Endocrine Intersections of Growth and Reproduction in *Oncorhynchus mykiss*

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Major Professor: James J Nagler, PhD

Authorization to Submit Dissertation

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Abstract

Post-spawned female rainbow trout were developed as a model for studying repeat spawning in steelhead kelts. Female trout were collected after spawning and separated into a standard maintenance ration group and a restricted ration group (20% of standard ration). Our intention was to induce an energy deficit that would arrest rematuration among restricted-ration fish, and then compare plasma levels and tissue gene expression for candidate endocrine biomarkers between non-rematuring and rematuring fish. Food-restriction arrested ovarian growth and development within 15–20 weeks, as evidenced by reduced ovarian growth and lower plasma estrogen levels. Food restriction also affected hepatic expression of the metabolic endocrine factors insulin-like growth factor and leptin, but not consistently across age classes, and did not affect circulating levels of nesfatin-1 or ghrelin. Next, we combined plasma hormone assays, physical measurements, and post-release tracking data for female steelhead kelts from a reconditioning program on the Yakima River to predict early detection of reproductive maturation. Rematuring kelts were longer and heavier at intake in spring; grew faster during summer reconditioning; and were longer, heavier, fatter, and of higher condition factor at autumn release. This work is the first known comparison of reconditioned steelhead with natural-origin in-river migrating steelhead, and shows that reconditioning projects yield bigger and fatter fish, with higher circulating levels of estrogen and similar circulating levels of vitellogenin compared to in-river migrating steelhead, and that tracking data suggests rematuring reconditioned kelts behave congruently with repeat spawning in the Upper Yakima River.

Vita

Lucius K Caldwell was born in Cohasset, MA. He received his BSc with a major in Biology and minor in Philosophy from McGill University, and his MS with a major in Food Science and Human Nutrition from University of Maine.

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Dedication

LKC dedicates this dissertation to his family: JKC, NGC, WAC, and KAK.

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Abbreviations

BPG ..... Brain-Pituitary-Gonad

FSH ..... Follicle-Stimulating Hormone

GnRH ..... Gonadotropin Releasing Hormone

GtH ..... Gonadotropin Hormone

GH ..... Growth Hormone

GSI ..... Gonadosomatic Index

HPG ..... Hypothalamus-Pituitary-Gonad

HPL ..... Hypothalamus-Pituitary-Liver

HSI ..... Hepatosomatic Index

IGF ..... Insulin-Like Growth Factor

IGFBP ..... Insulin-Like Growth Factor Binding Protein

k ..... Fulton's Condition Factor

LH ..... Luteinizing Hormone

PCR ..... Polymerase Chain Reaction

q-RT-PCR ..... Quantitative Reverse Transcriptase Polymerase Chain Reaction

SGR ..... Specific Growth Rate

Chapter 1: "General Introduction"

Iteroparity

Endocrine regulation of initial maturation (puberty) has received some attention among certain fishes of economic importance or intellectual interest [1,2,3,4]. However, rematuration and the physiological processes associated with gonadal recrudescence remain largely unstudied in fishes. This is unfortunate, because the fishes present an interesting case among vertebrates for the study of gonadal recrudescence: some fishes, such as salmonids, exhibit complete compensatory ovarian recrudescence following unilateral ovariectomy [5], and it is likely that similar mechanisms regulate recrudescence among rematuring iteroparous fishes.

While it makes sense to presume that the same endocrine factors regulate puberty and re-maturation [6], this assumption has largely gone untested with a few exceptions [7,8,9,10,11]. However, even if this is true, photoperiodic, growth, and nutritional thresholds may differ between the two processes. For instance, body size thresholds gating puberty have necessarily been met by the time of re-maturation; perhaps growth rate or nutritional stores are more important for subsequent reproductive efforts. Also, at the time of rematuration, at least some reproductive anatomy remains from puberty. This means that fewer somatic resources need to be diverted toward reproductive system growth and development, less time is required to prepare for spawning, and the reproductive system is essentially primed for recrudescing almost immediately after

spawning. This may result in higher circulating levels of sex steroids during subsequent reproductive efforts, as has been described in meagre (*Argyrosomus regius*) [12].

Salmonid fishes (Family Salmonidae) represent a unique group in which to study variation in life history and reproductive strategy for a number of reasons. Salmonids are capital breeders that invest heavily in reproduction by producing large, yolky eggs [13,14,15,16], and many migrate long distances to reproduce [17,18,19,20,21]. Reproduction occurs once per year, often under conditions of intense competition for nest sites and mates [22]. The combination of these traits means that salmonids invest heavily in reproduction, sometimes at the cost of their own life. As a group, salmonid fishes exhibit a diversity of life histories, and life history may be plastic within a species [23,24]. There are semelparous Pacific salmon (*e.g.*, Chinook salmon *Oncorhynchus tshawytscha*, sockeye salmon *O. nerka*), iteroparous trout (*e.g.*, cutthroat trout *O. clarkii*, brown trout *Salmo trutta*), and species that exhibit both life histories (*e.g.*, largely semelparous anadromous steelhead trout and iteroparous resident rainbow trout). Semelparity appears to be a shared derived character (synapomorphy) among salmonids [25,26,27,28], and evidence suggests that semelparity is confounded with anadromy in this group [13]. Migration to the ocean generally results in larger body size, higher quality gametes, and greater fecundity, but is often associated with death after spawning [25]. Costs associated with producing large, yolky, metabolically expensive eggs, and then migrating long distances, may inhibit the successful expression of iteroparity in animals that have been selected to produce such eggs. Still, some anadromous salmonids (notably Atlantic salmon, *Salmo salar*, and steelhead) do display iteroparity

[22,29,30,31]. Individuals among iteroparous salmonid species that have survived initial spawning are called kelts.

For numerous reasons, steelhead numbers have declined markedly over the last century [32,33]. The National Oceanic and Atmospheric Administration has identified five distinct population segments of steelhead from the interior (non-coastal) portion of the Columbia River Basin; all five are currently listed as threatened under the Endangered Species Act [34,35]. One strategy to increase returns of steelhead in the Columbia River Basin [36,37,38] involves capturing post-spawned steelhead during the spring, when they are outmigrating downstream, holding and feeding them in land-based tanks while they recover from spawning, and then releasing them in autumn with the assumption that these fish will migrate back upstream and spawn again [38]. These programs are known as "reconditioning."

The Columbia River Inter-Tribal Fish Commission (CRITFC) has ongoing projects to recondition steelhead kelts captured during their downstream migration at locations throughout the Columbia River Basin. Progress has been made in refining techniques and methods associated with these projects, and by some measures they have been successful [36,37,39]. Both collection and survival of steelhead kelts are heavily biased toward female fish. Consequently, nearly all of the fish released from reconditioning programs are female. However, the reproductive status of fish released after reconditioning is not known. Improvements to the reconditioning process could be made if fish could early and accurately be separated into groups based on their likelihood of re-maturing, with special care or additional treatments being provided solely to fish that require such action. It seems probable that endocrine signals or physical parameters

differ between fish that will re-mature and those that will skip the upcoming spawning season. Identification of these metrics would provide markers that could then be used to focus efforts and resources on fish that need and would respond to costly treatments. Toward this end goal, a series of studies were undertaken to determine the endocrine signals responsible for communicating information about nutritional and growth status from the periphery to the central reproductive axis in iteroparous *O. mykiss*. In order to conduct these studies in a tractable experimental model, post-spawned, domesticated female rainbow trout were developed as a model for studying repeat spawning in steelhead kelts.

Intersection of Growth & Reproduction

Nutritional status mediates trade-offs and informs the physiological underpinnings of life history decisions among salmonids. Atlantic salmon (Salmo salar) physiologically assess energy reserves during critical periods prior to metamorphosis and seaward migration (smoltification), smoltifying only if food within the stream environment is inadequate to support maturation [40]. Both brown trout and Atlantic salmon migrate more quickly and smoltify at a higher rate and more rapidly when fed reduced rations [40,41]. Hatchery raised steelhead *O. mykiss* that are raised under conditions of nutrient excess often “residualize”, remaining in the stream system as resident rainbow trout rather than outmigrating to the ocean [42,43]. It may be the case that when nutritional resources are abundant and adequate for maturation, no physiological motivation for

seaward migration exists. These examples suggest that, among salmonids, life history is highly plastic and regulated by environmental energy availability.

A link between energy balance and reproduction has long been observed in fishes [44], but the physiological mechanisms that underlie this phenomenon have remained unclear until rather recently. While a broad body of literature has established the major endocrine factors linking growth and reproduction in mammalian systems, until recently the fishes have been less studied. Furthermore, most fish research has focused on the metabolic regulation of initial sexual maturation (puberty) [45], rather than re-maturation. Among salmonids and other fishes, there is no evidence that size or age thresholds directly gate puberty [3]; instead, it appears that maturation is an adaptive and heritable response to environmental conditions (*e.g.*, photoperiod, temperature, nutrient availability) that functions to maximize reproductive success [46]. In salmonid fishes, age and size at maturity are products of the interaction of environmental conditions and genetic background, with maturation generally being regulated by inhibition [47]. This means that maturation proceeds when environmental food availability during critical periods of development allows an animal to acquire sufficient energy to accumulate somatic energy reserves that exceed genetically determined thresholds. If food energy is deficient, investment in reproductive development is arrested until the following year, when an individual's nutritional status is physiologically assessed anew [47,48].

As a group, the fishes exhibit a number of unique growth physiology characteristics. First, most fishes exhibit indeterminate growth. Although growth rate changes through life, and somatic growth decreases during puberty when investment to reproductive development consumes most of the energy budget, adult fishes continue to

grow throughout their lives [49,50]. Second, fish growth is hyperplastic rather than hypertrophic throughout life, partially due to elevated growth hormone–insulin-like growth factor (GH-IGF) tone through adulthood [50]. Finally, excess energy in fishes is often stored as muscle protein, rather than lipid or carbohydrate, as is the case among most terrestrial quadrupeds. This is the result of both fish anatomy and habitat characteristics. By depositing muscle tissue around the spinal cord, excess muscle does not restrict movement as it does in terrestrial mammals. Lipid storage would compromise fish buoyancy, and the utilization of stored lipids by fishes requires more O_2 than does the utilization of stored proteins [50]. For these reasons, growth physiology and endocrinology, and mechanisms of cross-talk between endocrine growth and reproductive axes often differ among fishes and terrestrial vertebrates.

In mammals, a vast number of endocrine factors regulating somatic growth have been characterized, the most important of which are those associated with the hypothalamus-pituitary-liver (HPL) endocrine growth axis. Additionally, numerous peripheral metabolic cues originating in fat and gastric cells interact with the HPL axis to stimulate or inhibit growth. The GH-IGF system, ghrelin, and leptin are key factors coordinating growth with nutritional status and energy availability. Recently, the peptide nesfatin-1 has emerged as a factor that regulates feeding behavior and reproductive function [51], although its role in fishes remains unclear.

Poikilotherms (*e.g.*, most fishes) appear to differ significantly from homeotherms (*e.g.*, mammals) in regard to the roles that most of these endocrine factors play. Hormones are generally named for the effect with which they are initially associated, and are often later discovered to be pleiotropic, exerting multiple effects in addition to the one

for which they are named. For example, in fishes, GH not only stimulates protein synthesis and growth [52], but also regulates lipolysis and gluconeogenesis [53], and affects osmoregulation by interacting with prolactin, IGFs, thyroid hormones, and cortisol, to direct the developmental trajectory of gill-crypt chloride cells [54,55,56,57,58]. Endocrine factors that share similarity across taxa in nucleotide or amino acid sequence, or in structure, may be thus similarly annotated, while having vastly divergent effects in different evolutionary groups. Considering that the divergence within the fishes between the Sarcopterygii (which gave rise to all tetrapods including mammals) and Actinopterygii (which gave rise to modern ray-finned fishes including salmonids and other teleosts) is estimated to have occurred 450 million years ago [59], we cannot assume hormones that regulate growth in mammals play identical roles in fishes.

Iteroparous anadromous salmonids fast for prolonged periods during migration to spawning areas, spawning, and return migration. Among species that have evolved to withstand periods during which food is not available, fasting may not represent the stress that is often assumed based on mammalian physiology. For instance, food-restricted salmonids exhibit reduced weight and condition [60], but not elevated cortisol [61]. For poikilotherms that regularly experience bouts of food deprivation preceding the spawning season, food restriction may actually activate the reproductive endocrine axis, stimulating reproductive development or behaviors [62].

Growth Hormone/Insulin-Like Growth Factor

The most important system regulating somatic growth in vertebrates is the GH-IGF system, which encompasses the hypothalamic peptides growth hormone releasing hormone (GHRH) and somatostatin (SST), the pituitary peptide GH, and both hepatic (systemic) and organ-specific (local) sources of IGFs and associated binding proteins (IGFBPs) [53,63,64,65]. In salmonid fishes, GH is highly pleiotropic and impacts a vast diversity of physiological endpoints including lipid distribution, organ size, plasma hormone levels and tissue gene expression for other metabolic and nutritional endocrine factors, osmoregulation, smoltification, reproduction, and immune function [66,67]. Among vertebrates, the IGFs [68] are the peripheral mediators of the growth-stimulating effects of GH [69]. In salmonids, the liver is the main site of IGF synthesis, which is stimulated primarily by GH [70]; the IGF1-stimulating effects of GH are inhibited by insulin, glucagon, and corticosteroids [71]. In rainbow trout, liver mRNA expression patterns of *igf1* and *igf2* are highly correlated with plasma levels of the respective peptides, IGF1 and IGF2 [72], suggesting that the IGFs are not appreciably stored within the liver, but are secreted as they are synthesized. Circulating IGF1 concentration has been suggested as the most promising biomarker of growth rate in fishes [73], and is highly correlated with growth rate in rainbow trout [66,72] and other salmonids [74,75]. IGF2 regulates growth during early development [72,76,77], sexual maturation [78,79,80,81], osmoregulation [82], and stress response [75].

Ration affects the GH-IGF system and can uncouple GH from growth. In a state of energy sufficiency, GH stimulates liver synthesis of IGFs, which circulate systemically

to act in an endocrine fashion promoting growth in those tissues expressing the IGF-receptors. In rainbow trout [83,84] and in coho salmon (*O. kisutch*) [85], GH injections only increase plasma IGF1 under conditions of nutrient sufficiency [83,86]. Under conditions of energy availability, GH upregulates muscle and other tissue-specific mRNA transcription of *igf* [50], supporting a paracrine growth-promoting role of the IGFs [87].

However, in a state of energy deficit, liver expression of the GH-R is down-regulated, uncoupling pituitary GH secretion from hepatic IGF-synthesis [86,88,89,90]. In rainbow trout, fasting induces a change in the overall role of GH, from one of growth promotion to one of lipid mobilization [52]. This decoupling mechanism explains why fasting depresses hepatic expression of *igf1* and plasma IGF1 levels [60,84], while simultaneously elevating circulating GH [91].

The GH-IGF system is regulated by a class of IGFBPs ranging from 24-44 kDA [92]. The members of the IGFBP family bind most plasma and extracellular IGFs, which have greater affinity for the IGFBPs than for the IGF-Rs or the insulin-R. A 21-23 kDa IGFBP present in plasma of salmonids and expressed predominantly in the liver has been determined by phylogenetic analysis to be one of two salmon homologues of IGFBP-1 [93,94]. In salmonid fishes, circulating IGFBP-1 levels are positively correlated with hepatic mRNA expression of *igfbp1*, suggesting that IGFBP-1 is not stored appreciably in the liver. Plasma IGFBP-1 levels are inversely correlated with growth rate, suggesting that IGFBP-1 antagonizes the mitogenic effects of IGF [93]. Food restriction increases circulating IGFBP-1 in Atlantic salmon [95], and corticosteroids up-regulate hepatic mRNA expression and secretion of *igfbp1* in coho salmon [96]. Taken together, these

results suggest that IGFBP-1 plays a role in stress physiology and is regulated in response to a variety of stressors including nutrition and reproduction in salmonids.

A well-established literature suggests that the GH-IGF system regulates the brain-pituitary-gonad (BPG) axis [81,97,98,99] and puberty among salmonids [53,100,101,102,103], and other fishes [104,105]. The GH-IGF system communicates growth and nutritional status to the upper tiers of the reproductive endocrine axis [102,106], and controls gonadal growth and development both directly and by affecting pituitary secretion of the gonadotropes [3].

In fishes, GH directly regulates gonadal steroidogenesis, as well as pituitary and hypothalamic function [100,107,108,109]. Likewise, IGF1 also modulates hypothalamic gonadotropin releasing hormone (GnRH) secretion, pituitary follicle-stimulating hormone (FSH) secretion, and gonadal steroidogenesis [110,111,112]. In salmonids, IGF1 directly regulates pituitary expression of gonadotropin subunit genes ($\alpha 2$, $lh\beta$, $fsh\beta$) [113] and increases pituitary sensitivity to GnRH [110,113], although the effect depends on reproductive stage [65,113,114]. Among male Chinook salmon [103,115] and maiden female rainbow trout [101], plasma IGF1 is elevated up to one year in advance of spawning among individuals that will mature, compared to individuals that will delay puberty. In female rainbow trout, this difference parallels differences in growth rates between maturing and non-maturing individuals, and precedes differences in plasma sex steroids [101,102].

The link between growth and reproduction is especially pronounced at certain reproductive phases, and it appears that the GH-IGF system plays a major role in puberty, gametogenesis, and fertility across diverse taxa, including fishes, amphibians, and

mammals [116]. In rainbow trout [117] and other fishes [118], 17β-estradiol eliminates or reduces the hepatic response to GH, depressing the systemic IGF response that usually follows GH release from the pituitary. Circulating testosterone tends to have the opposite effect, increasing peripheral sensitivity to the GH-IGF system. In this manner, the interaction between sex steroids and the GH system may be responsible for altering the effects of GH from those associated with increased growth to those associated with altered metabolism and increased reproductive development. Some critical threshold of body size or condition may partially underlie this shift [117].

Ghrelin

Ghrelin is a short peptide [119] first isolated from the stomach and small intestine of rats and humans [120] that stimulates release of GH from the pituitary, and is the only known gastro-intestinal orexigenic hormone [120]. In mammals and other vertebrates, ghrelin and *ghrl* mRNA are found at the highest concentrations in stomach and intestine [119]. Ghrelin exhibits a highly conserved GH-releasing effect, but the orexigenic effects of ghrelin differ widely across phyla [121,122], as do other regulatory roles of ghrelin. For example, ghrelin regulates pituitary prolactin secretion in fishes [123] and adrenal function in birds [124]. Ghrelin assumes diverse regulatory functions due to species-specific energetic demands, life histories, and ecology, and due to differences in metabolic needs between homeotherms and poikilotherms [125].

In fishes, ghrelin stimulates pituitary GH release in a dose-dependent fashion [123,126,127]. In rainbow trout, ghrelin injections have no effect or inhibit feeding

behavior [91,128], *i.e.*, opposite of what is observed in mammals, and ghrelin secretion is neither inhibited by feeding nor stimulated by fasting [91].

Ghrelin also regulates reproduction, by affecting all tiers of the BPG axis and in some cases by facilitating the intake of nutrients that are necessary for energetically funding a reproductive effort [129]. However, in fishes, ghrelin regulates reproductive physiology differently from its role described in mammals. While ghrelin is primarily inhibitory to mammalian reproduction, it appears to stimulate pituitary activity associated with reproduction in fishes. Although teleological evolutionary speculation is dubious, in this case there is a stark difference between the ecology and life history of mammals and that of fishes, and this discrepancy has largely been ignored in the literature. Many fishes undergo long periods of the year in which they volitionally fast, due to lack of food or to central nervous system mediated inhibition of feeding during spawning migrations [16,130,131], similar to migratory syndrome-associated behaviors described in birds and insects [132]. Reproduction is energetically costly for all species; however, fishes differ dramatically from mammals in the mechanisms by which they apportion nutrients to offspring. Mammals generally exhibit long gestation periods that often require nearly continuous feeding during pregnancy to support embryonic development, a scenario referred to as income breeding [133,134,135]. On the other hand, many fish species are capital breeders that accrue large energy reserves when food is plentiful, and later use these reserves to fund reproductive efforts during times of food scarceness [133,136]. It therefore seems reasonable that a molecule signaling “hunger” may have different roles in mammals and fishes.

The picture emerging from a body of comparative work suggests that ghrelin is a multifunctional peptide with effects on reproduction, blood sugar, insulin, learning, memory, cardiac function, and bone metabolism [137]. Results in trout and other salmonids suggest that ghrelin is involved in GH-associated metabolic effects, but not necessarily acute feeding behavior within this group of fishes.

Leptin

Leptin [138,139] is the peptide product of the obese (*ob*) gene [140]; in mammals, leptin is secreted primarily by adipose tissue [141]. Leptin plays a central role in the physiological maintenance of lipid homeostasis [140,142,143], communicating nutritional and lipid status from the periphery to the central nervous system [144]. In addition to regulating long-term energy homeostasis, leptin also appears to inhibit feeding. Generally, plasma leptin increases after food intake and participates in the generation of a satiety signal [145] to modulate feeding behavior [146], by crossing the blood-brain-barrier and interacting with the central nervous system [147].

Early evidence that leptin played a role in reproduction came from observations that leptin deficient (*ob*/*ob*) mice were infertile, and that leptin injections could rescue reproductive development and restore fertility in these mutants [148]. Leptin interacts with other metabolic hormones to permissively gate reproduction by informing the hypothalamus of fat availability [146], and by directly regulating pituitary and gonadal function [149].

Leptin has been identified in a number of fishes but the characterization of leptin in teleosts is ongoing [150,151,152] and its physiological function(s) remain unclear in this group. Some fishes express two leptins, which appear to have different physiological roles [153,154]. In contrast to mammals, which express leptin primarily in adipose tissue, rainbow trout [150,151] and many other fishes [65] express leptin primarily or exclusively in the liver. Indeed, the first description of fish leptin was in puffer (*Takifugu rubripes*), an animal that exhibits no distinct adipose tissue, but whose liver contains a high concentration of lipids, suggesting functional conservation of leptin expression location among vertebrates [150].

Among fishes, leptin appears to be a generalized stress signal, and may be involved in mitigating or buffering maladaptive physiological endpoints associated with the endocrine stress response [155,156,157,158,159,160,161]. From a comparative evolutionary perspective, it is interesting to speculate that leptin may historically have been a stress signal in a common ancestor of fishes and terrestrial vertebrates, with its current-day pleiotropic effects in different taxa representing a concerted response to relevant stressors in different ecological settings.

Among the salmonids, a body of evidence suggests that leptin plays a contrasting physiological role to that described in mammals, although some results are contradictory. In adult Atlantic salmon, long-term feed restriction (10 months) does not affect plasma leptin, but does inhibit *slepa1* mRNA expression in fat depositing tissues such as belly flap and white muscle, [154], suggesting a similar role of leptin in salmonids to that described in mammals. However, in juvenile Atlantic salmon, 7 weeks of feed restriction increases hepatic s*lepa1* mRNA expression and plasma leptin, opposite to observations in

mammals [162]. Similarly, rainbow trout fasted for 3 weeks exhibited elevated plasma leptin [163]. Taken together, these results suggest that, in salmonids, leptin assumes a different regulatory role under conditions of acute versus chronic feed restriction [164], possibly reflecting an allostatic response to what is essentially a hormetic stressor in this group of fishes [165]. Among salmonid fishes, high adiposity is associated with decreased feeding, supporting a lipostatic model for feed intake [163], but leptin does not appear to underlie this phenomenon [166].

The majority of information concerning the role of leptin in the regulation of reproduction comes from mammalian literature. In mammals, leptin is necessary—though not sufficient—for the onset of puberty and continued adult ovarian cycling [167,168]. Although the majority of work studying the effects of leptin on reproduction has focused on female puberty, there is some evidence that leptin inhibits testicular steroidogenesis, and may regulate pituitary and hypothalamic function in male mammals [169].

Leptin effects on reproduction in fishes are variable, and likely depend on ecology and life history. Leptin affects both growth and reproduction in fishes largely by acting on the hypothalamus and pituitary [170,171]. In rainbow trout, leptin stimulates pituitary secretion but not synthesis of gonadotropins, although the effect differed among stage of gamete development [172]. In burbot (*Lota lota*), low circulating leptin does not inhibit spawning. Through evolutionary time, reproduction may have become uncoupled from acute lipid status around the time of spawning among species like burbot and trout that normally undergo a seasonal fast prior to reproducing [173]. Evidence from mammals suggests a similar phenomenon in seasonally fasting mink (*Mustela vison*) [174].

Nesfatin-1

First described in rats [175], nesfatin (NEFA/nucleobindin2-encoded satiety- and fat-influencing protein) is an anorexigenic and metabolic protein synthesized and secreted by the hypothalamus. Nesfatins are peptides cleaved from the nucleobindin-2 gene product (NUCB2); nesfatin-1 represents amino acid residues 1-82 of NUCB2. Under conditions of starvation, *nucb2* gene expression and nesfatin-1 concentration in the hypothalamic paraventricular nuclei (PVN) are both reduced. ICV injection of nesfatin-1 reduces feeding, while injection of a nesfatin antagonist stimulates feeding. Appetite suppression by NUCB2 requires conversion (*i.e.*, cleavage) to nesfatin-1, and appears to act independently of leptin signaling in the hypothalamus, possibly involving the melanocortin signaling system [175].

Some evidence in fishes supports a conserved role of nesfatin-1 in regulating feeding behavior and energy homeostasis in response to food availability [176,177,178]. In goldfish (*Carassius auratus*), food deprivation reduces hypothalamic expression of *nucb2* mRNA and elevates hepatic expression of *nucb2* mRNA, with the net effect being reduced serum nesfatin-1 levels within one day of food deprivation [177]. However, it is hard to draw conclusions about nesfatin-1 from *nucb2* mRNA expression data, as this gene product has multiple cleavage products [175]. Also, evidence in mammals suggests that nesfatin-1 is synthesized in the pancreas [179]; in combination with the observation that some fishes expresses *nucb2* in hepatopancreas [178], it may be the case that reports of hepatic NUCB2/nesfatin in fishes that exhibit a combined hepatopancreas (*e.g.*, goldfish) actually represent hepatopancreatic expression. In goldfish, serum nesfatin-1

responds rapidly to feeding status, increasing within one hour of feeding, and then returning to fasting levels within three hours [177]. Also in goldfish, central and peripheral injections of nesfatin-1 inhibit food intake [177].

Objectives of Dissertation

The overall goal of the work contained in this dissertation is to understand the endocrine regulation of repeat spawning in *O. mykiss*, focusing on the phase of recovery and re-maturation immediately after spawning. Previous work has suggested that growth and nutrition affect this process. We suspected that, by nutritionally restricting a group of fish, we could induce the skip-spawner life history in a group of female fish, which could then be compared to a group of fully fed fish that would presumably re-mature. Because legal and political concerns heavily restrict the number of steelhead available for research and the type of research that is possible when using fish from an Endangered Species Act-listed population, iteroparous rainbow trout were developed as an intraspecific surrogate model organism.

The objectives of the work described in this dissertation were as follows:

1) Perform manipulative studies with rainbow trout to provide a base of information about how iteroparous salmonids respond to food restriction during the months immediately after spawning, as they prepare for another spawning effort. Construct a profile for metabolic hormones (hepatic expression of IGF1, IGF2, IGFBP1, and leptin; plasma levels of ghrelin and nesfatin-1) and reproductive hormones

(hypothalamic expression of GnRH3; pituitary expression of FSH and LH; and plasma levels of 17β-estradiol and 11-ketotestosterone) in post-spawned rainbow trout.

2) Determine which physiological parameters (*e.g.*, body length, mass, shape, adiposity) are associated with differences in the rate of re-maturation between fish fed a full-ration and fish fed a restricted-ration. Examine the roles of IGFs, leptin, ghrelin, and nesfatin-1 in signaling energetic capacity for reproduction to the HPG axis. Identify which indices of reproduction predict re-maturation, and how early this detection can accurately be made.

3) Combine plasma hormone assays, physical measurements, and post-release tracking data for female steelhead kelts from a reconditioning program within the Columbia River Basin (Yakima River) to predict early detection of reproductive maturation. An additional purpose of this project was to compare kelts sampled after reconditioning with natural-origin steelhead, which presumably comprise primarily maiden fish, on their spawning migration up the Yakima River.

Chapter 2: "Metabolic endocrine factors involved in spawning recovery and rematuration of iteroparous female rainbow trout (*Oncorhynchus mykiss*)."[1]

1. *Introduction*

In salmonid fishes, the physiological decision whether or not to mature is made during photoperiodically defined critical periods, which occur up to a year in advance of spawning [47]. During these critical periods, an individual fish physiologically assesses lipid stores, growth rate, and total body size to determine whether it will mature or not [115,180]. In general, sexual maturation proceeds normally unless certain genetically determined thresholds are not exceeded during these critical periods, in which case development is arrested or inhibited [47].

While a large literature has explored the physiological regulation and endocrine signaling involved in the initial maturation (puberty) of juvenile fishes [1,2,3,4,12,181,182,183], much less work has examined the regulation and signaling involved in the rematuration process among adult iteroparous fishes. Some salmonids invest heavily in both reproductive tissue and spawning behavior [17,18,184,185,186]; thus, post-spawned individuals are energetically depleted. After spawning, individuals must assess available energetic resources and make a commitment to remature or skip spawning ("resting", *sensu* [187]) in the upcoming year. It is unclear how this

[1] Caldwell, L. K., A. L. Pierce and J. J. Nagler (2013). "Metabolic endocrine factors involved in spawning recovery and rematuration of iteroparous female rainbow trout (*Oncorhynchus mykiss*)." General and Comparative Endocrinology 194: 124-132. See Appendix 1 for copyright information.

rematuration process differs from puberty, or how energetic thresholds and endocrine signals involved in initiating rematuration change with reproductive age.

The peripheral endocrine factors that communicate growth and nutritional status to the reproductive endocrine axis (hypothalamus-pituitary-gonads-liver) include the various components of the IGF system [106]. The IGFs are peripheral mediators of the growth effects associated with GH. In response to elevated plasma GH, the liver synthesizes and secretes IGF-I and IGF-II, which then circulate systemically *via* plasma [188]. While IGF-I is the primary post-natal growth factor in mammals, IGF-I and IGF-II both appear to be important post-natal growth factors in fishes [82,189], and have effects on reproduction [3,81]. A class of at least six IGFBPs, also synthesized primarily in the liver, regulates IGF activities [190]. The main inhibitory binding protein, IGFBP-1 diminishes IGF-I activity by modulating the interaction of IGF with the IGF-receptor; IGFBP-1 is produced during a wide variety of catabolic states [92,191]. In rainbow trout, the IGFs may act as peripheral signals of growth status that inform the reproductive endocrine axis, and IGFBP-1 may regulate the activity of IGFs and the IGF response to systemic GH.

Among mammals, the peptide hormone leptin is synthesized by adipose tissue constitutively, so that leptin synthesis is proportional to lipid reserves [141,144]. In this way, leptin functions as an adipostat, signaling the brain about the quantity of peripheral lipid reserves. Leptin also has a demonstrated role permissively gating the onset of puberty in mammals [167,192]. Among fishes, leptin is generally produced in the liver (also an energy storage organ), but the role of leptin is unclear [65]. Some researchers have reported that fish leptin acts similarly to mammalian leptin [163], while others have

reported an inverse role for leptin in fishes [164]. We suspect that leptin could be a positive peripheral metabolic signal that regulates reproductive development among iteroparous fishes such as rainbow trout.

The purpose of this study was to investigate the endocrine mechanisms that play a role in signaling energy status and growth rate to the central nervous system and reproductive endocrine axis during the hypothesized critical period immediately after spawning among iteroparous female rainbow trout. It was hypothesized that restricting food among a group of iteroparous female rainbow trout would inhibit rematuration following spawning; that liver *igf1*, *igf2*, and *slepA1* expression would increase during refeeding after spawning, and liver *igfbp1* expression would decrease during refeeding after spawning; and that some or all of these metabolic effects would be inhibited in fish fed a restricted ration. It was further hypothesized that the overall pattern would be similar among two-year-old and three-year-old fish, although energetic status of the two age classes after spawning might differ.

2. *Material and Methods*

2.1. *Animals*

Post-spawning female rainbow trout *Oncorhynchus mykiss* were purchased from Troutlodge (Sumner, WA) and transported to the University of Idaho (Moscow, ID). Fish had been manually strip-spawned 2 d prior to transport, and were fasted for one month (in the case of two-year-old trout) or two months (in the case of three-year-old trout) prior to spawning. Fish were held in 1,130 L tanks, in a recirculating system (flow

rate 14 L min^{-1} per tank, temperature 12 to 15°C following a seasonal profile). Experiments were conducted under approved protocols in accordance with the principles and procedures of the Animal Care and Use Committee, University of Idaho.

2.2. *Experiments*

For both experiments, treatments consisted of a control group, which was fed 0.5% total fish mass per day, and a restricted group, which was fed 0.1% fish mass per day. Fish were fed a commercial trout broodstock diet (6.4 mm pellets, Rangen, Inc., Buhl, ID). Rations were adjusted to compensate for a 24-h pre-sampling fast, fish numbers, and fish weight based on sampling data and mortalities. Fish were individually identified by PIT tags.

2.2.1. *Experiment 1: Three-year-old fish*

Three-year-old fish (post 2^{nd} spawning) were stocked into 6 tanks (25 February 2010, 26-27 fish per tank, average weight 1.35 kg). Tanks were randomly assigned control or restricted feeding treatments (*n*=3 tanks per treatment), and fish were sampled every 4 weeks. During sampling, all fish were anesthetized (60 mg L^{-1} tricaine methanosulfonate, buffered). Fish were weighed, fork length was measured, muscle lipid content was measured (Fish Fatmeter, Distell, Fauldhouse, UK), and blood (2 ml) was collected from the caudal vein using syringes that were pre-coated with heparin by aspirating and then dispensing 3.0 mL of 10 mg mL-1 ammonium heparin (Sigma-Aldrich) suspended in ultrafiltered H_2O. Plasma was separated by centrifugation and stored at -80 °C. At each sampling, ten fish (*n*=5 fish per treatment group) were lethally sampled. Livers and ovaries were dissected and weighed, and a liver sample was collected and snap-frozen in liquid N_2 for tissue gene expression analyses using

quantitative real-time reverse transcriptase polymerase chain reaction (q-RT-PCR). To reduce post-spawning mortality, fish were stripped of residual eggs and injected with oxytetracycline (Liquamycin®, Pfizer, Inc., New York, NY, 20 mg kg^{-1}) at the second sampling date. To control *Gyrodactylus* sp. gill parasites, fish were treated twice with praziquantel (Medisca, Plattsburgh, NY, water borne, 2.3 - 3.4 mg L^{-1}).

2.2.2. *Experiment 2: Two-year-old fish*

Two-year-old fish (post 1st spawning) were stocked into 12 tanks (3 March 2011, 26-27 fish per tank, average weight 1.13 kg). Tanks were randomly assigned control or restricted feeding treatments (*n*=6 tanks per treatment). Sampling was similar to Experiment 1, except that fish were sampled every 5 weeks, 12 fish were lethally sampled at each time point (*n*=6 fish per treatment group), and liver samples were collected in RNAlater (QIAGEN, Hilden, Germany) before being snap-frozen in liquid N_2. At the time of stocking, fish were stripped of residual eggs, injected with oxytetracycline, and treated with praziquantel as described above. In addition, fish were treated to control *Saprolegnia* (formalin 150 ppm and NaCl 2%, 1 hour static baths, repeated 3 times).

2.3. *RNA Extractions & cDNA Synthesis*

Liver samples were homogenized in 1.0 mL TRIzol® (Invitrogen™ , Life Technologies, Carlsbad, CA), and RNA was isolated following the TRIzol protocol, using three chloroform:isoamyl alcohol extractions and three 70% ethanol washes. Resuspended nucleic acid fractions were treated with DNase (TURBO™ DNA-free, Ambion®, Life Technologies, Carlsbad, CA), RNA purity was assessed by spectrophotometric absorbance (NanoDrop ND-1000, Thermo Fisher), and RNA

concentration was measured using the RiboGreen RNA assay kit (Invitrogen) with a fluorometer. 1 μg total RNA was reversed transcribed with the SuperScript III First-Strand Synthesis Kit (Invitrogen) using random hexamer primers. cDNA was diluted 1:5 in 1x Tris-EDTA.

2.4. *q-RT-PCR*

Quantitative real-time reverse transcriptase polymerase chain reaction (q-RT-PCR) primer sets were adapted from published sequences (Table 1). Specificity was confirmed by bioinformatic analysis, agarose gel electrophoresis of PCR products, and melting curve analysis of PCR products. To carry out the q-RT-PCR, sample cDNA was amplified in 96-well optical reaction plates (Invitrogen) containing 20 μL PCR reactions made up of 2μL cDNA, 10 μL *Power* SYBR® Green PCR Master Mix (Life Technologies), 6 μL H_2O, and 2 μL of a mix of forward and reverse primers at 2 pM each, in an Applied Biosystems™ ABI 7900HT real-time PCR system (Life Technologies) (2 min @ 50°C; 10 min @ 95ºC; 40 cycles of 15 sec @ 95ºC and 1 min @ 60ºC). Copy numbers in samples were quantified using standard curves of PCR amplicons. Three replicate PCRs were completed for each sample. Expression levels of target genes were normalized by dividing the copy number of the target gene by the copy number of the reference gene (EF1-α). The mean of the normalized expression level for the replicate PCRs is the value reported. q-PCR results were $\log_2$-transformed prior to statistical analysis.

2.5. *Data Analysis*

Specific growth rate for mass (SGR) was calculated as

$$ln[\frac{\text{mass (g)}_{\text{time 2}}}{\text{mass (g)}_{\text{time 1}}} \div \text{interval (d)}] \times 100$$ [193]. Fulton's condition factor (k) was calculated as

$\frac{\text{body mass (g)}}{\text{fork length (cm)}^3} \times 1000$ [194]. Fatmeter readings were validated by chemical analyses of muscle lipid content (Fig. 2.1); the correlation between the two measurements was high (linear regression, $r^2 = 0.58$; Tukey mean-difference plot, matched pairs correlation = 0.76), as previously found in other studies on salmonids [19,195]. Hepatosomatic index (HSI) was calculated as $\frac{\text{liver mass (g)}}{\text{body mass (g)} - \text{liver mass (g)}} \times 100$ [196]. Gonadosomatic index (GSI) was calculated as $\frac{\text{ovary mass (g)}}{\text{body mass (g)} - \text{ovary mass (g)}} \times 100$ [196]; GSI is a well established and long utilized metric of reproductive maturation [197,198] that peaks during spawning among rainbow trout and other salmonids [11,199]. Only data from fish that survived until being terminally sampled were included in statistical analyses for SGR, *k*, Fatmeter readings, GSI, and HSI. For SGR, *k*, Fatmeter readings, GSI, and HSI, among two-year-old trout at week 0, *n*=84; week 5, *n*=36; week 10, *n*=30; week 15, *n*=24; week 20, *n*=18 per treatment. For SGR, *k*, Fatmeter readings, GSI, and HSI, among three-year-old trout at week 0, *n*=46; week 4, *n*=18; week 8, *n*=13; week 12, *n*=8; week 16 *n*=3 per treatment). For q-RT-PCR data, among two-year-old trout, *n*=6 at all time point, and among three-year-old trout, *n*=5 at all time points except week 16, when *n*=3 per treatment.

Systematic tank differences were not detected within treatment for any variable (ANOVA, $p > 0.05$). Therefore, tank replicates were pooled and analyzed together. Two-way ANOVA was used to detect main and interaction effects (time, treatment, time x treatment). When ANOVA indicated a significant time effect, Tukey-Kramer Honestly Significant Difference tests (Tukey-Kramer HSD) were used to compare values at all time points within a given treatment. Within each time point, two-tailed t-tests were used

to detect treatment differences. Statistical analyses were performed within JMP® (Version 9, SAS Institute Inc., Cary, NC). Differences are reported as significant when $p < 0.05$.

3. *Results*

The feeding regime used here caused fish that were fed a restricted diet to arrest ovarian development and also affected metrics of growth and metabolism in both two- and three-year-old fish. Feeding treatments produced a difference in reproductive development during the period of recrudescence, as indicated by differences in mean relative ovarian mass (GSI) between treatment groups. GSI diverged between full-ration fish and restricted-ration fish toward the end of the experiment with two-year-old fish, but the experiment with three-year-old fish did not continue long enough to capture this effect (Fig. 2.2). Among two-year-old trout, control-ration fish exhibited significantly elevated GSI at week 30 compared to all previous weeks.

Specific growth rate was greater among control-ration fish than among restricted-ration fish at all time points in both two-year-old and three-year-old trout (Fig. 2.3). Fish fed the control-ration generally exhibited positive growth, while fish that were fed the restricted-ration generally exhibited negative growth. Between week zero and week five, control-ration three-year-old trout grew at nearly double the growth rate of control-ration two-year-old trout (three-year-old trout SGR=0.33±0.04, two-year-old trout SGR=0.17±0.03, p=0.004).

Fulton's condition factor (*k*) decreased over time in restricted-ration fish, which had lower *k* values than control-ration fish at all time points after the initial sampling in both two-year-old and three-year-old trout (Fig. 2.4). At week zero, *k* was significantly greater among two-year-old trout than among three-year-old trout.

Feed-restriction also affected muscle lipid content. Muscle lipid percentage increased over time among control-ration fish and decreased (two-year-old trout) or remained static (three-year-old trout) over time among restricted-ration fish. This led to greater muscle lipid level among control-ration fish than among restricted-ration fish, in both two-year-old and three-year-old trout (Fig. 2.5). At week zero, muscle lipid level was significantly greater among two-year-old trout than among three-year-old trout.

For both two-year-old and three-year-old trout, HSI was consistently greater among full-ration fish than among restricted-ration fish, although this difference was only significant at later time-points (Fig. 2.6). When two-year-old trout from weeks 5-20 or three-year-old trout from weeks 4-16 were pooled and analyzed together, control-ration fish exhibited significantly greater HSI than did restricted-ration fish. Among two-year-old fish, control-ration fish HSI did not change between the beginning and end of the experiment, but restricted-ration fish showed a decrease in HSI over time. Among three-year-old fish, control-ration fish showed an increase in HSI over time, while restricted-ration fish HSI did not change between the beginning and end of the experiment. At week zero, HSI was marginally greater among two-year-old trout than among three-year-old trout (p=0.05).

Among two-year-old trout, between week zero and week five, control-ration fish exhibited a slight decrease, and restricted-ration fish exhibited a slight increase, in hepatic

igf1 expression, leading to a difference between the two treatment groups at week five (Fig. 2.7). In contrast, among three-year-old trout, hepatic *igf1* expression was elevated in control-ration fish relative to restricted-ration fish at week eight. No difference was detected in hepatic *igf1* expression among time-points within either treatment group among either two-year-old or three-year-old trout.

Among two-year-old trout, hepatic *igf2* expression was elevated at week 15 compared to all other time points within control-ration group of fish; no difference in *igf2* expression was detected among time-points within the restricted-ration group of fish (Fig. 2.8). Among two-year-old fish, no difference in *igf2* expression levels was detected between the two treatment groups at any time point. Among three-year-old trout, hepatic *igf2* expression was higher among control-ration fish than among restricted-ration fish at week four. Although differences were not detected at any other sampling point, there was a trend for higher *igf2* expression in livers of control-ration fish across the entire experiment: when pooled, control-ration fish sampled at weeks 8-16 exhibited significantly higher *igf2* expression than did restricted-ration fish. No difference in hepatic *igf2* expression was detected among time-points within either treatment group.

Hepatic *igfbp1* expression tended to decrease over time among both age classes and treatment groups (Fig. 2.9). Among two-year-old trout, hepatic *igfbp1* expression decreased significantly over time within both treatment groups. Within control-ration fish, *igfbp1* expression was significantly lower at week 15 compared to week zero. Within both treatment groups, *igfbp1* expression was significantly lower at week 20 compared to week zero. Among three-year-old trout, hepatic *igfbp1* expression decreased significantly over time within control-ration but not restricted-ration fish.

Control-ration hepatic *igfbp1* expression was significantly reduced versus week zero at 12 and 16 weeks., No difference in hepatic *igfbp1* expression was detected between the two treatment groups at any time-point in either year.

No difference in hepatic *slepA1* expression was detected among time points for either treatment group within either age class (Fig. 2.10). Among two-year-old trout, during weeks 10-20, there was a trend of higher hepatic *slepA1* expression among control-ration fish, a difference that became significant at week 20. A similar trend was observed among three-year-old trout, although no difference was detected between treatment groups in hepatic *slepA1* expression at any time-point.

4. *Discussion*

The restricted-ration employed in these experiments affected nutrition sufficiently to arrest reproductive development among two-year-old trout, as evidenced by a divergence in GSI among two-year-old trout by the end of the experiment. This suggests that the nutritional limitation imposed upon the restricted-ration fish sufficed to induce a physiological trade-off between investment in continued survival and growth versus investment in reproductive development. While the experiment using three-year-old trout did not continue long enough to capture this arrest, it is clear from the experiment using two-year-old trout that between 15 and 20 weeks after spawning, the control-ration fish began to increasingly partition energy to the ovary for developing oocytes.

The feeding regime selected in this study also impacted fish growth and metabolism rapidly and dramatically. For both two-year-old and three-year-old post

spawned female rainbow trout, the control ration induced weight gain, while the restricted ration induced weight loss; SGR was higher among control-ration fish than among restricted-ration fish at nearly every time point. Moreover, among both two-year-old and three-year-old trout, *k* among restricted-ration fish decreased over time, while *k* among control-ration fish remained stable, leading to rapid divergence in body shape between rations. Muscle lipid content and HSI tended to increase among control-ration fish and remain stable or decrease among restricted-ration fish. These results suggest that control-ration fish were accumulating excess food energy and storing it in muscle and liver tissue, while restricted-ration fish were utilizing stored energy to support metabolism, but were not growing.

Both two-year-old and three-year-old trout began the experiment in an energy-depleted state, due to the energetic demands of fasting and spawning. However, several observations suggest that the two-year-old trout were less energy-depleted than the three-year-olds at the beginning of the experiment. Two-year-old trout had greater *k*, higher muscle lipid content, and greater HSI at the initial sampling than did three-year-old trout. This was likely at least partially due to the difference in the duration of pre-spawning fasting imposed by the production facility from which the fish were obtained. Interestingly, in the control-ration treatment over the first 10-week period post spawning, the three-year-old trout grew at nearly double the rate of two-year-old trout. This suggests a compensatory growth response [200] that was greater among three-year-old than among two-year-old trout, consistent with the greater state of energy depletion at intake observed among three-year-old than among two-year-old fish. Compensatory growth has been previously shown to vary in proportion to degree of feed restriction in rainbow trout

[201,202]. Further, although control-ration fish were fed the same ration in both years, three-year-old fish were able to grow significantly faster, suggesting greater growth efficiency. There was an age effect with respect to HSI only, in that 2-year old control-ration fish exhibited a trend of decreasing HSI after week five while the HSI of three-year old fish increased. This may be due to greater metabolism of liver lipids in the 2-year-old fish.

Among control-ration fish, the different age classes of fish exhibited different hepatic *igf* expression responses to refeeding. Among three-year-old trout hepatic *igf1* expression in control-ration fish exhibited a significant increase leading to levels elevated above those observed in restricted-ration fish, whereas no changes over time or between treatments were found in two-year-old fish. A similar expression pattern was observed with hepatic *igf2* expression: among two-year-old trout, there was no difference detected in hepatic *igf2* expression between control-ration and restricted-ration fish. However, among three-year-old trout, there was a consistent trend of higher hepatic *igf2* expression among control-ration fish compared to restricted-ration fish. The difference in pattern of hepatic *igf* expression between the age classes may be due to differences in initial condition and subsequent compensatory growth between the two age classes after spawning. Given that plasma IGF-I levels reflect growth rate in *O. mykiss* [203] and other teleosts [73], it is likely that the elevated hepatic *igf1* mRNA observed in control-ration three-year-old fish over the first eight weeks is associated with the greater compensatory growth observed among this age class, as has been previously described in *O. mykiss* [201,204] and other salmonid fishes [205]. The observation that hepatic *igf2* expression remained elevated among 3-year-old control-ration fish supports the hypothesis that IGF-

II in rainbow trout is regulated by nutritional status and involved in coordinating compensatory growth during refeeding [90]. Work in Atlantic salmon has demonstrated that IGF-II is more sensitive than IGF-I to food nutrient content [157].

Among both two-year-old and three-year-old trout, no change over time in hepatic *igf1* or *igf2* expression occurred among restricted-ration fish. As all fish were fasted prior to spawning, it may be the case that liver *igf* expression was already reduced to basal levels among incoming fish, and the restricted-ration was not sufficient to increase hepatic *igf* expression above basal levels. During fasting, plasma IGF-I decreased to ~40% of fed levels over two weeks and then remained constant in juvenile Chinook salmon (*Oncorhynchus tshawytscha*), suggesting that a basal plasma IGF-I level exists in salmonid fishes [71]. In the present study, restricted fish were fed a less than maintenance ration, suggesting that a greater than maintenance ration may be required to increase liver *igf* expression from basal levels during refeeding. Under conditions where anabolic growth is not occurring, the relationship between plasma IGF-I levels or hepatic *igf* expression and growth may become discordant [63].

Both two-year-old and three-year-old control-ration fish were fed a standard broodstock ration sufficient to support rematuration, which appears to have proceeded unchecked among this group (Fig. 2.2). Current models suggest that these fish would have initiated rematuration during the time period covered in the present study [206,207], and that a metabolic indicator such as IGF-I may play a permissive role in the initiation of maturation or rematuration [113,208]. Thus, it is interesting that no post-spawning increase in hepatic *igf1* mRNA was found in two-year-old fish. This suggests that an increase in liver *igf1* transcription after spawning is not required to initiate rematuration

in rainbow trout. Further, a significant increase in hepatic *igf2* mRNA did occur 15 weeks after spawning in two-year-old control-ration fish. A trend toward elevation in hepatic *igf2* mRNA occurred over this time period in three-year-old control-ration fish. Therefore, our data are consistent with the possibility that IGF-II rather than IGF-I plays a permissive role in the initiation of rematuration in rainbow trout. Alternatively, it is possible that fish may initiate rematuration without an increase in either IGF.

The hepatic *igfbp1* expression patterns observed were similar in both age groups and feeding treatments, with high hepatic *igfbp1* expression at week 0 followed by decreasing hepatic *igfbp1* expression over the course of the experiment, suggesting that hepatic *igfbp1* was increased during spawning. To our knowledge, this is the first report of a spawning elevation in hepatic *igfbp1* expression. In teleost fishes, hepatic *igfbp1* expression is rapidly upregulated in response to a variety of catabolic conditions including fasting [reviewed in 92], and expression is stimulated by plasma corticosteroids [96], which are elevated in salmonids during spawning [209,210]. The lack of difference in hepatic *igfbp1* expression between treatment groups may be due to regulation of IGFBP-1 at a level other than transcription. Work with Atlantic salmon [189], demonstrated that fish fasted for 14-days exhibited lower plasma IGFBP-1 compared to control fish, with no difference in hepatic *igfbp1* expression between the groups. Regardless, our results suggest that decreasing hepatic *igfbp1* expression or plasma IGFBP-1 levels may provide an indicator of recovery from spawning, as has been found for other stressors [191].

In both age classes, no difference in hepatic *slepA1* expression was detected among time points for either treatment group. However, among two-year-old trout,

hepatic *slepA1* expression was elevated in control-ration versus restricted-ration fish at the final time point. Among three-year-old trout, no obvious pattern in *slepA1* was apparent. Leptin physiology differs substantially between fishes and mammals. The role of leptin in salmonids is unclear [211], but hepatic leptin expression appears to be upregulated in response to various stressors, including food restriction [154,162,166] and temperature challenge [157]. Our results do not support regulation of hepatic *slepA1* expression by either recovery from spawning or nutritional status in post-spawning rainbow trout.

In conclusion, a feeding regime that significantly impacted the growth and lipid levels of post spawning rainbow trout was utilized to look for age-related effects manifested in metabolic endocrine indicators. Most nutrient restriction studies in salmonids have employed rapidly growing juvenile fish. This is the first study we are aware of to examine nutritional endocrinology in post-spawning salmonids. Intriguingly, the IGFs emerged as being influenced by age, with the older three-year-old fish displaying a greater response to treatment than did the two-year-old fish.

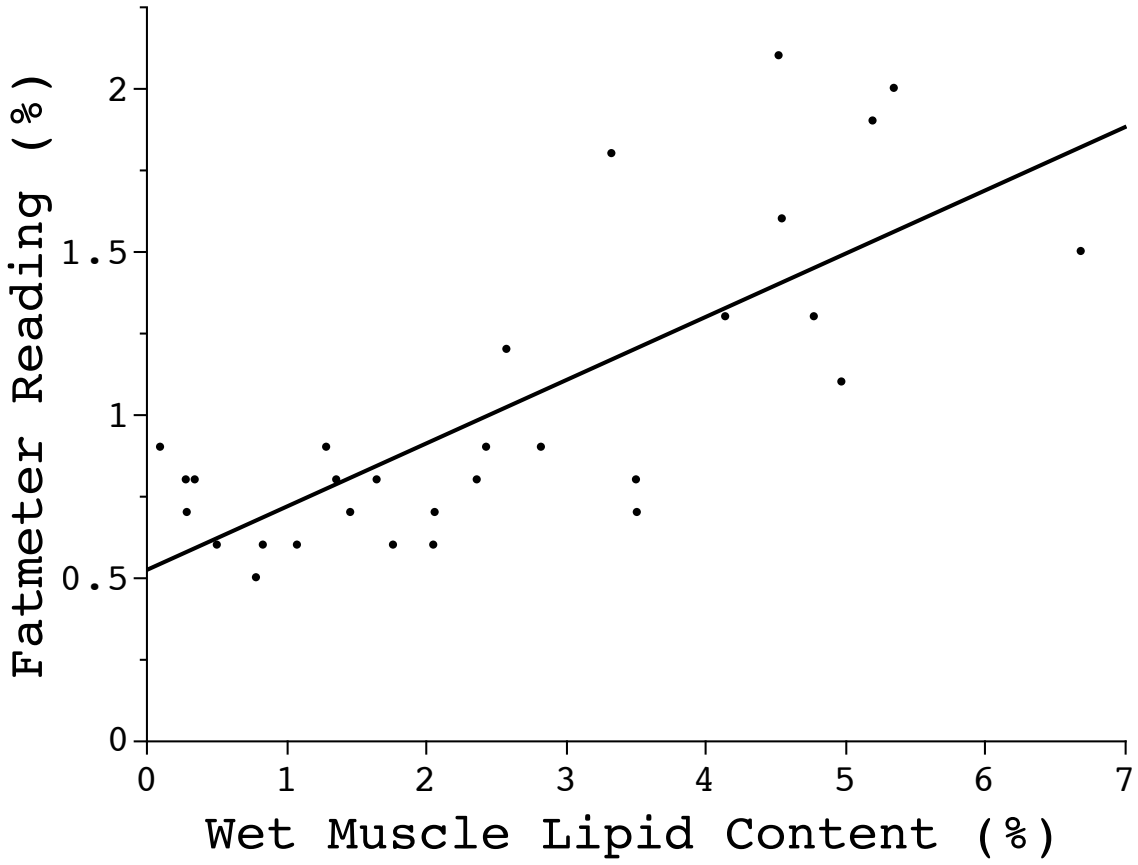

Figure 2.1. Relationship between Fatmeter readings and biochemical assay of wet muscle lipid content in female rainbow trout. The line shows least squares linear regression (r^2 = 0.58, p<0.0001).

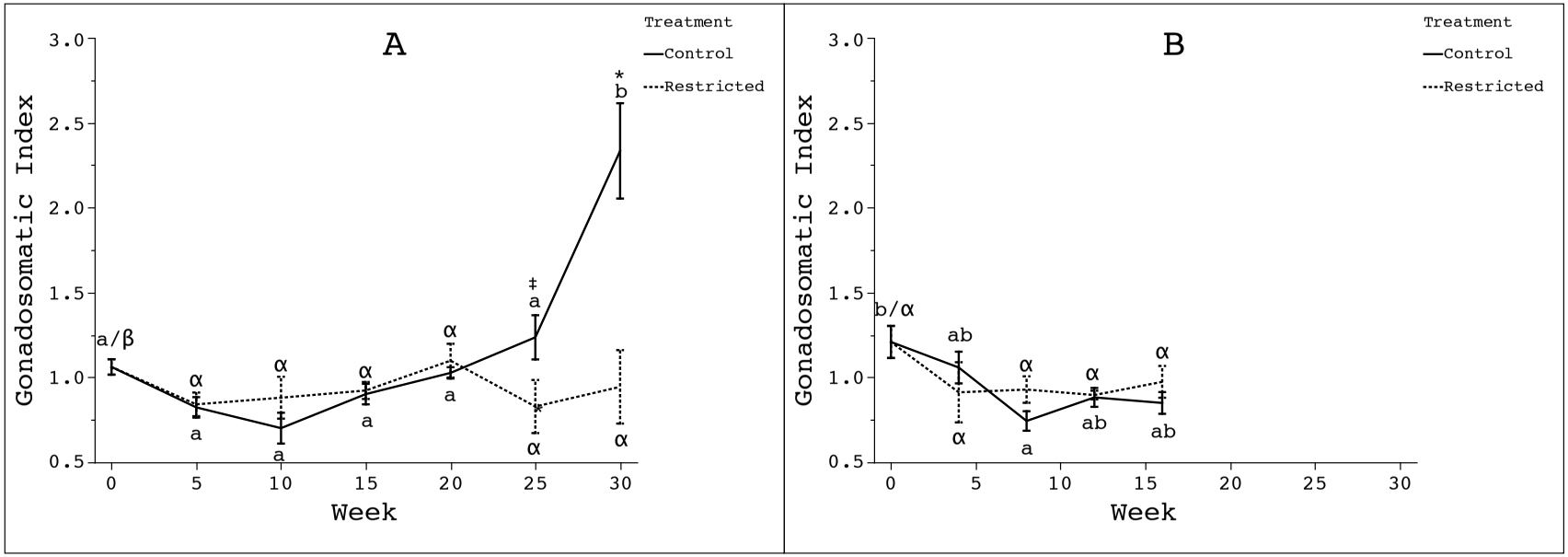

Figure 2.2. Mean (±SEM) gonadosomatic index (GSI) over time in female rainbow trout (A: two-year-old; B: three-year-old) fed a control-ration or restricted-ration. Treatment means differ significantly ($p < 0.05$) at time-points marked "*". Within each age class, time-points within a treatment group sharing the same letter or letters are not significantly different ($p \geq 0.05$).

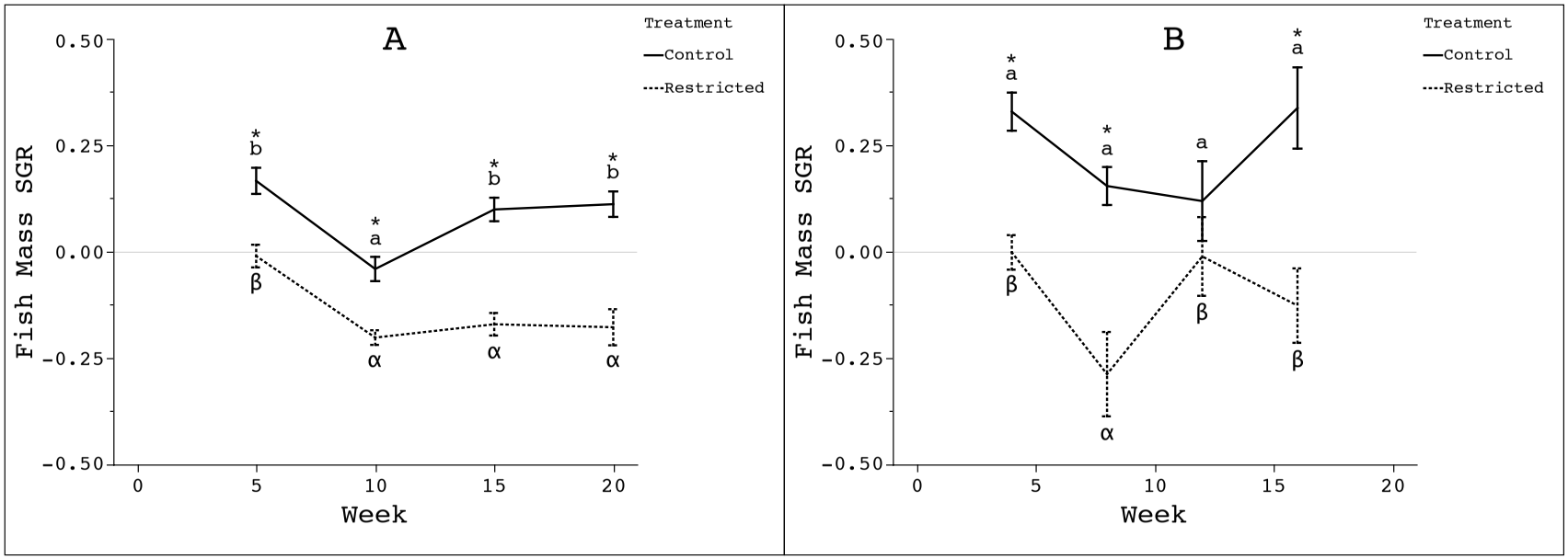

Figure 2.3. Mean (±SEM) fish mass specific growth rates (SGR) over time in female rainbow trout (A: two-year-old; B: three-year-old) fed a control-ration or restricted-ration. Growth rate over the preceding interval of four (B) or five (A) weeks is shown at each time-point. See Figure 1.2 for an explanation of what each symbol signifies.

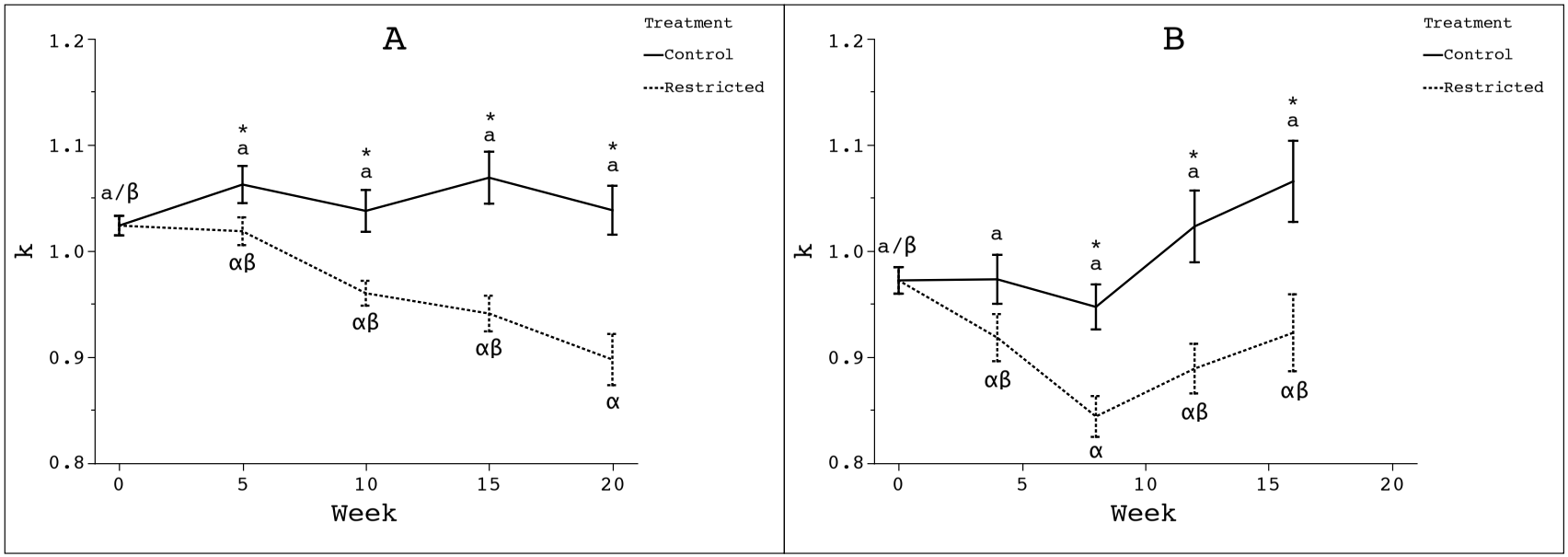

Figure 2.4. Mean (±SEM) condition factor (k) over time in female rainbow trout (A: two-year-old; B: three-year-old) fed a control-ration or restricted-ration. See Figure 1.2 for an explanation of what each symbol signifies.

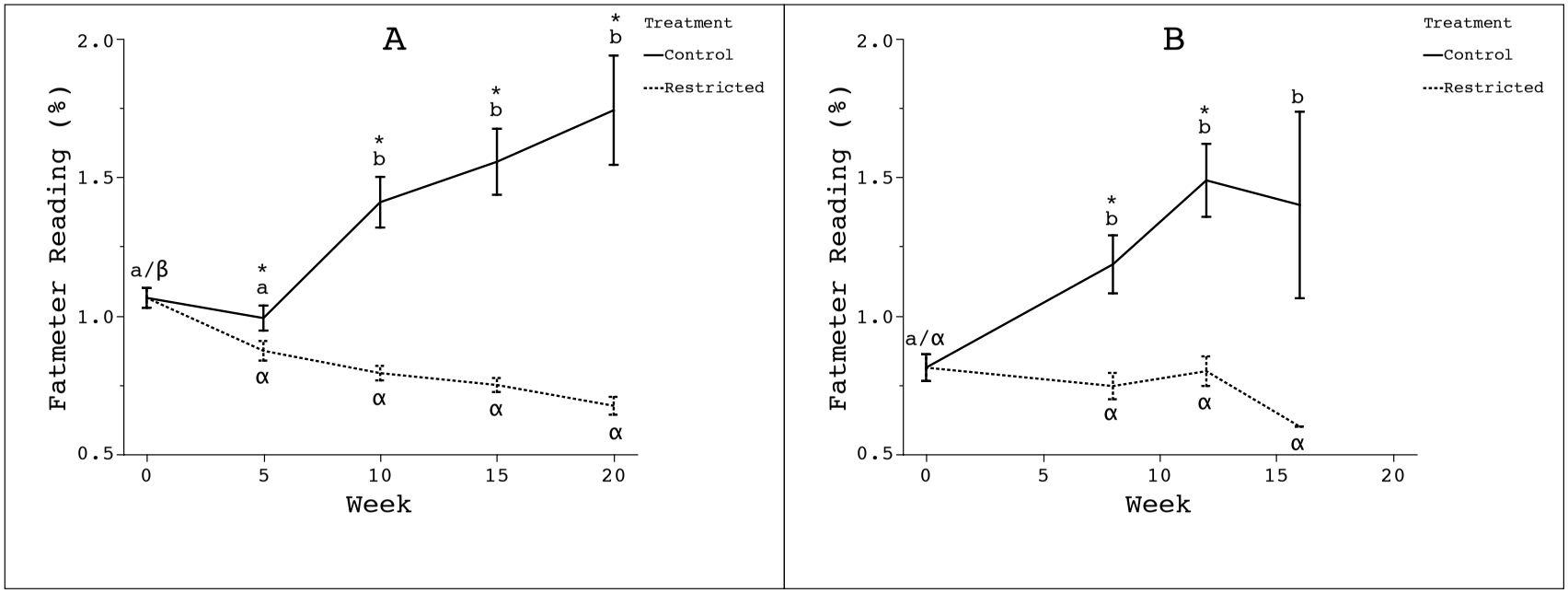

Figure 2.5. Mean (±SEM) Fatmeter readings over time in female rainbow trout (A: two-year-old; B: three-year-old) fed a control-ration or restricted-ration. Data are not present for four-week sampling point in panel B. See Figure 1.2 for an explanation of what each symbol signifies.

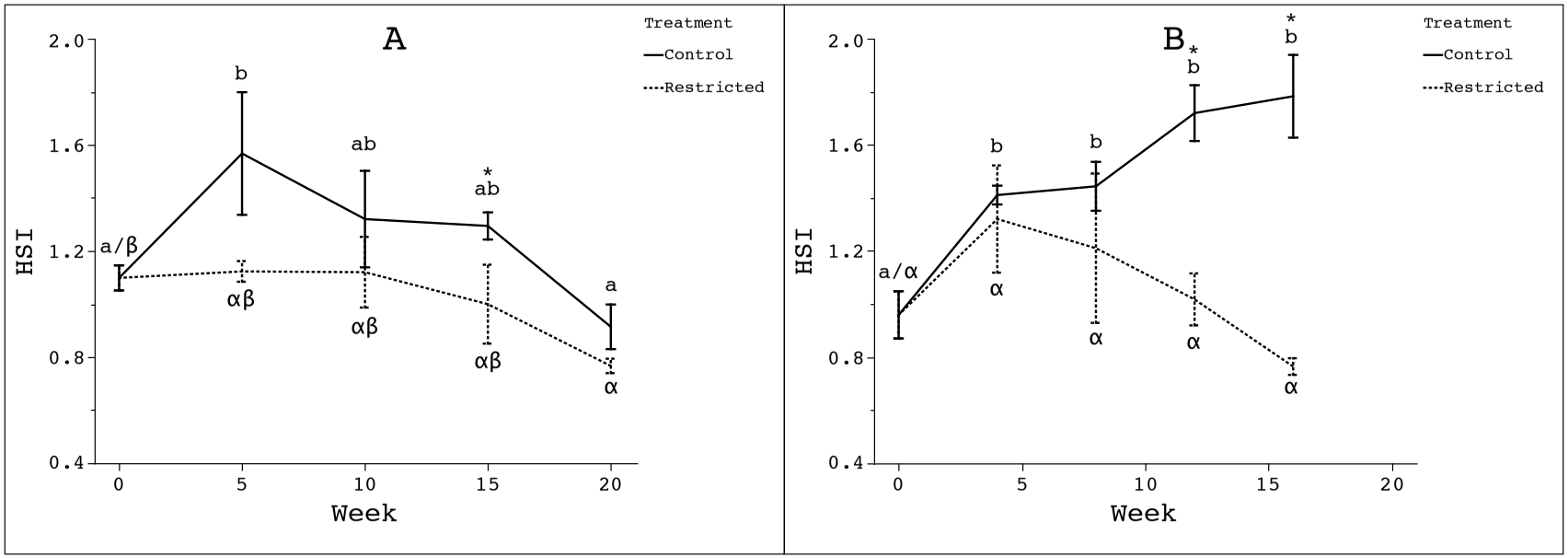

Figure 2.6. Mean (±SEM) hepatosomatic index (HSI) over time in female rainbow trout (A: two-year-old; B: three-year-old) fed a control-ration or restricted-ration. See Figure 1.2 for an explanation of what each symbol signifies.

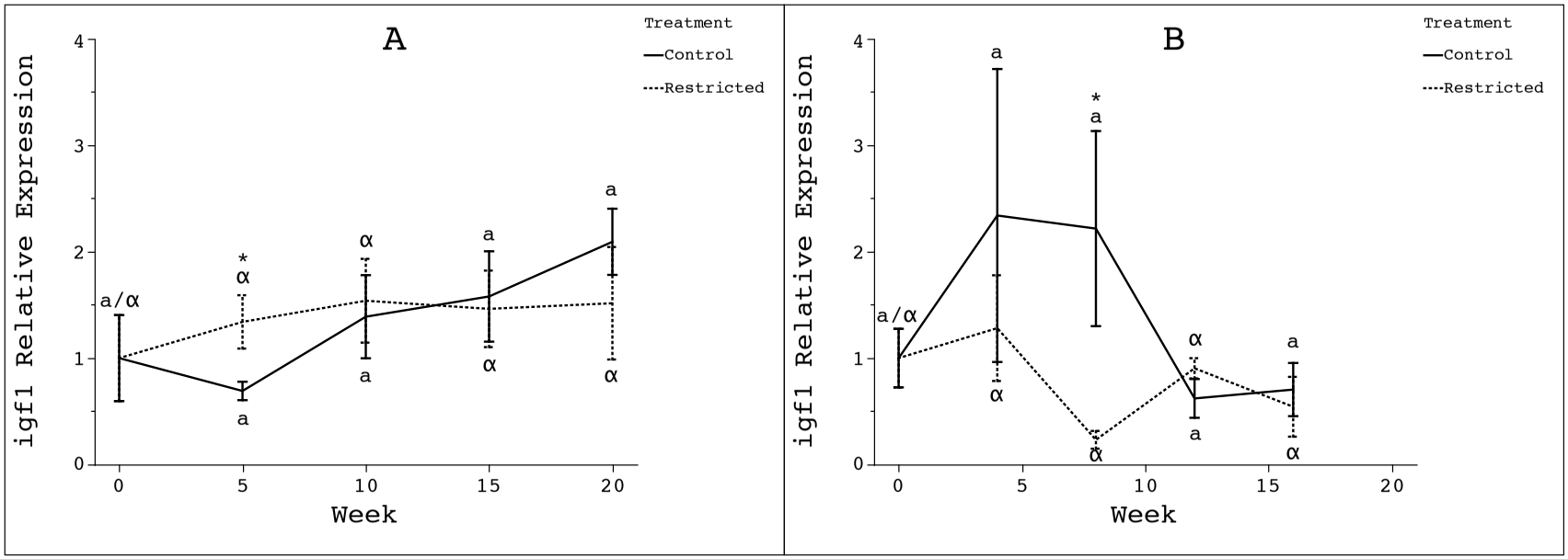

Figure 2.7. Mean (±SEM) liver insulin-like growth factor 1 (*igf1*) mRNA levels over time in female rainbow trout (A: two-year-old; B: three-year-old) fed a control-ration or restricted-ration. See Figure 1.2 for an explanation of what each symbol signifies.

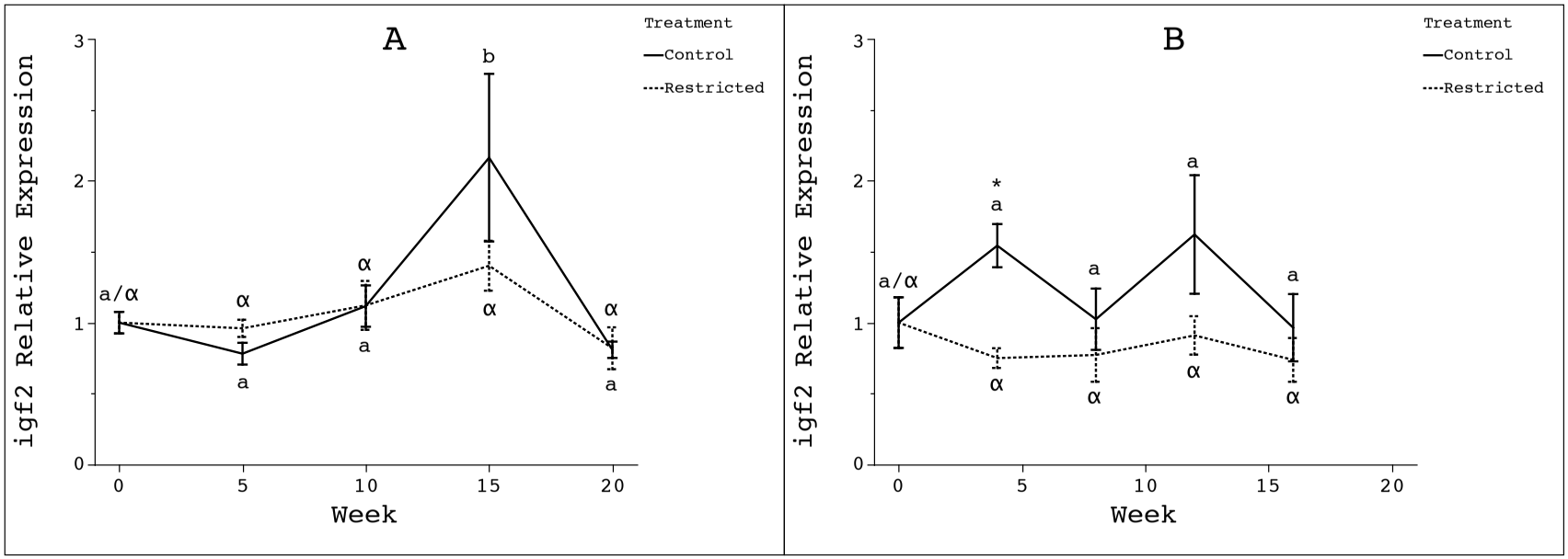

Figure 2.8. Mean (±SEM) liver insulin-like growth factor 2 (*igf2)* mRNA levels over time in female rainbow trout (A: two-year-old; B: three-year-old) fed a control-ration or restricted-ration. See Figure 1.2 for an explanation of what each symbol signifies.

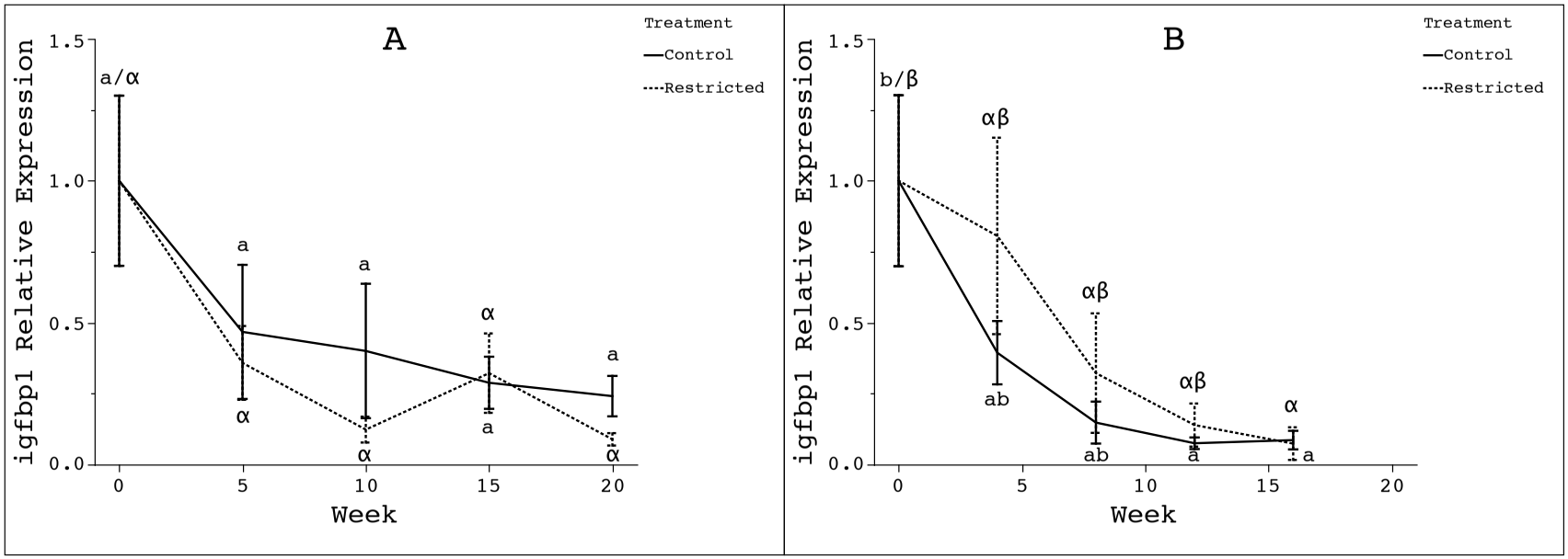

Figure 2.9. Mean (±SEM) liver insulin-like growth factor binding protein 1 (*igfbp1*) mRNA levels over time in female rainbow trout (A: two-year-old; B: three-year-old) fed a control-ration or restricted-ration. See Figure 1.2 for an explanation of what each symbol signifies.

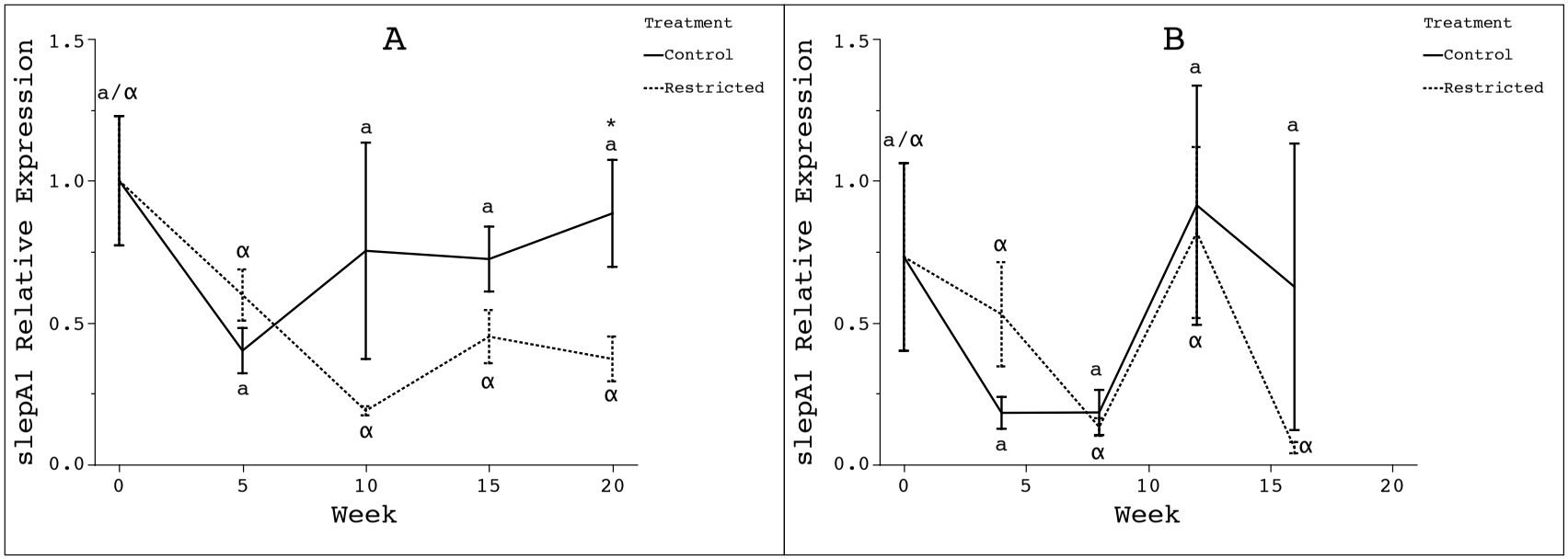

Figure 2.10. Mean (±SEM) liver salmon leptin A1 (*slepA1)* mRNA levels over time in female rainbow trout (A: two-year-old; B: three-year-old) fed a control-ration or restricted-ration. See Figure 1.2 for an explanation of what each symbol signifies.

Chapter 3: "Plasma nesfatin-1 is not affected by long-term food restriction and does not predict rematuration among iteroparous female rainbow trout (*Oncorhynchus mykiss*)."[2]

Introduction

The reproductive endocrine (BPG) axis has been characterized for some fishes [1,4,181] including salmonids [212], and the basic mechanism by which the BPG axis secretes hormones to regulate initial maturation (puberty) in salmonid species are well understood [60]. However, there is a dearth of information concerning endocrine regulation and coordination of gonadal recrudescence (rematuration). While it is reasonable to suspect that puberty and rematuration are regulated similarly, this hypothesis has mostly gone untested. Furthermore, although the role of energetics in puberty is well described in many animals [213,214,215] including fishes [110,180,216], how metabolic and nutritional status regulate successive reproductive efforts has largely been overlooked.

In mammals, nesfatin-1 is an 82-amino acid hormone cleaved from the nucleobindin-2 (*nucb2*) gene product that is secreted primarily from the hypothalamus and adipose tissue, and that has both anorexigenic and growth inhibiting effects [175]. In

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[2] Caldwell, L. K., A. L. Pierce, L. G. Riley, C. A. Duncan and J. J. Nagler (2014). "Plasma nesfatin-1 is not affected by long-term food restriction and does not predict rematuration among iteroparous female rainbow trout (*Oncorhynchus mykiss*)." PLoS ONE 9(1): e85700.

rats, short-term (24-hour) fasting leads to a decrease in circulating levels of nesfatin-1, which return to normal levels upon refeeding [217]. In addition to behavioral and metabolic effects, nesfatin-1 appears to inform the reproductive endocrine axis regarding nutritional status [218]: in pigs, nesfatin-1 injections elevated plasma LH levels [219]. In goldfish, *nucb2* mRNA is widely expressed in the brain and peripheral tissues, with the highest expression levels found in the liver and pituitary [177], nesfatin-1 injections reduce feeding [177], and nesfatin-1 suppresses the BPG axis at all three tiers [51]. Taken together, these results suggest that circulating nesfatin-1 may act to integrate metabolic or feeding status with reproductive development.

The metabolic peptide hormone ghrelin is synthesized and secreted from the stomach of vertebrates during an energy deficit [220]. In concert with other circulating endocrine factors, ghrelin coordinates the metabolic response to fasting and has behavioral effects such as stimulating appetite in salmonid fishes [128,157]. Ghrelin and nesfatin-1 colocalize in stomach and hypothalamus of goldfish [176]. In unfed goldfish, i.c.v. nesfatin-1 injection suppressed preproghrelin and ghrelin receptor mRNA expression in forebrain, suppressed ghrelin and *nucb2* mRNA expression in hypothalamus, and suppressed *nucb2* mRNA expression in forebrain [176]. Along with leptin and other regulatory metabolic hormones, ghrelin acts at multiple levels of the BPG axis to inhibit reproduction, *e.g.*, by suppressing pituitary LH and testicular androgen secretion [149]. Ghrelin acts directly on zebrafish follicles to inhibit both basal and MIH-induced germinal vesicle breakdown [221]. Ghrelin abnormalities have been implicated in a variety of human reproductive disorders [137,222]. Thus, ghrelin may act as a peripheral metabolic signal that negatively regulates reproductive development under

conditions of nutritional deficit among iteroparous fishes such as rainbow trout [45,129,214].

Among salmonid fishes, the onset of puberty occurs up to a year in advance of spawning [103,223]. The current working hypothesis states that puberty in salmonids is ultimately regulated by energy availability [45,214]. When energy is deficient, some peripheral metabolic hormone inhibits or arrests reproductive development [129,221]. When energy is sufficient, some peripheral metabolic hormone stimulates or permits the reproductive axis to proceed with reproductive development [51,224]. Upon receiving the stimulatory or permissive signal from such a metabolic cue, maturation is initiated in the brain by gonadotropin releasing hormones (GnRHs). GnRHs originate primarily in the hypothalamus [225] and act hierarchically to stimulate pituitary release of the GtHs, FSH and LH [226]. In female fishes, the GtHs stimulate growth and development of the ovaries [65], and also activate steroidogenic pathways culminating in secretion of both the estrogenic sex steroid 17β-estradiol (E2) and the androgenic sex steroid 11-keto-testosterone (11-KT) [2,3]. Increasing levels of circulating E2 both initiate and signal ovarian maturation [227,228]. Recent work suggests a role for 11-KT in early oocyte growth among various species of teleost fishes [229,230,231]; *in vitro* evidence suggests such a role for 11-KT in salmonids [232].

The purpose of this study was to determine how energy availability after spawning affects plasma levels of nesfatin-1, ghrelin, and sex steroid hormones in rematuring female rainbow trout (*Oncorhynchus mykiss*). It was hypothesized that by restricting food availability in a group of female trout, these fish would become energy deficient and thus arrest reproductive development as individuals adopted a non-

consecutive year or "skip-spawner" life history that has been described in fishes [187,233]. It was also hypothesized that this energy deficit would be initially reflected by increased plasma ghrelin and decreased plasma nesfatin-1 [187,233]. It was further hypothesized that this skip-spawner life history decision would be detectible first by reduced pituitary levels of *fsh-β* mRNA, then by reduced circulating levels of 11-KT, and finally by reduced circulating levels of E2.

Materials & Methods

1. *Animal handling and husbandry*

See Chapter 1.

2. *Experiments*

See Chapter 1.

3. *RNA Extractions & cDNA Synthesis*

See Chapter 1.

4. *Quantitative real-time reverse-transcriptase polymerase chain reaction*

A quantitative real-time reverse-transcriptase PCR (q-RT-PCR) primer set (Table 1) was designed using ABI Primer Express 3.0 software (Life Technologies, Carlsbad, CA) to amplify a 60 bp fragment of the annotated *O. mykiss fsh-β* sequence (NM_001124586). Specificity was confirmed by bioinformatic analysis, agarose gel electrophoresis of PCR products, and melting curve analysis of PCR products. To carry out the q-RT-PCR, sample cDNA was amplified in 96-well optical reaction plates (Invitrogen) containing 20 μL PCR reactions made up of 2μL cDNA, 10 μL *Power*

SYBR Green PCR Master Mix (Life Technologies), 6 μL H_2O, and 2 μL of a mix of forward and reverse primers at 2 pM each, in an Applied Biosystems ABI 7900HT real-time PCR system (Life Technologies) (2 min @ 50°C; 10 min @ 95ºC; 40 cycles of 15 sec @ 95ºC and 1 min @ 60ºC). Copy numbers in samples were quantified using standard curves of PCR amplicons. Positive and negative controls were included on each plate. Three technical replicate PCRs were completed for each sample. The mean of the mRNA copy number for the three replicate PCRs is the value reported. q-RT-PCR results were $\log_2$-transformed prior to statistical analysis.

5. *17β-Estradiol Radioimmunoassay (RIA)*

5.1. *Solvent Extraction*

Plasma samples were extracted with methyl tert-butyl ether (MTBE) (Fisher Scientific, Hampton, NH) by combining 100 µL plasma with 4.0 mL MTBE and vortexing for 1 min [234]. Samples were incubated at room temperature for 7 min to allow phase separation to occur and then the aqueous phase was frozen. The solvent fraction was decanted, equilibrated 10 min at room temperature and incubated at 55°C until all solvent had volatilized (approximately 2 hour). A second extraction of the remaining aqueous fraction from each plasma sample was performed, using 3.0 mL MTBE, and pooled with the first extract. Dried extracts were resuspended in 250 µL E2 zero calibrator solution from the E2 RIA kit (Coat-A-Count, Siemens, Munich, Germany). Average extraction efficiency was 83%, as determined by RIA values for extracted versus unextracted assay standards included with the RIA kit.

5.2. *RIA*

Resuspended plasma extracts were analyzed in duplicate for E2 concentration using an antibody-coated tube E2 radioimmunoassay (RIA) kit (Coat-A-Count, Siemens, Munich, Germany), per the manufacturer's instructions. Sensitivity for the assay is reported to be 8 pg mL^{-1}. Positive and negative controls were included in all assays.

6. *11-keto testosterone Enzyme-linked Immunosorbent Assay (EIA)*

6.1. *Solvent Extraction*

Plasma samples were extracted with anhydrous diethyl ether (JT Baker, Avantor Performance Materials, Inc.; Center Valley, PA, USA) by combining 100 µL plasma with 2.0 mL diethyl ether and vortexing for 1 min [235]. Samples were incubated at room temperature for 7 min to allow phase separation to occur, and then the aqueous phase was frozen. The solvent fraction was decanted. A second extraction of the remaining aqueous fraction from each plasma sample was then performed, using 2.0 mL diethyl ether, and pooled with the first extract. Diethyl ether extracts were dried down in a 49ºC water bath under a gentle stream of N_2 directed *via* a nitrogen evaporator manifold (N-EVAP 112; Organomation Associates, Inc; Berlin, MA). Dried extracts were resuspended in 1000 µL EIA buffer from the 11-KT EIA kit (described below). Extraction efficiency ranged from 90-102%, as determined by RIA values for extracted versus unextracted assay standards included with the RIA kit.

6.2. *EIA*

Resuspended plasma extracts were analyzed in duplicate for 11-KT concentration using an antibody-coated 96-well plate based enzyme-linked immunosorbent assay (EIA)

kit (Cayman Chemical Company; Ann Arbor, MI). Sensitivity for the assay is reported to be 1 pg mL^{-1}. Positive and negative controls were included in all assays.

7. *Ghrelin RIA*

Plasma ghrelin concentrations were measured in duplicate using a ghrelin radioimmunoassay established and validated for use in fish by Riley et al. [236], with the following minor variations. One hundred μL of rat ghrelin standards and plasma samples were incubated with 200 μL anti-rat ghrelin (from H. Hosoda) at a dilution of 1:750,000. The anti-rat ghrelin (a.a. 1-11) recognizes the octanoylated epitope (biologically active region) of ghrelin [237] and detects only the biologically active forms of ghrelin (ghrelin-C8 and ghrelin-C10). After incubation at 4°C overnight, 100 μL of ^{125}I-human ghrelin (Millipore, St. Charles, MO) was added before an additional overnight incubation at 4°C. Finally, 100 μL anti-rabbit IgG goat serum at 1:35 (with 10 % polyethylene glycol) was added, incubated for 2 hour at room temperature, and then centrifuged at 3200 x g for 60 min to separate free and bound tracers. Radioactivity of aspirated pellet was then quantified using a gamma counter (Packard, Palo Alto, CA). Intra- and inter-assay CV's were 6.2 and 9.8%, respectively. Positive and negative controls were included in all assays

8. *Nesfatin-1 EIA*

8.1. *Parallelism of trout plasma dilution*

A rat nesfatin-1 EIA (EK 003-22; Phoenix Pharmaceuticals) was used for measuring nesfatin-1 immunoreactivity (nesfatin-1) in rainbow trout plasma. Although this kit has been previously validated for use in fish (goldfish [177]), the following procedure was conducted to establish its use in rainbow trout. A representative sample of

female trout plasma was serially diluted 1.5 fold in 1x nesfatin-1 EIA buffer and measured using the nesfatin-1 EIA; displacement of label by standard and by trout plasma were then compared (Fig. 3.1). A four-parameter logarithmic regression line was fit to the relationship between displacement and plasma volume. Displacement of rat nesfatin-1 by trout plasma was parallel to the standard curve, indicating the presence of an immunologically similar protein in trout plasma. Displacement was linear over the range 1.3μL - 9.9µL trout plasma ($R^2 = 0.99$).

8.2. *Measuring nesfatin-1 levels in trout plasma*

Rainbow trout plasma samples were assayed for nesfatin-1 by diluting them 1:10 in 1x EIA buffer to bring values within the linear range of the kit, as described above. Samples were run in duplicate, and had intra-sample CV values <10%. Positive control samples were all within the range specified in literature accompanying the kit, and negative controls were included for all assays.

9. *Data Analysis*

Only data from fish that survived until being terminally sampled were included in statistical analyses. Systematic tank differences were not detected within treatment for any variable (ANOVA, $p > 0.05$). Therefore, tank replicates were pooled and analyzed together. For normality and homoscedasticity requirements of ANOVA and post-hoc tests, plasma hormone data were log-transformed prior to analysis. Two-way ANOVA was used to detect main and interaction effects (time, treatment, time*treatment). When ANOVA indicated a significant time effect, Tukey-Kramer Honestly Significant Difference (Tukey-Kramer HSD) tests were used to compare values at all time points within a given treatment. Within each time point, two-tailed t-tests were used to detect

treatment differences. Statistical analyses were performed with JMP (Version 9, SAS Institute Inc., Cary, NC). Differences are reported as significant when $p < 0.05$.

Results

As described in Chapter 1, the feeding regime used here affected metrics of growth, metabolism, and reproduction in both two- and three-year-old rematuring female rainbow trout. However, in both experiments there was no significant difference in plasma nesfatin-1 immunoreactivity between the treatment groups at any time point (Fig. 3.2). Within each experiment, a two-way ANOVA determined that "week" was a significant effect, but neither "treatment" nor the "treatment*week" interaction were significant effects. Plasma nesfatin-1 immunoreactivity covaried significantly in the two treatment groups (ANCOVA, $p<0.0001$), suggesting that plasma nesfatin-1 was not affected by treatment. While the feeding regimes used here did not yield differences in trout plasma nesfatin-1 immunoreactivity between treatments, plasma nesfatin-1 immunoreactivity did change over time both within each treatment individually and when the feeding treatments were pooled and considered together. In both two-year-old and three-year-old trout, there was a marked increase over time in plasma nesfatin-1 immunoreactivity, before levels declined toward the end of the experiment. Similarly, no difference in plasma levels of acylated-ghrelin (ghrelin) was detected either among time points within treatment groups for a given age class, or between treatment groups at any time-point for a given age class (Fig. 3.3).

The feeding treatments produced a significant difference in reproductive development during this period of initial ovarian recrudescence, as indicated by plasma E2 levels (Fig. 3.4): among two-year-old trout fed the control-ration, plasma E2 concentration was elevated compared to restricted-ration fish by week ten and continued to increase dramatically over the course of the experiment. Plasma E2 levels showed a similar but non-significant elevation in control versus restricted-ration fish among three-year-old trout starting at week eight. Interestingly, at week zero, three-year-old trout exhibited plasma levels of E2 that were an order of magnitude greater than those observed among two-year-old trout, before decreasing significantly as the experiment progressed.

Among two-year-old trout, pituitary *fsh-β* mRNA expression decreased from weeks 0-25 within restricted-ration fish, while *fsh-β* expression increased from weeks 10-30 within control-ration fish (Fig. 3.5). This trend led to a significant difference between the treatment groups: restricted-ration fish exhibited higher pituitary *fsh-β* mRNA levels shortly after spawning, at week 10, and control-ration fish exhibited higher pituitary *fsh-β* mRNA levels later, at weeks 25-30. Among three-year-old trout, pituitary *fsh-β* mRNA expression did not significantly change over time within either treatment group, and pituitary *fsh-β* mRNA expression levels did not significantly differ between the two groups at any time point.

Plasma concentration of 11-KT (Fig. 3.6) showed a similar trend within both year classes: plasma 11-KT levels were elevated at week zero before precipitously declining and remaining low through the remainder of the experiment. Among two-year-old trout, plasma 11-KT diverged at week 25, with control-ration fish exhibiting significantly

elevated plasma 11-KT levels compared to restricted-ration fish; the experiment with three-year-old trout was presumably not long enough to capture this effect, as no difference was detected.

Discussion

The results presented in Chapter 1 showed that the control-ration was sufficient to maintain positive growth rates, while the restricted-ration was sufficient to maintain near-zero or negative growth rates. The results in this chapter provide evidence that plasma nesfatin-1 levels are not affected by this long-term feeding restriction in post-spawned female rainbow trout; no difference in plasma nesfatin-1 levels was detected between the control-ration and restricted-ration fish at any time point in two-year-old or three-year-old trout. Additionally, circulating nesfatin-1 levels observed during the months after spawning do not predict rematuration in rainbow trout: restricted-ration fish failed to undergo initial sexual maturation, but nonetheless exhibited plasma nesfatin-1 levels that were similar to control-ration fish. While i.p. injections of nesfatin-1 have been shown to inhibit the reproductive endocrine axis in fish [51], the observed inhibitory effect may be due to relatively high (*i.e.*, pharmacological) dosage, and thus not accurately reflect the native (*i.e.*, physiological) role of circulating nesfatin-1. Alternatively, nesfatin-1 may act differently in fish with group-synchronous ovary development (rainbow trout) compared to fish with asynchronous ovary development (goldfish). Plasma nesfatin-1 levels did exhibit a pattern suggesting nesfatin-1 may be regulated seasonally in rainbow trout, with

a peak occurring near the summer solstice (Fig 3.2A). Further studies are required to determine the significance of seasonal changes in trout plasma nesfatin-1 levels.

Similarly, within both age classes, plasma ghrelin levels did not differ significantly between treatment groups at any time point. Among two-year-old trout fed the restricted-ration, plasma ghrelin did significantly increase between week 20 and week 30. However, plasma ghrelin levels also exhibited a transient spike among two-year-old trout fed the control-ration around week 25. Among three-year-old trout, the later increase in plasma ghrelin (between week 20 and week 30) was not observed; this is likely the result of the experiment using three-year-old trout not running long enough. Taken together, these findings do not support a role for ghrelin as an indicator of long-term nutritional status in rainbow trout. Although these data do not support the original hypothesis, this contradiction with previous results in other systems may be partially explained by the observation that ghrelin physiology differs substantially between fishes and mammals [238,239]. For instance, in rainbow trout, ghrelin administration does not increase feed intake, and plasma ghrelin levels are reduced during fasting [91,128]. Also in rainbow trout, *in vitro* ghrelin treatment of gastrointestinal tissue does not affect GI contractility [240]. In channel catfish, neither plasma ghrelin nor stomach *ghrl* mRNA expression changes with feeding status [241]. In two species of sturgeon [242], and in grass carp [243], ghrelin appears to play a principally developmental role. It appears that more work needs to be done to clarify the disparate roles of ghrelin among non-mammalian vertebrates. However, these results do not support regulation of plasma ghrelin levels by either recovery from spawning or nutritional status in post-spawned rainbow trout.

The experimental design used in this study affected nutrition sufficiently to arrest reproductive development among two-year-old trout, as evidenced by a divergence in plasma E2, GSI (Chapter 1), plasma 11-KT and pituitary *fsh-β* mRNA expression. This suggests that the nutritional limitation imposed upon the restricted-ration fish sufficed to induce a physiological trade-off between investment in continued survival and growth versus investment in reproductive development. While the experiment using three-year-old trout did not continue long enough to capture this arrest, it is clear from the experiment using two-year-old trout that between 15 and 20 weeks after spawning, the control-ration fish began to increasingly partition energy to the ovary for developing oocytes.

At intake, three-year-old trout exhibited plasma E2 levels that were approximately an order of magnitude greater than those observed in two-year-old trout (Fig 3.4), while ovarian masses were similar between the two age classes (Chapter 1). This difference in circulating E2 levels may be due to differences in the duration of pre-spawn fasting used by the facility from which trout were obtained (as described in Methods section), or to altered clearance of steroids by the liver [244,245]. Previous work suggests that animals may experience an aging-associated decrease in the rate of steroid clearance via liver conjugation and hydroxylation reactions [246,247], or an increasing sequestration of steroids by sex hormone-binding globulin [248], or some diffuse age-related effects associated with a diminished basal metabolic rate similar to that described in humans [249,250] and other animals [251]. Regardless, this difference between age classes was transient: two-year-old and three-year-old fish exhibited similar plasma E2 levels by the second sampling date.

The results in Chapter 1 described how this feeding regime induced remodeling of organs and a general redistribution of energy stores, with control-ration fish accumulating lipid in muscle tissue and increasing liver mass. Interestingly, differences in plasma E2 between the two treatment groups were already significant at week ten and presumably were present sometime between weeks five and ten of the experiment. This would indicate that a trajectory of reproductive rematuration for the following year's spawning effort had been at least partially determined approximately six weeks after spawning (as fish were obtained approximately one week after spawning), similar to the current understanding of the progression of puberty in salmonid fishes [103]. This implies that the energy deficit incurred during spawning and feeding conditions during the period immediately after spawning interact to determine whether rematuration is initiated within an approximately six week window. Restricted ration fish appear to have arrested rematuration before significant energy was invested in ovarian development.

Classic reproductive endocrine axis theory [4,212] predicts that an increase in secretion of pituitary (*i.e.*, FSH) factors should precede an increase in plasma E2. However, among control ration fish, we detected no elevation in pituitary *fsh-β* mRNA (Fig. 3.5) expression prior to elevation of plasma E2 levels (Fig. 3.4). *In vitro* work suggests that FSH is regulated at the level of both transcription and secretion by the interaction of GnRH hierarchical stimulation and E2 feedback [252], and thus, pituitary expression of *fsh-β* may not be directly correlated with circulating levels of FSH peptide [253]. In addition, our ability to detect treatment differences in pituitary *fsh-β* mRNA was less than for plasma E2 due to the number of lethal and non-lethal samples at each time point (*e.g.*, for two-year-old trout at week 10, n=60 for plasma E2, while n=12 for

pituitary *fsh-β* mRNA). Interestingly, within two-year-old trout, pituitary *fsh-β* mRNA expression was significantly higher among restricted-ration fish than control-ration fish between weeks 10 and 15; plasma E2 levels show the opposite trend and were elevated among control-ration fish during this time. This combination of observations is explained by the well-documented inhibitory tone of sex steroids on pituitary gonadotropin synthesis [254,255,256]. Among control-ration fish, increasing levels of plasma E2 exert negative feedback inhibition of *fsh-β* expression, as has been described in rainbow trout [257] and other fishes [258,259]. Conversely, the lower plasma E2 levels observed among restricted-ration fish around this time releases pituitary *fsh-β* mRNA transcription from inhibition, causing an apparent increase in pituitary *fsh-β* mRNA levels among restricted-ration fish.

In a recent *in vitro* study in coho salmon, 11-KT stimulated growth of late perinucleolar stage follicles, suggesting a role for this androgen in early ovarian development [232]. However, it is not clear whether circulating or local levels of 11-KT are physiologically relevant in this scenario. In the present study, plasma 11-KT levels dropped rapidly between zero and five weeks after spawning and did not differ between treatment groups until week 25, which was 15 weeks after plasma E2 diverged. Therefore, our results do not support the hypothesis that plasma 11-KT stimulates ovarian rematuration during the period immediately following spawning. However, it cannot be ruled out that local 11-KT (*i.e.*, 11-KT produced in the ovary) may be involved in stimulating early ovarian development immediately after spawning, in a paracrine fashion. Between 20 and 25 weeks after spawning, treatment groups within the two-year-old trout diverged, with control ration fish exhibiting 2.4-fold elevated plasma levels of

11-KT versus restricted-ration fish by 30 weeks. This elevation in plasma 11-KT coincided with a significant elevation in GSI among control ration fish (Chapter 1), suggesting that plasma 11-KT among maturing female fish may be of ovarian origin, as has previously been proposed [260]. Both age classes of trout started the experiment at the first sampling point immediately after spawning with elevated plasma 11-KT (Fig. 3.6), supporting the proposed role for 11-KT in female salmonid spawning physiology and behavior [228,261,262].

In conclusion, by restricting food availability in a group of female rainbow trout, growth and energy partitioning were both affected, with restricted-ration fish generally existing in a catabolic state and control-ration fish existing in an anabolic state for the duration of the experiment. Furthermore, the treatments were sufficient to induce differences in gonadal recrudescence between the two treatment groups: while restricted-ration fish arrested ovarian growth, control ration fish accumulated ovarian tissue until the end of the experiment (Chapter 1). Differences in ovary size were preceded by differences in circulating levels of E2, which diverged between one and two months after the start of the experiment. Although pituitary secretion of FSH presumably drives the increase in ovarian steroidogenesis underlying elevated plasma E2, our results suggests that this purported increase in FSH is not regulated at the level of transcription during the months immediately after spawning. In addition, our study provides no evidence to support the notion that circulating 11-KT is involved in early maturation of recrudescing female rainbow trout. Finally, food-restriction and the subsequent difference in reproductive trajectory did not measurably affect plasma levels of nesfatin-1 or ghrelin, suggesting that circulating levels of neither peptide link metabolic status to reproduction

in *O. mykiss*. Future work should focus on elucidating the different roles of endocrine and paracrine nesfatin-1, as well as clarifying differences in chronic and acute nesfatin-1 responses to nutrient availability.

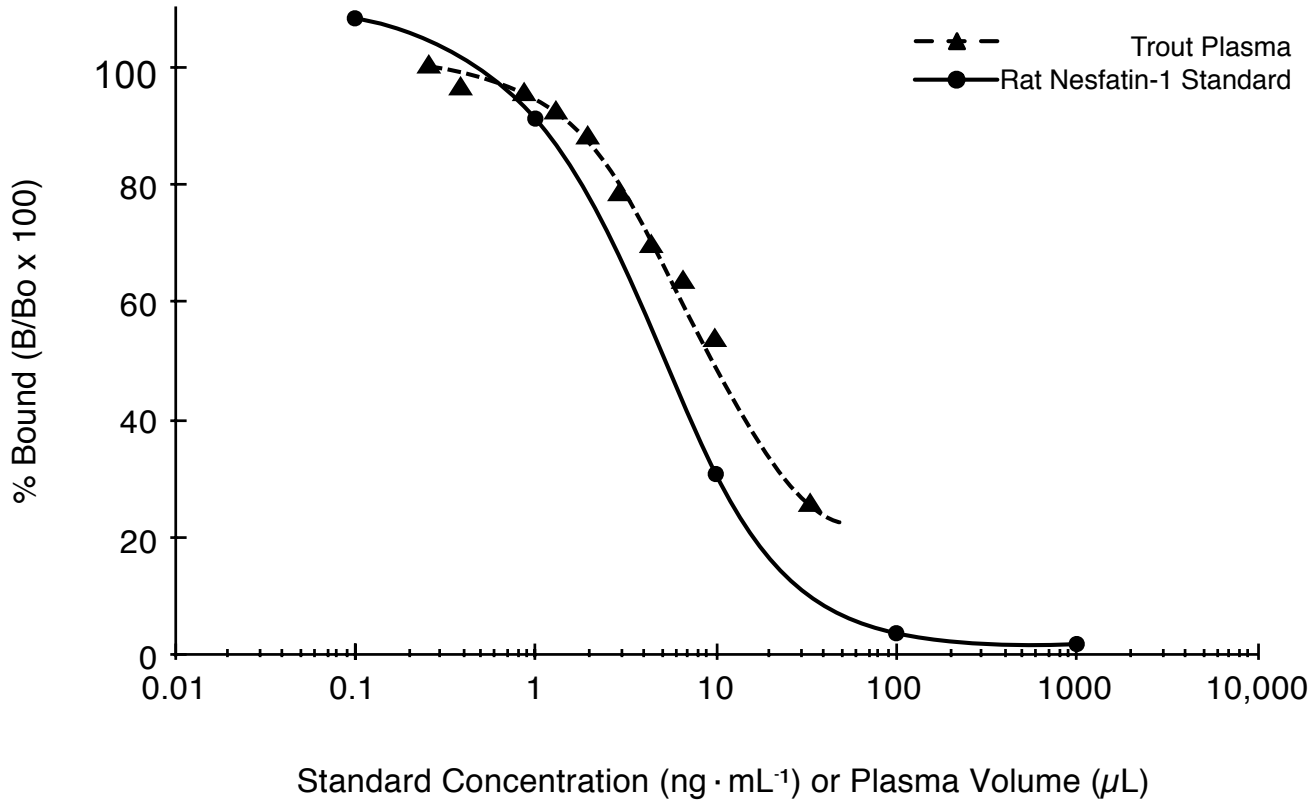

Figure 3.1. **Parallel displacement of rat nesfatin-1 by a serial dilution of trout plasma.** Displacement curve showing parallelism of the rat nesfatin-1 standard curve (circle points, solid line) and a dilution series of female rainbow trout plasma displacing rat-nesfatin-1 (triangle points, dashed line) in the nesfatin-1 EIA. Horizontal axis depicts volume of trout plasma (μL) used in the assay or concentration of rat-nesfatin-1 standard ($ng \cdot mL^{-1}$).

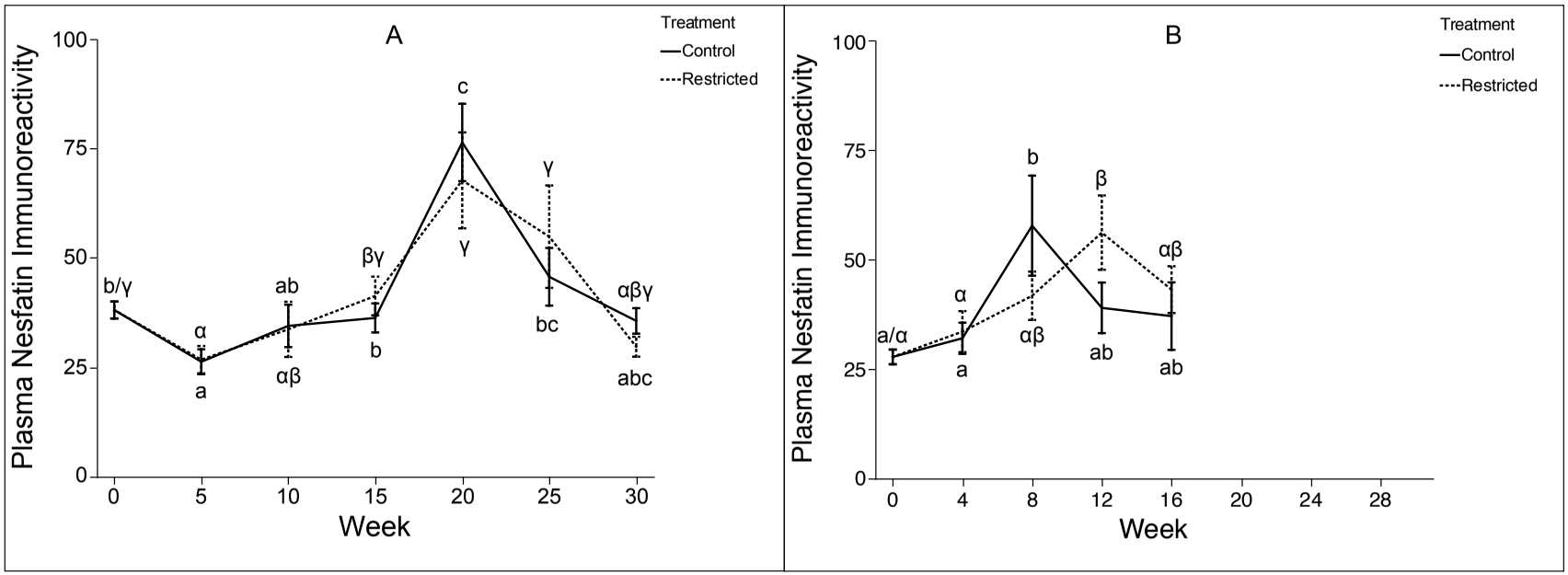

Figure 3.2. **Plasma nesfatin concentrations in female rainbow trout recovering from spawning.** Mean (±SEM) plasma nesfatin-1 concentration over time in female rainbow trout (A: two-year-old; B: three-year-old) fed a control-ration (CR) or restricted-ration (RR). Mean values differ significantly between treatments (two-tailed *t*-test, $p<0.05$) at times marked "*" and marginally (two-tailed *t*-test, $p<0.1$) at times marked "‡". Within each figure, times sharing the same letter are not significantly different (Tukey's HSD, $p\geq0.05$). Latin letters (a, b, c, …) refer to CR fish; Greek letters (α, β, γ, …) refer to RR fish. Samples sizes per treatment group are as follows. Figure A: Week 0, n=42; Week 5, n=36; Week 10, n=30; Week 15, n=24; Week 20, n=18; Week 25, n=12; Week 30, n=6. Figure B: Week 0, n=25; Week 4, n=20; Week 8, n=15; Week 12, n=10; Week 16, n=5.

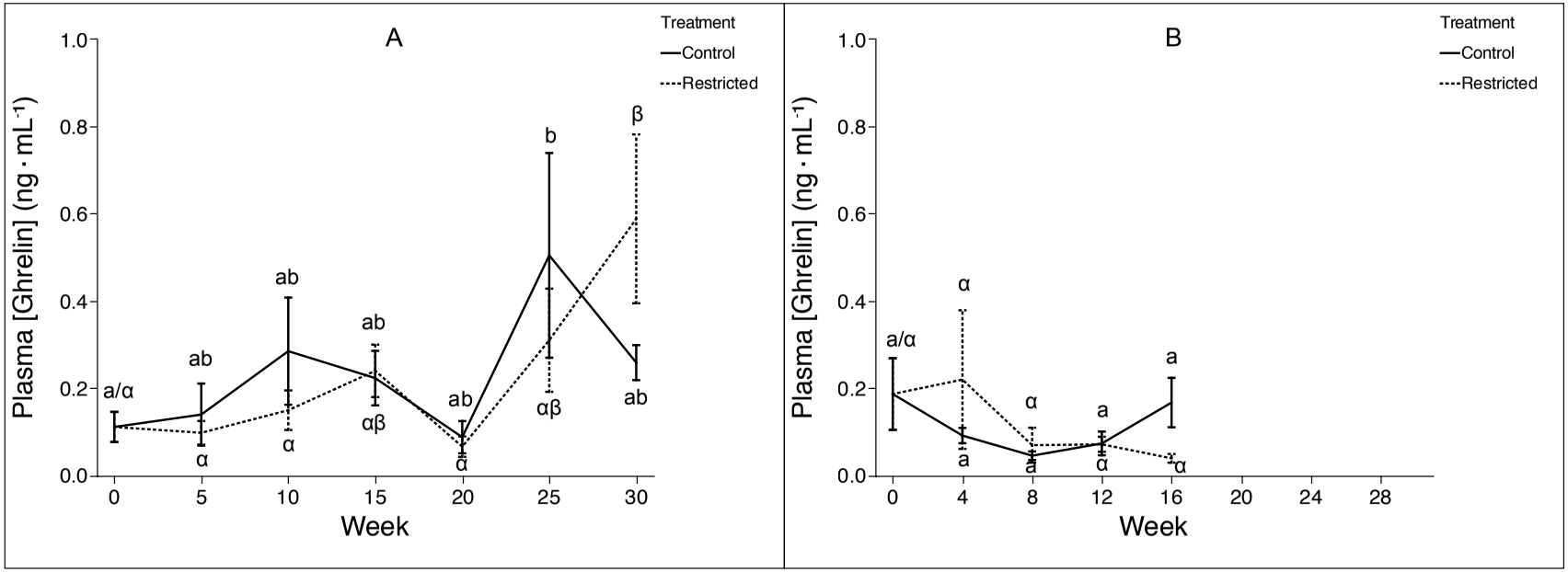

Figure 3.3. **Plasma ghrelin concentrations in female rainbow trout recovering from spawning.** Mean (±SEM) plasma acylated ghrelin levels over time in female rainbow trout (A: two-year-old; B: three-year-old) fed a control-ration or restricted-ration. See Figure 2.2 for an explanation of what each symbol signifies. Figure A: *n*=6 per treatment. Figure B: Week *n*=5 per treatment.

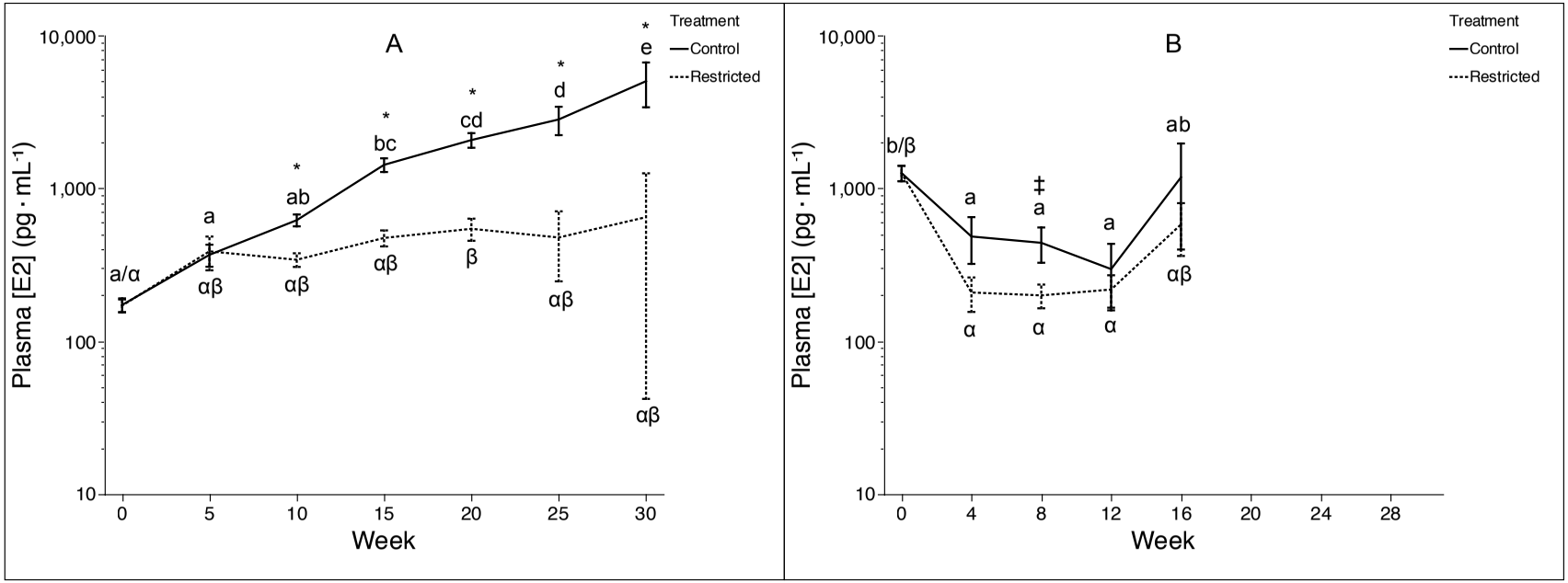

Figure 3.4. **Plasma estrogen concentrations in female rainbow trout recovering from spawning.** Mean (±SEM) plasma 17β-estradiol concentration over time in female rainbow trout (A: two-year-old; B: three-year-old) fed a control-ration or restricted-ration. See Figure 2.2 for an explanation of what each symbol signifies. Figure A: Week 0, *n*=42 per treatment; Week 5, *n*=36 per treatment; Week 10, *n*=30 per treatment; Week 15, *n*=24 per treatment; Week 20, *n*=18 per treatment; Week 25, *n*=12 per treatment; Week 30, *n*=6 per treatment. Figure B: Week 0, *n*=25 per treatment; Week 4, *n*=20 per treatment; Week 8, *n*=15 per treatment; Week 12, *n*=10 per treatment; Week 16, *n*=5 per treatment.

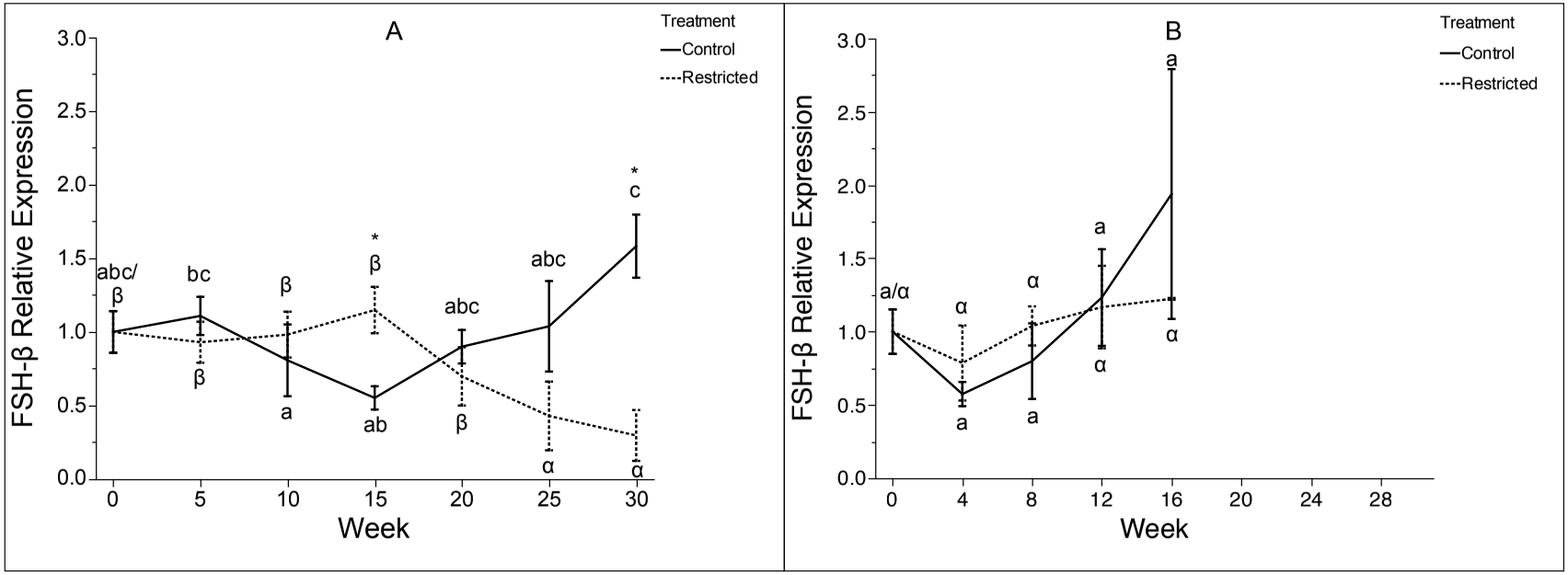

Figure 3.5. **Pituitary *fsh-β* mRNA expression in female rainbow trout recovering from spawning.** Mean (±SEM) normalized ratio of pituitary *fsh-β* to *β-actin* mRNA levels over time in female rainbow trout (A: two-year-old; B: three-year-old) fed a control-ration or restricted-ration. See Figure 2.2 for an explanation of what each symbol signifies. Figure A: *n*=6 per treatment at all time points. Figure B: *n*=5 per treatment at all time points.

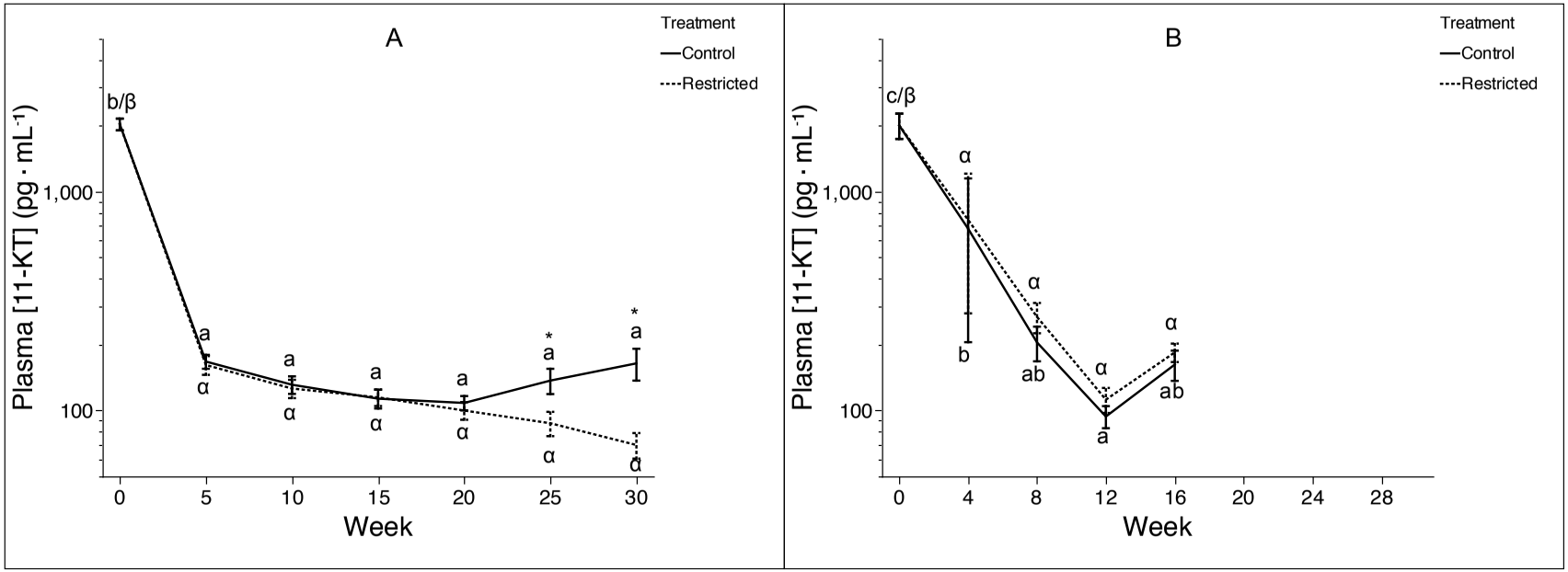

Figure 3.6. **Plasma androgen concentrations in female rainbow trout recovering from spawning.** Mean (±SEM) plasma 11-keto testosterone concentration over time in female rainbow trout (A: two-year-old; B: three-year-old) fed a control-ration or restricted-ration. See Figure 2 for an explanation of what each symbol signifies. Figure A: Week 0, *n*=42 per treatment; Week 5, *n*=36 per treatment; Week 10, *n*=30 per treatment; Week 15, *n*=24 per treatment; Week 20, *n*=18 per treatment; Week 25, *n*=12 per treatment; Week 30, *n*=6 per treatment. Figure B: Week 0, *n*=25 per treatment; Week 4, *n*=20 per treatment; Week 8, *n*=15 per treatment; Week 12, *n*=10 per treatment; Week 16, *n*=5 per treatment.

Gene	Accession Number	Direction	Sequence	Product Size
fsh-b	AB050835	Fwd	AGAGCTGCGATTGCATCAAA	61 bp
		Rev	GCCATGCTTATGCGATCACA	

Table 3.1 – Primer sequence data for q-RT-PCRs.

Chapter 4: "Reconditioned Steelhead Kelts Exhibit Similar Endocrine Biomarkers Compared to In-River Migrating Steelhead"

1. *Introduction*

Oncorhynchus mykiss is a salmonid fish native to northwestern North America that exhibits high levels of variability and plasticity of life history [23,24,263]. *O. mykiss* may live as resident rainbow trout or may adopt one of many life history patterns that involve a migration to the ocean and return to natal stream for spawning (anadromy) [264,265]. Collectively, these anadromous life history forms are known as steelhead. Steelhead are native to the North American west coast and to the interior west of the continental divide, from Alaska to the Mexican Baja California peninsula [23]. Steelhead typically spawn in smaller tributaries that feed mainstem rivers during the months of early spring through early summer [23,263]. All *O. mykiss* are capable of repeat spawning (iteroparity); however, many steelhead do not survive spawning [263]. Steelhead (and other anadromous salmonids) that do survive initial spawning are called kelts. Kelts may recover during the year after initial spawning and spawn again in the following year (consecutive spawners), or they may delay rematuration for one or more years (skip-spawners) [33,187].

Repeat spawning steelhead are ecologically important for two reasons: 1) life history diversity within a species may buffer populations against otherwise potentially catastrophic effects of stochastic events [266], and 2) repeat-spawning fish often contribute more than twice the lifetime reproductive output of once-spawning fish, and thus represent a way to sustain and possibly recover threatened or endangered

populations [263]. As salmonids grow and age, the potential lifetime reproductive output of a given individual increases at a rate greater than predicted by fecundity at the initial spawning, due to two inherent features of salmonids: indeterminate growth and size-dependent fecundity. All salmonids exhibit indeterminate growth, meaning that they continue to increase in size throughout their life; theoretically, there is no upper limit to attainable body size, given enough food and protected from predation and disease [50]. Salmonids also exhibit size-dependent fecundity, meaning that the bigger a fish grows, the more gametes it produces [267]. Thus, when steelhead survive initial spawning and grow before the next spawning season, those kelts that do repeat spawn contribute more than twice the number of offspring in their lifetime than fish that spawn only once [263]. The potential ecological contribution of these repeat spawning kelts in rebuilding threatened wild populations is considerable.

In addition to differences in migratory behavior, one major difference between rainbow trout and steelhead life history forms is the degree to which individuals repeat spawn (parity) in the wild. Resident rainbow trout are highly iteroparous: approximately 25-50% of resident trout survive initial spawning and spawn multiple times throughout their life [267]. Historical records of iteroparity rates among steelhead are unreliable, but steelhead may have exhibited iteroparity rates as high as 20-40% in certain systems [268]. Atlantic salmon (*Salmo salar*) is a closely related species that provides a comparable model for steelhead. Unlike Pacific salmon, Atlantic salmon are iteroparous: in the wild as many as 10-20% may survive the initial spawning effort to repeat spawn, and there are reports of individual fish surviving to spawn up to six times in the wild [29]. Steelhead iteroparity rates have decreased throughout the Columbia River Basin, but

especially in upper watersheds like the Yakima River Basin, largely due to anthropogenic factors [38]. Current estimates of repeat spawning rates for steelhead in the Columbia River Basin are low (0.5-9%) [268]. Female kelts have a higher survival rate after initial spawning and generally contribute more than males towards population growth rates [263].

Although most kelts attempt outmigration (perhaps as many as 70%), few actually return to spawn repeatedly [36,37]. One strategy that has been tested to increase returns of salmonids [31,269,270,271,272,273,274], and that has been applied to steelhead in the Columbia River Basin [36,37,38] involves capturing post-spawned steelhead during the spring, when they are oumigrating downstream, holding and feeding them in land-based tanks while they recover from spawning, and then releasing them in autumn with the assumption that these fish will migrate back upstream and spawn again [38]. These programs are known as "reconditioning." Previous work has shown that kelt reconditioning produces a substantial proportion of rematuring kelts, that reconditioned kelts spawn in the wild, and that these spawning efforts produce viable progeny [37]. However, direct comparisons of the reproductive status of reconditioned kelts and natural-origin, in-river migrating steelhead (IRMS) have not been conducted. As the efficacy and long-term sustainability of reconditioning programs have been contested [263,275], comparisons between reconditioned kelts and IRMS are needed, to show that steelhead kelt reconditioning programs generate fish that are physiologically comparable to natural-origin steelhead undertaking spawning migrations, and to help justify further pursuance of kelt reconditioning as a conservation measure. Also, improvements to reconditioning methods could be made if it is possible to early and accurately separate

fish into groups based on likelihood of rematuring, with special care or additional treatments being provided solely to fish that require such action. It seems probable that endocrine signals or physical parameters differ between kelts that will remature and those that will not during the upcoming spawning season. Identification of these metrics could provide markers that could be used to both increase the proportion of successfully rematured kelts and to focus efforts and resources on fish that need and would respond to costly treatments.

The goals of this study were to monitor the reproductive status steelhead kelts from the YRB reconditioned at Prosser, WA; to directly compare the reproductive and energetic status of reconditioned kelts and IRMS moving upriver at the time of kelt release following reconditioning in October; and to explore relationships between parameters measured in October and VSP segments as determined by tracking for both IRMS and reconditioned kelt steelhead. We combined plasma hormone assays, physical measurements, and post-release tracking data for steelhead in the reconditioning program at Prosser, WA, to determine how early kelt rematuration can be detected, what parameters accurately predict rematuration, and which physical and endocrine parameters differ among steelhead from the different VSP segments within the YRB. We hypothesized that kelts would be bigger than IRMS, which are predominantly maiden fish. Also, since reconditioned kelts do not undergo the long-distance spawning migration that in-river fish do, we hypothesized that reconditioned kelts would be in better condition, as indication by Fulton's condition factor (k), and would have greater concentrations of muscle lipid. Since evidence suggests that continuous swimming can inhibit reproductive investment by diverting energy towards somatic maintenance

[20,276], we hypothesized that reconditioned kelts would exhibit plasma levels of 17β-estradiol, the primary fish estrogen and an established marker of reproductive development in *O. mykiss* (Chapter 2) that were higher than IRMS. In salmonid fishes and other lecithotrophic vertebrate, 17β-estradiol stimulates liver synthesis of the egg precursor protein vitellogenin [277,278], which circulates *via* the blood and is taken up by developing ovaries to fuel oocyte growth [279].

A substantial literature documents many aspects of salmonid physiology and endocrinology. However, most of this work describes changes in organismal biology occurring during initial sexual maturation, often using hatchery-origin fish in a laboratory or aquaculture setting [280,281]. A smaller portion of work has examined endocrine and other physiological processes during maturation and spawning migrations of natural-origin salmonids [282,283,284,285]. Much less work has focused on the physiology and endocrinology of recovery from spawning and gonadal recrudescence preceding repeat spawning efforts. To our knowledge, this is the first study comparing physiological and endocrine factors between natural-origin steelhead recovering from spawning and natural-origin IRMS.

2. ***Materials and Methods***

2.1.A. *Steelhead Kelt Collection, Husbandry, Handling, Sampling, Release:*

The Yakima River Basin (YRB) encompasses the Yakima River and associated tributaries located in south-central Washington [38] (Fig. 4.1). The YRB drains a 1.6M

hectare area including high and wet Cascade Mountain forests, transitional eastern Cascade slopes, and low and dry Columbia Basin shrub steppe, before flowing into the Columbia River near Richland, WA [38,286]. Steelhead returning to the Yakima River spawn in the tributaries upstream of Prosser Dam, including Satus Creek, Toppenish Creek, and the many first and second order tributaries located in the upper reaches of the YRB [286]. In late spring and early summer, YRB steelhead kelts outmigrate down the mainstem Yakima and ultimately encounter Prosser Dam, where some of the fish (less than 50%) are diverted into the Chandler Juvenile Monitoring Facility (CJMF). Inside CJMF, a separator screen sorts juvenile smolts from adult kelts, and the latter are evaluated for inclusion in the reconditioning program located on-site. Detailed methods associated with collection, husbandry, sampling, and release of kelts at the Prosser, WA facility have been described previously [38]. After separation, kelts were transferred to a temporary holding tank, anesthetized with buffered tricaine methanesulfonate, weighed, measured for fork length (FL), and implanted with a uniquely coded PIT tag injected into the pelvic girdle [287,288,289]. Fish are then treated for parasitic gill copeopods with emamectin or ivermectin, and transferred to covered 23 m^3 circular tanks fed 13.8ºC well water at a rate of 570-950 L/min. Kelts were started on frozen krill for 4-6 weeks, after which 6.0-mm pellet feed (Bio-Oregon, Longview, WA) was introduced. Feed was administered 3–5 times daily, and fish were fed until satiation, corresponding to a rate of 1–2% body weight per day. In October prior to release, reconditioned steelhead were weighed, measured for FL, measured for muscle lipid content with a microwave-based fatmeter (Fish Fatmeter, Distell, Fauldhouse, UK), blood sampled, and scanned for PIT tags.

2.1.B. *Sampling of In-River Migrating Steelhead*

A Denil-type fish passage ladder [290,291] is in place at Prosser Dam [292], allowing upstream migrating fishes to navigate what would otherwise be an impassible structure. During the upriver spawning migration in autumn months (26 September-19 November), natural-origin IRMS were captured as they negotiated this structure, and sampled on site. IRMS were weighed, measured for FL, measured for muscle lipid content with the fatmeter, blood sampled, and scanned for PIT tags. If no tag was detected, fish were implanted with a uniquely coded PIT tag injected into the pelvic girdle. Measurements were taken and samples collected from kelts and IRMS at slightly different times. However, all fish were sampled within approximately one month of each other (October 2012), during what was their upstream (spawning) migration, making these fish comparable.

2.2. *Tracking*

Two remote instream PIT detection antenna arrays are located below the known spawning distributions of the Satus Creek and Toppenish Creek populations. The Toppenish Creek array is located approximately 400 m upstream and the Satus Creek array is located approximately 2.5 km upstream from their respective confluences with the Yakima River. Additionally, all fish are trapped and scanned for PIT tags during upstream migration through Roza Dam, north of Yakima, WA. Based on PIT array detections, fish were divided into VSP segments corresponding to tributaries in which

they appear to spawn; all fish detected at or above Roza dam are considered Upper Yakima fish. There were no PIT arrays operating during 2012 in the Naches River, the largest VSP segment of the Yakima steelhead population. Tracking data was also used to infer behaviors congruent with successful spawning and survival. If a fish was detected migrating past an upstream array at the mouth of a tributary during the spawning season, and spent at least two weeks in that tributary before being detected migrating past the downstream array at the tributary mouth, it was presumed that these fish 1) spawned in that tributary, 2) survived the spawning effort, and 3) swam downstream out of the tributary into the mainstem of the Yakima River. Most fish that were detected migrating downstream after entering a tributary had spent more than 2 weeks in that stream; a very small number of fish spent only 2-5 days in the tributary, which is hard to reconcile with spawning behavior [293]. Also, many fish were only detected migrating past the upstream arrays; while these fish may have successfully spawned and survived the spawning effort, numerous alternate hypotheses could describe these behaviors, which prevented us from drawing conclusions about post-spawn survival of such fish. Using this PIT tag data, it is impossible to infer failed spawning and death with any reasonable measure of specificity, but it may be possible to sensitively infer success and survival.

2.3. *Plasma hormone assays*

Blood plasma was obtained from whole blood samples by centrifugation, after which plasma samples were immediately frozen on dry ice until returning to University of Idaho, where samples were stored at -80ºC until analysis. Per kit recommendations, all

samples were ether extracted prior to assay, as described in Chapter 2. Plasma 17β-estradiol was measured by EIA (Cayman Chemical Company, Ann Arbor, MI; Item No. 582251), following kit directions; samples were measured in duplicate. Plasma vitellogenin was measured by homologous EIA (Biosense, Bergen, Norway; Product No. V01004402), as previously described [37]; samples were measured in duplicate.

2.4. *Statistical analysis*

197 steelhead kelts that survived reconditioning until release in autumn were included in analyses. However, not all measurements were available for each individual at every sampling point. 171 kelts were measured for fork length and mass at intake; all 197 kelts were blood sampled and measured for fork length, mass, and fatmeter reading, at release. In some cases, total blood drawn was insufficient for all assays; therefore, 196 kelts were assayed for plasma 17β-estradiol, but only 185 kelts were assayed for plasma vitellogenin. 46 reconditioned kelts were detected at PIT arrays upstream of the release site in tributaries of the Yakima River.

129 putative female IRMS were sampled at the Denil trap near Prosser, WA in autumn 2012. Of these fish, 2 appeared to be males based on extremely low plasma 17β-estradiol and secondary sex characteristics (coloration, head morphology) noted during sampling. Of the 127 IRMS included in analyses, all were blood sampled and measured for fork length, mass, and fatmeter reading. In some cases, total blood drawn was insufficient for all assays; therefore, all 127 IRMS were assayed for plasma 17β-estradiol, but only 125 IRMS were assayed for plasma vitellogenin.

Plasma concentrations of 17β-estradiol and vitellogenin were $\log_{10}$-transformed prior to analysis.

Reconditioned kelts were categorized as rematuring or non-rematuring based on multivariate cluster analysis. Ward's minimum variance method [294] was used to hierarchically group fish into one of two clusters (rematuring and non-rematuring) based on plasma 17β-estradiol and vitellogenin. Cutoffs for inclusion as rematuring were identified to be 1 ng/ml 17β-estradiol and 0.4 mg/ml vitellogenin.

A multivariate correlation analysis was performed to determine the relationship of measurements taken at release among both rematuring and non-rematuring reconditioned kelts. Pearson's correlation coefficients and associated significance probabilities were generated for each pairwise relationship among the parameters measured at release.

Based on the cluster analysis groupings generated using plasma 17β-estradiol and vitellogenin, parameters measured in rematuring kelts were compared directly to those measured in both non-rematuring kelts and IRMS. As these comparisons were used to test hypotheses about two groups for which we had no prior hypotheses about the direction of differences, and as the two pairs of groups are orthogonal, two-tailed *t*-tests were used.

In cases where more than two groups were simultaneously compared, ANOVA was used to detect significant differences; in the event of a significant ANOVA ($p<0.05$), Tukey's HSD multiple comparison tests were used to determine which groups were different. Differences are reported as significant when $p<0.05$. Condition factor was calculated as described in Chapter 1. All statistical analyses were performed using JMP9 (SAS Institute, Cary, NC). All ranges provided refer to mean ± SD.

3. ***Results***

3.1. *Reconditioned kelts from Prosser 2012:*

Steelhead kelts reconditioned during summer 2012 at the Prosser, WA facility robustly and discretely separated into two groups based on the multivariate clustering analysis using both plasma 17β-estradiol and plasma vitellogenin: rematuring fish exhibited plasma 17β-estradiol > 1 ng/mL and plasma vitellogenin > 0.5 mg/mL (Fig. 4.2). During this year, 148/185 (80%) reconditioned kelts that survived until release, and were assayed for both 17β-estradiol and vitellogenin, had rematured. When 11 additional kelts were categorized based on plasma 17β-estradiol data only ([17β-estradiol] > 1 ng/ml), 8 additional kelts appeared to be rematuring and 3 appeared to be non-rematuring, making the total 156/196 (80%).

3.1.a. *Parameters measured in reconditioned kelts at intake*:

Kelts that ultimately rematured entered the reconditioning program bigger than kelts that did not remature (Fig. 4.3). Rematuring kelts were 4% longer (a mean difference of 2.5 cm; two-tailed *t*-test, $p = 0.02$) and 15% heavier (a mean difference of 290 g; two-tailed *t*-test, $p = 0.007$) than non-rematuring kelts at intake. Condition factor at intake was similar between rematuring and non-rematuring kelts: rematuring kelts had 2% higher condition factor than non-rematuring kelts at intake (a mean difference of 0.2;

two-tailed *t*-test, $p = 0.49$). Rematuring and non-rematuring kelts arrived at Prosser at similar times (mean intake date of rematuring kelts was April 29 ± 22 days, mean intake date of non-rematuring kelts was April 27 ± 24 days; two-tailed *t*-test, $p = 0.60$).

3.1.b *Parameters measured in reconditioned kelts at release:*

Kelts that rematured during reconditioning grew faster than kelts that did not remature (Fig. 4.4); upon release after reconditioning, rematuring kelts were bigger, fatter, and of higher condition factor than kelts that did not remature (Fig. 4.5). Rematuring kelts had 32% greater SGR than non-rematuring kelts over the course of reconditioning (two-tailed *t*-test, $p < 0.0001$), and were 7% longer than non-rematuring kelts (a mean difference of 4.6 cm; two-tailed *t*-test, $p < 0.0001$), 41% heavier (a mean difference of 1,084 g; two-tailed *t*-test, $p < 0.0001$), had 26% more muscle lipid content (a mean difference of 1.1% lipid; two-tailed *t*-test, $p = 0.005$) and 15% higher condition factor (a mean difference of 1.5; two-tailed *t*-test, $p < 0.0001$) than non-rematuring kelts at release.

Among non-rematuring reconditioned kelts, there was a significant positive pairwise correlation between CF and both plasma vitellogenin and plasma 17β-estradiol, between SGR and plasma vitellogenin, and between fish mass and plasma 17β-estradiol at the time of release (Table 1). Among rematuring reconditioned kelts, there was a significant positive pairwise correlation between most physical measurements and both plasma vitellogenin and plasma 17β-estradiol at the time of release (Table 2).

3.1.c *Comparisons within Reconditioned Kelts by VSP Segment*:

All 43 kelts detected entering spawning tributaries were rematuring as determined by plasma 17β-estradiol and vitellogenin, so it is impossible to evaluate reconditioning success as a factor of VSP segment.

Date of intake for reconditioning (outmigration timing) was significantly different among kelts from the different VSP segments (ANOVA, $p < 0.0001$). Satus Creek kelts arrived on their post-spawn, downstream (seaward) migration earliest, followed by Toppenish Creek kelts, with Upper Yakima kelts arriving last (Fig. 4.6). Kelts outmigrating from Satus Creek had a mean arrival date at Prosser Dam of April 6 ± 10 days, two and a half weeks earlier than kelts outmigrating from Toppenish Creek (April 24 ± 15 days, Tukey's HSD, $p < 0.0002$) and over a month earlier than kelts outmigrating from tributaries in the Upper Yakima (May 11 ± 1 day, Tukey's HSD, $p < 0.0002$). Run timing was similar between kelts outmigrating from Toppenish Creek and kelts from tributaries in the Upper Yakima (Tukey's HSD, $p < 0.13$). Following release in autumn after reconditioning, the date of first detection at the mouth of the respective tributaries upstream of Prosser Dam was similar among kelts from the different VSP segments (ANOVA, $p = 0.16$).

3.2. *Comparison of Reconditioned Kelts to Wild Migrating Steelhead:*

Rematuring kelts were bigger and had higher circulating levels of estrogen compared to IRMS (Fig. 4.5). Compared to IRMS, rematuring kelts were 3.5% longer (a mean difference of 2.3 cm; Tukey's HSD $p = 0.005$), 35% heavier (a mean difference of 0.95 kg; Tukey's HSD $p < 0.0001$), 43% fatter (mean difference of 1.6%; Tukey's HSD $p < 0.0001$), exhibited 22% higher condition factor (mean difference of 1.03; Tukey's HSD $p < 0.0001$), 152% higher plasma 17β-estradiol (mean difference of 17.5 ng/mL; Tukey's HSD $p < 0.0001$), and similar plasma vitellogenin levels (rematuring kelts plasma vitellogenin = 12.960 ± 0.508 mg/mL, IRMS plasma vitellogenin = 12.819 ± 0.555 mg/mL; *i.e.*, rematuring reconditioned kelts had 1% higher plasma vitellogenin than IRMS; Tukey's HSD, $p = 0.98$). Compared to IRMS, non-rematuring reconditioned kelts were similar in length (non-rematuring kelts = 63.2 ± 1.0 cm, IRMS = 65.2 ± 0.6 cm; Tukey's HSD, $p = 0.16$), mass (non-rematuring kelts = 2.6 ± 0.1 kg, IRMS = 2.7 ± 0.1 kg; Tukey's HSD, $p = 0.78$), and fatmeter (non-rematuring kelts = 4.2 ± 0.3 %, IRMS = 3.7 ± 0.2 %; Tukey's HSD, $p = 0.26$); however, non-rematuring kelts had higher condition factor (non-rematuring kelts = 10.2 ± 0.2, IRMS = 0.6 ± 0.1; Tukey's HSD, $p = 0.049$), lower plasma 17β-estradiol (non-rematuring kelts = 0.196 ± 4.4 ng/mL, IRMS = 24.6 ± 2.5 ng/mL; Tukey's HSD, $p < 0.0001$), and lower plasma vitellogenin (non-rematuring kelts = 0.043 ± 1.015 mg/mL, IRMS = 12.819 ± 0.555 mg/mL; *i.e.*, IRMS had 297-fold higher plasma vitellogenin than non-rematuring kelts; Tukey's HSD, $p < 0.0001$).

3.3. *In-River Migrating Steelhead – Comparison of VSP Segments*

When sampled at the Prosser Dam Denil trap in October 2012, IRMS from the various VSP segments exhibited similar values for fork length (ANOVA, $p = 0.31$), fish mass (ANOVA, $p = 0.24$), fatmeter (ANOVA, $p = 0.68$), plasma 17β-estradiol (ANOVA, $p = 0.28$), and plasma vitellogenin (ANOVA, $p = 0.22$). IRMS destined for the Upper Yakima (including IRMS that were detected entering Swauk, Taneum, and Teanaway Creeks) exhibited higher condition factor than fish destined for tributaries lower in the basin (Fig. 4.7). Upper Yakima IRMS exhibited 7% higher condition factor compared to IRMS destined for Satus Creek (mean difference of 0.32; Tukey's HSD, $p = 0.02$) and 7% higher condition factor compared to IRMS destined for Toppenish Creek (mean difference of 0.31; Tukey's HSD, $p = 0.04$).

If IRMS and reconditioned kelts are pooled, then first detection upstream reflects general expectations based on migration logistics (Fig. 4.8). Satus Creek steelhead and Toppenish Creek steelhead both arrived at their respective tributaries on a similar date (Feb 9 ± 45 days and Jan 25 ± 40 days, respectively; Tukey's HSD, $p = 0.24$), which was significantly earlier (Tukey's HSD, $p \leq 0.0002$) than steelhead destined for the Upper Yakima (March 30 ± 19 days).

4. *Discussion*

Of 196 female steelhead kelts released after reconditioning at Chandler Juvenile Monitoring Facility (CJMF) in October 2012, 80% were rematuring, as determined by

plasma levels of the estrogen 17β-estradiol and the yolk precursor protein vitellogenin. This is the highest maturation percentage recorded to date at this facility [38]. Compared to IRMS migrating upstream in October, reconditioned kelts that had rematured were longer and heavier, and exhibited higher plasma 17β-estradiol and similar plasma vitellogenin. Increases in length and mass through the reconditioning program may seem like an obvious result of kelts being at least one year older than IRMS. However, it should be noted that non-rematuring reconditioned kelts were similar to IRMS in both length and mass, suggesting that significant growth is not the inevitable outcome of aging among steelhead kelts. From a project standpoint, it is assuring that reconditioned kelts accrue muscle lipid and exhibit both higher condition factor and higher plasma 17β-estradiol than IRMS.

To our knowledge, this is the first report of a physiological comparison between rematuring kelts and IRMS, the latter of which comprise a majority of maiden fish along with a much smaller proportion (approximately 1.6%) of naturally occurring kelts [292]. For the purposes of evaluating steelhead kelt reconditioning projects throughout the Columbia River Basin, the most relevant physiological comparisons are between reconditioned and naturally occurring steelhead kelts. However, the low incidence and prevalence of natural kelts in the Upper Columbia River, along with the technical challenges associated with non-lethally determining spawning history for a given fish, logistically preclude such a comparison. The next most salient comparison is between reconditioned kelts and in-river steelhead that are migrating upstream, collected from similar locations, and sampled at similar stages of reproductive development. Due to very low numbers of naturally occurring repeat-spawning steelhead kelts in the Upper

Columbia River, we have assumed that all IRMS thus sampled are maidens. However, this group may contain a small number of naturally occurring repeat spawning steelhead kelts.

Circulating estrogen and egg protein levels unequivocally show that the reconditioned steelhead kelts determined to be rematuring were developing on a trajectory congruent with reproduction in the following spring. However, concentrations of these plasma biomarkers do not enable complete separation between rematuring and non-rematuring kelts until at least late summer [36]. Earlier determination of the particular reproductive trajectory fish are on could help improve both efficacy and efficiency of reconditioning programs. To better understand the factors that influence rematuration and associated biomarkers, measurements at intake, when outmigrating kelts are captured at CJMF after spawning, were compared between kelts that ultimately rematured after reconditioning and those that did not, to assess parameters that could be used to predict rematuration. Kelts that ultimately rematured were longer and heavier at intake, possibly reflecting greater somatic energy reserves that are not detectible by measuring muscle lipid content. Previous work has shown that longer steelhead kelts [295] and Atlantic salmon [296] have higher somatic energy reserves compared to shorter fish. Although perhaps underwhelming, this finding suggests that reconditioning practices increasing growth among kelts should also improve rematuration percentages.

After spawning in the spring, outmigrating kelts arrive at CJMF in discrete groups. Date of intake for reconditioning was significantly different among kelts from the different viable salmon population (VSP) segments, with Satus Creek kelts arriving earliest, followed by Toppenish Creek kelts, and later by Upper Yakima kelts. Spawn

timing in the VSP segments generally follows elevation, with Satus Creek fish spawning earliest, followed by fish from Toppenish Creek and then the fish from the Upper Yakima [292]. The arrival date of kelts at CJMF is consistent with this pattern: Steelhead that spawn in lower elevation tributaries closer to Prosser Dam arrive earlier, while steelhead that spawn at higher elevations in the Yakima basin further from Prosser Dam arrive at CJMF later. Kelts that remature during reconditioning are longer and heavier when they are collected at CJMF during their post-spawn downstream migration in the spring.

When sampled on their upstream migrations during the autumn prior to spawning, IRMS from the various VSP segments in the upper Yakima generally exhibited similar physical and endocrine measurements. However, fish undergoing longer migrations (*i.e.*, fish that were detected later) had higher condition factor when sampled at the Prosser Denil trap, although fatmeter readings did not differ among the IRMS VSP segments (data not shown). Fish undergoing longer migrations are likely subjected to strong selection pressures for acquiring substantial energy reserves in order to successfully complete a spawning migration [20,21,297]. It may be the case among steelhead that body shape reflects a more integrated measure of energy reserves, which include hepatic lipids and glycogen, visceral adipose tissue, and belly flap lipids, in addition to muscle lipid stores, the only energy reserve assessed with the fatmeter [298].

As a group, salmonids exhibit a synergistic combination of indeterminate growth [50] and size-dependent fecundity [17]. Together, these traits are responsible for an emergent phenomenon: repeat-spawning salmonids contribute more than double the number of offspring in their lifetime than salmonids who spawn only once [263]. Repeat-spawning steelhead thus have the potential to contribute meaningfully toward population

recovery. Ensuring the survival of repeat and skip-spawning steelhead kelts will also contribute to the maintenance of salmonid life history diversity, an ecological property thought to buffer populations against devastating crashes associated with life in a stochastic environment [299,300,301]. For these reasons, kelt reconditioning programs that generate repeat spawning females are a viable short-term strategy to mitigate the present decline in steelhead populations throughout the Columbia River Basin. This work provides the first known comparison of reconditioned steelhead with natural-origin in-river migrating steelhead (IRMS), and shows that reconditioning projects yield bigger and fatter fish, with higher circulating levels of estrogen and similar circulating levels of vitellogenin compared to IRMS, and that those reconditioned kelts determined to be rematuring by plasma 17β-estradiol and vitellogenin analysis do behave congruently with repeat spawning in the Upper Yakima River.

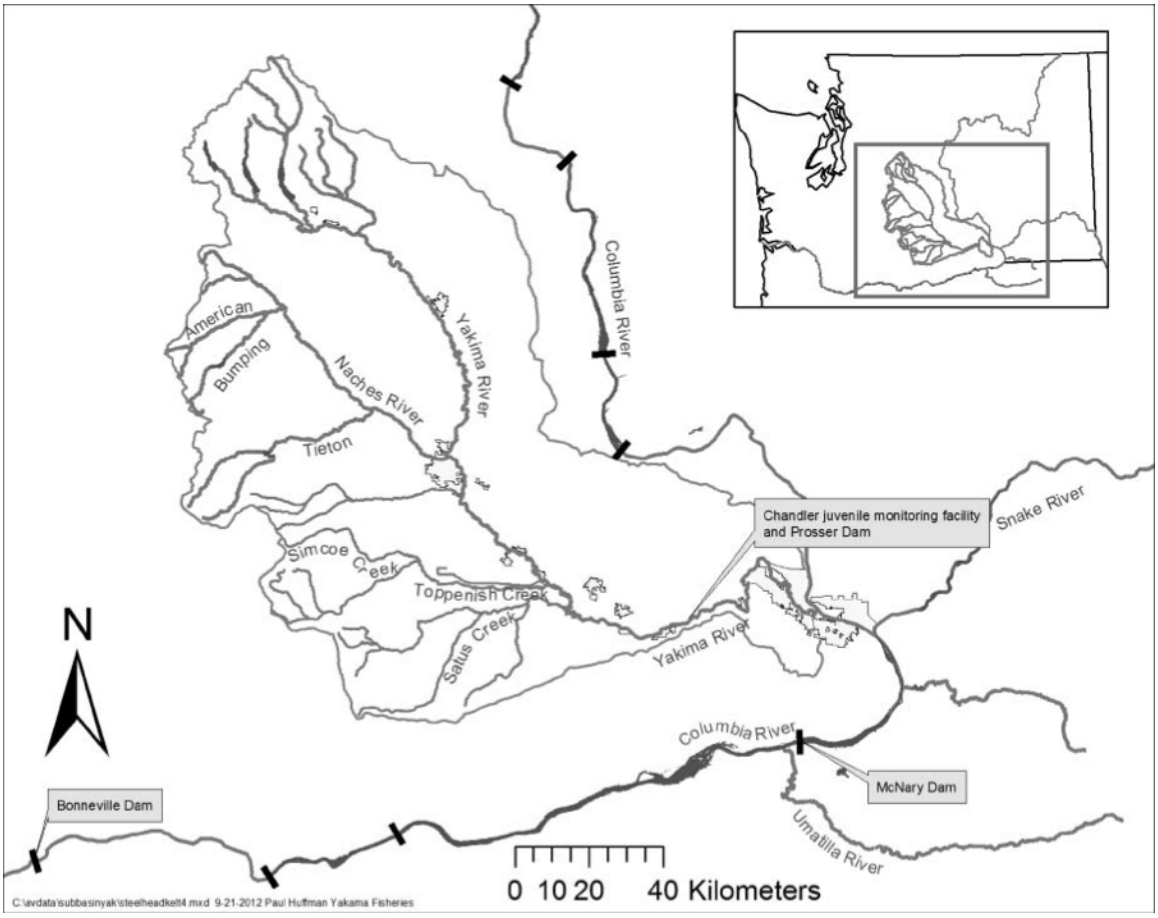

Figure 4.1. Map of Yakima River Basin, including location of kelt reconditioning facility at Chandler Juvenile Monitoring Facility, near Prosser, WA. From Hatch (2013).

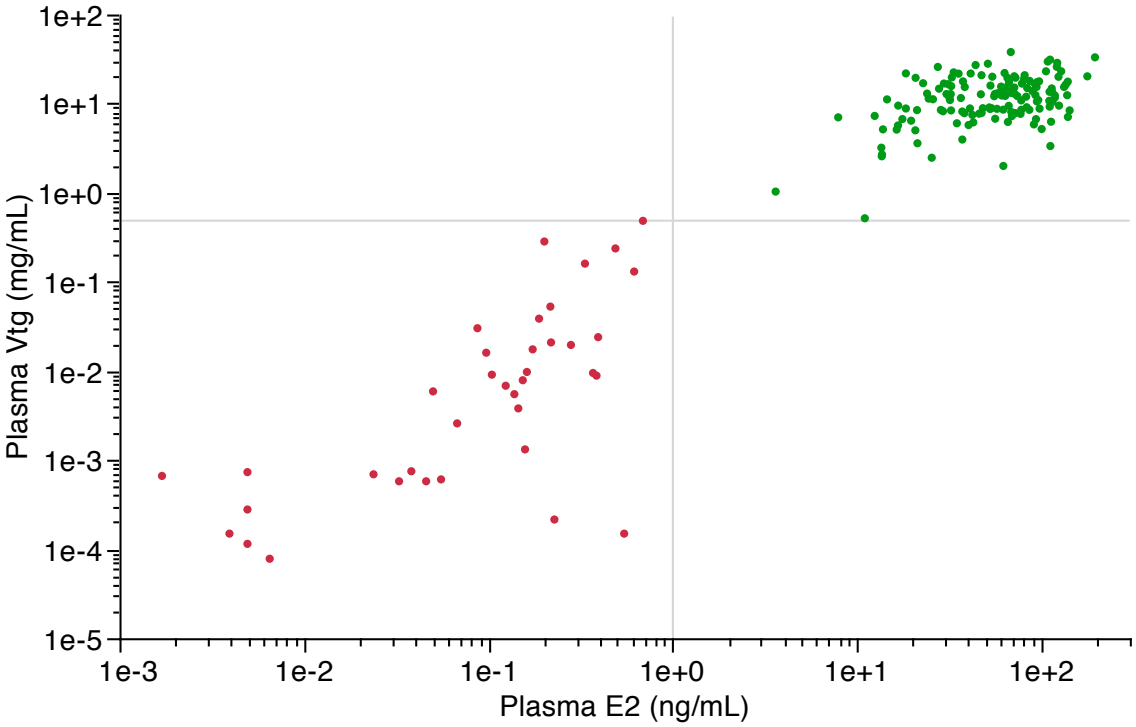

Figure 4.2. Plasma concentration of vitellogenin plotted as a factor of plasma concentration of 17β-estradiol in kelts reconditioned at the Prosser, WA facility during 2012. Red indicates fish determined to be non-rematuring, and green indicates fish determined to be rematuring, based on multivariate cluster analysis. Estimated cutoff concentrations of 1 ng/mL for 17β-estradiol and 0.4 mg/mL for vitellogenin are indicated by grey lines.

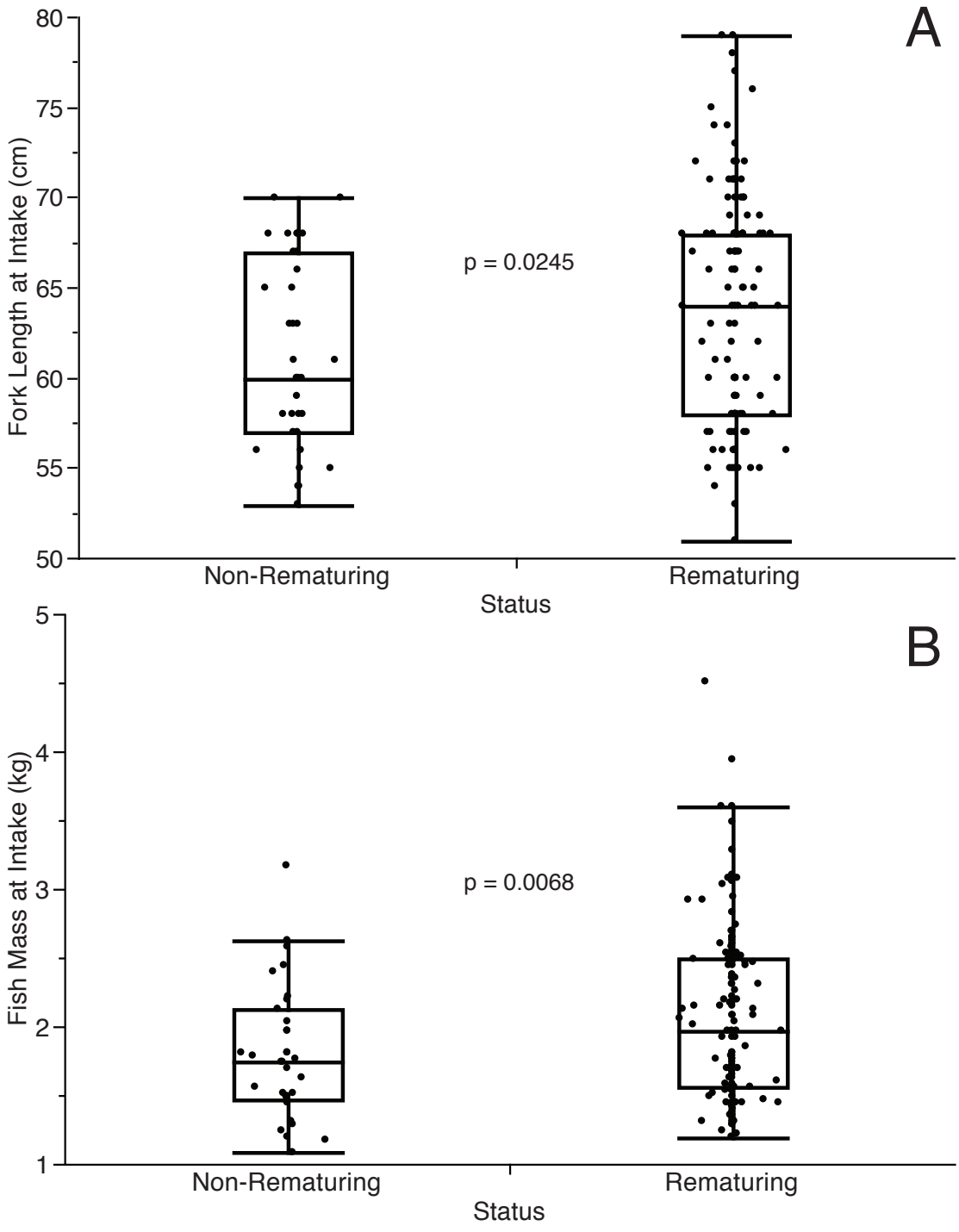

Figure 4.3. Comparisons of fork length (A) and total fish mass (B) at intake in Spring 2012 between reconditioned kelts that ultimately rematured and those that did not at Prosser, WA.

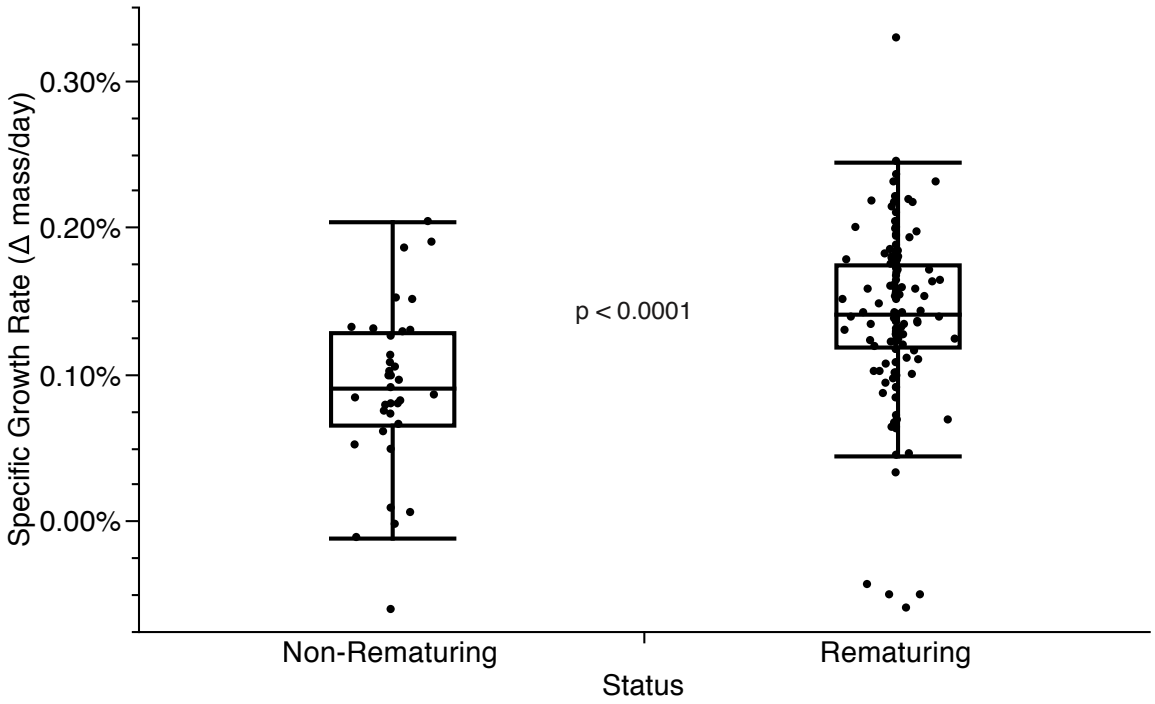

Figure 4.4. Comparison of growth rate during 2012 reconditioning between kelts that ultimately rematured and those that did not at Prosser, WA. 2-tailed *t*-test, $P<0.0001$.

γ

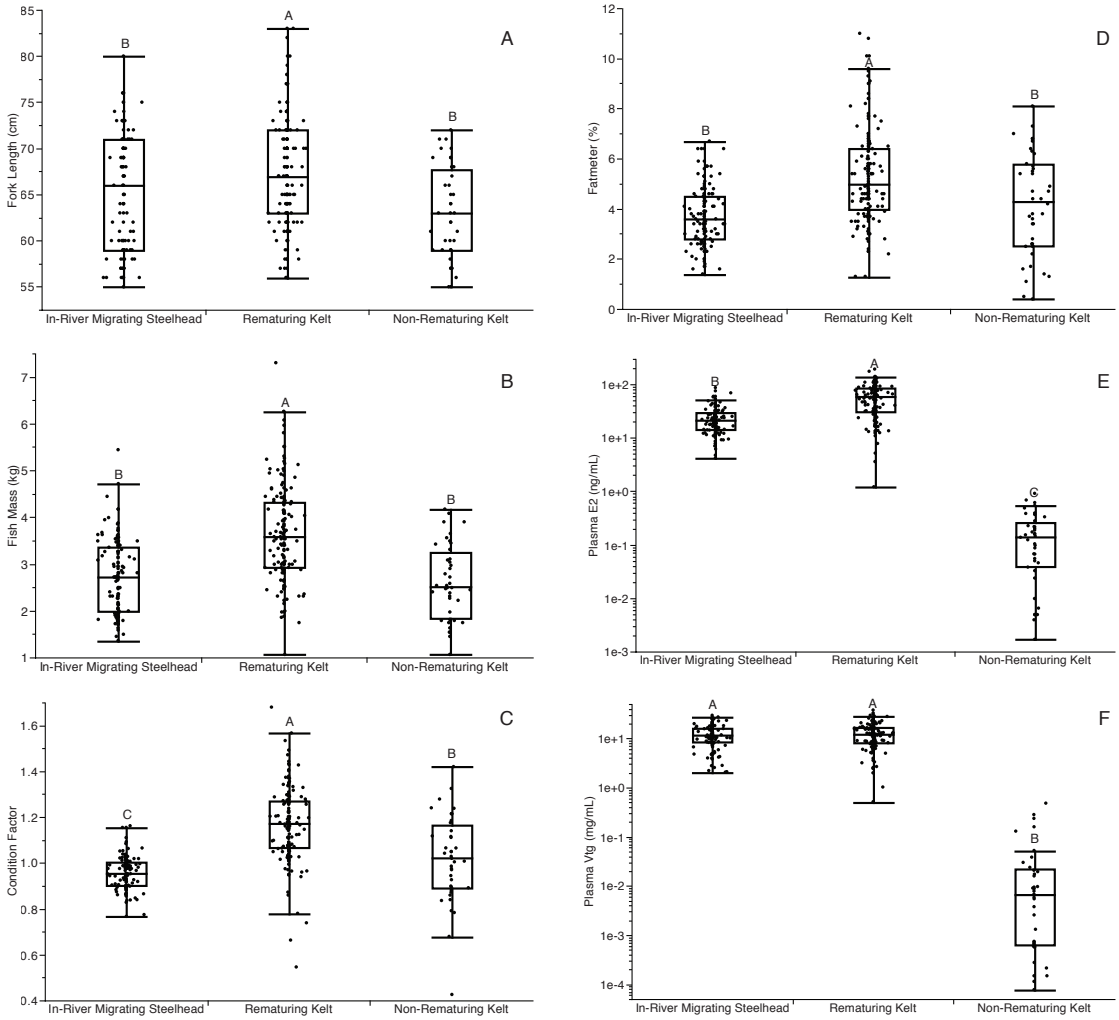

Figure 4.5. Comparison of fork length (A), fish mass (B), condition factor (C), fatmeter (D), plasma 17β-estradiol (E), and plasma vitellogenin (F) measured in Autumn 2012 among IRMS, reconditioned kelts that rematured, and reconditioned kelts that did not remature. Groups sharing a letter are not significantly different.

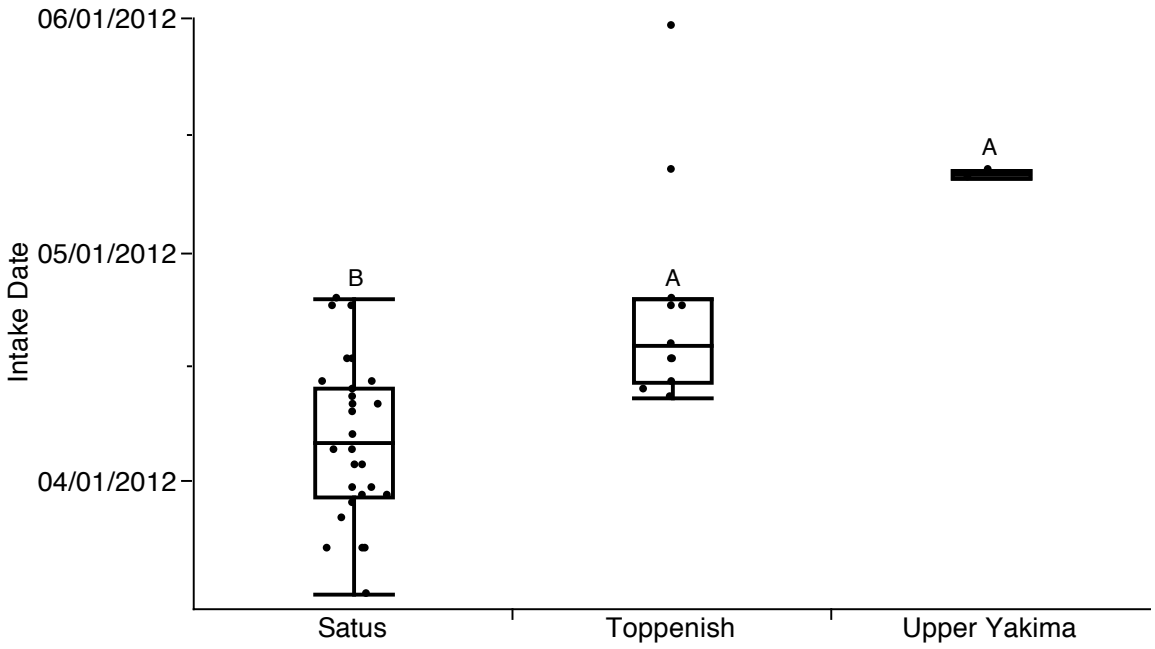

Figure 4.6. Comparison of intake date for kelts entering the reconditioning program at Prosser, WA, by VSP segment. Groups sharing a letter are not significantly different.

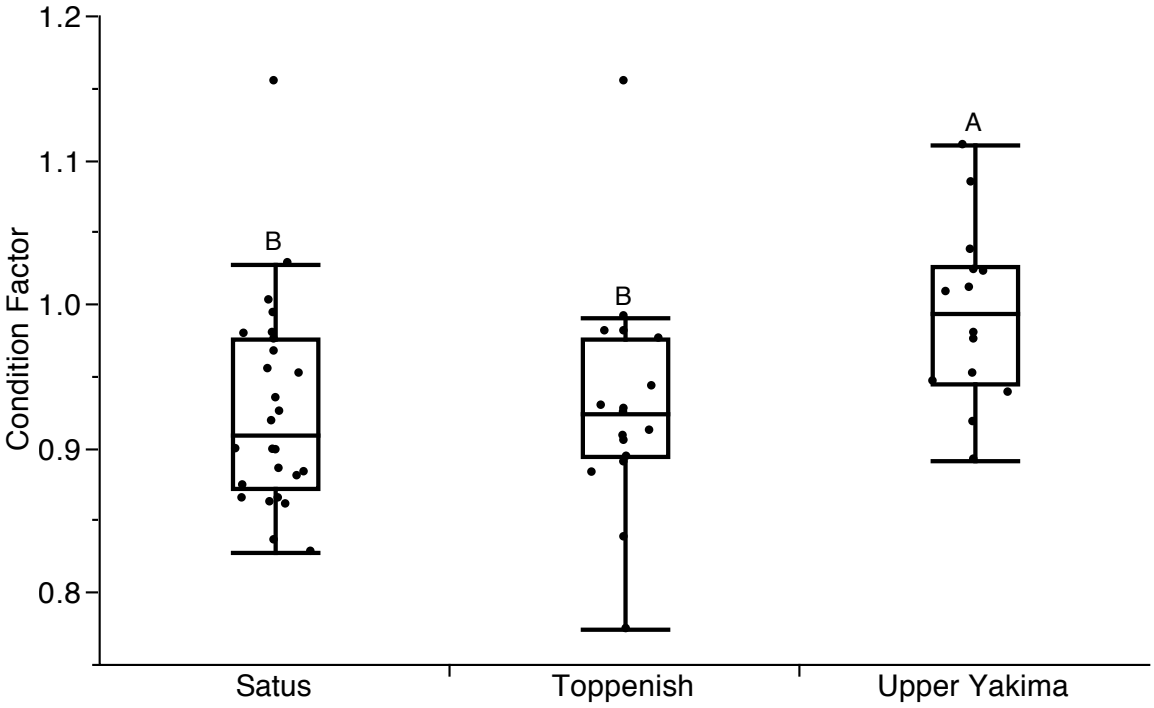

Figure 4.7. Comparison of condition factor among VSP segments of IRMS. Groups sharing a letter are not significantly different.

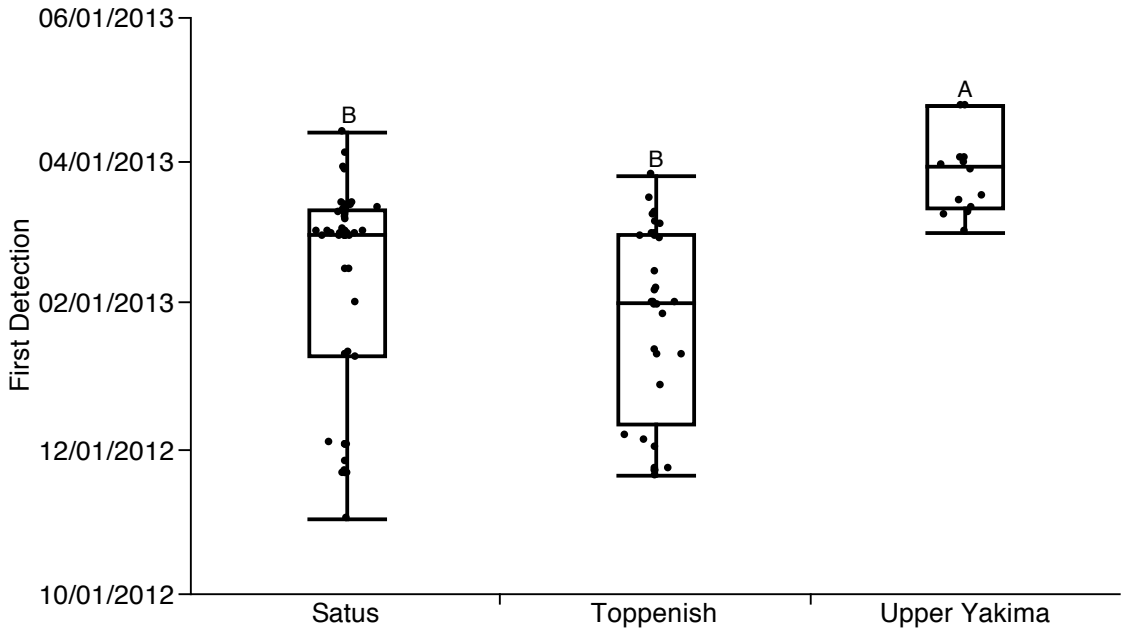

Figure 4.8. Comparison of first upstream detection among VSP segments of combined IRMS and reconditioned kelts. Groups sharing a letter are not significantly different.

	FL at Release	Mass at Release	SGR during reconditioning	*k* at release	Fatmeter at release	Plasma E2 at release	Plasma vtg at release
FL at Release	1.0000	**0.7986**	0.1545	0.0636	0.1054	0.1403	-0.0301
Mass at Release	**<0.0001**	1.0000	**0.5107**	**0.6401**	**0.5254**	**0.3208**	0.2182
SGR during reconditioning	0.3757	**0.0017**	1.0000	**0.7933**	**0.8049**	0.2986	**0.4041**
***k* at release**	0.6965	**<0.0001**	**<0.0001**	1.0000	**0.8071**	**0.3452**	**0.4270**
Fatmeter at release	0.5176	**0.0005**	**<0.0001**	**<0.0001**	1.0000	0.3025	**0.4194**
Plasma E2 at release	0.3879	**0.0435**	0.0815	**0.0291**	0.0578	1.0000	**0.7011**
Plasma vtg at release	0.8598	0.1944	**0.0218**	**0.0084**	**0.0098**	**<0.0001**	1.0000

Table 4.1. Correlations of parameters measured at Autumn 2012 release in non-rematuring steelhead kelts. Values in shaded cells represent Pearson's correlation coefficients; values in un-shaded cells represent associated significance probabilities. Bolded cells represent significantly correlated variables (p<0.005).

	FL at Release	Mass at Release	SGR during reconditioning	*k* at release	Fatmeter at release	Plasma E2 at release	Plasma vtg at release
FL at Release	1.0000	**0.8798**	0.0038	-0.1199	-0.0969	**0.1699**	**0.2780**
Mass at Release	**<0.0001**	1.0000	**0.3325**	**0.3427**	0.0719	**0.2534**	**0.3862**
SGR during reconditioning	0.9655	**<0.0001**	1.0000	**0.7048**	**0.5850**	**0.2576**	**0.4086**
***k* at release**	0.1359	**<0.0001**	**<0.0001**	1.0000	**0.3747**	**0.0027**	**0.3265**
Fatmeter at release	0.2288	0.3722	**<0.0001**	**<0.0001**	1.0000	0.1474	**0.2992**
Plasma E2 at release	**0.0339**	**0.0014**	**0.0026**	**0.0027**	0.0664	1.0000	**0.4578**
Plasma vtg at release	**0.0006**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0002**	**<0.0001**	1.0000

Table 4.2. Correlations of parameters measured at autumn 2012 release in rematuring steelhead kelts. Values in shaded cells represent Pearson's correlation coefficients; values in un-shaded cells represent associated significance probabilities. Bolded cells represent significantly correlated variables ($p<0.005$)

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