an excess production of α over TSH β in euthyroid and hypothyroid animals, pituitary cell cultures, and murine thyrotropic tumors (Carr *et al.*, 1985; Shupnik *et al.*, 1989). In mammals, both α and TSH β subunit levels are reduced by thyroid hormone treatment; however, there is a greater effect on the β than on the α subunit (Chin *et al.*, 1985b; Carr *et al.*, 1985; Shupnik *et al.*, 1989). In our study, T3 treatment reduced TSH β mRNA levels after four weeks, but α subunit levels increased at two weeks and then declined to initial levels at four weeks.

This increase in α subunit mRNA level, as an initial response to the low T3 treatment, is paradoxical in a euthyroid animal. In mammals, low doses of T3 cause increases in TSH mRNAs only in severe hypothyroidism (D'Angelo *et al.*, 1976; Franklyn *et al.*, 1987).

Induction of a hypothyroid state was accomplished after four weeks of methimazole treatment of salmon. In the hypothyroid salmon both TSH β mRNA and α mRNA levels increased significantly; β increased approximately 250% and α approximately 50%. However, the absolute increase in TSH β was approximately 5.6 pg/μg DNA and for α-subunit was approximately 15 pg/μg DNA. Since the α and β probes were of equivalent size, there was approximately a threefold greater production of α than β mRNA in the hypothyroid state. Assuming that the increase in subunit mRNAs was limited to thyrotrophs, then α mRNA was produced in excess of β mRNA. This excess of α mRNA implies that the synthesis of β subunit may be rate-limiting for production of TSH, if the level of mRNA is directly related to protein synthesis.

It is well established in mammals that the α and TSH β genes contain thyroid response elements (TREs), which consist of specific DNA sequences that mediate transcriptional activation or repression by thyroid hormone-receptor complexes (Chin *et al.*, 1993). Recently, Suzuki *et al.* (1995) identified

a TRE in the α subunit gene of chinook salmon. Thus, the specific mechanism whereby thyroid hormones regulate TSH mRNA expression in teleosts is likely to be similar to that found in tetrapod vertebrates.

In summary, we have successfully designed an RPA that allows us to quantify, for the first time, TSH β mRNA expression in a teleost. The assay was specific, sensitive, rapid and required a relatively small quantity of RNA. The RPA for salmon TSH β and a similar assay for the salmon pituitary α subunit were used to confirm that salmon TSH transcription may be regulated by thyroid hormones operating in a negative feedback manner as they do in mammals. Future studies will use this assay for quantifying changes in steady-state levels of TSH mRNA during the parr-to-smolt transformation and reproductive maturation of salmon, in addition to studies of hypothalamic factors regulating salmon TSH.

Chapter 4

The pituitary-thyroid axis during the parr-smolt transformation of coho salmon, *Oncorhynchus kisutch*: Quantification of TSH, TSH mRNA and thyroid hormones.

A. INTRODUCTION

Thyroid activity is regulated through the hypothalamic-pituitary-thyroid axis. In short: the hypothalamus stimulates production and release of thyroid stimulating hormone (TSH) by the pituitary. TSH, in turn, stimulates secretion of thyroxine (T4) by the thyroid, which is subsequently converted to the more biologically active triiodothyronine (T3). In mammals it is believed that thyroid status is maintained by a balance between pituitary stimulation via TSH and negative feedback from the thyroid hormones acting back at the level of the hypothalamus and the pituitary gland (Eales and Brown, 1993).

In fish it has been demonstrated that injection of TSH causes an increase in plasma thyroid hormone levels (Chan and Eales, 1976; Grau and Stetson, 1977; Milne and Leatherland, 1980; Specker and Richman, 1984; Specker and Schreck, 1984; Grau *et al.*, 1985; Specker and Kobuke, 1987; Swanson and Dickhoff, 1987; Moriyama *et al.*, 1997). However, the lack of a reliable assay for quantifying fish TSH has impeded efforts to understand the role of endogenous TSH in controlling the thyroid. To further our understanding of thyroid regulation in fish, we have recently developed an

RNase protection assay for pituitary TSH β mRNA (Chapter 3; Larsen *et al.*, 1997) and a radioimmunoassay for salmon pituitary and plasma TSH (Moriyama *et al.*, 1997).

The parr-smolt transformation of salmonid teleosts is characterized by a dramatic increase in thyroid activity; originally shown histologically (Hoar, 1939) and later through measurement of plasma thyroid hormone levels (Dickhoff *et al.*, 1978b, 1982; Nishikawa *et al.*, 1979; Grau *et al.*, 1981). Thus, smoltification is an excellent model for studying thyroid activity in teleost fish. The objective of this investigation was to quantify steady-state levels of pituitary TSH β mRNA, pituitary and plasma TSH and plasma thyroid hormone levels. These data were then used to examine the interrelationships among these various parameters during this period of increased thyroid activity.

B. MATERIALS AND METHODS

Animals

In November, 1993, approximately 5000 coho salmon, *Oncorhynchus kisutch*, eggs were obtained from the broodstock that returns yearly to the University of Washington, School of Fisheries hatchery (Seattle, WA) and reared at the Northwest Fisheries Science Center (Seattle, WA). A sub-sample of approximately 1000 fish was reared through smoltification (approximately

16 months post-hatch) in 1.3 m diameter cylindrical fiberglass tanks with flow through dechlorinated municipal water, and fed standard rations of Biodiet Grower Pellets (Bioproducts, Warrenton, OR). The fish were maintained under natural photoperiod; temperature ranged from 8 °C (January) to 15 °C (August).

Sampling protocol

From January to May of 1994, 20 fish were randomly selected for analysis at approximately one week intervals and sampled during the same time of day; 1300-1600 hrs-approximately 19 hrs after last feeding. The fish were individually anesthetized in a buffered solution of 0.05% tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA) and measured for fork length (mm) and weight (g), visually assessed for stage of smolt development (1 = parr, 2 = transitional, 3 = smolt) (modified from Gorbman *et al.*, 1982) and sampled for pituitary glands and plasma. Blood samples were collected from severed caudal vessels into heparinized Natelson tubes (VWR Scientific), centrifuged, and stored frozen at -80°C until analyzed by radioimmunoassay (RIA). Pituitary glands were removed, frozen in liquid nitrogen, and stored at -80°C until analyzed by either radioimmunoassay (RIA) or RNase protection assay (RPA).

Condition factor (CF) represents the relative change in weight

compared to body length of a fish as calculated by the following formula, CF = [weight (g) / length $(mm)^3$] X 100,000. During smoltification, a characteristic decrease in CF is typically reported (Hoar, 1988).

Sample analysis

Due to the relatively small quantity of plasma obtained from the 20 individual fish at each sampling date, equal volumes of plasma were pooled from two fish, of similar developmental stage, for a total of 10 plasma pools. Plasma pools were analyzed for T4 and T3 by the method of Dickhoff *et al.* (1978b, 1982) and TSH by the method of Moriyama *et al.* (1997). Pituitary TSH content was determined from six of the 20 pituitaries collected at each sampling date as described by Moriyama *et al.* (1997). Pituitary TSH β mRNA was determined by the method of Larsen *et al.* (1997). The small size of the individual pituitaries, and the relatively small quantity of nucleic acid per pituitary necessitated pooling of pituitary glands for TSH β transcript determination. Thus, at each date, four replicates of three pituitaries each were analyzed.

Statistical Analysis

To assess seasonal changes in morphology as well as hormone and mRNA levels, all data were subjected to analysis of variance (ANOVA) followed by the Fisher Protected Least Significant Difference Test (Dowdy and

Weardon, 1991). Furthermore, to determine the feedback relations among the various endocrine factors, regression analysis was conducted. Since some endocrine data were determined from pooled samples, all regressions were conducted simply on the mean values at each date. All statistical analyses were conducted using the Statview 4.0 program (Abacus Concepts, Inc.). Statistical significance was set at a level of $\alpha = 0.05$.

C. RESULTS

Smolt Morphology

Changes in body morphology and appearance during the parr-smolt transformation are presented in Fig. 11. Fork length (Fig. 11A) and body weight (Fig. 11B) remained unchanged in January, increased steadily throughout early Spring (Feb-May) and sharply increased in late May. The change in condition factor and general appearance were used as indices of smolt development. Condition factor (Fig. 11C) showed a characteristic decline throughout the smoltification period from a peak value of 1.32 in January to a low value 1.16 in April. Stage of smolt development is presented in Fig. 11D. In January, fish were either parr or transitional with an average stage score of approximately 1.5. From February to late April most fish were scored as transitional (Stage 2). In May, the fish were all considered smolts (Stage 3).

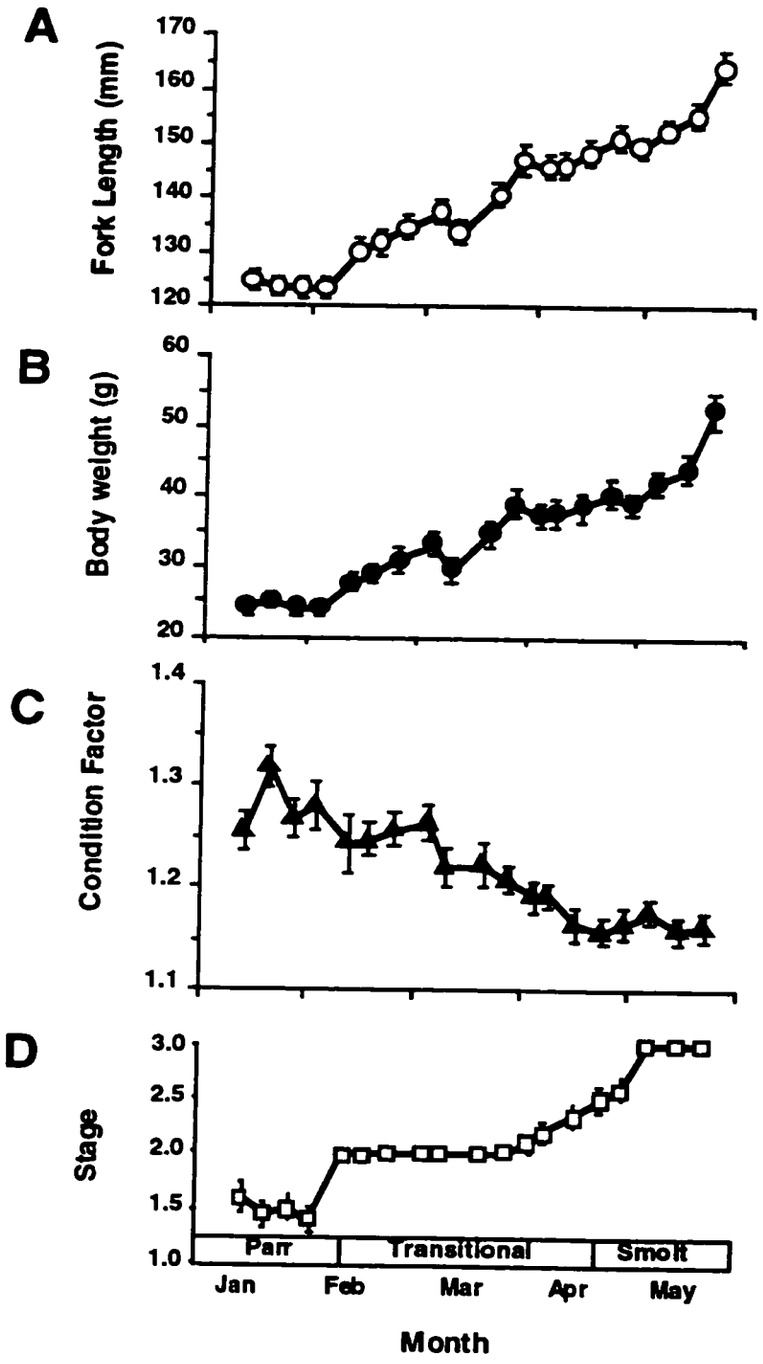


FIGURE 11. Fork length (A), body weight (B), condition factor (C), and stage of smolt development (D) of coho salmon during parr-smolt development. Mean values ± s.e. (n=20).

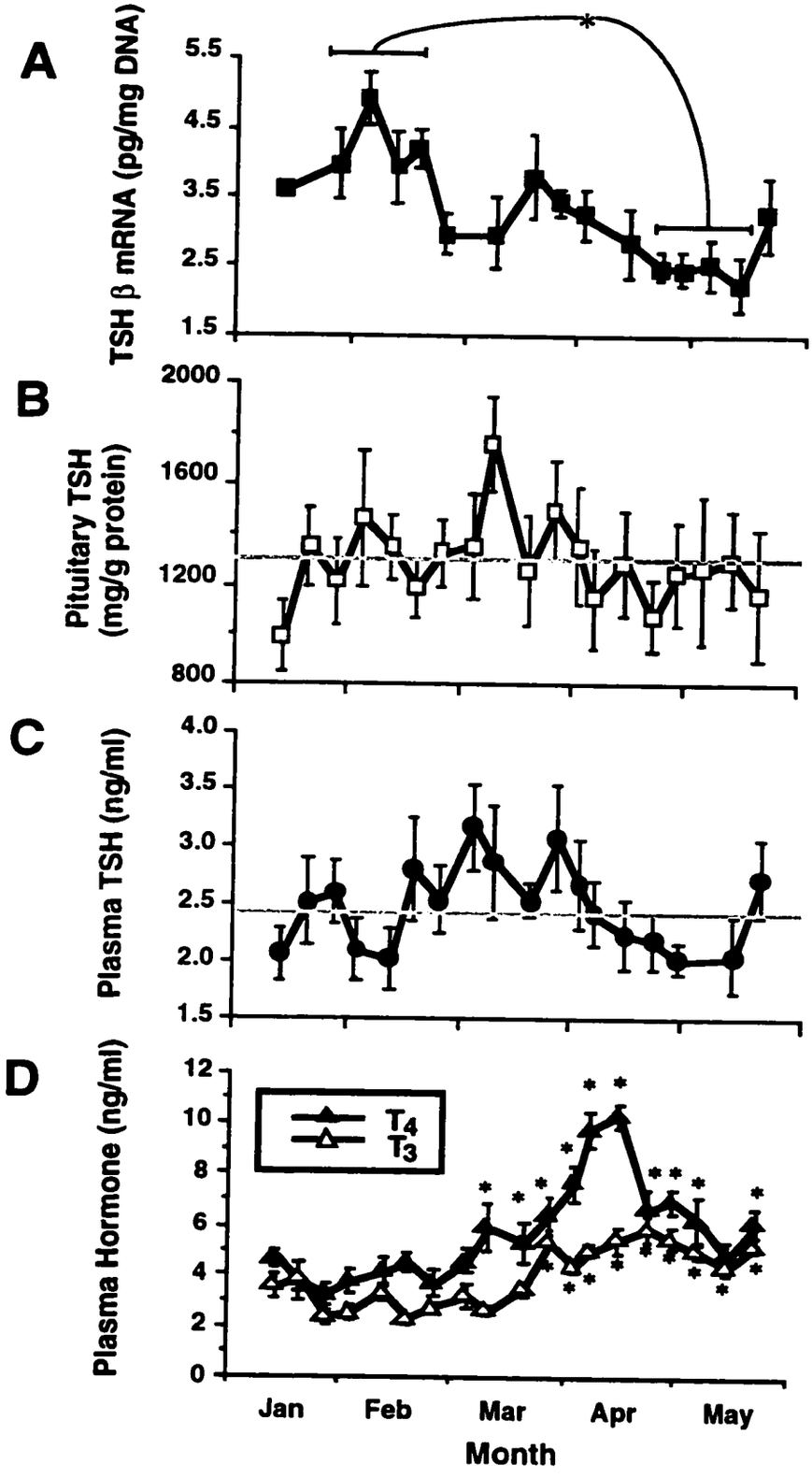
Smoltification associated changes in endocrine parameters

TSH β mRNA levels varied significantly during the smoltification period (p = 0.0004) (Fig. 12A). A peak TSH β mRNA level of 4.9±0.38 pg/μg DNA was observed in parr in early February followed by a steady decline throughout the spring to a low of 2.3±0.4 pg/μg DNA in smolts in May. The bracketed values in late January and early February were significantly greater than the bracketed values in late April and early May. All other values were intermediate.

Pituitary TSH content is presented in Fig. 12B. There appears to be some variation in the mean pituitary TSH levels; ranging from a low of 993.4±144.5 (mg/g protein) in January to a peak of 1760.5±189.3 (mg/g protein) in March followed by a steady decline in April to 1073.0±143.3 (mg/g protein). To illustrate this trend, the grand mean of all samples (1289.8±44 mg/g protein) is represented by the horizontal line. However, due to high variability among samples at each date, there was no overall significant difference among dates (p = 0.7763).

Plasma TSH levels are presented in Fig. 12C. Similar to the pituitary TSH content, there was no statistically significant overall difference in plasma TSH levels (p = 0.3027). However, it is noteworthy, that the plasma TSH levels were consistently above the grand mean of 2.48±0.08 (ng/ml) from late February to early April, just prior to the T4 elevation (Fig 12D). Furthermore,

FIGURE 12. Pituitary TSH β mRNA (A), pituitary TSH (B), plasma TSH (C) and plasma T4 and T3 (D) levels of coho salmon during parr-smolt development. Mean values ± s.e. n=4 replicates of pituitaries pooled from 3 fish for TSH β mRNA, n=6 for pituitary TSH, n=10 replicates of plasma pooled from 2 fish each for plasma TSH, T4 and T3. For TSH β mRNA (A), bracketed values in late January and early February were significantly different than bracketed values in late April and early May. All other values were intermediate. For pituitary and plasma TSH levels (B and C) the grand mean of all values is indicated by the horizontal lines at 1289.8 mg/g protein and 2.48 ng/ml respectively. For plasma T4 and T3 (D), asterisks indicate values that are significantly higher than levels in late January and February.


A
TSH β mRNA (pg/mg DNA)
5.5
4.5
3.5
2.5
1.5
B
Pituitary TSH (mg/g protein)
2000
1600
1200
800
C
Plasma TSH (ng/ml)
4.0
3.5
3.0
2.5
2.0
1.5
D
Plasma Hormone (ng/ml)
12
10
8
6
4
2
0
T4
T3
Jan
Feb
Mar
Apr
May
Month

mean plasma TSH levels declined to levels below the grand mean in April and May, at a time when the thyroid hormone levels were highest (Fig.12D).

In contrast to the plasma TSH levels, both plasma T4 and T3 levels increased significantly during smoltification (T4: $p = 0.0001$, T3: $p = 0.0001$) (Fig. 12D). Plasma T4 levels remained relatively low (approx. 4.0 ng/ml) during January and February, increased dramatically, starting in early March to a peak level in April (10.2 ng/ml), and subsequently declined in late April and May (approx. 6.0 ng/ml). Plasma T3 levels were relatively low, as well, from January to early March (approx. 3.0 ng/ml). In late March; however, the plasma T3 levels increased significantly, to approximately 5.0 ng/ml, and remained stable through May. Thus, during the smoltification period, plasma TSH levels remained relatively constant while plasma thyroid hormone levels were quite dynamic. Furthermore, the dramatic decline in plasma T4 levels seen in May was not accompanied by a corresponding decline in plasma T3.

Regression analysis

To investigate the interrelationships among the various parameters examined in this study, regression analysis was performed (Table 1). Plasma T3 levels were positively correlated with plasma T4 ($R^2=0.52$, $p=0.0005$). In contrast, plasma T3 levels were negatively correlated with pituitary TSH β mRNA ($R^2=0.46$, $p=0.004$) (Fig. 13). Plasma T4 levels were significantly

TABLE 1. Regression analysis of various endocrine parameters of the pituitary-thyroid axis of coho salmon, *Oncorhynchus kisutch*, measured during the parr-smolt transformation. Pit.TSH mRNA = pituitary TSH mRNA; Pit.TSH = pituitary TSH; Pl.TSH = plasma TSH. n = 16-19 per comparison.

Parameter Comparison	r^2	*p*-value
T_3 *vs* T_4	0.52	0.0005
T_3 *vs* Pit. TSH mRNA	0.45	0.004
T_3 *vs* Pit.TSH	0.11	0.1757
T_3 *vs* Pl.TSH	0.03	0.47
T_4 *vs* Pit. TSH mRNA	0.27	0.0411*
T_4 *vs* Pl.TSH	0.00	0.8515
T_4 *vs* Pit.TSH	0.02	0.55
Pit.TSH mRNA *vs* Pit.TSH	0.01	0.7488
Pit.TSH mRNA *vs* Pl.TSH	0.02	0.6303
Pit.TSH *vs* Pl.TSH	0.16	0.1066

*see results for explanation of stepwise multiple regression model

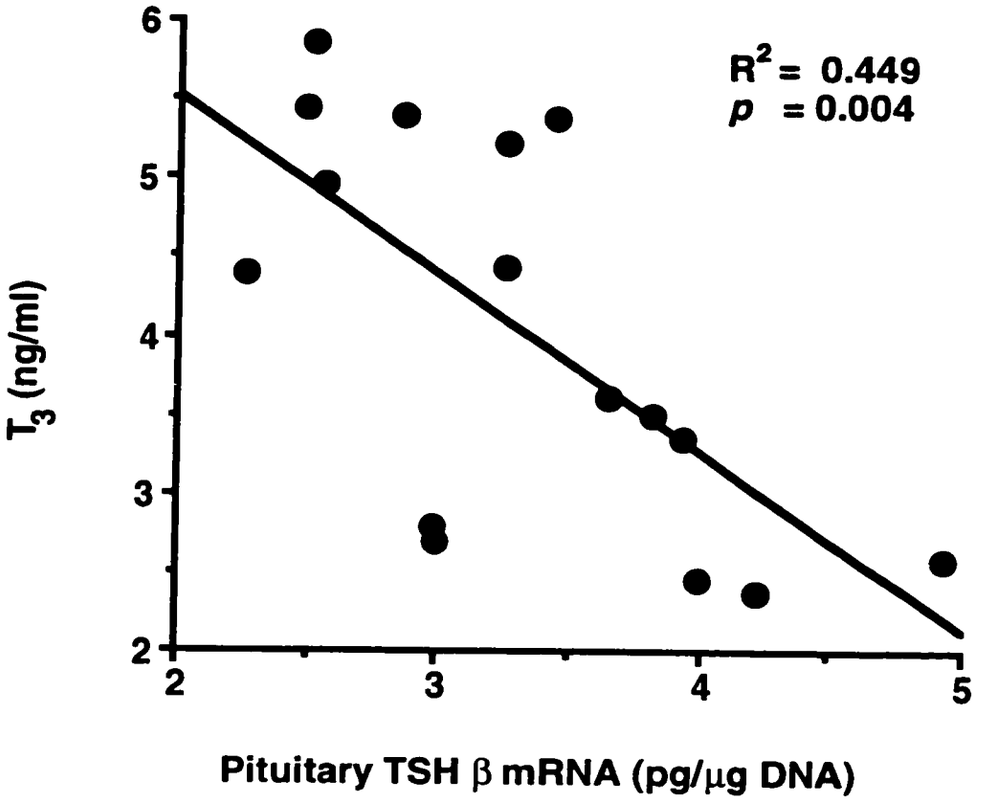


FIGURE 13. Regression analysis of pituitary TSH β mRNA versus plasma T3. Analysis is based on mean levels at each date. n=16. p=0.004.

negatively correlated with pituitary TSH β mRNA ($R^2 = 0.27$, $p=0.04$) as well. However, when a multiple regression model was constructed for TSH β mRNA using plasma T3 and T4 levels, the stepwise procedure did not include T4 in the model, as it did not significantly increase the correlation found between T3 and TSH β mRNA. Thus, the significant regression of T4 with TSH β mRNA is driven by the positive correlation of plasma T3 with T4. All other relationships were not significant including plasma TSH levels with both plasma T4 and T3.

D. DISCUSSION

It has been established through radioiodine uptake studies (Eales, 1969), histology (Hoar, 1939; Nishioka *et al.*, 1982), and RIA of plasma thyroid hormones (Dickhoff *et al.*, 1978b; 1982; Nishikawa *et al.*, 1979; Grau *et al.*, 1981; Specker *et al.*, 1992) that the thyroid gland is activated during salmonid smoltification. Similarly, in this investigation, we once again demonstrated a dramatic increase in plasma T4 and a significant increase in plasma T3 levels (Fig. 12D) in association with the transitional period of smolt development (Fig. 11D). During late spring, as the fish were becoming fully smolted, the plasma T4 levels dropped to near presmolt levels while the plasma T3 levels remained elevated. One might anticipate that the dramatic increase in plasma T4 levels would be preceded by increases in both pituitary

and plasma TSH. However, neither measure of TSH activity showed a statistically significant elevation prior to the increase in plasma T4 (Fig. 12B and C). It is worth noting that there is some suggestion of a modest increase in TSH activity in March, when average peak levels of both pituitary and plasma TSH were approximately 70-80% above the grand mean, followed by a steady decline in April and May to levels consistently below the grand mean. But, due to high variability among the pooled plasma samples, these elevations were not statistically significant. Thus, there may be a small increase in TSH secretion just before the T4 elevation, but its magnitude is below our level of assay precision and experimental error.

The lack of a dramatic change in pituitary and plasma TSH coupled with the significant increase in plasma T4 during smoltification corroborates the histological findings of Nishioka *et al.* (1982). These investigators demonstrated that during smoltification of coho salmon, cells from the thyroid gland were categorized as being very active cytologically. In addition, the pituitary cells responsible for production of prolactin, adrenocorticotropin and growth hormone were also categorized as being either active or very active as well. However, during this same period the pituitary thyrotrophs were categorized as inactive in parr and stunts (Clark and Nagahama, 1977; Folmar *et al.* 1982) and more importantly, only variably active in freshwater- and seawater-adapted smolts. Thus, both previous histological evidence and the pituitary and plasma TSH levels measured in this investigation suggest

that the activity of the thyrotrophs shows relatively little change during smoltification.

Numerous studies have established that injection of TSH causes an increase in plasma thyroid hormone levels in fish (Chan and Eales, 1976; Grau and Stetson, 1977; Milne and Leatherland, 1980; Specker and Richman, 1984; Specker and Schreck, 1984; Grau *et al.*, 1985; Specker and Kobuke, 1987; Swanson and Dickhoff, 1987; Moriyama *et al.*, 1997). While it is clear that the teleost thyroid is controlled by TSH, the dose-response varies depending on season (photoperiod) and physiological state of the animal (Milne and Leatherland, 1980; Specker and Richman, 1984; Specker and Schreck, 1984; Grau *et al.*, 1985; Specker and Kobuke, 1987; Swanson and Dickhoff, 1987). In coho salmon a developmental change in the sensitivity of the thyroid to TSH occurs during smoltification; being low in January, then increasing throughout smoltification in May (Specker and Schreck, 1984; Swanson and Dickhoff, 1987). An exception to this trend was observed in April when basal levels of T4 were elevated and the thyroid appeared unresponsive to increasing doses of TSH. Swanson and Dickhoff (1987) noted that the thyroid may be operating at maximal secretory capacity and unable to respond to additional stimulation during the period of maximal plasma T4 level. In the current investigation the mean pituitary and plasma TSH levels show a similar pattern of change during smoltification with lowest levels in January and highest in March. However, the lack of a very dramatic change in either

pituitary or plasma TSH levels would support the idea that the increase in plasma T4 levels seen during smoltification might result from a photoperiodically induced change in the sensitivity of the thyroid to stimulation by a relatively constant amount of endogenous TSH rather than an increase in TSH secretion from the pituitary. Alternatively, the increase in plasma thyroid hormone levels observed during smoltification may result from a change in clearance rate as well (Specker *et al.*, 1984). The fish from this study were sampled approximately weekly in an effort to avoid missing some large transient increase in TSH which might be responsible for the increase in T4. Thus, it is unlikely that we missed some critical period during our sampling schedule.

In contrast to the TSH protein levels, the steady state TSH β mRNA levels from this study were relatively dynamic, being highest during early winter and declining throughout smoltification, coinciding with the increase in thyroid hormones. This is supportive of thyroid hormone negative feedback (Fig. 12A). In Chapter 3 (also see Larsen *et al.* , 1997) we demonstrated that after 4 weeks of feeding T3 treated food to sub-adult coho salmon, there was a significant decline in steady-state levels of TSH β mRNA, supporting a negative feedback relationship between plasma T3 levels and pituitary TSH β mRNA. In this investigation, the increase in plasma T3 levels in the spring (late March through May) (Fig. 12D) was negatively

correlated with a significant decrease in pituitary TSH β mRNA (Fig. 12A, Table 1 and Fig. 13). However, the plasma T4 levels (Fig. 12D), which increased during late March and then declined in late April and May, did not show the same significant negative correlation (Table 1). These data are supportive of a negative feedback role for T3, but not T4, in regulation of TSH transcription during smoltification.

Negative feedback of the thyroid hormones on the pituitary in teleosts has been well established using indirect methods (for review see Eales, 1979; Eales and Brown, 1993). Furthermore, we previously demonstrated that injection of T3 in sub-adult coho salmon induced a significant decrease in plasma TSH levels (Moriyama *et al.* 1997) of similar magnitude (from approx. 3.0 down to 2.0 ng/ml) to the low levels of plasma TSH seen in April and May from this study (Fig. 12C). Thus, direct confirmation of a negative feedback relationship between plasma T3 and plasma TSH has been established and the magnitude of change in TSH in response to increased plasma T3 levels is comparable between the two studies. However, unlike the suppressive effect of T3 on TSH shown previously, in this investigation the decline was not statistically significant.

In mammals it is generally believed that thyroid activity is regulated through the hypothalamic-pituitary-thyroid axis. This "central control model" involves hypothalamic secretion of thyrotropin releasing hormone (TRH) which stimulates release of TSH from the pituitary. TSH, in turn,

stimulates secretion of T4 which is subsequently converted to the more biologically active T3. According to this model, thyroid status is determined by the quantity of free T4, which is maintained by a balance between pituitary stimulation and negative feedback from the thyroid hormones acting on the hypothalamus and the pituitary gland (Eales and Brown, 1993).

In contrast to the central control model of gnathostomes, Dickhoff and Darling (1983) suggested that thyroid regulation by the pituitary in cyclostomes was secondary to other regulatory mechanisms such as modifications of thyroid hormone metabolism and distribution, receptor number, and events following receptor activation. Similarly, in teleosts, Eales and Brown (1993) have proposed that thyroid activity is regulated, at least in part, via a "peripheral control model" whereby thyroid status is dictated by a tissue specific T3 set-point or the "availability of T3 to receptor sites in specific tissues". The T3 set-point is dependent primarily on T4 to T3 conversion rate, although hormone degradation rate and change in tissue sensitivity may play roles as well.

In some respects, the results from this investigation support this peripheral control model. First, both pituitary and plasma TSH levels show only minimal (in fact not statistically variable) changes during smoltification, while plasma thyroid hormone levels change dramatically. Thus, the increase in T4 may be a result of a relatively constant quantity of TSH stimulating a more sensitive thyroid (depending on season) to secrete T4.

Second, while the T3 and T4 levels do appear to mirror one another in late March, when both hormones increase, they do not in late April when T4 declines, but T3 remains elevated. Several investigators have shown that during the parr-smolt transformation, there can be major increases in plasma T4 with little change in T3 (Fok and Eales, 1984; Leatherland, 1985; Leatherland *et al.*, 1987; Eales and Brown, 1993). The lack of coordination between T4 and T3 is suggestive that the T3 may be tightly regulated at some peripheral level independent of the quantity of circulating T4.

Finally, support for the peripheral control model comes from the observation that the plasma T4 levels were not negatively correlated with either TSH β mRNA, pituitary or plasma TSH whereas plasma T3 was negatively correlated with TSH β mRNA. In the central control model, T4 levels drive the system and one might anticipate that increased T4 would negatively correlate with transcription and/or translation of TSH. In this study only T3 was negatively correlated with TSH transcription. This finding supports a more significant role for T3 over T4 in the regulation of the pituitary-thyroid axis of teleosts.

In conclusion, this investigation is the first to directly quantify TSH activity during the parr-smolt transformation of coho salmon. Steady-state levels of TSH β mRNA were used as an index of TSH transcription. TSH β mRNA levels were elevated in late winter and declined throughout the

spring. The decline in transcription was correlated with increased plasma T3, suggesting a negative feedback relationship between the two. Pituitary and plasma TSH levels were used as indices of translation/storage and secretion of TSH, respectively. While mean levels of both pituitary and plasma TSH varied from low levels in January and peak levels in March before declining in late spring, the changes were not statistically significant. In contrast both plasma T4 and T3 showed characteristically dramatic increases during smoltification. Thus, such changes in thyroid status may result from a smoltification-associated change in thyroid sensitivity and/or change in hormone clearance rate as opposed to a significant increase in plasma TSH. Finally, these data appear to support the peripheral control model of thyroid regulation for teleost fish described by Eales and Brown (1993).

Summary

The development of quantification techniques for TSH activity has allowed for direct investigation of the regulatory properties of the hypothalamic-pituitary-thyroid axis in fish. Results from this dissertation have been used to construct the model depicted in Figure 14. We used a previously developed radioimmunoassay for salmon TSH to establish, *in vitro*, that a corticotrophin-like (CRH) molecule may be responsible for hypothalamic stimulation of TSH secretion in fish, similar to that which is observed in metamorphosing amphibians. It may be proposed that both the thyroid and interrenal axis of fish are regulated by a common hypophysiotropin. We developed an RNase protection assay (RPA) for quantification of steady state levels of TSH β mRNA. Coupled with a related RPA for total α subunit mRNA, we established that, as in mammals, the TSH subunits are differentially regulated by negative feedback from the thyroid hormones. Finally, through quantification of both transcription and translation of TSH during salmonid smoltification; a period of heightened thyroid activity, we provide evidence to suggest that the thyroid may be activated most significantly by a change in its own sensitivity to a relatively constant level of TSH production. Future studies may address the role of CRH in the regulation of TSH subunit transcription and the endocrine factors involved in regulating seasonal changes in hormonal sensitivity of the pituitary-thyroid axis.

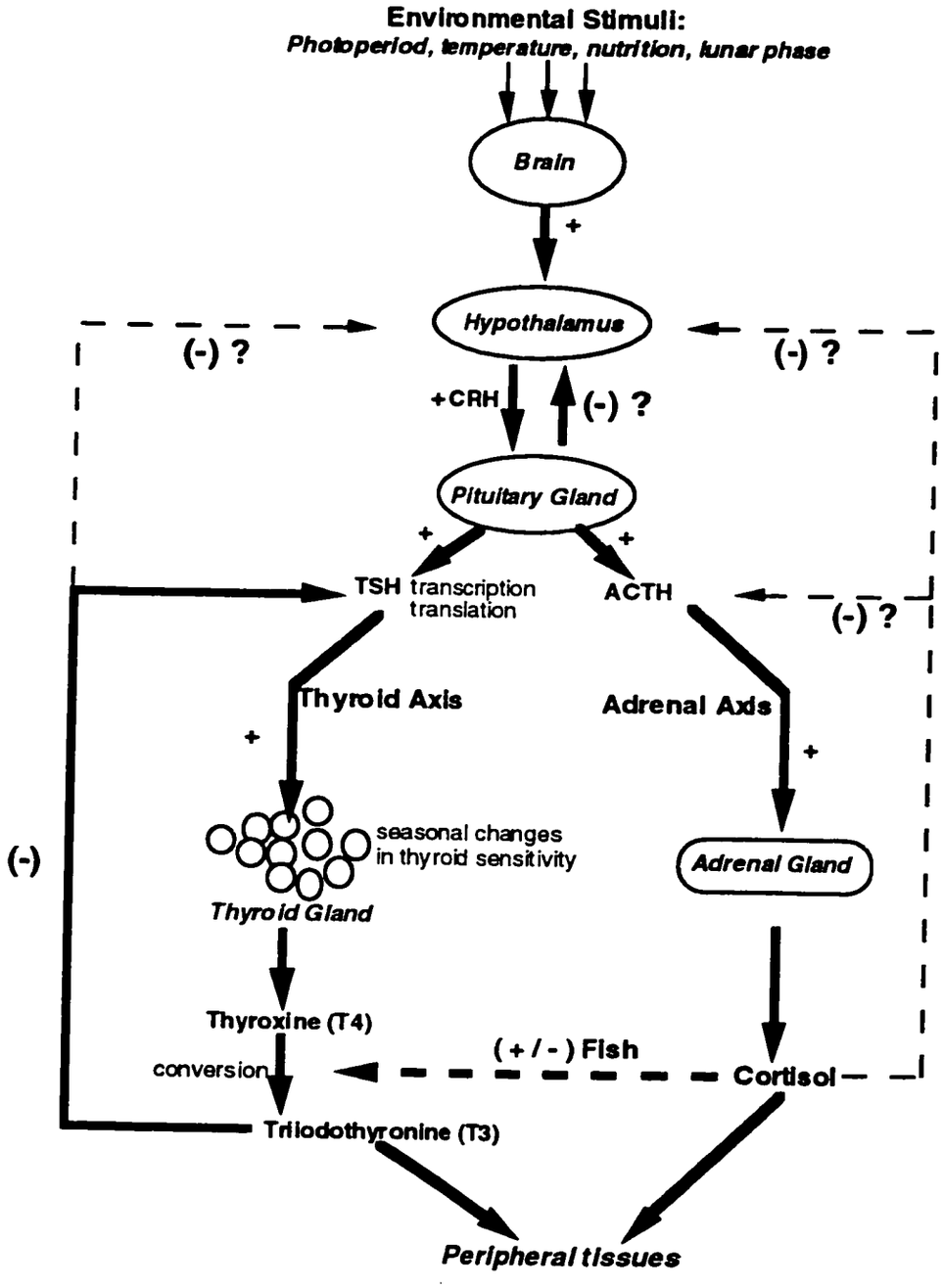


FIGURE 14. A proposed regulatory model of the hypothalamic-pituitary-thyroid axis in salmonid teleosts. Regulatory pathways are designated as either positive (+) or negative (-) based on our current knowledge. Pathways which are believed to exist, but have not been directly demonstrated are indicated by (?).

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