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Seasonal Changes in CRF-I and Urotensin I Transcript Levels in Masu Salmon: Correlation with Cortisol Secretion During Spawning

Christian G. Westring¹, Hironori Ando^{2,*}, Takashi Kitahashi⁴, Ramji Kumar Bhandari³, Hiroshi Ueda³, Akihisa Urano^{2,3}, Robert M. Does¹, Anna A. Sher¹, and Phillip B. Danielson¹

¹ University of Denver, Department of Biological Sciences, Denver, Colorado 80210

² Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo, Hokkaido 060-0809, Japan

³ Field Science Center of Northern Biosphere, Hokkaido University, Sapporo 060-0809, Japan

⁴ Division of Marine Biological Sciences, Graduate School of Fisheries Science, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

Abstract

Pacific salmon employ a semelparous reproductive strategy where sexual maturation is followed by rapid senescence and death. Cortisol overproduction has been implicated as the central physiologic event responsible for the post-spawning demise of these fish. Cortisol homeostasis is regulated through the action of hormones of the hypothalamus-pituitary-interrenal (HPI) axis. These include corticotropin-releasing factor (CRF) and urotensin-I (UI). In the present study, masu salmon (*Oncorhynchus masou*) were assayed for changes in the levels CRF-I and UI mRNA transcripts by quantitative real-time PCR (qRT-PCR). These results were compared to plasma cortisol levels in juvenile, adult, and spawning masu salmon to identify specific regulatory factors that appear to be functionally associated with changes in cortisol levels. Intramuscular implantation of GnRH analog (GnRHa) capsules was also used to determine whether GnRH influences stress hormone levels. In both male and female masu salmon, spawning fish experienced a 5–7 fold increase in plasma cortisol levels relative to juvenile non-spawning salmon. Changes in CRF-I mRNA levels were characterized by 1–2 distinctive short-term surges in adult masu salmon. Conversely, seasonal changes in UI mRNA levels displayed broad and sustained increases during the pre-spawning and spawning periods. The increases in UI mRNA levels were positively correlated ($R^2 = 0.21$ male and 0.26 female, $p < 0.0001$) with levels of plasma cortisol in the pre-spawning and spawning periods. Despite the importance of GnRH in sexual maturation and reproduction, the administration of GnRHa to test animals failed to produce broad changes in CRF-I, UI or plasma cortisol levels. These findings suggest a more direct role for UI than for CRF-I in the regulation of cortisol levels in spawning Pacific salmon.

Corresponding author: Tel.: +1-303-871-3561; Fax: +1-303-871-3471. E-mail address: cwestrin@du.edu (C.G. Westring).

*Present Address: Laboratory of Advanced Animal and Marine Bioresources, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka 812-8581, Japan

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Keywords

Pacific salmon; Senescence; Hypothalamus-Pituitary-Interrenal Axis; cortisol; Gonadotropin-Releasing Hormone; Corticotropin-Releasing Factor; Urotensin-I; Semelparity

1. Introduction

Teleost reproduction and senescence follows one of two pathways. The reproductive strategy of the majority of fishes is iteroparity which involves an extended reproductive life-span during which fish spawn repeatedly and undergo gradual senescence. In semelparity, the alternative strategy employed by Pacific salmon sexual maturation is followed by rapid senescence and death (Finch 1989; Rose 1991). It is the neuroendocrine mechanisms involved in the regulation of these events that remain to be elucidated.

The hypothalamus-pituitary-interrenal (HPI) axis is a neuroendocrine network responsible for modulating a broad range of physiological functions such as chronic and reproductive stress (Axelrod and Reisine 1984; Chrousos and Gold 1992; Lovejoy and Balment 1999; Carruth, Dores et al. 2000). Regulation of the HPI axis is a complex process which involves interaction among suprahypothalamic brain centers and the integration of multiple feedback signals (Seeman and Robbins 1994).

Initial studies of the CRF family of peptides began during the early 1980's. The mature 41-residue polypeptide hormone was localized to the mammalian hypothalamus where it acted to control the synthesis and release of adrenocorticotrophic hormone (ACTH) from corticotropes in the anterior pituitary (Vale, Spiess et al. 1981). Some studies have suggested that CRF is the primary hypothalamic hormone controlling the stress axis in teleost fish (Wendelaar Bonga 1997). CRF binds to corticotropes in the anterior pituitary thereby stimulating the synthesis and release of ACTH from the pituitary *pars distalis*. ACTH, in turn, binds to interrenal cells to induce the release of cortisol in the head kidney (Metz, Geven et al. 2005). Cortisol then mediates several functions, including: a) the suppression of ACTH release via negative feedback to corticotrophic cells; and b) the activation of cortisol-sensitive neurons which tonically inhibit CRF neurons in the hypothalamus (Bentley 1998). The proper regulation of cortisol synthesis and release is an essential component of the HPI axis in order to maintain physiological homeostasis. Cortisol also mediates several other physiological functions; including the metabolism of macromolecules (carbohydrates, lipids, and proteins), osmoregulation, immunosuppression, arterial hypertension, and several other housekeeping functions (Bentley 1998; Mommsen P. T 1999; Barton and Vijayan 2002).

Among teleosts, another hormone which has been implicated in the regulation of interrenal cortisol release is the 41-residue polypeptide urotensin-I (UI) (Kelsall and Balment 1998; Barsyte, Tipping et al. 1999). UI was first characterized in the white sucker (*Catostomus commersoni*) (Lederis, Letter et al. 1982; Lederis, Vale et al. 1982) and was recognized as a member of the CRF family of hormones. Similar to other corticotropin releasing factors, UI is expressed in both the hypothalamus and the caudal neurosecretory system, although UI transcript levels in the urophysis are significantly greater (Barsyte, Tipping et al. 1999; Lu, Dow et al. 2004; Craig, Al-Timimi et al. 2005). The subsequent cloning of the UI ortholog, urocortin, from several mammalian species demonstrated the high sequence similarity between these peptides, suggesting a conserved evolutionary role among CRF-like peptides in vertebrates. Despite this high degree of sequence homology between UI, UII, and UC, a clear function has yet to be determined for these peptides (Vaughan, Donaldson et al. 1995; Waugh, Anderson et al. 1995; Robinson, Tellam et al. 1999). Among the many possible functions that have been postulated are osmoregulation, vasoconstriction, regulation of food intake

(anorexigenic agent), locomotion, and possibly interrenal cortisol release (Bentley 1998; Kelsall and Balment 1998; Lovejoy and Balment 1999; Mommsen P. T 1999).

The sequence similarity between UI/urocortin and the CRF proteins suggests a possible role for this hormone in the chronic stress response (Barsyte, Tipping et al. 1999; Lovejoy and Balment 1999). This is a role that is supported by a range of experimental studies. For example, intraperitoneal injections of UI isolated from the White Sucker (*Catostomus commersoni*) increases circulating plasma cortisol levels in goldfish. UI administration has been shown to stimulate cortisol secretion from interrenal/head kidney preparations in trout and flounder in a concentration-dependant manner (Kelsall and Balment 1998). UI treatment of a superfused goldfish anterior pituitary cell column appears to stimulate ACTH release which in turn leads to higher cortisol levels (Fryer, Lederis et al. 1983; Arnold-Reed and Balment 1994; Kelsall and Balment 1998; McQuillan, Lokman et al. 2003). UI has even been demonstrated to stimulate intermediate pituitary melanotropes to release α -MSH and ACTH (Tran, Fryer et al. 1990). Thus, there is a reasonable body of evidence to suggest that UI may be able to influence plasma cortisol levels.

In the present study, therefore, we employed quantitative real-time PCR (qRT-PCR) to test the hypothesis that seasonal changes in the levels of hypothalamic CRF-I and UI mRNAs in masu salmon (*Oncorhynchus masou*) are correlated with the elevated plasma cortisol levels that are thought to be responsible for the post spawning demise of Pacific salmon. Furthermore we sought to determine whether or not GnRH has an influence on seasonal changes in CRF-I and UI mRNA levels by comparing GnRHa-treated salmon to an untreated control group.

2. Material and Methods

2.1. Experimental Fish

All experiments were conducted at the Toya Lake Station for Environmental Biology, Hokkaido University. *O. masou* of the Mori hatchery strain were obtained from the Mori branch of the Hokkaido Fish Hatchery (Hokkaido, Japan). The Mori hatchery strain retains its natural homing ability but the fish grow, mature, and reproduce entirely in fresh water. A detailed description of these fish has been published previously (Bhandari, Taniyama et al. 2003).

Fish that served as a stock for experimental animals were reared in circular outdoor 1400 L tanks fed by a flow of spring water under natural photoperiod. Water temperatures ranged from 9.8°C to 12.1°C. Experimental animals were reared from eggs collected from 20 female salmon and maintained in outdoor tanks at a density of 500 fish/tank. Since the sex of juvenile salmon could not be determined, male and female fish were kept in the same tanks throughout the 3-year duration of the study. Experimental fish were fed dry trout pellets (Oriental Feed Industry, Yokohama, Japan) equivalent to 2% of body weight/day. Feeding rates were adjusted monthly to correct for increases in body mass.

2.2. GnRHa Treatment and Sample Collection

GnRHa implantation and sampling of experimental animals began in April when fish were 17 months old. Implantations were performed one week prior to the collection and sacrifice of experimental animals. This GnRH implantation and collection regimen (Bhandari, Taniyama et al. 2003) was performed monthly until the fish reached 34 months of age in September of the following year. During the spawning period in September, the implantation-collection regimen was performed every other week. Fish that are 17 – 25 months of age (post hatching) are classified as 1+; those 25 – 35 months of age are classified as 2+.

A total of 380 fish were used for the present study. For each sampling, 20 fish were randomly selected from the same cohort, and anesthetized using 0.01% tricaine methanesulfonate

(MS222) and 0.01% sodium bicarbonate. From these 20 fish, 10 fish were implanted in the dorsal muscle with a 2 mm GnRHa capsule containing 75µg of [D-Gly¹⁰, D-Ala⁶, Pro⁹]-GnRH ethylamide in an ethylene vinyl acetate copolymer matrix (Zohar, Goren et al. 1990; Zohar 1996). The choice of this dosage is the standard operating procedure for GnRHa administration that has been validated by the Urano laboratory for use on salmonids. This dosage has been shown to increase both the LH subunit mRNA and plasma 17α,20β-dihydroxy-4-pregnen-3-one (DHP) levels in maturing sockeye salmon (*O. nerka*) (Fukaya 1998; Kitahashi 1998). The remaining 10 animals (sham-implanted controls) received a blank copolymer matrix. GnRHa-treated fish were maintained in separate aquaria from the sham-implanted control fish.

Both GnRHa- and sham-implanted fish were sacrificed seven days after implantation and separated by sex. Although 20 fish were sacrificed each month, the number of males versus females within each group varied because gender cannot be determined by external examination in juvenile and non-spawning salmonids. Rather it must be determined by internal examination of the gonads. Fish were anesthetized with 0.01% MS222 buffered with an equal amount of sodium bicarbonate, and checked by gentle abdominal pressure to determine whether they spermiated or ovulated. The fork length and body weight were recorded and blood samples were collected from the caudal vein. Blood samples were centrifuged at 3000 rpm for 15 min to obtain plasma which was stored at -30°C until assayed for cortisol. Sample fish were sacrificed by decapitation. Whole brains were removed and trimmed to prepare forebrain samples. They were cut frontally at the middle of the telencephalon, and posteriorly at the anterior end of the *saccus vasculosus*. Therefore, the forebrain samples contained the posterior half of the telencephalon, diencephalon, and mesencephalon including a part of the optic tectum (Figure 1). Samples were flash frozen in liquid nitrogen and stored at -80°C for later use. Gonads were removed and weighed in order to estimate their maturity based on the gonadosomatic index ($GSI = \text{gonad weight/body weight} \times 100$).

2.3. Assaying Plasma Cortisol Levels

Plasma cortisol levels were determined by time-resolved fluoroimmunoassay (Yamada H 1997). Briefly, ether-extracts containing serum steroid hormones were dried under nitrogen gas, reconstituted into 1 ml of assay buffer (0.2 g BSA, 0.01 g Thimerosal, 200 ml 0.05M Borate Buffer), and stored at -30°C until use. The samples were applied to 96-well microtiter plates in which BSA-conjugated antigen was immobilized by physical adsorption. Wells were incubated with primary antibody overnight at 37°C. After 3 washes, the assay plates were incubated with Europium (Prunet, Boeuf et al.)-labeled IgG. (BSA-conjugated antigen, antibody and Eu-labeled IgG were kindly provided by Dr. H. Yamada, Kitasato University.) Following stringent washes to remove unbound Eu-labeled IgG, the intensity of fluorescence from dissociated Eu was measured with a time-resolved fluorometer (1234 Delfia fluorometer, Wallac Oy Inc., Turku, Finland). Standard curves in each assay were plotted and values were calculated automatically by the instrument software. The sensitivity of the assay was 24 pg/ml. The intra- and inter-assay coefficients of variation (CV) in the present study were 4.0 and 19.4%, respectively.

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from individual brain samples by the guanidium thiocyanate hot phenol-chloroform method (Chirgwin, Przybyla et al. 1979). Poly(A) RNA for cDNA synthesis was isolated from brain tissue by direct capture onto oligo dT₂₅ coated paramagnetic beads (Novagen, Madison, WI) following the procedure of (Jakobsen 1994). First-strand cDNA for use in qRT-PCR was synthesized from total RNA and primed from the poly(A) tail using oligo dT₁₂₋₁₈ primers and Multiscribe Reverse Transcriptase (PE Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. First-strand cDNA for use in molecular cloning of UI and CRF-I was synthesized from total RNA and poly(A) mRNA, respectively. All cDNA

synthesis reactions employed SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol using an anchor dT₁₇: (5'-GACTCGAGTCGGATCCATCGA[T]₁₇-3') primer.

2.5. Molecular Cloning

A partial-length CRF-I cDNA was cloned using a 3'Rapid Amplification of cDNA Ends (RACE) approach (Frohman, Dush et al. 1988). A non-degenerate primer, KMV-F2 (5'-CATAGCAACAGAAAAATGATG-3'), based on the known CRF-I sequence from sockeye salmon (*O. nerka*) (Ando, Hasegawa et al. 1999) served as the gene-specific forward primer. Anchor 56 (5'-GACTCGAGTCGGATCCATCGA-3') was used as the reverse primer.

A partial-length cDNA for UI was cloned from urophysial cDNA using a reverse-transcriptase PCR approach. A degenerate forward primer, UI-F3 (5'-CC[C/T]GT[C/T]CC[C/T][C/T]TI[A/G]TICT[C/G]CT-3' where "I" represents deoxyinosine and brackets contain mixed bases), and a degenerate reverse primer, UI-R2 (5'-GTG[G/T]AA[A/G]GTGAGGTC[A/G]ATGGA-3') were designed by comparative sequence analysis of UI cDNAs from rainbow trout (*O. mykiss*, Accession AJ005264), goldfish (*Carassius auratus*, Accession AF129115), and carp (*Cyprinus carpio*, Accession M11671).

PCR amplified products were visualized on a 2%, 0.5X TBE, EtBr stained agarose gel. Amplicons from 3'RACE were excised from a 2% TAE gel, purified, and ligated into the pGEM-T vector system (Promega, Madison WI). Following electroporation into *Escherichia coli* DH5α cells, transformants were screened for inserts by PCR and positive clones containing appropriate size inserts were sequenced using a CEQ8000 capillary sequencer (Beckman-Coulter, Fullerton, CA). Sequences were analyzed for similarity to known genes using the BLAST algorithm (Altschul, Madden et al. 1997).

2.6. Quantitative Real-time PCR Assay

qRT-PCR was performed using the TaqMan[®] 5' nuclease assay chemistry on an ABI Prism[®] 7900HT Sequence Detection System with SDS software 2.1.1 (PE Applied Biosystems, Foster City, CA). Quantitative analyses were performed using standards of known concentration ranging from 5×10^2 – 5×10^9 copies/μl for CRF-I and 2×10^0 – 2.2×10^6 copies/μl for UI. For CRF-I, mRNA standards were transcribed by MAXIscripT T3 RNA polymerase (Ambion, Austin, TX) from plasmids containing the CRF-I cDNA from sockeye salmon. For UI, DNA standard DNA was directly amplified from plasmids using primers that flanked a partial-length UI cDNA insert. Size-purified amplification products were quantified by UV spectroscopy and serially diluted to yield qRT-PCR standards of known concentration. qRT-PCR primers and fluorogenic probes are listed in Table 1.

All standards were amplified in triplicate, and all experimental samples were assayed in duplicate. mRNA concentrations were expressed as copies/μl. A quantitative amplification control was also included. This consisted of a single stock of cDNA from masu salmon brain tissue. This quantitative amplification control was included on each plate run during this study to estimate the coefficient of variation (CV) for differences in amplification efficiency between runs. In order to estimate the total amount of transcribed cDNA for downstream quantification purposes, all total RNA samples were normalized by two independent methods. After RNA isolation from tissue, UV spectrophotometric quantitation at 260nm was used to help equalize the amount of input RNA in each RT reaction. As a secondary control, quantitation of β-actin was used to more accurately normalize data for differences in the initial cDNA concentration. Although β-actin levels may vary under exceptional conditions (*e.g.*, rapidly regenerating tissues and blastomers, (Hassin 1998; Bustin 2000; Tricarico 2002)) it displays stable levels in most other tissues. Masu salmon β-actin mRNA levels were quantified using the same

protocol described above with gene specific standards ranging from 1.7×10^1 – 1.79×10^7 copies/ μ l. β -actin primers and fluorogenic probes were designed based on the published masu salmon sequence (Accession AB111056) Table 1.

2.7 Statistical Analysis

In order to test the significance of changes in levels of plasma cortisol, CRF-I, and UI mRNAs over time, data were analyzed with a repeated measures ANOVA (JMP-IN 2005). A full model that investigated the effect of sex, treatment (GnRHa-treated vs. the control), and the interaction between the two across sampling months (“time”) was run for each of the response variables (plasma cortisol, CRF-I, and UI mRNAs). Where interactions were non-significant, they were omitted from the final model. All response variables were tested for normality and homogeneity of variance, and log-transformed where necessary. Some months had to be excluded from the analysis due to lack of an adequate sample size (i.e. less than or equal to 3 replicates for a sex*treatment*date combination). These omissions were minimal except in the case for GSI, thus it was decided that pairwise comparisons using t-tests would be used for dates of interest, rather than repeated measures ANOVA for this variable. Interpretation of the results included considering differences over time within each group (“within subject tests”) as well as between groups (i.e. sex, treatment), using repeated measures as replicates (“between subjects tests”). *Post hoc* analyses of differences between specific time periods were subject to a Bonferroni adjustment to determine significance levels. The potential for CRF-I, GSI and UI mRNAs to explain cortisol levels within individuals was investigated using stepwise selection multiple regression (JMP-IN 2005).

3. Results

3.1 Growth and Development in Experimental Animals

Analyses of fork length and body weight indicated broad concordance between the sham-implanted control and GnRHa-implanted groups for both male and female samples (data not shown). In general, fork length and body weight for males and females increased gradually until peaking in 34-month-old fish (i.e., 2+ fish in September). Although not statistically significant, there was a trend among the GnRHa-treated fish toward accelerated increases in fork length in maturing 2+ males and body weight in vitellogenic 2+ females.

Precocious maturation of many of the 1+ sham-implanted control male fish was seen from late summer to early autumn (Fig. 2a). As a result, there were an insufficient number of non-precocious +1 GnRHa- and sham-implanted control males remaining for meaningful statistical analysis. Thus, non-precocious fish were excluded from further consideration. The increase in GSI of precocious 1+ males began in late July, while that of the 2+ GnRHa- and sham-implanted control males began one month earlier (i.e., at the end of June). Finally, two of five 2+ sham-implanted controls and all of the 2+ GnRHa-implanted males were found to spermiolate in late August. The remaining 2+ males spermiolated in early September.

In contrast, the relatively low and stable GSI of +1 females indicated the absence of precocious maturation in these fish (Fig. 2b). In 2+ control female fish, however, an increase in the GSI began at the end of July and continued through early September. This was one month later than for 2+ males. It should be noted that although 2+ GnRHa-implanted female fish showed a significant increase in GSI between late August and early September, this did not appear to accelerate ovulation which didn't occur until late September when the fish were fully matured.

3.2 Plasma Cortisol Levels

Seasonal plasma cortisol levels for control and GnRHa-treated masu salmon are shown in Figure 3. A repeated measures ANOVA revealed significant changes in cortisol levels over

time ($df=4/10$; $F=36.25$; $p<0.0001$), with no significant difference in response of males versus females or between GnRHa- and sham-operated controls (either between or within subjects tests). Plasma cortisol levels remained stable (approx. 50ng/ml) in both male and female salmon age 17 – 26 months (*i.e.*, from the start of the study in April through December of the same year), but at 27 months of age (January), 2+ adult masu salmon experienced a spike in plasma cortisol levels. A statistically significant 5-fold increase in plasma cortisol levels was observed in both males and females relative to the previous month's levels (Fig. 3a and 3b). Within one month, plasma cortisol returned to basal levels in all fish and remained there for one month. Adult 2+ masu salmon then displayed broad and sustained increases in plasma cortisol levels during the pre-spawning and spawning period (*i.e.* March through September). Post hoc tests on differences over time showed that during this period, plasma cortisol levels in 2+ males showed a statistically significant ($p<0.01$) 7-fold increase relative to the pre-March levels. However, no significant difference was observed between GnRHa- treated and control fish (Figure 3a). In 2+ female salmon, a significant ($p<0.01$) 7-fold increase in plasma cortisol levels was also observed beginning in March, reaching a maximum in August and remaining relatively steady through the end of the spawning season in late September (Figure 3b).

3.3 Molecular Cloning of CRF-I and UI

In order to quantify the levels of brain CRF-I and UI mRNAs by qRT-PCR, cDNAs for these genes were cloned from whole brain extracts from masu salmon. Based on the resulting sequences, homologous primers and probes were designed for use in 5' nuclease quantitation assays. Sequencing and BLAST analysis of 24 subcloned cDNAs amplified by 3' RACE using a non-degenerate primer based on the known CRF-I sequence from sockeye salmon (*O. nerka*) resulted in the identification of a partial masu salmon CRF-I cDNA (Genbank AY998975). The consensus nucleotide sequence and conceptual amino acid translation for the 479 bp partial cDNA (Figure 7) revealed an open reading frame of 36bp and a 3' UTR of 443 bp containing a canonical polyadenylation signal (AATAAA) 12 bp upstream of the poly(A) tail. The nucleotide sequence displayed 96% global identity with the previously cloned CRF-I ortholog from *O. mykiss* including the 3' UTR (AJ566334).

Sequencing and BLAST analysis of 24 subcloned cDNAs amplified by RT-PCR using degenerate primers based on the known UI sequence from rainbow trout (*O. mykiss*), goldfish (*Carassius auratus*), and carp (*Cyprinus carpio*) resulted in the identification of a partial masu salmon UI cDNA (Genbank AY571961). The consensus nucleotide sequence and conceptual amino acid translation for the 355 bp partial cDNA (Figure 8) revealed an open reading frame that encoded 118 amino acids which displayed 89% global identity with the *O. mykiss* UI ortholog.

3.4 Seasonal mRNA levels of CRF-I and UI in the Hypothalamus

In order to determine seasonal variations in mRNAs encoding CRF-I and UI, a qRT-PCR approach was employed. Quantitation standards of known concentration and the inclusion of a common cDNA control on all reaction plates made it possible to accurately determine the inter- and intra-plate CV. For CRF-I, UI, and β -Actin, the intra-assay CV was 0.54–2.75, 1.29–2.49, and 0.48–1.09%, respectively. The inter-assay CV was 3.20, 9.82, and 5.06% respectively. Assay sensitivity was 2.0×10^1 copies/ μ l for CRF-I, 2.0×10^0 copies/ μ l for UI, and 1.79×10^2 copies/ μ l for β -actin. Quantification of β -actin mRNAs in both sham operated controls and GnRHa treated fish did not show statistically significant changes in expression throughout the lifecycle of the fish. Furthermore, there was no difference in β -actin mRNA levels between control fish and GnRHa treated fish (data not shown).

Seasonal CRF-I mRNA levels for control and GnRHa-treated masu salmon are shown in Figure 4. Repeated measures ANOVA revealed a significant three-way interaction between

sex*treatment*time (df=6/3; $F=8.63$; $p<0.05$), suggesting that males and females responded differently to GnRHa across months. Up to two distinctive surges in CRF-I levels were observed in adult masu salmon. By 30 months of age (April), both 2+ sham-implanted control and GnRHa-treated female salmon showed statistically significant increases (3.2 and 6.1 fold respectively) in CRF-I mRNA levels relative to the preceding month. During the same period, however, only 2+ GnRHa-treated male salmon displayed a spike (5.1 fold) in CRF-I levels while sham-implanted control fish remained unchanged (Fig. 4a and 4b). Within one month, CRF-I levels returned to baseline levels in all fish and remained there until the onset of spawning in September when a second spike in expression was observed in all fish regardless of sex or GnRHa treatment. The second surge of CRF-I in male fish peaked in early September while in female fish it remained high through late September. In male fish, CRF-I levels increased 4.3 fold in the GnRHa-treated group and 3.3 fold in the control group relative to the preceding month. The increase in female salmon appeared to be only slightly lower at 3.2 fold and 2.5 fold, respectively.

Seasonal UI mRNA levels for control and GnRHa-treated masu salmon are shown in Figure 5. Repeated measures ANOVA revealed significant differences in response to GnRHa treatment over time (treatment*time: df= 6/4; $F=11.09$; $p<0.02$), with no significant difference between sexes. Pooled male and female salmon showed lower levels of UI mRNA on average in GnRHa- treated salmon versus controls in January, but the reverse in April (Figure 5a). Overall, adult 2+ masu salmon experienced broad and sustained increases in UI levels during the pre-spawning and spawning period, (*i.e.* April through September). In both females and 2 + males, UI mRNA levels showed a statistically significant 4-fold increase relative to pre-April levels.

The stepwise regression analysis on CRF-I, GSI and UI mRNAs identified UI mRNA and GSI as factors associated with temporal cortisol levels by the following relationship for a) females ($R^2 = 0.26$, $p<0.0001$) and b) males ($R^2 = 0.21$, $p<0.0001$):

$$\text{a. } y = 3.55 + x_1(101.47) + x_2(0.74)$$

$$\text{b. } y = (3.63) + x_1(154.47) + x_2(0.29)$$

Where $y=\ln(\text{cortisol}+1)$, $x_1=\ln(\text{UI}+1)$, and $x_2=\ln(\text{GSI}+1)$. The positive relationship between UI mRNA levels and plasma cortisol in pre-spawning and spawning masu salmon was an especially strong (Figure 6A and 6B).

4. Discussion

4.1 Understanding the Role of Chronic Stress Response in Semelparous Reproduction

In most organisms, the expression of glucocorticoid and mineralocorticoid hormones plays a critical role in maintaining proper physiological homeostasis. In teleosts, cortisol can act as a mineralocorticoid and glucocorticoid, targeting both glucocorticoid receptor(s) (GR) and mineralocorticoid (Arai, Assil et al.) receptors(s) (Knoeb, Fitzpatrick et al. 1996; Teitsma, Anglade et al. 1998; Greenwood, Butler et al. 2003; Sturm, Bury et al. 2005). Under normal circumstances, cortisol release is associated with stress, gluconeogenesis, metabolic function, immune suppression, and osmoregulation in several species of fish. Since the late 1950s, studies on Pacific salmon have suggested that the deleterious effects of prolonged hypersecretion of the stress hormone cortisol (*e.g.*, muscle atrophy, renal degeneration and immune deficiency) are responsible for the post spawning demise of Pacific salmon (Robertson and Wexler 1957; Idler, Ronald et al. 1959; Phillips, Holmes et al. 1959; Robertson and Wexler 1959; Robertson and Wexler 1960; Robertson and Wexler 1962; Donaldson and Fagerlund 1968; Fagerlund and Donaldson 1969; Alrubaian, Danielson et al. 1999). In juvenile Pacific salmon, plasma cortisol levels play a critical role in parr-smolt transformation and downstream

migration (Barton, Schreck et al. 1985; Young, Bjornsson et al. 1989). These physiological changes prepare the developing parr for the changing environmental conditions encountered during adaptation from freshwater to saltwater. As demonstrated in previous studies, juvenile masu salmon (April through November, 1+ fish) experienced slightly elevated plasma cortisol levels consistent with parr-smolt transformation, despite their captive rearing (Barton, Schreck et al. 1985; Young, Bjornsson et al. 1989). Prior to sexual maturity, plasma cortisol levels returned to normal, demonstrating that a functional regulatory mechanism exists for controlling hypercorticism in sexually immature fish. Recognizing that cortisol homeostasis in many other organisms is primarily regulated by the activity of the hypothalamus-pituitary-adrenal (interrenal) axis, there are several candidate regulatory components that could be involved in the loss of this regulation in semelparous salmon during spawning. These include: the cortisol biosynthetic pathway itself; the expression of the hypothalamic neuropeptides, CRF-I, AVT, and UI along with the POMC-derived hormones from the pituitary which stimulate cortisol release; and the negative feedback mechanisms involving these same neuropeptides and polypeptide hormones. Perturbation of these components of the HPI axis may be responsible for the apparent hypercorticism seen in spawning pacific salmon.

Given the evidence that CRF-I and UI are able to induce the secretion of cortisol as well as their involvement in a negative feedback loop for cortisol synthesis, these neuropeptides represent front-line candidates for a central role in the development of sustained hypercorticism. Of particular interest was whether changes in CRF-I and/or UI mRNA levels were correlated with changes in serum cortisol in the months leading up to spawning (*i.e.*, from 26 months of age through the end of the spawning season). In fact, evidence of a correlation may provide valuable insight for delineating the underlying molecular events and pathways involved in the regulation of the chronic stress response in semelparous fish. Finally, the impact of GnRH α treatment was investigated to determine whether increases in circulating GnRH affect mRNA levels encoding CRF, UI, and/or plasma cortisol levels. These data show that GnRH α treatment did induce a response in CRF-I and UI levels, but these responses varied by time of year, sex, or both. In masu salmon, it has been demonstrated that treatment with GnRH α was associated with increased levels of GTH α 2 and LH β subunit mRNAs in the pituitary of both male and female masu salmon (Kitahashi, Bhandari et al. 2004). This GnRH-induced increase in mRNA levels was also correlated with increases in plasma steroid levels and sexual maturation during the pre-spawning period. Given the broad impact of GnRH on key regulatory factors involved in reproduction, the correlation, or lack thereof, between GnRH activity and changes in the HPI axis may provide added insight into the complex underlying mechanisms by which salmonids respond to reproductive stress. The absence of a strong, consistent positive reaction to the treatment, especially by cortisol levels, was unexpected.

4.2 Cortisol Levels vs. Expression of Hypothalamic Neuropeptides

Although CRF-I and UI belong to the same multigene family, and the observed increases in CRF-I and UI levels in the year leading up to spawning were of the same magnitude, the level of CRF-I mRNA was 12.0 to 24.6 fold higher than that of UI mRNA (normalized to endogenous β -actin mRNA levels, data not shown). The physiological significance of this difference in terms of cortisol regulation remains to be determined. Beyond the sequence homology of the CRF family of peptides, several studies suggest both UI and CRF may bind a common class of CRF receptors. Comparative studies on the CRF-type I receptor (R1) demonstrated equivalent binding by CRF, UI, urocortin (UC), and sauvagine (SVG) ((Chang, Pearse et al. 1993; Chen, Lewis et al. 1993; Kishimoto, Pearse et al. 1995; Lovenberg, Chalmers et al. 1995; Vaughan, Donaldson et al. 1995; Arai, Assil et al. 2001; Pohl, Darlison et al. 2001). Furthermore, studies on the CRF-type II (R2) receptor in brown bullhead catfish (*Ameiurus nebulosus*) (Arai, Assil et al. 2001) and chum salmon (*Oncorhynchus keta*) (Pohl, Darlison et al. 2001) demonstrated similar binding affinity by CRF and UI to CRF(R2). Not only were

these receptor-specific patterns of UI vs. CRF binding confirmed in studies in teleost fish, but also a third CRF receptor from catfish was shown to bind CRF with a 5-fold greater affinity than UI and SVG (Arai, Assil et al. 2001).

Given the differential binding affinities associated with specific CRF receptor subtypes, it might be reasonable to postulate that this compensates for differences in the physiological potency of these neuropeptides. It bears pointing out, however, that while an abundance of research demonstrates the ability of UI to bind and activate CRF receptors *in vitro*, the *in vivo* relevance of these studies is questionable. For example, detailed immunohistochemical studies of UI and CRF secretion in the brain seem to indicate that these two related neuropeptides are restricted to separate neuronal systems and that there is a lack of UI immunoreactive neurons in the rostral adenohypophysis where contact with corticotropes would typically occur (Yulis, Lederis et al. 1986). This apparent incongruity between the findings of *in vitro* and *in vivo* studies suggests the correlation between UI expression and plasma cortisol levels in spawning Pacific salmon reflects that role of an alternate regulatory pathway. In the brain, UI may act at the level of the nucleus *lateralis tuberis* where both CRF and UI neurons coexist and which has been implicated as having a role in ACTH secretion (Fryer and Peter 1977). Conversely, UI may impact CRF levels in a completely ACTH-independent manner by directly stimulating the release of cortisol by interrenal/head kidney tissues (Arnold-Reed and Balment 1994; Kelsall and Balment 1998). Such a role may suggest interplay between UI secreting neurons in the brain and the large quantities of UI found in the urophysis which could lead to a surge in plasma cortisol levels similar to those seen in spawning Pacific salmon.

Another intriguing, and potentially far more informative difference, is seen in an analysis of the seasonal mRNA levels of CRF-I and UI in 2+ masu salmon. While it is recognized that individual fish mature at somewhat different rates and that this can increase the apparent variability within sample groups, this inter-individual variability did not mask the statistically significant changes in mRNA levels that appear in the months leading up to and during spawning. For example, changes in CRF-I levels in 2+ masu salmon in the year leading up to spawning are characterized by one or two distinct and short-lived (1 month) spikes in mRNA transcript levels. The first of these occurs in April (male and female GnRH α treated fish) and the second occurs in late summer towards the onset of the spawning period (all males and females, with a more sustained peak for females). In sharp contrast to this, UI mRNA levels in 2+ fish are characterized by steady increases beginning in early spring and continuing through much of the spawning season for both sexes. However, despite the fact that a short lived spike (January, 1+ fish) in plasma cortisol exists, it does not appear to correlate with either CRF-I or UI mRNA expression. These findings suggest it would be overly simplistic to associate either of these two neuropeptides in all increases in plasma cortisol levels. Although UI was significantly correlated with plasma cortisol in this study, its levels explained less than 30% of cortisol level variation for either the male or female salmon measured. In fact, it appears that the regulation of the HPI axis on the secretion of these short lived spikes in plasma cortisol levels prior to spawning may be under the regulation of several components of the HPI axis; where the steady increase in UI expression during spawning may just be one of the factors which lead to hypercorticism in spawning Pacific salmon.

It bears pointing out, however, that while increased mRNA expression is usually indicative of increased protein expression, mRNA transcript levels can really only be said to reflect the potential for increased levels in protein activity. A direct assay of proteins would be needed to confirm this association.

These divergent patterns of expression may provide important insight for the formulation of hypotheses about the mechanisms responsible for broad and sustained elevation in plasma

cortisol levels. While there is evidence from iteroparous species that ACTH, angiotensin II (AII), UI and UII are all capable of stimulating cortisol release from interrenal preparations of trout and flounder (Arnold-Reed and Balment 1994; Kelsall and Balment 1998), this may not reflect the role CRF in spawning semelparous masu salmon. The seasonal pattern of sequential increases in UI levels is consistent with a stimulatory role leading to a sustained elevation of plasma cortisol. The transitory spikes in CRF-I mRNA levels, however, are not consistent with such a role. In fact, these spikes occur long after the rise in plasma cortisol begins. This difference between the CRF-I vs. UI is also borne out in a statistical analysis of these patterns. UI and not CRF-I mRNA levels contributed to a model explaining plasma cortisol levels. While these results point to UI as potentially playing a critical role in the significantly elevated cortisol levels in spawning salmon, the fact that both UI and CRF-I spike in late summer suggests that CRF-I may play a complementary role in this process. In fact, some studies have suggested that the action of UI and CRF may be additive (Fryer, Lederis et al. 1985). If true, it is possible that the occurrence of a spike in CRF-I mRNA levels near the end of the spawning season may contribute to chronic hypercorticism.

A second difference between the seasonal expression patterns for CRF-I and UI may provide further grounds for speculation on the regulation of cortisol levels in spawning masu salmon. Previous studies of the HPI axis have indicated the existence of a negative feedback loop which mediates ACTH release in vertebrates (Fryer, Lederis et al. 1984). These *in vitro* studies demonstrated that cortisol inhibits the ACTH-releasing activity of UI and CRF in goldfish pituitary cells. Such a negative feedback loop may play an important role in maintaining cortisol homeostasis so as to ameliorate the deleterious impact of chronically elevated cortisol levels.

It may be reasonable to postulate that negative feedback involves a suppression of UI and/or CRF mRNA levels. The short-lived duration of the observed spikes in CRF-I transcript levels seen in masu salmon, therefore, would be consistent with the existence of a functional negative feedback mechanism. In a high-cortisol environment, any increase in CRF-I expression should be rapidly reversed as appears to be the case. Conversely, UI transcript levels appear to be relatively unresponsive to the presence of high levels of cortisol; thus suggesting an “inability” of UI to respond appropriately to negative feedback signals. Might this be part of the underlying mechanism responsible for a sustained elevation in plasma cortisol levels in spawning masu salmon? In fact, studies on several species of fish have demonstrated that acute hypersecretion of the stress hormone cortisol decreases CRF expression under varying conditions of osmoregularity, food intake, locomotion, or prolonged restraint (Pepels, Van Helvoort et al. 2004; Craig, Al-Timimi et al. 2005). Despite the informative nature of these studies on the stress regulation of the HPI axis on CRF and/or UI, little information is known about the expression of CRF and UI under chronic stress in spawning Pacific salmon. Perhaps the failure to ameliorate sustained periods of hypercorticism in spawning Pacific salmon is a result UI induced secretion of cortisol in an ACTH-independent manner *in vivo*.

In summary, the current study quantified seasonal changes in mRNA levels for CRF-I and UI in masu salmon. Variations in the levels of these neuropeptide transcripts were compared to plasma cortisol levels for the same period to identify potential correlations. While plasma cortisol levels began to rise months prior to spawning, CRF-I levels remained relatively stable throughout the lifecycle of masu salmon until the onset of spawning in September. In contrast to this, an increase in UI mRNA levels was observed in pre-spawning and spawning masu salmon. This pattern was closely correlated with the changes in plasma cortisol levels during the same time period. Despite the importance of GnRH in sexual maturation and reproduction, the administration of GnRH α to test animals failed to produce broad changes in the CRF-I, UI or plasma cortisol levels - aside from a mild stimulatory effect on CRF-I levels in 2+ salmon (April and September). While recognizing that the regulation of cortisol levels in spawning Pacific salmon may be influenced by a combination of factors, including CRF binding proteins,

receptor binding affinity, and the feedback mechanisms within the HPI axis, these findings suggest that UI may play a more direct role than CRF-I in the regulation of cortisol levels in spawning Pacific salmon. Test of the latter hypothesis would be to analyze CRF-I and UI mRNA levels in iteroparous salmonids such as the steelhead trout. By taking this comparative approach, it should be possible to gain further insight into the contrasting reproductive strategies of semelparous and iteroparous fish.

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Figure 1.

Illustration of the salmonid brain and the anatomical regions that were used in this experiment. Whole brains were trimmed frontally at the middle of the telencephalon, and posteriorly at the anterior end of the *saccus vasculosus*. Forebrain samples contained the posterior half of the telencephalon, diencephalon, and mesencephalon including a part of the optic tectum.

Figure 2.

Seasonal changes in gonadosomatic index (GSI) in male (Figure 2A) and female (Figure 2B) masu salmon. Data are presented as mean $\pm$ SEM. Precocious maturation of 1+ sham-implanted control male fish was seen from late summer to early autumn. The GSI of +1 females indicated the absence of precocious maturation. 2+ control female fish showed an increase in GSI that began at the end of July and continued through early September. The spawning period for 2+ masu salmon in the current study was September. Sampling months marked with an “*” or “**” indicates a significant difference between sampling months at $p < 0.05$ and $p < 0.01$, respectively. “+” indicates a significant difference between GnRH α -treated salmon and sham-operated controls at the $P < 0.05$ level.

Figure 3.

Seasonal changes in plasma cortisol levels in male (Figure 3A) and female (Figure 3B) masu salmon. Data are presented as mean $\pm$ SEM. As this is a post hoc test, significance levels are adjusted thus: $\alpha'' = \alpha / (\# \text{ comparisons})$ with $\alpha = 0.05$, therefore significance for this test was $0.05/2$ ($p < 0.025$). There were no statistically significant differences between GnRH α - and sham-operated controls for either sex. The spawning period for 2+ masu salmon in the current study was September. “***” indicates a significant difference ($p < 0.01$) between sampling months. “+” indicates significant difference between the control and GnRH α treated group ($p < 0.01$).

Figure 4. Seasonal changes in corticotropin-releasing factor I (CRF-I) mRNA levels in male (Figure 4A) and female (Figure 4B) masu salmon. Data are presented as mean $\pm$ SEM. As this is a post hoc test, significance levels are adjusted thus: $\alpha' = \alpha / (\# \text{ comparisons})$ with $\alpha = 0.05$, therefore significance for this test was $0.05/2$ ($p, 0.025$). Prior to spawning, male masu CRF-I mRNA levels increased 3.3 to 4.3 fold relative to the preceding month, while female salmon increased 2.5 to 3.2 fold. Spawning period for 2+ masu salmon in the current study was September. “*” indicates significant difference between sampling months ($p < 0.025$), “**” ($p < 0.01$). “+” significant difference ($p < 0.05$), between GnRH-treated fish and control group. Only one comparison (Figure 5A) was made and thus no Bonferroni adjustment.

Figure 5. Seasonal changes in urotensin I (UI) mRNA levels in male (Figure 5A) and female (Figure 5B) masu salmon. Data are presented as mean $\pm$ SEM. Adult 2+ masu salmon experienced broad and sustained increases in UI levels from April through September. The spawning period for 2+ masu salmon in the current study was September. “*” indicates significant difference ($p < 0.05$) between sampling months.

Figure 6.

Scatter plots of plasma cortisol vs. UI mRNA levels for male (Figure 6A) and female (Figure 6B) masu salmon. A regression analysis of these data indicate a positive correlation ($R^2 = 0.18$ for male fish and 0.16 for female fish) between changes in UI mRNA levels and plasma cortisol levels in pre-spawning and spawning masu salmon ($p < 0.0001$).

Figure 7.

Nucleotide sequence alignment of masu salmon (*Oncorhynchus masou*) and rainbow trout (*Oncorhynchus mykiss*) CRF-I. Conserved regions are highlighted in grey and sequence differences are indicated with bold letters. Nucleotide sequences for masu salmon and rainbow trout mRNAs encoding CRF-I are available at Genbank Accession No. AY998975 and AF296672 respectively.

Figure 8.

Nucleotide sequence alignment of masu salmon (*Oncorhynchus masou*) and rainbow trout (*Oncorhynchus mykiss*) UI. Conserved regions are highlighted in grey and sequence differences are indicated with bold letters. The nucleotide sequence for the masu salmon and rainbow trout mRNAs encoding UI is available at Genbank Accession No. AY571961 and AJ005264 respectively.

Table I

Sequences for synthetic oligonucleotide primers and fluorogenic probes used for quantification of mRNA encoding CRH and UI in masu salmon (*Oncorhynchus masou*) Quantitative Real-time PCR.

CRF-I:**Forward primer** 5'-TCC TGG ACA GCT CGA TCA AAC-3'**Reverse primer** 5'-TGA ATG AAG TGC CTG GTC ACA-3'**Probe** 6FAM-CTT TAG AGT GTG AAG GGA TAG TGT TGT CGA CCA-TAMRA**UI:****Forward primer** 5'-AGT TTC ATT GTC TGG AAG GCT ACA-3'**Reverse primer** 5'-GCC TCG ACC CCC ATG AAG-3'**Probe** VIC-TGT CAT TGC CTG GTG GCG GTC TT-TAMRA**Beta-Actin:****Forward primer** 5'-TGT ATG CCA ACA ATG TCC TGT CT-3'**Reverse primer** 5'-CTT GAT TTT CAT GGT GCT TGG A-3'**Probe** TET-TTG CAG ACC GTA TGC AGA AGG AGA T-TAMRA