Endocrine growth regulation in salmon: mechanisms of nutritional control of the growth axis.

Andrew Lee Pierce

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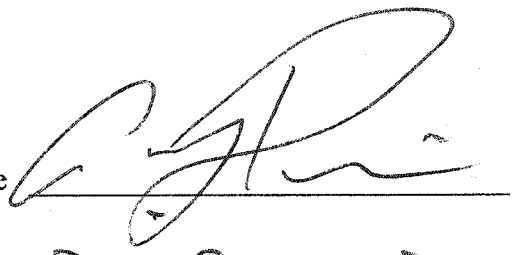
Signature 

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Chair of Supervisory Committee:

Walton W. Dickhoff

Reading Committee:

Walton W. Dickhoff

Penny Swanson

Thomas P. Mommsen

Date: Dec 8, 2003

University of Washington

Abstract

Endocrine growth regulation in salmon: mechanisms of nutritional control of the growth axis

Andrew Lee Pierce

Chair of Supervisory Committee:
Professor Walton W. Dickhoff
School of Aquatic and Fishery Sciences

Vertebrate growth is regulated by the growth endocrine axis, which consists of pituitary growth hormone (GH), liver insulin-like growth factor-I (IGF-I), and IGF binding proteins (IGFBPs). Studies were conducted to characterize mechanisms of nutritional regulation of the salmon growth axis. The time course of the growth axis response to Fasting, Maximum, or Control rations was assessed. GH increased in Fasted salmon. Fasted GH first increased moderately (days 1-12) and then dramatically (days 15-29), suggesting that the GH response to fasting has two phases. Plasma IGF-I and 41-kDa IGFBP (putative IGFBP-3) were lower in Fasted salmon from day 4 onward, suggesting that liver GH resistance developed within 4 days. Liver IGF-I mRNA was lower in Fasted salmon from day 6 onward, and correlated with plasma IGF-I, suggesting that liver IGF-I production regulates plasma IGF-I level. Basal plasma insulin was lower in Fasted salmon from day 6 onward, but did not respond strongly to fasting.

Primary hepatocyte culture was used to study the regulation of salmon liver IGF-I production. Hepatocyte IGF-I mRNA decreased over 48 hours in culture. GH always

stimulated IGF-I mRNA. The IGF-I mRNA response to GH was biphasic, declining from maximal at 5 x 10^{-7} M GH, suggesting that GH signal transduction in salmon is similar to mammals. Medium IGF-I (5 x 10^{-9} M), insulin (10^{-6} M), glucagon (10^{-6} M), triiodothyronine (10^{-7} M), dexamethasone (10^{-6} M), and glucagon-like peptide (10^{-6} M), did not affect basal IGF-I mRNA. However, dexamethasone, insulin, and glucagon reduced the IGF-I mRNA response to GH, indicating that hepatocyte GH sensitivity is regulated by metabolic hormones in salmon. Dexamethasone inhibited the GH response at concentrations of 10^{-12} M and above, insulin at 10^{-9} M and above, and glucagon only at pharmacological concentrations (10^{-6} M). Inhibition of IGF-I production by dexamethasone is a mechanism for growth inhibition by stress. The sensitivity of salmon hepatocytes to dexamethasone suggests glucocorticoids tonically regulate the salmon growth axis. Inhibition by insulin is the inverse of what is found in mammals, suggesting that the role of insulin in growth regulation differs between salmon and mammals.

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GLOSSARY OF ABBREVIATIONS

AA	Amino acid
ALS	Acid labile subunit
cDNA	Coding DNA
CIMPR	Cation-independent mannose 6 phosphate receptor
Daf-2	Nematode insulin-like receptor
Dex	Dexamethasone, a synthetic glucocorticoid hormone
GH	Growth hormone
GHBP	Growth hormone binding protein
GHR	Growth hormone receptor
Glu	Glucagon
GLP	Glucagon-like peptide
HBSS	Hanks's balanced salt solution
HNF	Hepatocyte nuclear factor, a liver-enriched transcription factor
HSI	Hepatosomatic index
IGFBP	Insulin-like growth factor binding protein
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
IGF1R	IGF type 1 receptor
IGF2R	IGF type 2 receptor
InR	Fruit fly insulin-like receptor
Ins	Insulin
INSR	Insulin receptor
INSRR	Insulin receptor related receptor
IRS	Insulin receptor substrate
mRNA	Messenger RNA
PCR	Polymerase chain reaction
PRL	Prolactin
RIA	Radioimmunoassay
ROS	Reactive oxygen species
RPA	RNase protection assay
RPMI	Roswell Park Memorial Institute cell culture medium
RT	Reverse transcription
SL	Somatolactin
STAT	Signal tranducer and activator of transcription
TaqMan	Real-time quantitiative RT-PCR technique
T_3	Triioodothyronine, a thyroid hormone

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Dedication

To my parents, Dr. Kenneth L. Pierce and Linda W. Pierce.

Introduction

The dramatic life cycle of Pacific salmon has long fascinated humans. Hatched from pea-sized and smaller eggs in stream gravels, salmon parr spend a variable amount of time in fresh water, some living a troutlike life in streams for one or more years, and then migrate to the ocean as silvery smolts, where they range across the entire north Pacific as the region's top pelagic predator. Adults return to their natal streams after two to eight years to spawn and die, at which point individuals of some species may weigh over 50 pounds. Growth plays a central role in the salmon life cycle. Growth influences survival and feeding opportunities at the parr stage, the successful transition to the ocean as smolts, the time spent in the ocean, and reproductive timing, strategies, and success (Groot *et al.* 1991). Research on the physiological regulation of growth in salmon will aid in understanding the salmon life cycle.

Pacific salmon have considerable ecological, cultural, and economic value. Fish populations have declined sharply over the past century, due to human caused habitat degradation and excessive harvest. This has led to efforts to conserve and manage wild stocks, the construction of numerous hatcheries intended to conserve existing stocks and to increase production of fish for the fishery, and the development of salmon aquaculture. While the goals of these efforts differ, growth is an important factor in all of them. Research on salmon growth physiology has applications in salmon management and conservation, hatchery science, and aquaculture.

Animal growth responds to environmental conditions. Nutritional status, seasonal variables, reproductive status, stress and disease, and social/behavioral factors influence

growth. In vertebrates, neuroendocrine systems have developed to regulate the growth of individuals in accordance with environmental conditions. As might be expected for a process as important in the life of any animal as growth, endocrine growth regulation is highly complex. The primary endocrine system in growth regulation is the growth or somatotropic axis. The growth axis consists of growth hormone (GH), insulin-like growth factor-I (IGF-I), and associated binding proteins and receptors.

This dissertation presents research aimed at understanding the regulation of the salmon growth axis by nutritional factors. The first chapter reviews endocrine growth regulation in mammals and fishes, including brief coverage of evolutionary aspects, a fish-oriented introduction to the burgeoning field of regulation of aging and lifespan, and a section on applications of growth axis research in fishes. Subsequent chapters present results of experimental work, and are structured as publishable units. Since these sections must be able to stand on their own, there is some redundancy, particularly in the introductions. Chapter 3 is in press (Pierce *et al.* 2003; *General and Comparative Endocrinology*), Chapter 2 is in review (*Journal of Endocrinology*), and Chapter 4 will be submitted for publication soon.

Chapter 1: Endocrine growth regulation literature review

THE GROWTH AXIS IN MAMMALS

Growth hormone (GH) is a 22 kiloDalton (kDa) protein hormone produced by the pituitary gland. GH is a member of the cytokine family, evolutionarily and structurally related to the pituitary hormone prolactin as well as erythropoietin and the interleukins (Kopchick *et al.* 1999). Pituitary GH secretion is regularly pulsatile and increases during sleep in mammals (Tannenbaum *et al.* 1979). Characteristics of the GH secretion pattern such as pulse height and baseline GH levels are sexually dimorphic in rodents, and the male and female GH secretion patterns differentially regulate gene expression in the liver (Tannenbaum 1993; Tannenbaum *et al.* 2001). Pituitary GH secretion is regulated by the hypothalamic region of the brain. While hypothalamic regulation of GH secretion is complex, primary inhibitory control is exerted by somatostatin and stimulatory control by GH releasing factor (GRF) in most mammalian species (Tannenbaum 1993). GH secretion is also regulated by peripheral factors. The recently discovered acylated peptide hormone ghrelin is produced by the stomach, and stimulates pituitary GH secretion (Horvath *et al.* 2001; Kojima *et al.* 2001). Ghrelin also strongly stimulates appetite. Circulating IGF-I significantly inhibits GH secretion directly at the level of the pituitary and via the hypothalmus (Berelowitz *et al.* 1981; Tannenbaum *et al.* 1983).

GH acts on target tissues through a specific cell surface receptor, the growth hormone receptor (GHR). The GHR is a member of the cytokine receptor family (Edens *et al.* 1998; Schwartzbauer *et al.* 1998). The receptor is a single chain protein with a single transmembrane domain, an extracellular ligand binding domain, and intracellular

signaling domains. GH signal transduction begins when a single GH molecule simultaneously binds two GHRs. The resulting dimerization of receptors begins the intracellular signal transduction cascade. This unusual mechanism predicts that concentration/response curves to GH and other cytokines will be bell shaped or biphasic, as is found to be the case (Ilondo *et al.* 1994; Frank 2002; Helander *et al.* 2003). The GH signal transduction pathway proceeds through activation of Janus Kinases (JAKs), which in turn activate Signal Transduction and Activator of Transcription (STAT) second messengers. Activated STATs form dimers and translocate to the nucleus, where they activate gene transcription (Kopchick *et al.* 1999).

GH circulates bound to a specific binding protein, which is generated by proteolysis of the GHR or alternative splicing of the GHR mRNA transcript (Edens *et al.* 1998). The GH binding protein is present at relatively low levels, does not bind GH more strongly than the GHR, and is not thought to play a major role in growth physiology (Baumann 2001).

GH has both metabolic and growth stimulatory effects. GH acts in opposition to insulin to release stored energy by promoting lipolysis and increasing blood levels of free fatty acids, decreasing glucose uptake and metabolism, and causing cellular insulin resistance (Kopchick *et al.* 1999). Like insulin, GH stimulates amino acid flux across cell membranes. In chickens, the energy mobilizing effects of GH appear to predominate over the hormone's growth promoting effects (Buyse *et al.* 1999). GH does not seem to function in prenatal development: tissue levels of GHR are low prenatally, and animals with disrupted GH signaling are normal size at birth. However, GH is essential for

normal postnatal growth. Animals with disrupted GH signaling display postnatal growth defects, but are viable and fertile (Zhou *et al.* 1997). Several examples of naturally occurring GH deficient or GH insensitive dwarf animals have been described (Hull *et al.* 1999), and transgenic animals overexpressing GH are larger than normal due to rapid postnatal growth (Kopchick *et al.* 1999; Bartke *et al.* 2002).

Insulin-like growth factor-I and –II (IGF-I and IGF-II) are 7.5 kDa peptide hormones which are highly conserved throughout the vertebrates (Daughaday *et al.* 1989; Jones *et al.* 1995; Stewart *et al.* 1996). As their names imply, IGF-I and IGF-II are members of the insulin superfamily of proteins. The structure of the IGFs is similar to insulin, except the C chain of the peptide is not removed during processing. Thus, the IGFs are single chain peptides. In addition, mature IGFs have a D domain not found in insulin, and an E domain of the prohormone is cleaved during peptide processing. IGFs are produced by nearly all cell types, but the major source for circulating (endocrine) IGFs is the liver. IGFs are secreted by the constitutive pathway; there is no evidence for storage within cells (D'Ercole *et al.* 1987).

Endocrine IGF-I is chiefly regulated by GH and nutritional status. GH stimulates hepatic IGF-I gene expression and production in healthy animals in good nutritional status, consistent with IGF-I's role as the major mediator of postnatal GH dependent growth. Gene knockout and transgenic studies have shown that IGF-I is essential for GH dependant postnatal growth (Efstratiadis 1998; Lupu *et al.* 2001; Nakae *et al.* 2001). IGF-II, in contrast, is chiefly regulated by developmental stage. IGF-II gene expression and plasma levels are highest during prenatal development, and decrease in most species

after birth, consistent with IGF-II's role as an important factor in prenatal development. Disruption or enhancement of IGF-II signaling affects prenatal, but not postnatal growth (Wolf *et al.* 1998; Nakae *et al.* 2001). However, considerable species variation exists between mammals in the amount of circulating IGF-II present after birth, and the function of this post-natal IGF-II is not clear (Daughaday *et al.* 1989; Wolf *et al.* 1998).

Both IGF-I and IGF-II act through the same cell surface receptor, the type I IGF receptor (IGF1R). The IGF1R is a tyrosine kinase receptor related to the insulin receptor. Mature IGF1R is a tetramer of 2 alpha and 2 beta chains linked by disulfide bonds. The extracellular alpha subunits form the ligand-binding pocket, and the beta subunits contain transmembrane and signaling domains. Signal transduction is similar between the insulin receptor and the IGF1R. Ligand binding causes tyrosine autophosphorylation in intracellular domains, which is followed by assembly of a signaling complex around the activated receptor. Proteins such as the insulin-receptor substrates (IRSs) and Src-homology containing (SHC) proteins are recruited to the signaling complex, and in turn activate a number of second messenger cascades (Jones *et al.* 1995; Blakesley *et al.* 1999). A second receptor, the IGF-II receptor (IGF2R) is thought to function only in the clearance of IGF-II from the plasma. The IGF2R is identical with the cation-independent mannose-6-phosphate receptor (CIMPR), which functions in endocytotic and lysosomal pathways. This receptor lacks intracellular signaling domains, and has no known IGF signaling function; it is thought to simply take up and degrade IGF-II.

Like GH, the IGFs have both metabolic and growth stimulatory effects. However, growth stimulation is clearly the major role of the IGFs, and metabolic effects

may be secondary to growth stimulation, or mediated by the insulin receptor (Jones *et al.* 1995; Stewart *et al.* 1996). IGF-I is a powerful mitogen, and has been shown to stimulate proliferation in a wide variety of cell types (Florini *et al.* 1996; Stewart *et al.* 1996; Baserga *et al.* 1999). In fact, it has been proposed that IGF-I is involved in all proliferative responses in all cell types (Florini *et al.* 1996). As well as stimulating cell division, IGF-I also causes increases in cell size or hypertrophy, possibly due to stimulation of DNA synthesis and induction of ribosomal RNAs (Baserga *et al.* 1999). In living mammals, tissue growth results from the balance between mitogenesis and apoptosis, or cell death, with hypertrophy playing a minor role in most tissues. IGF-I inhibits apoptosis in a wide variety of cell types (Florini *et al.* 1996; Stewart *et al.* 1996; Baserga *et al.* 1999). Growth stimulation by IGF-I is chiefly due to its mitogenic and anti-apoptotic effects. Consistent with these effects, excessive IGF-I signaling can transform cells, i.e. cause cells to acquire the ability to form tumors (Stewart *et al.* 1996; Baserga *et al.* 1999).

Both *in vivo* and in tissue culture, administration of GH and IGF-I together is often more effective in stimulating growth than either hormone alone. A "dual effector hypothesis" has been proposed to explain the synergistic effect. GH was hypothesized to stimulate cell differentiation, and IGF-I to stimulate proliferation (Green *et al.* 1985). However, along with its proliferative effects, IGF-I stimulates differentiation of principle cell types in muscle, bone, cartilage, fat, and the nervous system (Florini *et al.* 1996; Stewart *et al.* 1996). Hence the dual effector hypothesis has not been confirmed as it was originally stated. IGF-I seems to be capable of stimulating both proliferation and

differentiation. The phrase "dual effector theory" continues to be used loosely to refer to the synergism between GH and IGF-I. The reasons for the synergism are not clearly understood, but could involve direct GH effects, local IGF-I production, or effects of IGF binding proteins.

The vast majority (more than 99%) of IGF-I and -II found in the blood circulates bound to specific IGF binding proteins (IGFBPs) (Rajaram *et al.* 1997; Hwa *et al.* 1999; Mohan *et al.* 2002). There are at least six different IGFBPs in mammals, named IGFBP-(1-6). Unlike the GHBP, IGFBPs are not related to either IGF1R or IGF2R. IGFs bind to IGFBPs with greater affinity than the IGF1R, so IGFBPs may strongly influence IGF activity. IGFBPs decrease the clearance rate of IGF-I, regulate the availability of IGF-I to different tissues, associate with cell surface and extracellular matrix proteins, and can both potentiate and inhibit the growth stimulatory activity of the IGFs. By far the most abundant IGFBP in the circulation of mammals is IGFBP-3, which forms a ternary complex of 150 kDa with IGF-I and a third protein termed the acid-labile subunit (ALS). The ternary complex is too large to pass out of the vascular compartment, and constitutes a storage pool of IGF-I. IGFBP-3 is generally thought to be growth stimulatory, and liver IGFBP-3 production is decreased by fasting and stimulated by GH. IGFBP-1 and IGFBP-2 are the second and third most abundant IGFBP found in the blood. These IGFBPs are growth inhibitory: IGFBP-1 is rapidly induced by fasting and stress, and inhibited by insulin (Ketelslegers *et al.* 1996). IGFBP physiology is extremely complex: there are IGF-I independent effects of some IGFBPs; there is a large family of IGFBP

related proteins, which bind IGFs with lower affinity; there are specific proteases which cleave IGFBPs; and IGFBPs are regulated by phosphorylation.

Research on the growth axis has been guided by the somatomedin hypothesis. Now more than thirty years old and widely accepted (in various forms), the somatomedin hypothesis states that circulating IGF-I (formerly known as somatomedin) of hepatic origin mediates the growth promoting effects of GH (Daughaday *et al.* 1999). The finding that IGF-I exerts physiologically significant negative feedback on GH secretion at the pituitary and hypothalmus completes the picture by making the somatomedin hypothesis into an endocrine axis (Berelowitz *et al.* 1981; Tannenbaum *et al.* 1983). Negative feedback of the target gland hormone on the pituitary hormone is a classic endocrine mechanism for maintaining homeostasis in hypothalamic-pituitary-target gland endocrine axes. Thus, the somatomedin hypothesis has developed into the growth endocrine axis, in which pituitary GH stimulates liver IGF-I production, which mediates the growth stimulatory effects of GH and feeds back to inhibit pituitary GH secretion. Since the pituitary and the liver are linked in a feedback loop, primary changes in circulating levels of either GH or IGF-I will cause secondary changes in secretion and blood levels of the other hormone.

Since its original formulation, the somatomedin hypothesis has been modified to account for local production of IGF-I, GH resistance, and the effects of IGFBPs. While the liver produces most of the IGF-I found in the circulation, most other tissues also produce IGF-I. Non-hepatic IGF-I production is regulated by GH under some circumstances (Le Roith *et al.* 2001). The relative contributions of local and endocrine

IGF-I to mammalian growth are currently under debate (Le Roith *et al.* 2001; Lupu *et al.* 2001). Further, circulating IGF-I levels do not simply follow GH levels. The response of the liver to GH is strongly influenced by nutritional status and other aspects of the health of the animal. Under catabolic conditions, the liver becomes resistant to GH stimulation of IGF-I production and plasma IGF-I levels fall. Finally, the complexity of the IGFBP system seems to indicate that IGFBPs must play an important role in growth regulation. Plasma binding capacity may limit plasma IGF-I levels, and local IGFBP production, binding to ECM and cell surfaces, and proteolytic cleavage may significantly modulate local IGF-I actions.

Liver GH resistance appears to be a key point of regulation of the growth axis. During fasting, circulating IGF-I levels decline in all species studied, and GH increases in all species studied except rats (Straus 1994; Thissen *et al.* 1994; Estivariz *et al.* 1997). GH declines during fasting in the rat (Tannenbaum *et al.* 1979). GH also declines in streptozotocin diabetic rats, the opposite of the GH increase found in other diabetic mammals (Tannenbaum 1981). The reasons for these differences are not known, but they suggest that rat studies on growth regulation must be interpreted with caution. The pattern of decreased plasma IGF-I and increased plasma GH is indicative of GH resistance (Thissen *et al.* 1994; Ross *et al.* 1995). GH resistance occurs in a variety of catabolic states, including fasting or calorie restriction, feeding of diets deficient in protein or lacking in essential amino acids, and sepsis and post-surgical trauma. This response is adaptive during catabolic states, since increased GH acts to release stored energy, while low IGF-I prevents growth. GH resistance has been studied by Thissen

and colleagues (Thissen *et al.* 1994; Ketelslegers *et al.* 1995; Thissen *et al.* 1999; Beauloye *et al.* 2002). The decrease in liver GH sensitivity is the primary defect in GH resistance; the increase in circulating GH is due to relaxation of negative feedback from IGF-I. The cellular basis for GH resistance within hepatocytes involves both reductions in GHR level and downregulation of post-receptor GH signaling. The recently described suppressors of cytokine signaling (SOCS) proteins are one mechanism for post-receptor regulation of hepatocyte GH sensitivity. SOCS proteins inhibit GH and other cytokine signaling by interfering in second messenger cascades (Krebs *et al.* 2001). In rats, fasting causes GH resistance and induces SOCS3 in hepatocytes (Beauloye *et al.* 2002).

The physiological mechanisms leading to liver GH resistance are not fully understood. Diabetic mammals are GH resistant, and the GH resistance of diabetes is normalized by restoration of portal insulin levels, suggesting that insulin plays a significant role (Griffen *et al.* 1987; Hanaire-Broutin *et al.* 1996; Landau *et al.* 2000). Primary hepatocyte culture studies have suggested that insulin, thyroid hormones, and low levels of glucocorticoid hormones enhance hepatocyte GH responsiveness (Tollet *et al.* 1990; Boni-Schnetzler *et al.* 1991; Villafuerte *et al.* 1992; Krishna *et al.* 1996). Glucagon and high levels of glucocorticoids suppress GH-stimulated or basal IGF-I production by mammalian hepatocytes (Denver *et al.* 1994; Beauloye *et al.* 1999). Interleukins associated with infection strongly inhibit the hepatocyte IGF-I response to GH (Thissen *et al.* 1999). Additional studies have found direct roles for nutrients in the regulation of hepatocyte GH sensitivity. Glucose positively regulates hepatocyte GH sensitivity in mammalian hepatocytes (Brameld *et al.* 1995; Goya *et al.* 1999). Overall

amino acid level positively regulates hepatocyte GH sensitivity (Brameld *et al.* 1999; Thissen *et al.* 1999; Wheelhouse *et al.* 1999). Specific amino acids have been suggested to play a role: deletion of proline, threonine, tryptophan, arginine, phenylalanine, valine, and methionine from hepatocyte culture medium have been found to reduce hepatocyte IGF-I response to GH or GHR gene expression (Harp *et al.* 1991; Brameld *et al.* 1999; Stubbs *et al.* 2002).

Evolution of the Growth Axis from Invertebrates to Mammals

Insulin-like (i.e. insulin and/or IGF) ligands, receptors, and second messenger systems are conserved across metazoans. Functional characteristics such as the responsiveness of insulin-like signaling to nutritional conditions, and roles in the regulation of nutrient metabolism, growth and development, reproduction, and aging are remarkably well conserved (Smit *et al.* 1996; Leevers 2001; Longo *et al.* 2003). GH family cytokine hormone signaling appears to be limited to the vertebrates; the genome of the tunicate *Ciona intestinalis* does not contain any GH family receptors (Dehal *et al.* 2002), but GH is found in lampreys and all more derived vertebrates (Rand-Weaver *et al.* 1993; Kawauchi *et al.* 2002). This suggests that insulin-like signaling may be of more basic importance in the growth axis than GH signaling.

The availability of genome sequences enables us to say what components of the insulin-like signaling system are and are not present in a variety of animals. The genome of the fruit fly (*Drosophila melanogaster*) contains a single insulin-like receptor (InR) and 7 separate insulin-like peptides, and the nematode (*Caenorhabitans elegans*) has a single insulin-like receptor (Daf-2) and 37 insulin-like peptides (Leevers 2001; Tatar *et*

al. 2003). A single insulin-like receptor has been found in molluscs (Roovers *et al.* 1995). Searches of the genome of the tunicate *Ciona intestinalis* yield a single insulin-like receptor and a single ligand (http://genome.jgi-psf.org/ciona4/ciona4.home.html). There may be species differences within the tunicates, since McRory and Sherwood (1997) found two ligand genes in the tunicate *Chelyosoma productum*, which were classified as related to insulin and IGF. The cephalochorodates are derived relative to the tunicates, and in the cephalochorodate *Branchistoma* (formerly *Amphioxius*), a single insulin-like peptide (Chan *et al.* 1990) and a single insulin like receptor (Pashmforoush *et al.* 1996) have been identified. Thus, in invertebrates, the data suggest that a single receptor mediates insulin-like signaling, while there are often multiple ligands. Tatar et al. (2003) propose that the multiple ligand gene duplications in invertebrates enabled the evolution of complex tissue and temporally specific insulin-like ligand gene expression. In this way, insulin-like signaling could be tightly regulated even though there is only one receptor. As the authors state, an implication of this view is that insulin-like signaling acts locally in invertebrates. Consistent with local action, invertebrate insulin-like peptides are expressed in neurons rather than the digestive tract (Smit *et al.* 1996; Leevers 2001; Longo *et al.* 2003).

On the basis of the arrangement of the insulin-family genes in the human genome, it has been proposed that IGF-I arose from insulin by a simple tandem gene duplication, while IGF-II arose from IGF-I as a consequence of a chromosomal duplication (Patton *et al.* 1998; Reinecke *et al.* 1998). A phylogenetic analysis of chordate insulin-related proteins found that the insulin and IGF-like sequences from the tunicate *Chelyosoma*

productum group with *Branchiostoma* insulin-like peptide, in a different clade than either vertebrate insulins or vertebrate IGFs (Patton *et al.* 1998). This suggests that these primitive chordate ligands are descendants of invertebrate insulin-like peptides. Insulin and IGF ligands are distinct in the hagfishes (Nagamatsu *et al.* 1991; Patton *et al.* 1998). The data and phylogenetic analysis are consistent with the gene duplication leading to the divergence of insulin and IGF-I ligands occurring during the transition from chordates to primitive vertebrates (Reinecke *et al.* 1998). However, given the large number of invertebrate insulin-like ligands, it is clear that an understanding of the divergence of the receptors mediating insulin and IGF signaling is more functionally important. Interaction with different receptors is one likely reason for the divergence of insulin and IGF ligands, but not the only possible one. A single insulin-like receptor is present in *Branchiostoma*, which responds similarly to mammalian insulin and IGF-I (Pashmforoush *et al.* 1996). The insulin and IGF receptors are distinct in the hagfishes (Pashmforoush *et al.* 1996). Thus, the ligand data and a limited amount of receptor data are consistent with a scenario in which insulin and IGF signaling diverged during vertebrate evolution between the cephalochorodates and the agnathans.

In the mouse and human, there are 4 insulin-like receptors; the insulin receptor (INSR), IGF1R, the non-signaling IGF2R and the orphan insulin related receptor (INSRR). The first three receptors have crucial functions in metabolism, growth, and development, and gene knockouts have lethal or severe phenotypes (Nakae *et al.* 2001; Kulkarni *et al.* 2002). It is reassuring to find that a knockout of the INSRR has no effect on phenotype (Kitamura *et al.* 2001). Mammals have fewer insulin-like ligands per

receptor than invertebrates. The ligands in mammals are the familiar IGF-I, IGF-II, and insulin (2 genes), as well as two insulin-like peptides.

The receptor duplication giving rise to separate INSR and IGF1R enabled separate roles for insulin and IGF-I signaling to evolve in vertebrates (Kelley *et al.* 2000). In mammals, both insulin and IGF-I act as endocrine hormones at a distance from where they are produced. The advantage to this might be that these nutrient-sensitive hormones are produced in the pancreas and liver, directly downstream from where nutrients are absorbed in the gut, and where the best information is available on nutritional and metabolic status. Consistent with this idea, mechanisms for sensing nutritional and metabolic status are present in insulin and IGF-I secreting cells.

In mammals, insulin is a regulator of short-term nutrient metabolism, with an indispensable role in glucose homeostasis. Insulin has an endocrine mode of action: circulating insulin is produced only in the beta cells of the pancreas, and is carried through the blood to its target tissues, the most important being liver, muscle, and fat. Insulin levels are regulated minute to minute by adjustment of beta cell secretion in response to blood glucose levels and other nutritional signals. Mammalian beta cells sense glucose flux through glycolytic pathways and adjust insulin secretion and synthesis accordingly (Thorens 2003). For this to work, clearance of insulin must also be rapid, which may explain why there are no binding proteins for insulin. IGF-I, in contrast, is a long-term mediator of protein and bone growth. IGF-I has both endocrine and local modes of action (Daughaday *et al.* 1999; Le Roith *et al.* 2001; Lupu *et al.* 2001). Endocrine IGF-I is produced by hepatocytes. Arguably, hepatocyte IGF-I production is

regulated in a manner analogous to insulin regulation. Hormones related to nutritional and metabolic status regulate IGF-I production, as do blood nutrient levels (Thissen *et al.* 1999). In the endocrine mode of IGF-I action, slow clearance of IGF-I due to the presence of IGFBPs leads to the large pool of IGF-I in vertebrate blood. This pool of stored IGF-I is much slower to change in response to nutritional status than insulin, which might serve to integrate the growth stimulation signal over time. This function, however, is achieved by IGFBP-3 and the ALS; it does not explain the strikingly complex system of IGFBPs that exists. The local mode of action is preserved from invertebrates to mammals for IGF-I, but not for insulin. Local IGF-I may perform functions that are not performed by endocrine IGF-I in processes such as embryonic and postnatal development, wound healing, and bone growth. Regulation of tissue IGF-I gene expression in mammals is complex, with multiple transcription initiation sites and alternative splicing pathways (Rotwein 1999).

IGFBPs are one branch of a large family of secreted cysteine rich factors (the TIC superfamily), which is divided into the twisted gastrulation (TSG) proteins, the IGFBPs, and the Connective tissue growth factor/Cysteine-rich 61/Nephroblastoma overexpressed (CCN) family of growth factors (Vilmos *et al.* 2001). TSG proteins are found in invertebrates and vertebrates, while the CCN and IGFBP branches are verifiably present only in vertebrates (Vilmos *et al.* 2001). Thus, the functions of TIC superfamily proteins, possibly including IGFBPs, predate the emergence of endocrine insulin-like signaling. IGFBPs have non-IGF dependent functions in mammals, which are only beginning to be explored (Hwa *et al.* 1999; Lee *et al.* 2002; Mohan *et al.* 2002). Secreted proteins with

homology to IGFBPs which bound specifically to insulin and IGF have been found in molluscs (Weiss *et al.* 2001) and insect cell lines (Doverskog *et al.* 1999). While far from established, one view is that the development of the IGFBPs in their IGF binding roles in vertebrates resulted from the change from local to endocrine mode of action for insulin-like peptides during evolution from invertebrates to vertebrates (Upton *et al.* 1992; Reinecke *et al.* 1998; Tatar *et al.* 2003). The IGF-dependent functions of vertebrate IGFBPs could then be regulating IGF clearance, preventing crosstalk between insulin and IGFs, and enabling tissue-specific regulation of IGF signaling (Reinecke *et al.* 1998; Hwa *et al.* 1999; Kelley *et al.* 2000; Kelley *et al.* 2002).

IGF-I and IGF-II exert their biological effects through a single receptor, the IGF1R. Thus, IGF-I and IGF-II signaling are not separated to the same degree as IGF and insulin signaling. However, IGF-I and IGF-II actions are temporally separated: IGF-II acts during embryonic development, whereas IGF-I mediates post-natal GH dependent growth (D'Ercole 1999). In mice, knocking out the IGF-II gene does not affect postnatal growth (Efstratiadis 1998; Lupu *et al.* 2001), and knocking out the GHR gene does not affect prenatal growth (Zhou *et al.* 1997; Lupu *et al.* 2001). The IGF2R does not signal, but functions to downregulate IGF-II signaling during development by clearing IGF-II. In mammals, IGF-II and the IGF2R are reciprocally imprinted (IGF-II maternal allele and IGF2R paternal allele expressed, Barlow 1993), suggesting a function for IGF2R in placental development.

THE GROWTH AXIS IN FISHES

The major components of the growth axis, i.e. GH, IGF-I, and associated binding proteins and receptors, have been identified in fishes (Duan 1998; Mommsen 1998; Reinecke *et al.* 1998; Nicoll *et al.* 1999; Perez-Sanchez *et al.* 1999; Kelley *et al.* 2000; Perez-Sanchez 2000; Mommsen 2001a, b). A number of differences exist between the growth axis in fish versus mammals, some of which suggest that the fish growth axis may be simpler. However, GH and IGF-I also have functions such as osmoregulation in fishes that are not found in mammals. The fish growth axis is primarily regulated by nutritional status, but additional environmental variables such as photoperiod and temperature also play roles.

The GH gene has been cloned and GH protein isolated from many fish species, including salmon (Rand-Weaver *et al.* 1993; Kawauchi *et al.* 2002). GH secretion by fish pituitary somatotrophs is under multiple inhibitory and stimulatory controls (reviewed in Peter *et al.* 1995). In salmonids, primary control is inhibitory and is exerted by somatostatin (Le Bail *et al.* 1991). As in mammals, GH release in fish occurs in secretion bursts, but unlike mammals, episodes of GH secretion in fish occur at irregular intervals (Zhang *et al.* 1994; Gomez *et al.* 1996). Basal GH levels in-between secretion episodes in immature rainbow trout (*Oncorhynchus mykiss*) in fresh water were found to be very low compared to those of other vertebrates (Gomez *et al.* 1996). Along with GH and the related prolactin, the fish pituitary secretes an additional hormone in the GH family named somatolactin (SL) (Rand-Weaver *et al.* 1992b). The function of SL is not yet clear, however, it has been proposed to regulate metabolism (Company *et al.* 2001;

Mingarro *et al.* 2002). GH has been proposed to be the evolutionary progenitor of the GH/PRL/SL family (Kawauchi *et al.* 2002).

High-affinity, low capacity GH binding sites have been demonstrated in fish tissues, indicating that the GHR is present (Gray *et al.* 1991; Gray *et al.* 1992; Perez-Sanchez *et al.* 1994b; Peter *et al.* 1995). A rainbow trout GHBP has been isolated, and immunoprecipitation experiments suggest that it is related to the GHR as in mammals (Sohm *et al.* 1998). Recently, the GHR cDNA has been cloned in a number of fish species (Calduch-Giner *et al.* 2001; Lee *et al.* 2001; Calduch-Giner *et al.* 2003; Fukada *et al.* 2003; Tse *et al.* 2003). A truncated form of the GHR mRNA without the intracellular domains required for signaling has been reported in turbot (*Scophthalmus maximus*); this might give rise to the GHBP as in some mammals (Calduch-Giner *et al.* 2001).

IGF-I has been cloned, and the circulating protein isolated in numerous fish species (Duan 1998; Mommsen 1998; Plisetskaya 1998; Nicoll *et al.* 1999; Kelley *et al.* 2000; Moriyama *et al.* 2000). As in mammals, liver IGF-I gene expression and plasma IGF-I levels are strongly regulated by GH and nutritional status (Cao *et al.* 1989; Moriyama *et al.* 1994; Perez-Sanchez *et al.* 1994a; Hashimoto *et al.* 1997; Kermouni *et al.* 1998; Shimizu *et al.* 1999a; Kajimura *et al.* 2001). The fish IGF-I gene is smaller than that of mammals and birds (17 Vs 50 to 80 kb), and it has a single promoter, a single transcription initiation site, and a single polyadenylation site, unlike the multiple such elements in mammals and birds (Duguay *et al.* 1994b; Rotwein 1999). Evidence to date suggests that fish tissues other than the liver do not respond strongly to GH with increases in local IGF-I production: only liver, gill, and possibly kidney may be strongly

responsive (Sakamoto *et al.* 1993b; Duguay *et al.* 1994b; Duguay *et al.* 1996; Duan 1998). Therefore, the relative contributions of local and circulating IGF-I to growth may be skewed toward circulating IGF-I in fish. However, muscle IGF-I gene expression was increased, albeit not greatly, by GH injection in salmon and tilapia (*Oreochromis mossambicus*; Duguay 1993; Kajimura *et al.* 2001), and in a recent study muscle IGF-I mRNA was strongly increased by refeeding (Chauvigne *et al.* 2003). Thus, mechanisms for growth regulation by both local and endocrine IGF-I appear to exist in fish.

IGF-I exerts negative feedback on pituitary GH secretion in fish (Perez-Sanchez *et al.* 1992; Blaise *et al.* 1995; Rousseau *et al.* 1998; Fruchtman *et al.* 2000). This effect is strong, highly specific for GH, and appears to be of major biological significance in the regulation of GH, suggesting that the feedback loop between the pituitary and liver that constitutes the growth axis is fully functional in fish.

Less information is available on fish IGF-II than IGF-I, but cDNA sequence and protein are available for a number of species (Duguay *et al.* 1996; Gentil *et al.* 1996; Shamblott *et al.* 1998; Vong *et al.* 2003). No IGF-II gene was found in the hagfishes; IGF-II is first known to be present in an elasmobranch, leading to the hypothesis that IGF-II diverged from IGF-I along the evolutionary lineage between the hagfishes and elasmobranchs (Duguay *et al.* 1995). IGF-II appears to regulate non-GH dependent growth early in development in fishes as in mammals, and declines in importance after hatching. IGF-II gene expression is elevated during embryonic development and declines after hatching (Duguay *et al.* 1996; Duan 1998). Plasma IGF-II does not respond strongly to GH injection or fasting in fishes, suggesting that IGF-II does not play a vital

role in the postnatal GH-dependent growth (Gentil 1996). Muscle IGF-II gene expression was not regulated by refeeding in rainbow trout (Chauvigne *et al.* 2003), and circulating IGF-II did not relate to growth rate (Gabillard *et al.* 2003a). However, one study found GH-regulated gene expression of IGF-II in the pyloric cecae and liver of rainbow trout (Shamblott *et al.* 1995). Plasma levels of IGF-II in salmonids are ~10 fold higher than levels of IGF-I (Gentil *et al.* 1996; Gabillard *et al.* 2003a). The physiological function of the large amount of circulating IGF-II in fishes remains a mystery. Assays for fish IGF-II have only recently become available (Gentil *et al.* 1996; Degger *et al.* 2001), and should lead to clarification of the functions and regulation of IGF-II in fish.

Specific IGF-I binding has been demonstrated in fish tissues, indicating that the IGF1R is present in fish (Drakenberg *et al.* 1993; Gutierrez *et al.* 1995). The IGF1R cDNA has been cloned in a number of species (Chan *et al.* 1997; Funkenstein *et al.* 1997; Maures *et al.* 2002a; Nakao *et al.* 2002). As in mammals, the IGF1R mRNA is widely distributed in fish, with high levels in muscle and bone tissue, consistent with a role in growth stimulation. IGF1R levels are low in fish liver, as in mammals. This may be a mechanism for preventing autocrine action of IGF-I on hepatocytes. Several reports claim that the fish IGF2R does not bind IGF-II (Upton *et al.* 1992; Drakenberg *et al.* 1993; Gutierrez *et al.* 1995; however see Mendez *et al.* 2001). Binding of IGF-II to the IGF2R seems to be a recent evolutionary event, occurring only in mammals (Reinecke *et al.* 1998), which suggests a unique role for this receptor in the development of placental vertebrates.

Plasma IGF binding activity has been demonstrated in fishes, indicating the presence of IGFBPs (Kelley *et al.* 1992; Shimizu *et al.* 1999a; Kelley *et al.* 2002). IGFBPs are present in the hagfishes (Upton *et al.* 1992), suggesting that they have performed their IGF binding roles since the divergence of insulin and the IGFs. Western ligand blotting studies have shown that most fish have 3 to 4 detectable IGFBPs (Shimizu *et al.* 1999b; Kelley *et al.* 2000). Less than 0.5% of circulating IGF-I in salmon is found in the free form, unbound by IGFBPs (Shimizu *et al.* 1999a). Most circulating IGF-I in fishes is found in an ~50 kDa acid dissociable complex (Moriyama *et al.* 1994; Perez-Sanchez *et al.* 1994a; Siharath *et al.* 1996; Shimizu *et al.* 1999a; Kelley *et al.* 2000). The IGFBP in this complex has been isolated from salmon serum (Shimizu *et al.* 2003a; Shimizu *et al.* 2003b). Based on its molecular weight (41 kDa), glycosylation, stimulation by GH, and inhibition by fasting, this IGFBP is homologous to mammalian IGFBP-3. Plasma levels of the salmon 41 kDa IGFBP correlate strongly with total plasma IGF-I, suggesting that they are regulated in concert (Beckman *et al.* 2003b; Shimizu *et al.* 2003a). Fish do not appear to have a 150 kDa ternary IGF-I/IGFBP-3/ALS complex (Shimizu *et al.* 1999a; Kelley *et al.* 2000). An IGFBP similar to mammalian IGFPB-1 in terms of molecular weight and regulation has been found in fish, including salmon (Siharath *et al.* 1996; Shimizu *et al.* 1999a; Park *et al.* 2000; Kelley *et al.* 2001; Maures *et al.* 2002b). Recent molecular cloning indicates that this IGFBP is indeed a fish homolog of mammalian IGFBP-1 (M. Shimizu, unpublished data).

GH and IGF-I stimulate growth in fishes, as in mammals. Many studies have shown growth stimulation by GH administered by various techniques (McLean *et al.*

1993; Mommsen 1998). IGF-I administration has also been shown to stimulate growth. Administration of IGF-I to coho salmon by implanted osmotic minipumps stimulated growth in a dose-dependent manner (McCormick *et al.* 1992). High IGF-I levels led to hypoglycemia and death, probably due to IGF-I interactions with the insulin receptor. Recently, weekly IGF-I injections were shown to stimulate growth in a dose-dependent manner in tilapia (Chen *et al.* 2000). IGF-II injections also stimulated growth, but required a higher dose for maximum stimulation. Exogenous GH stimulates growth more effectively than exogenous IGF-I, suggesting that GH and IGF-I act synergistically, as in mammals (Duan 1998; Moriyama *et al.* 2000). GH and IGF-I stimulate hyperplastic and hypertrophic muscle growth in fish, but the relative contributions of the two hormones to the many processes involved have not been delineated (Mommsen 2001a).

IGF-I stimulation of fish growth has been shown *in vitro* by bioassay techniques. Recombinant bovine IGF-I stimulated cartilage sulfation in long-jawed mudsucker (*Gillichthys mirabilis*) explants (Gray *et al.* 1991). Cartilage sulfation is a measure of cartilage growth, and a standard bioassay for IGF activity. Stimulation of sulfation showed an interesting concentration-dependent response to IGF-I. Explants from hypophysectomized (no GH) fish had low basal sulfation rates, which increased linearly with increasing IGF-I. Intact fish (normal GH) had a higher basal rate, with a parabolic dose response curve to IGF-I. GH injected hypophysectomized fish (high GH) had a higher basal rate, and a parabolic dose response curve with peak response at a lower IGF-I level. Gray and Kelley interpreted these results as GH increasing tissue IGF-I sensitivity, a possible mechanism for GH/IGF-I synergism.

The four receptors that mediate insulin/IGF signaling in mammals are found in the genome of the pufferfish (*Fugu rubripres*). The conservation of the orphan INSRR from fish to mammals suggests that this receptor does have a function. Two homologous IGF1R transcripts were found in zebrafish (*Danio rerio*) and flounder (*Paralichthys olivaceus*), and two homologous INSR transcripts were found in zebrafish, consistent with the tetraploid genomes of these species (Maures *et al.* 2002a; Nakao *et al.* 2002). As previously discussed, both insulin and IGF-I are present in the hagfishes, as are the INSR and IGF1R (Nagamatsu *et al.* 1991; Pashmforoush *et al.* 1996). While the signaling molecules are conserved from fish to mammals, functional aspects of the role of insulin appear to differ substantially. The metabolic and growth stimulatory roles of insulin in fishes have been well studied (Mommsen *et al.* 1991; Duguay *et al.* 1994a; Plisetskaya 1995; Mommsen 1998; Plisetskaya 1998; Mommsen 2001a; Hemre *et al.* 2002). Briefly, glucose homeostasis is much less important in the protein-centered metabolism of carniverous fishes such as salmon than in mammals. Fish clear a glucose load very slowly, in spite of relatively high circulating insulin levels, and tolerate levels of hypoglycemia that would be fatal in mammals without apparent ill effects. Amino acids are more potent insulin secretagogues in fishes than glucose, and insulin stimulates amino acid uptake and metabolism. Insulin appears to play a greater role in protein anabolism in fishes than in mammals. The relative abundance and affinity of muscle insulin and IGF-I receptors varies phylogenetically. Fish and other ectotherm muscle contains more IGF1R than INSR, while bird and mammal muscle contains more INSR than IGF1R (Navarro *et al.* 1999; Planas *et al.* 2000). This could relate to the decreased

importance of muscle glucose metabolism in fish, and/or to the indeterminate growth of fish (Mommsen 2001b). The insulin receptor is also less specific for insulin in fish than in mammals, resulting in a greater potential for IGF-I to act through the insulin receptor (Moon *et al.* 1996).

Nutritional status is a primary regulator of the growth axis in fish, as in mammals. Liver IGF-I gene expression and total plasma IGF-I decrease during fasting in all fish species studied (Duan *et al.* 1993c; Niu *et al.* 1993; Matthews *et al.* 1997b; Moriyama *et al.* 1999; Meton *et al.* 2000; Moriyama *et al.* 2000; Perez-Sanchez 2000; Baker *et al.* 2003; Uchida *et al.* 2003). In salmon, plasma IGF-I adjusts to ration within 2 to 4 weeks (Moriyama *et al.* 1999; Larsen *et al.* 2001b; Pierce *et al.* 2001). GH increases during fasting in most studies (Sumpter *et al.* 1991; Johnsson *et al.* 1996; Moriyama *et al.* 1999). In gilthead sea bream (*Sparus aurata*), GH is inversely related to ration over a relatively wide range of rations (Perez-Sanchez *et al.* 1995), whereas in salmonids, GH does not appear to be increased until rations are reduced below maintenance (Storebakken *et al.* 1991; Pierce *et al.* 2002; Gabillard *et al.* 2003b). GH increased again at very high rations in gilthead sea bream and tilapia; this increase may be related to a reduction in growth efficiency at very high rations (Perez-Sanchez *et al.* 1995; Toguyeni *et al.* 1996). GH may change transiently in response to a change in feeding level (Pierce *et al.* 2001; Uchida *et al.* 2003). GH increased and then decreased over 4 h after feeding in goldfish (*Carassius auratus*; Himick *et al.* 1995), consistent with the stimulation by feeding found in rainbow trout (Boujard *et al.* 1993). Some of the negative or contradictory results in GH response between studies may be due complex temporal patterns in the GH

response to feeding, as well as the episodic nature of GH secretion and the resulting high variability in plasma GH levels.

Nutritional status appears to regulate the growth axis by modulating liver GH sensitivity in fishes (Perez-Sanchez *et al.* 1999; Kelley *et al.* 2000). Gray *et al.* (1992) found hepatic GH binding was reduced in fasted coho salmon. Perez-Sanchez *et al.* (1994b) found reduced hepatic GH binding in fasted gilthead sea bream, and liver GH binding was positively related to ration over rations from 0 to 2.7 % body weight per day in gilthead sea bream (Perez-Sanchez *et al.* 1995). Regulation of IGF-I level is probably more complex than simple hepatic GHR up- or down- regulation. Gray *et al.* (1992) found that GHR downregulation in fasted coho salmon required 3 weeks, whereas decreased IGF-I activity was found within 5 d. In a recent study, liver IGF-I gene expression and plasma IGF-I levels were decreased in fasted coho salmon relative to controls within 2 weeks, whereas liver GHR gene expression did not differ between control and fasted fish over this time period (Fukada *et al.* 2003). These findings suggest that the post-receptor GH resistance found in mammals also occurs in fish.

As in mammals, hepatic GH resistance occurs in a variety of catabolic states in addition to fasting in fishes (Perez-Sanchez *et al.* 1999). Liver IGF-I gene expression and circulating IGF-I were reduced in spite of increased GH in coho salmon suffering from growth arrest due to premature transfer to saltwater, a phenomenon known as stunting (Duan *et al.* 1995). Liver membrane GH binding was reduced in stunted salmon (Gray *et al.* 1992), and in hypophysectomized mudsuckers (Gray *et al.* 1991). In fish hepatocyte culture, thyroid hormones stimulated IGF-I gene expression independently of GH in

salmon and tilapia hepatocytes, and insulin potentiated the response to GH after 6 h in culture in salmon hepatocytes (Duan *et al.* 1992; Schmid *et al.* 2003). The signal transduction pathway from the GHR to IGF-I gene expression appears to be conserved between mammals and salmon. The salmon IGF-I promoter was activated by GH treatment in a mammalian hepatoma cell line, after reconstruction of the pathway by co-expression of the GHR (rat), the GH signal transducer STAT5 (mouse), and the liver-enriched transcription factor HNF-1α (sheep) (Meton *et al.* 1999). Thus, mechanisms mediating GH resistance in hepatocytes may also be conserved from fish to mammals.

The fish growth axis has roles in addition to growth regulation. Growth axis hormones control osmoregulation in fish. GH is the primary hormone promoting saltwater osmoregulation in fishes (McCormick *et al.* 1991; Sakamoto *et al.* 1993a; McCormick 1996; Sakamoto *et al.* 1997). GH and IGF-I may act synergistically to promote seawater adaptation in salmonids (Madsen *et al.* 1993; Sakamoto *et al.* 1993a; Mancera *et al.* 1998). The pituitary hormone prolactin, which is closely related to GH, is the primary stimulator of freshwater osmoregulation (Grau *et al.* 1990; Yada *et al.* 1994). Seawater adaptation is one of the physiological, morphological, and behavioral changes associated with smoltification, the metamorphosis of stream dwelling parr into ocean going juvenile salmon. GH and IGF-I levels peak, along with many other hormones, in smoltifying salmon (Dickhoff 1993). GH and IGF-I appear to be important regulators of smoltification (Dickhoff *et al.* 1997; Beckman *et al.* 1998b; Moriyama *et al.* 2000). This may explain the relationship between growth rate and plasma IGF-I level at the time of release and smolt quality in hatchery salmon (Beckman *et al.* 1999).

In fish, the growth axis appears to regulate reproductive maturation at multiple levels (Baker *et al.* 2000; Shearer and Swanson 2000; Okuzawa 2002). Similar to mammals, plasma IGF-I levels may function as a "gate" for puberty. Salmon plasma IGF-I may influence the maturation decisions made during critical decision windows in conceptual models of maturation developed by Thorpe and colleagues (Thorpe *et al.* 1998). In addition, maturing male salmon have highly elevated plasma IGF-I, possibly produced by the developing testes (Shearer and Swanson 2000; D. Larsen and P. Swanson, unpublished data). Fish growth axis hormones are also involved in immune response (Perez-Sanchez 2000). GH and IGF-I appear to stimulate hematopoeisis and immune function in fish (Calduch-Giner *et al.* 1995).

Factors other than nutritional status regulate the growth axis in fish. Seasonal variables such as photoperiod and temperature are probably best-studied in salmonids. GH follows photoperiod, peaking in midsummer and very low through the winter (Marchant *et al.* 1986; Young *et al.* 1989; Perez-Sanchez *et al.* 1994a; Bjornsson 1997; McCormick *et al.* 2000). IGF-I may peak near the spring and autumn equinoxes; in some cases regulated by GH and in others not; this peak may be associated with smoltification (Dickhoff *et al.* 1997; Moriyama *et al.* 1997; Beckman *et al.* 2000) (Beckman *et al.* 1998b; Pierce *et al.* 2002; Beckman *et al.* 2003b). Low temperatures decrease IGF-I and modulate photoperiod effects on the growth axis (McCormick *et al.* 2000; Larsen *et al.* 2001a), and high temperatures increased plasma GH in a recent study (Gabillard *et al.* 2003b). Stress and disease increase GH and decrease IGF-I (Nicoll *et al.* 1999). In humans and other mammals, exercise increases both GH and IGF-I (Borst *et al.* 2001);

studies in salmonids have found an acute exercise induced increase in GH (Barrett *et al.* 1988; Kakizawa *et al.* 1995); however, moderate exercise over an 11 month period did not change plasma IGF-I or GH levels in masu salmon (*Oncorhynchus masou masou*; Azuma *et al.* 2002). Finally, GH seems to increase with dominance status and may stimulate risky foraging behavior in stream dwelling salmonids (Bjornsson 1997).

The Growth Axis in the Regulation of Aging and Lifespan

Recent research on the regulation of aging and lifespan in nematodes, flies, and mice has shown that insulin-like signaling is deeply implicated in this process (Hursting *et al.* 2003; Longo *et al.* 2003). A model organism with indeterminate growth is notably absent from research in this area.

One way of conceptualizing the mechanistic tradeoffs involved in the regulation of lifespan is in terms of "life history modes" (Tatar *et al.* 2003). According to this analysis, the chief life history modes are the reproductive mode, in which metabolism is upregulated, and a stress resistant mode, in which metabolism is downregulated and the cellular and systemic stress response activated. In addition, animals with indeterminate growth must also have a growth mode, in which metabolic energy is invested in somatic growth. Further, unlike nematodes, flies and mice, there are many animals (for example salmon), which expend a great deal of metabolic energy on activities such as migration. Thus, an extension of the analysis might group reproduction, growth, and activity as life history modes in which metabolism is upregulated, versus a stress resistant life history mode in which metabolism is downregulated and stress responses activated.

The main proposed mechanistic tradeoff in the regulation of lifespan is between metabolism and stress resistance. At the cellular level, degenerative changes in DNA and proteins may result in apoptosis, cellular senescence, or malignancies. The generation of reactive oxygen species (ROS) during cellular respiration underlies many of the processes involved, including DNA and protein damage (Hekimi *et al.* 2003). Thus, the induction of cellular stress resistance responses such as the production of ROS quenchers (e.g., superoxide dismutase), heat shock proteins, and the reduction of transcription rate by DNA silencing (e.g., yeast Sir-2) tend to retard cellular degeneration. Conversely, since many ROS are produced in mitochondria by the operation of the electron transport chain, upregulation of cellular oxidative metabolism should tend to promote degenerative changes. Consistent with this idea, manipulation of the metabolic rate of fishes and marine invertebrates by changing temperature results in a negative correlation between calculated metabolic rate and lifespan (Gillooly *et al.* 2001). However, considerable variation remains. ROS play essential roles in cellular signaling and immune defense (nitrous oxide and superoxide anion, respectively), so they cannot be completely eliminated. The balance between ROS production and cellular antioxidant mechanisms will determine the degree of cellular damage by oxidative stress.

In nematodes, flies, and mice, insulin-like signaling inhibits the cellular stress response. Inhibition of insulin-like signaling at any point extends lifespan (Lithgow *et al.* 2003; Longo *et al.* 2003; Tatar *et al.* 2003). Calorie restriction has been found to extend lifespan in diverse species (Hursting *et al.* 2003; Longo *et al.* 2003). The downregulation of insulin-like signaling is thought to be primarily responsible for the extension in

lifespan; upregulation of glucocorticoids may be secondarily responsible. There appear to be strong developmental interactions in the insulin-like effects on lifespan. Increased GH/IGF-I during development from birth to reproductive maturation may shorten lifespan, which might explain the negative association between body size and lifespan found within species of dogs and other mammals (Patronek *et al.* 1997). Early life IGF-I and adult body size were increased and lifespan decreased in domesticated mice as compared with a wild strain (Miller *et al.* 2002). However, declines in GH and IGF-I during adulthood may cause some symptoms of aging (Bartke *et al.* 2003). Epidemiological evidence associates high blood IGF-I levels with increased incidence of cancer (Hursting *et al.* 2003).

Recent studies using genetic techniques have shown that there are strong tissue-specific and sex-specific interactions in the regulation of lifespan by insulin-like signaling. An adipose-specific knockout of the insulin receptor reduced fat accumulation and extended lifespan by 18% in mice (Bluher *et al.* 2003). Interestingly, these mice were inferred to have a higher metabolic rate than controls, suggesting that metabolic rate might not be the primary point of control in this case. Mortality associated with obesity may be more important. Mice with reduced IGF-I signaling ($IGF1R^{+/-}$) showed a 33% increase in lifespan in females only (Holzenberger *et al.* 2003). Metabolic rate did not differ between $IGF1R^{+/-}$ mice and controls, but $IGF1R^{+/-}$ females were more resistant to oxidative stress than controls. Male $IGF1R^{+/-}$ mice showed reduced glucose tolerance, which could explain the sex difference. Alternatively, a sexually dimorphic trait such as GH pulsatility or gonadal steroids could be responsible. Gonadal steroids are proposed to

interact in the regulation of lifespan in all three model species (Tatar *et al.* 2003), as well as in fish (Dickhoff 1989). These are only a few examples from the voluminous recent literature on insulin-like signaling and aging.

The regulation of lifespan in fishes has been explored from an evolutionary standpoint (Reznick *et al.* 2001; Reznick *et al.* 2002). Fishes are strongly over represented among animals that live more than 100 years. Fishes do senesce, but the onset of senescence is delayed in fishes relative to other classes of animals (Reznick *et al.* 2002). As argued by Reznick (2002), this may be an evolutionary consequence of indeterminate growth in fishes. Because of indeterminate growth, fecundity increases with age in fish. The evolutionary payoff for survival to older ages is increased, which could explain the evolution of delayed senescence. Thus, the growth pattern of a fish species determines the selective pressures applied to physiological mechanisms implicated in the regulation of lifespan. Indeterminate growth is a continuum rather than a binary opposition in fishes. Unlike mammals and birds, fishes continue to grow after reproductive maturation, but the rate of growth varies. Small tropical species typically have much more determinate growth patterns than large cold-water species (Dutta 1994). In keeping with their tropical origins, zebrafish show quite determinate growth for a fish, and are hence a non-representative model for growth and lifespan studies (Mommsen 2001b). The length of senescing zebrafish at death increased only slightly with age (Gerhard *et al.* 2002). In contrast, in the ferox trout, a highly predatory morph of brown trout (*Salmo trutta*), the increase in length with age was robust and linear from 2 to 16

years, showing that at least in the wild, the growth of salmonids can be truly indeterminate (Mangel *et al.* 2001).

No work has been done on the involvement of insulin-like signaling in the regulation of lifespan in fishes. Based on the role of insulin-like signaling in invertebrates and mammals, an obvious comparative question is would be: How is it that fish can have upregulated insulin-like signaling, allocating resources to metabolism and growth rather than stress resistance, and yet not show the degenerative changes associated with senescence? Clearly, a species with robust indeterminate growth such as the salmonids should be used to address this question. If it is true that fish can have upregulated growth without the costs in terms of aging, a number of biochemical mechanisms could be proposed as explanations. With their lower metabolic rate and toleration of hypoxia, fishes may be exposed to less oxidative stress than homeotherms. Further, it is possible that fishes have routes for disposal of ROS that are not available to land animals. Fishes can eliminate nitrogenous wastes directly as ammonia into the water. Depending on ROS reactivity, chemical properties, location of production, and the potential presence of carrier molecules, similar routes could be used for their elimination. Endocrine mechanisms may also be involved in the hypothesized resistance of fishes to senescence. Recently described anti-cancer effects of the trout IGF-I E-peptides could be a mechanism for controlling the negative effects of IGF-I (Chen *et al.* 2002). Other potential endocrine mechanisms might involve differences in the roles of insulin and glucose in fishes as opposed to tetrapods, potential differences in the effect of insulin/IGF-I signaling on the cellular stress response, and differences in tissue

characteristics associated with the indeterminate, allometric growth pattern of fishes. For example, the persistent neurogenesis found in the retina of teleosts, but not in mammals, has been linked to the indeterminate growth of fish through the presence of IGF-I sensitive stem cells in the teleost retina (Otteson *et al.* 2003).

The semelparous reproductive mode provides both opportunities and obstacles in the use of fish as models of lifespan regulation. Death after spawning is a compelling example of rapid senescence, but it is unclear how representative this process is of normal aging (Dickhoff 1989). In semelparous salmonids, the decision to mature is indirectly a decision to die, and thus lifespan is determinate and regulated. Pacific salmon are semelparous, but the closely related rainbow and cutthroat trout are iteroparous. Iteroparity has been known for some time to occur under some conditions in chinook salmon, and an evolutionary continuum has been proposed between semelparity and iteroparity in salmonids (Unwin *et al.* 1999). The death of Pacific salmon, lampreys, and eels after spawning has been reviewed from a mechanistic standpoint (Dickhoff 1989). Gonadectomy extends life in salmon, and GnRH injection accelerates death, suggesting that processes leading to death are stimulated by reproductive steroids or other gonadal factors. The interrenal is activated in spawning lampreys and salmon, suggesting that cortisol is involved. In one mechanistic model of post-spawning death in salmon, reproductive steroids increase the production of cortisol, which at high levels leads to catabolism of tissues and immune suppression. In another model, proteolytic enzymes and other factors released during ovulation damage organs within the body cavity. Support for the mediation of death by cortisol is provided by a comparison of

semelparous (4600 g migratory adult) and iteroparous (75 g mature parr) male chinook salmon, in which total and free cortisol were considerably higher in semelparous fish (Barry *et al.* 2001). Recently, aging-related pathologies were assessed in iteroparous female guppies (Reznick *et al.* 2001). Comparisons of old and young laboratory raised guppies showed that a variety of conditions associated with inflammation of the viscera and body cavity were increased in old female guppies (oophoritis, granuloma, enteritis, melano-macrophage hyperplasia, pigment deposition, myocardial vacuolation, and pleuroperitonitis). This pattern is consistent with degenerative changes resulting from reproductive effort, especially considering that guppies are livebearers. Waste products, mechanical stresses, and proteolytic enzymes from the developing young could affect the viscera of the mother. Visceral pathologies are also consistent with cortisol mediated tissue catabolism and infection secondary to immune suppression. Thus, evidence from iteroparous guppies seems consistent with the cortisol mediated and ovulatory enzyme models proposed for the death of Pacific salmon after spawning (Dickhoff 1989). Therefore, it is reasonable to hypothesize that aging related degenerative changes in fish are a consequence of the reproductive life history mode. The use of fish models might enable evaluation of the relative importance of reproduction and growth in the regulation of lifespan. Reproduction and growth are closely linked by the cessation of growth at puberty in animals with determinate growth, whereas the linkage may be looser in fishes.

Applications of Growth Axis Research in Fish

Investigators working on the growth axis in fishes may be motivated by intellectual curiosity, but they are funded based on potential applications of their

research. Established applied research programs in salmon focus on the modulation of growth in order to control reproductive maturation and smolting in hatchery fish (e.g., Swanson 1995; Dickhoff *et al.* 1997; Beckman *et al.* 1999; Beckman *et al.* 2000; Shearer *et al.* 2000). The basic idea is to reproduce in the hatchery the essential features of the nutritional and other conditions experienced by wild fish. Another major applied area is growth enhancement in aquacultured species. GH transgenesis has enhanced growth in a variety of species (Du *et al.* 1992; Devlin *et al.* 1994; Hernandez *et al.* 1997; Martinez *et al.* 2000; Maclean *et al.* 2002), however, this technology is unpalatable to consumers at present, and there are valid concerns about the effect of transgenic escapees on wild populations. In the following paragraphs, less developed applications are reviewed: the use of blood IGF-I level as an index of the nutritional status or growth rate of fish, and the use of blood levels of lower molecular weight IGFBPs as indicators of stress levels.

Total plasma IGF-I is established as an indicator of nutritional status, and more generally of anabolic/catabolic status, in human clinical settings and in other mammals (Straus 1994; Thissen *et al.* 1994; Estivariz *et al.* 1997; Thissen *et al.* 1999). Total plasma IGF-I clearly adjusts to reflect nutritional status in fish, including salmon (Moriyama *et al.* 1994; Perez-Sanchez *et al.* 1995; Matthews *et al.* 1997a; Beckman *et al.* 1998b; Duan 1998; Moriyama *et al.* 1999; Perez-Sanchez *et al.* 1999; Moriyama *et al.* 2000; Larsen *et al.* 2001b; Pierce *et al.* 2001; Baker *et al.* 2003; Uchida *et al.* 2003). An important point from these studies is that the change in IGF-I is sustained. IGF-I moves to a new level based on nutritional status and stays there, superimposed on developmental and seasonal changes. Other nutritionally regulated hormones such as insulin and GH, in

contrast, often change transiently or in a complex way in response to a change in nutritional status (Navarro *et al.* 1993; Himick *et al.* 1995; Navarro *et al.* 1995; Moriyama *et al.* 1999; Uchida *et al.* 2003). Thus, the available data support the possibility of the use of plasma IGF-I as an index of nutritional status in fish. One complicating factor is that increased dietary or body fat levels may increase blood IGF-I levels independently of energy intake (Shearer *et al.* 1997; Pierce *et al.* 2002), and another is the increased blood IGF-I levels found in maturing male salmon (Shearer and Swanson 2000; D. Larsen and P. Swanson, unpublished data).

The use of IGF-I as an index of nutritional status is different from its use as an index of growth rate. Mammalian studies on nutritional status and IGF-I have often been done in adults, which are no longer growing. Extending results on nutritional regulation of IGF-I in fish to claim a relationship between total plasma IGF-I and growth rate requires an assumption that nutritional status and growth rate are closely related. Under normal circumstances in immature fish this assumption may be true, but it is rarely tested. Another common assumption is that larger individuals from the same cohort have higher growth rates. While this is true over the lifetime of the fish in question, it is becoming clear that IGF-I best reflects growth rate over a period of 1 to 4 weeks. The relationship between growth rate over this short period and growth rate over the lifetime of the fish is not known, however, particularly in wild fish, growth rates probably vary considerably over time. To unequivocally show a relationship between total plasma IGF-I and growth rate, it is necessary to measure growth rate.

To use plasma IGF-I level as a growth index, the time period over which it is desired to assess growth rate must be specified. Bioenergetic modelers are able to measure short-term (days) energy intake by stomach fullness, and long term (months) growth by otolith and scale studies. However, there is currently no easily measurable proxy for energy intake and growth over periods of about 2 weeks (M. Mazur, D. Beauchamp, personal communication). Therefore, one potential purpose for IGF-I as a growth index would be as a proxy for growth rate over this period. Results thus far in salmon are consistent with adjustment to nutritional status within a 1 to 4 week period (Moriyama *et al.* 1994; Beckman *et al.* 1998b; Moriyama *et al.* 1999; Larsen *et al.* 2001b; Pierce *et al.* 2001; Baker *et al.* 2003; Beckman *et al.* 2003b).

To provide useful information on patterns of variation in growth rate in wild fish, an index of growth rate should be able to detect differences between the growth rates of individuals. To assess whether this is possible, it is essential to measure the growth rates of individual fish over a single time period. The relationship between individual IGF-I levels and growth rates can then be analyzed using linear or nonlinear regression. To my knowledge, three published studies have shown data of this sort, with similar linear correlation coefficients (Pierce *et al.* 2001 coho salmon, $r^2 = 0.62$; Beckman *et al.* 2003b coho salmon, $r^2 = 0.36$-0.68; Uchida *et al.* 2003 tilapia, $r^2 = 0.55$). In fish that had been serially sampled for blood (two blood draws), a strong correlation between total plasma IGF-I and growth rate was only found in fish on maintenance rations, suggesting that stress and ration interact in determining the strength of IGF-I/growth rate relationship (Pierce *et al.* 2001). The mechanism for this interaction is likely to involve differences in

plasma IGFBP profiles, since both stress and nutritional status affect IGFBPs. The establishment of reliable techniques for IGFBP extraction and IGF-I radioimmunoassay were essential in showing these relationships (Shimizu *et al.* 2000), since the same correlation using an earlier IGF-I assay was considerably weaker (A. Pierce, unpublished data). Groundbreaking studies in which corresponding treatment differences in growth rate and IGF-I were shown opened up this area (Moriyama *et al.* 1994; Duan *et al.* 1995; Perez-Sanchez *et al.* 1995; Duan 1998; Moriyama *et al.* 2000), however, the "correlations" between growth rate and IGF-I described in these studies are not the same as the correlations required to validate IGF-I as an index of growth rate. In its statistical sense, "correlation" implies a continuous relationship between two variables, and the desired correlation in this case would be a monotonically increasing (preferably linear) function, whereas a treatment difference in an ANOVA is a discontinuous step function. Other studies have used tank average IGF-I levels, and combined growth rates and IGF-I levels measured over different time periods into a single regression (e.g., Beckman *et al.* 1998b; Mingarro *et al.* 2002). Again, this shows a general relationship between IGF-I and growth rate, but it is not correct to interpret these data as showing that IGF-I has been validated as a growth index, because the data points do not represent individual fish, and IGF-I and growth rate were not measured over a single time period. Beckman (2001) discussed these points and reached similar conclusions. The crucial relationship is between individual fish growth rates over a single time period and blood IGF-I levels. A complicating factor is that IGF-I shows strong seasonal and developmental changes in salmonids, which sometimes correspond with changes in growth rate and sometimes do

not (Moriyama *et al.* 1997; Beckman *et al.* 1998a; Beckman *et al.* 2000; Larsen *et al.* 2001b; Pierce *et al.* 2002; Beckman *et al.* 2003a). The slope of the linear regression line and strength of the correlation between IGF-I and growth rate may change seasonally (Beckman *et al.* 2003b). Recently, an assay has become available for salmon 41 kDa IGFBP, putative IGFBP-3 (Shimizu *et al.* 2003a). The addition of information on circulating IGFBP levels is likely to improve our ability to estimate growth rates of fish based on a blood sample.

Another application of growth axis research in fish is the use of lower molecular weight IGFBPs (IGFBP-1: 22-24 kDa, and putative IGFBP–2: 28-30 kDa) as indices of stress level (Kelley *et al.* 2001; Kelley *et al.* 2002). Cortisol is the primary hormone in the endocrine stress response in fish, but cortisol levels change rapidly enough that stress associated with capture complicates interpretation (Barton *et al.* 1991; Wendelaar Bonga 1997; Mommsen *et al.* 1999). In jack mackerel, blood levels of the 24 and 30 kDa IGFBPs increased with increasing stress level on Western ligand blots (Kelley *et al.* 2001). IGFBP-1 levels increased very strongly after saltwater transfer in some coho salmon (M. Shimizu, unpublished data). Injection of cortisol caused increases in 24 and 30 kDa IGFBPs within 2 h in tilapia, which were sustained through 8 h and had returned to baseline by 24 h (Kajimura *et al.* 2003). Cortisol and dexamethasone injection also increased low molecular weight IGFBPs in gobies (Flores and Kelley, unpublished data, cited in Kelley *et al.* 2001). Dexamethasone strongly increased media levels of IGFBP-1 and increased levels of putative IGFBP-2 in primary salmon hepatocyte culture (A. Pierce and M. Shimizu, unpublished data). Thus, blood levels of low molecular weight

IGFBPs show promise as indices of stress level, probably because blood IGFBP levels integrate cortisol stimulation of hepatocyte and other cell type production of IGFBPs over a time period of minutes to hours. The establishment and validation of radioimmunoassays for these IGFBPs will make data in this area more quantitative and enable comparison between studies.

Chapter 2: Time course of the growth axis response to fasting and increased ration in chinook salmon (*Oncorhynchus tshawytscha*).

INTRODUCTION

The fasting response is universal among animals. With limited resources in varying environments, animals must partition energy between growth, reproduction, activity, and basal metabolism. During fasting, somatic growth is curtailed, and energy is mobilized from tissues to support metabolism. In vertebrates, the effect of nutritional status on growth is mediated by the growth endocrine axis. The growth stimulatory hormone, insulin-like growth factor-I (IGF-I), is at the center of this highly complex regulatory system (reviewed in Rosenfeld *et al.* 1999). The liver produces circulating or endocrine IGF-I, while other tissues produce locally acting IGF-I. IGF-I circulates bound to a number of IGF binding proteins (IGFBPs), which slow the clearance of IGF-I, modify IGF-I effects on target tissues, and exert IGF-I independent effects. Most IGFBPs are produced by the liver. The most abundant circulating IGFBP in mammals is IGFBP-3, which acts as a plasma reservoir for IGF-I. Growth hormone (GH) stimulates liver production of IGF-I and IGFBP-3 (Rajaram *et al.* 1997). GH is secreted in a pulsatile manner by the pituitary gland, under regulation by hypothalamic and peripheral factors, and IGF-I exerts significant negative feedback on GH (Tannenbaum 1993). IGF-I and GH act through membrane-bound receptors, the IGF type I receptor (IGF-IR) and growth hormone receptor (GHR).

In all mammals studied, fasting causes a decrease in IGF-I, and in all except rats, an increase in GH (Tannenbaum 1993; Thissen *et al.* 1994; Thissen *et al.* 1999). This may be adaptive in that low IGF-I limits growth, whereas high GH mobilizes stored energy by stimulating lipolysis. The increase in GH does not cause increased IGF-I production because liver IGF-I production becomes resistant to stimulation by GH (Thissen *et al.* 1994). Liver IGF-I production is the most important point of regulation of plasma IGF-I levels (Le Roith *et al.* 2001). In fasting and other catabolic states, the liver becomes resistant to GH, due to downregulation of hepatocyte GH signaling at the receptor and post-receptor level (Thissen *et al.* 1999; Beauloye *et al.* 2002). The endocrine mechanisms leading to GH resistance are not well understood. Hepatocyte culture studies in mammals and birds have shown that pancreatic, stress, and thyroid hormones can regulate hepatic GH sensitivity and IGF-I production, as can levels of nutrients such as amino acids and glucose (Tollet *et al.* 1990; Houston *et al.* 1991; Denver *et al.* 1994; Brameld *et al.* 1999; Thissen *et al.* 1999). However, the sequence of events *in vivo* leading to the development of liver GH resistance during fasting is not well established. Further, the relative importance of additional points of regulation such as blood IGF-I binding capacity is not known. More generally, then, the sequence of events leading to decreased plasma IGF-I and increased GH during fasting is not well understood.

Comparisons between different vertebrate classes in the operation of the growth axis should aid in identifying the most conserved and hence presumably the most

important functional relationships. The growth axis in teleost fishes is similar to other vertebrates (Duan 1998; Mommsen 1998; Kelley *et al.* 2000; Moriyama *et al.* 2000; Perez-Sanchez 2000). Core features of the growth axis, such as GH stimulation of IGF-I, negative feedback of IGF-I on GH, and the presence of multiple IGFBPs, have been shown in fish. Several considerations make fish an attractive group for studies of the growth axis. First, most fish species including salmon grow continuously through most of their lives, unlike land vertebrates (Dutta 1994; Mommsen 1998). Interactions between nutritional status and determinate growth patterns are less of an issue in fish than in common experimental mammals. Second, fishes are ectothermic, with a metabolic rate considerably lower than comparable endotherms. This should slow down the process of physiological adaptation to fasting, making it more experimentally accessible. Finally, many fish species, including salmon, undergo prolonged fasting as part of their normal life cycle (Navarro *et al.* 1995). Pathological changes found in moribund animals would be expected to occur much later in fish than in common experimental mammals during fasting.

We propose a model in which nutritional control of the growth axis in salmon is peripheral, exerted at the liver. During fasting, changes in nutrient or nutrient-sensitive hormone levels lead to the development of hepatic GH resistance, which causes a decrease in IGF-I production and subsequently plasma IGF-I. Relaxation of negative feedback then causes an increase in pituitary GH secretion. This model predicts that IGF-I should decrease before GH increases in the fasting response. Existing studies on

the growth axis response to fasting in salmon do not have sufficient resolution to determine if the time course is consistent with this model. Indeed, such information is rare in any vertebrate species. Therefore, we performed a feeding experiment, assessing the response of juvenile chinook salmon to a change in nutritional status. Fish were fed fasting, control, or maximum rations and sampled at frequent intervals.

MATERIALS AND METHODS

Animals and Treatments

Willamette strain chinook salmon were raised from eyed eggs in our hatchery at the Northwest Fisheries Science Center, Seattle, WA, U.S.A. Fish were held in recirculating water, with temperature ranging from 11.6 to 12.5 °C, under simulated natural photoperiod. Fish were fed BioOregon-Starter pellets for an initial period, and then fed a high protein, low fat diet beginning 20 Feb as part of a larger experiment (54% protein, 7% fat, 19.7 kJ/g, diet formulation and feeding protocol given in Alcorn *et al.* 2003). Fish in this experiment were fed at 52% of *ad libitum* ration, set by feed intake in the larger experiment. Fish were divided into 6 1.3-m cylindrical tanks (2 tanks per treatment; 160 fish per tank) on 29 July, sampling began 13 Aug, and feeding treatments began 20 Aug (day 0). Fish in the Control treatment continued on 52% ration, food was withheld from fish in the Fasted treatment, and Maximum ration fish were fed to excess. Fish were fed daily after sampling.

Sampling

All fish were sampled at days –7, -3, 0, 1, 2, 3, 4, 6, 8, 12, 15, 23, and 29; the Control and Maximum treatments were continued for days 36 and 46. Experimental feeding treatments were begun immediately after sampling on day 0. Sampling took place from 8 to 10:00 AM; thus, the day 1 sample was taken 22-24 h after experimental rations were begun. During sampling, 10 fish were removed from each tank, fatally anesthetized with buffered MS-222 (tricane methanesulfonate), weighed, fork length measured, and heparinized blood collected. Blood was centrifuged, hematocrit noted, and plasma stored at –80 °C. Livers were removed, weighed, and snap frozen in liquid nitrogen. The sex, maturation status, and any indications of disease were noted for each fish.

Assays

Radioimmunoassays (RIAs). Plasma GH was assayed according to Swanson (1994), except recombinant coho salmon (*O. kisutch*) GH (GroPep Pty Ltd, Adelaide, Australia) was used as tracer and standard, Pansorbin (Calbiochem) was used as a precipitant, and 1% normal rabbit serum was added to buffers to increase the efficiency of precipitation. Four GH assays were run, with intraassay coefficient of variation (CV) 10.0% and interassay CV 5.3%. Samples with high GH values beyond the range of the RIA curve were diluted and reassayed. Total plasma IGF-I was assayed after acid-ethanol extraction of IGFBPs according to Shimizu et al. (2000). Three IGF-I assays were run, with intraassay CV 6.0% and interassay CV 5.7%, including the extraction step. Plasma insulin was assayed according to Plisetskaya et al. (1986), with native coho

salmon insulin used as tracer and standard, and pansorbin precipitation. Three insulin assays were run, with intraassay CV 8.6% and interassay CV 2.3%. Plasma 41-kDa IGFBP was assayed in a single RIA according to Shimizu et al. (2003a), with intraassay CV 3.6%. Due to inadequate plasma volumes, plasma was pooled prior to assaying for the 41-kDa IGFBP (equal volumes from each fish, n = 3 to 4 pools per treatment per time point). For all RIAs, all of the samples from a given sampling day were run within a single assay, except for those samples reassayed for GH.

TaqMan assay. Liver IGF-I gene expression was measured in the Control and Fasted groups on selected sampling days with a TaqMan quantitative RT-PCR assay (Pierce *et al.* 2003). TaqMan PCR was performed on an Applied Biosystems (ABI) 7700 Sequence Detector, using the standard cycling conditions and primer and probe concentrations, with 25 µl/well PCR mixture made from ABI Universal PCR Master Mix. For each assay on each plate, a serial dilution of sample cDNA was run to assess PCR efficiency. IGF-I gene expression levels were normalized to expression level of acidic ribosomal phosphoprotein P0 using an efficiency corrected relative expression method (Pierce *et al.* 2003). Four TaqMan assays were run, for days 0 and 22, 2 and 4, 6 and 8, and 12. Control and Fasted samples from each sampling point were run in the same assay. Due to potential variation between runs of the TaqMan assay, IGF-I gene expression levels are only compared between treatment groups, and not over time.

Data Analysis

Condition factor (CF) was calculated as 100 x weight (g) x length (cm) $^{-3}$. Hepato-somatic index (HSI) was calculated as 100 x liver weight / body weight. An epizootic of bacterial kidney disease (BKD: disease organism *Renibacterium salmoninarum*) occurred during the experiment. Fish significantly affected by BKD as identified by physical examination, i.e. fish with kidney lesions, ascites, or petichial hemorhage, had low hematocrits and high HSIs. Therefore, fish with hematocrit below 27.5 or HSI above 2.5 were excluded from the data prior to analysis (15.9% of fish total, number excluded not significantly different between treatments). Precociously mature male fish (n=3) were also excluded from the data.

Results were considered statistically significant at $p < 0.05$. Treatment and time effects on physiological variables were examined by multi-way ANOVA, followed by the Student-Newman-Keuls test, or an unpaired t-test. There were no significant within-treatment tank effects, so the two tanks for each treatment were pooled. GH data were square root transformed prior to analysis to achieve homogeneity of variance. GH outliers were identified by an extreme studentized deviate procedure (maximum two outliers in each treatment at each time point, Rosner 2000). Linear regression and Pearson's correlation coefficient were used to investigate relationships between variables.

RESULTS

Fish body weight, CF, and liver weight responded to feeding treatment (Figure 2.1). No differences were found in physical parameters between treatment groups prior

to day 1. Fasted fish did not change significantly in weight from day 0 onward. Control and Maximum ration fish increased in weight throughout the experiment. Fasted fish weight was significantly lower relative to fed groups from day 6 onward. Fork length was similar to body weight in terms of treatment differences and changes over time (data not shown). CF was elevated in Maximum ration fish relative to Controls at days 1 and 2. No further differences in CF between the fed treatments were found, except at day 22, where Maximum ration fish had higher CF. CF was significantly lower in Fasted fish relative to Control and Maximum ration fish from day 2 onward. CF decreased over time in Fasted fish from day 0 onward. Liver weight was significantly lower in Fasted fish versus Maximum ration from day 1 onward, and versus Control ration from day 3 onward. Liver weight decreased significantly in Fasted fish from day 0 to day 1, after which no significant changes occurred. Liver weight was consistently higher in Maximum ration fish than Controls from day 1 onward. This difference was only significant at day 3, however, combining days 1 – 46, liver weight was significantly higher in Maximum ration fish. Liver weight increased in the fed treatments up to day 22, after which it did not change significantly. No sex differences were found in immature fish in any physical or endocrine parameter.

Some high GH values were identified as outliers (Figure 2.2a). Outliers were excluded from the GH profile over time to reduce the effect of these extreme values on the data (Figure 2.2b). Outliers occurred equally in all treatments (Control 8 outliers, Fast 6, Maximum 6; Chi-squared test of independence, $p = 0.89$). However, GH values

greater than 50 ng/ml occurred exclusively in fasted fish, and increased in frequency with increasing duration of fasting. The GH assay was repeated on many of these samples to confirm results, and there was no indication that the assay did not work properly (for values > 50, RIA binding 32-80% of binding without added standard).

GH did not differ significantly between feeding treatments or over time in Control and Maximum ration fish (Figure 2.2b; average 2.99 ± 0.20 ng/ml). GH was anomalously elevated in Fasted fish prior to beginning feeding treatments at day –7. After beginning feeding treatments, GH was consistently higher in Fasted fish from day 1 onward. The elevation in Fasted fish was significant versus both other treatments from day 4 onward. At days 1, 2, and 3, the contrast between Fasted fish and either or both of the fed treatments narrowly missed significance. Since Control and Maximum ration GH levels were never different, the combined fed treatments were compared to Fasted. In this comparison, Fasted fish had significantly higher GH levels at all time points from day 1 onward. GH increased significantly over time in Fasted fish. Levels were maintained at ~10 to 20 ng/ml from days 1 to 12, and then increased sharply from day 15 to 29, attaining levels of 169 ± 77 ng/ml at the final sampling of Fasted fish.

Components of the IGF system responded to feeding treatments (Figure 2.3). Total plasma IGF-I did not differ between treatments in pre-treatment samples. After beginning feeding treatments, Fasted fish had significantly lower plasma IGF-I levels from day 4 onward (Figure 2.3a). IGF-I decreased in Fasted fish up to day 15, and then remained relatively constant. There was little difference between plasma IGF-I levels in

Control and Maximum ration fish. From day 12 onward, plasma IGF-I was slightly but consistently elevated in Maximum ration fish compared with Controls; this difference was only significant at day 29, but combining days 12-46, Maximum ration fish had significantly higher plasma IGF-I levels than Controls. Plasma IGF-I increased over time in fed fish, with a peak at day 29 (18 Sept) in Control and Maximum ration fish.

Plasma 41-kDa IGFBP did not differ significantly between treatments from days 0 to 3. Fasted fish had significantly lower levels of 41-kDa IGFBP from day 4 onward, and levels of this IGFBP decreased over time in Fasted fish. There was no consistent difference between Control and Maximum ration fish in levels of this IGFBP, and little or no change over time.

Liver IGF-I gene expression levels were decreased by fasting. Expression levels were measured for selected days in Control and Fasted fish only. IGF-I expression levels did not differ at day 0; at day 2 Fasted fish had significantly lower expression levels; at day 4 expression levels did not differ; and at days 6, 8, 12, and 22 Fasted fish had significantly lower liver IGF-I gene expression levels.

Plasma insulin did not differ between treatment groups prior to beginning feeding treatments (Figure 2.4). Insulin was significantly elevated in Maximum ration fish versus Controls at days 1 and 2, after which it decreased to Control values. Insulin did not differ significantly between Maximum and Control fish at any other time points, except days 15 and 22 when Maximum ration insulin was higher, and day 29 when Control was higher. In Fasted fish, insulin was lower than in the fed treatments from day 6 onward, however,

this difference was not significant versus either or both of the fed treatments at days 8, 22, and 29. Insulin changed little over time in Fasted fish, whereas in both fed treatments, an increasing trend was observed, with a peak at day 36 (Sept 25) followed by a decline.

Correlations between selected variables are shown in Table 2.1. Body weight was positively correlated with plasma IGF-I levels at all time points. This correlation was also significant within each treatment group, with a few exceptions (Control: days 6 and 22 not significant, Fast: day 6, Maximum: days 0, 3, and 29). Liver IGF-I gene expression was positively correlated with plasma IGF-I at all time points where gene expression was measured, except day 4. Significant negative correlations between GH and plasma IGF-I or liver IGF-I gene expression were found at a few time points. Significant positive and negative correlations between plasma insulin and plasma IGF-I or GH were found at 6 or 5 of the 15 time points, respectively.

Discussion

This study provides the first example of the detailed time course of the response of the growth axis to fasting in a teleost fish. Overall, the results fit expectations, with total plasma IGF-I, liver IGF-I gene expression, and plasma 41-kDa IGFBP declining during fasting, and GH increasing. GH increased before IGF-I decreased during fasting, underlining the importance of the development of liver GH resistance in the fasting response.

Fasting reduced CF and liver weight within 2 days, suggesting that metabolism was changed within this time. However, Fasted fish did not lose significant body weight through 29 days, and mortality was minimal (1 fish). Thus, although Fasted fish were affected by experimental treatment, they were not starving. Chinook salmon used in the study had low fat levels for hatchery fish (3 - 5% by weight, Alcorn *et al.* 2003), and hence probably responded rapidly to food deprivation. CF is a measure of fish body shape, which is higher in individuals with a larger girth in relation to their length. CF increased in fish fed Maximum rations at days 1 and 2. This was probably due to hyperphagia and increased gut fullness in these fish. CF subsequently declined in Maximum ration fish, consistent with the operation of appetite limitation mechanisms (Lin *et al.* 2000). The separation between Control and Maximum ration fish in body size was not as great as expected. This may have been due to decreased feed consumption by fish with BKD, resulting in increased ration size for healthy fish in the Control treatment.

GH release in salmonids is episodic (Gomez *et al.* 1996), as in other teleosts (Zhang *et al.* 1994). GH values were highly variable in the present study. Low levels presumably were from fish sampled between secretion episodes, whereas the higher individual values are from fish sampled during a secretion episode. Very high GH values over 50 ng/ml were found only in fasted fish, and increased in number with duration of fasting (Figure 2.2a). Given the large number of Control and Maximum ration fish sampled, if such high values occurred in the fed treatments, they would have been found.

This implies that GH pulse height during secretion episodes is increased by fasting in salmon.

Treatment average GH was low in the Control and Maximum ration groups (1-6 ng/ml), and did not differ between these treatments or over time, in agreement with previous studies finding that dietary manipulation above maintenance rations in salmonids did not change GH levels (Storebakken *et al.* 1991; Pierce *et al.* 2001). Fasting, in contrast, clearly elevated GH. There were two phases in the GH response to fasting: an initial mild GH elevation to 10-20 ng/ml beginning 1 day after fasting was initiated, followed by a strong increase beginning after day 12 and continuing through day 29. These phases have not been previously described, to our knowledge. The present study does not provide any information on the physiological function of the two phases in GH response; however, the phases might relate to stages in nutrient mobilization during fasting. GH levels found in previous studies are generally consistent with levels during the initial mild GH elevation (Sumpter *et al.* 1991; Duan *et al.* 1993c; Johnsson *et al.* 1996; Moriyama *et al.* 1999). The strong increase in GH occurred when IGF-I had reached its lowest level, so relaxation of negative feedback from IGF-I could account for this increase. However, the initial mild elevation in GH is not consistent with regulation by total plasma IGF-I, since total plasma IGF-I did not change until day 4. Changes in plasma GH in less than 1 day in response to feeding level have been described in goldfish (*Carassius auratus*; Himick *et al.* 1995). The initial mild GH elevation found in the present study could be due to regulation of GH secretion by ghrelin

or IGFBP-1. The recently discovered acylated peptide hormone ghrelin stimulates pituitary GH secretion, is released by the stomach during food deprivation, and causes subjective hunger in mammals (Kojima *et al.* 2001). Recent work shows ghrelin exists and stimulates GH secretion in fish (e.g., Unniappan *et al.* 2002; Kaiya *et al.* 2003). IGFBP-1 is a growth inhibitory IGFBP produced by hepatocytes that increases rapidly during stress and food deprivation (Thissen *et al.* 1994; Frystyk *et al.* 1999). An IGFBP with similar molecular weight and regulation has been found in salmon (Shimizu *et al.* 1999a). An increase in plasma IGFBP-1 could inhibit IGF-I signaling to the pituitary and result in increased GH secretion. Alternatively, changes in circulating levels of glucose, lipids, or other metabolic fuels could be responsible.

Fasting decreased plasma IGF-I; overall, the response of plasma IGF-I began at day 4 and was completed by about 2 weeks. Previous studies in salmon found plasma IGF-I adjusted to ration in 2 to 4 weeks (Moriyama *et al.* 1999; Larsen *et al.* 2001b; Pierce *et al.* 2001). Maximum ration IGF-I did not separate clearly from Controls, however, from day 12 and thereafter Maximum ration fish consistently had higher plasma IGF-I levels. The plasma IGF-I response was slower than in mammals and chickens, where plasma IGF-I responds to fasting or refeeding within 1-2 days (Isley *et al.* 1983; Goldstein *et al.* 1991; Frystyk *et al.* 1999; Beccavin *et al.* 2001). This difference is likely due to the slower metabolic rate of fish. There was no hint of any plasma IGF-I response to elevated GH in the Fasted group over days 1-3. At day 4, Fasted plasma IGF-I was significantly decreased in spite of elevated GH. These observations suggest that GH

resistance developed over days 1-3 and was established by day 4. The continued decrease in Fasted plasma IGF-I from days 4 to 12, during which GH continued to be elevated, implies that IGF-I clearance was greater than production during this time, and suggests an increase in GH resistance. The strong increase in Fasted GH from days 15 to 29 had no effect on plasma IGF-I, suggesting that these fish were no longer GH responsive in terms of plasma IGF-I level. From day 15 onward, Fasted plasma IGF-I held steady at a remarkably consistent 39.8 – 40.1% of Control levels. Comparable relatively constant residual levels of plasma IGF-I were found during 4 – 8 weeks of fasting in masu salmon (*O. masou*; Moriyama *et al.* 1999), and coho salmon (Larsen *et al.* 2001b). This residual 40% of plasma IGF-I does not appear to be subject to regulation by nutritional status.

Liver IGF-I mRNA levels decreased in Fasted fish relative to Controls during fasting, with a time course similar to that of plasma IGF-I. Decreases in liver IGF-I gene expression during fasting have been found in salmon (Duan *et al.* 1993c), and other teleosts (Matthews *et al.* 1997b; Meton *et al.* 2000; Uchida *et al.* 2003). The consistent positive correlation between liver IGF-I gene expression and plasma IGF-I level (Table 2.1) supports a significant role for liver IGF-I production in the regulation of plasma IGF-I level. The liver is the source of most endocrine IGF-I in mammals (Le Roith *et al.* 2001), and very likely this also holds in fish (Duan 1998; Moriyama *et al.* 2000). At day 2, the significantly lower liver IGF-I gene expression in Fasted fish compared to Controls could be an early manifestation of the development of GH resistance. One might expect

changes in liver IGF-I mRNA to precede changes in plasma hormone level. At day 4, liver IGF-I mRNA was not significantly lower in Fasted fish, and this is also the only day when the correlation between liver gene expression and plasma hormone level was not significant ($p = 0.81$). This was the time point at which significant decreases in plasma IGF-I and the 41-kDa IGFBP first appeared. One potential explanation of this situation might be that at this point in the response, when the most abundant IGFBP in salmon blood begins decreasing, plasma IGF-I is regulated by blood binding capacity to a greater extent than by liver IGF-I production.

Plasma levels of the 41-kDa IGFBP declined during fasting, in a manner similar to the decline in plasma IGF-I. A decrease in this IGFBP during fasting has previously been shown in salmon (Shimizu *et al.* 1999a; Shimizu *et al.* 2003a). Based on its high abundance in the blood, inhibition by fasting, stimulation by GH, molecular weight, and glycosylation, this IGFBP is functionally homologous to mammalian IGFBP-3 (Shimizu *et al.* 1999a; Shimizu *et al.* 2003a; Shimizu *et al.* 2003b). IGFBP-3 declines during fasting in mammals (Thissen *et al.* 1994). The salmon 41-kDa IGFBP declined in Fasted fish in the face of increasing GH levels, suggesting that liver production of the 41-kDa IGFBP became GH resistant during fasting. In mammals, IGFBP-3 is not produced by hepatocytes, but instead by liver macrophages (Kupffer cells, Zimmermann *et al.* 2000). The cellular location of production of the salmon 41-kDa IGFBP is not known at present. Correlations between plasma levels of IGF-I and the 41-kDa IGFBP could not be tested in the current study because IGFBP was measured in plasma pools; however, high

correlations between individual fish plasma IGF-I and 41-kDa IGFBP levels have been found (Beckman *et al.* 2003b; Shimizu *et al.* 2003a). Together with the similarity in fasting response, these data provide evidence that total plasma IGF-I and plasma 41-kDa IGFBP are regulated in concert.

The insulin response to feeding treatment was assessed because insulin regulates liver GH sensitivity in mammals. However, basal insulin did not respond strongly to feeding treatment. Due to increases over time in the fed treatments, Fasted insulin was significantly lower than Control at most time points from day 6 onward. The small fasting effect on basal insulin seems unlikely to play a major role in the salmon growth axis response to fasting. Correlations between basal insulin and plasma IGF-I were positive, but weak and inconsistent. Previous studies in salmon found decreases in plasma IGF-I or liver IGF-I mRNA before any change in insulin during fasting, and basal insulin sometimes did not decrease (Duan *et al.* 1993c; Baker *et al.* 2003). The effect of fasting on basal insulin varies seasonally in fishes (Navarro *et al.* 1995). There may be differences between salmon and endotherms in the role of insulin in the fasting response. In primary salmon hepatocytes, insulin did not potentiate the IGF-I gene expression response to GH (A. Pierce, in preparation), unlike mammalian and avian hepatocytes (Tollet *et al.* 1990; Houston *et al.* 1991; Thissen *et al.* 1999). Regulation of blood glucose is of less importance in the protein-centered metabolism of carnivorous fishes than in mammals and birds (Plisetskaya *et al.* 1993; Mommsen 1998). Nonetheless, alternative scenarios in which insulin functions similarly to mammals cannot be

excluded. Postprandial as opposed to basal insulin, or changes in hepatic portal insulin which do not appear in the general circulation (Plisetskaya *et al.* 1989), might regulate the liver in the salmon fasting response. The increase in insulin in Maximum ration fish on days 1 and 2 coincided with an increase in CF in this treatment. Both are probably due to hyperphagia and increased gut fullness. Insulin responds to feeding in salmonids, increasing from basal levels within minutes and remaining elevated for ~12 h after a meal (Navarro *et al.* 1993). Increased feed consumption after the switch to Maximum ration would be expected to slow digestion, and could result in postprandial elevations in insulin 22-24 h after the last meal, when fish were sampled.

In the Control and Maximum ration groups, plasma IGF-I and insulin increased to peaks near the end of the study. Plasma IGF-I peaked on 18 Sept. A peak in IGF-I near the autumn equinox was found in another experimental group from the same larger experiment (Pierce *et al.* 2002). An autumn peak in IGF-I has been found in masu salmon (Moriyama *et al.* 1997), and autumn increases in IGF-I have been found in coho salmon (Beckman *et al.* 2003b). The physiological significance of the autumn IGF-I peak is not yet clear. High variability in plasma insulin levels in the fed groups occurred near the time of the autumn IGF-I peak, and the highest plasma insulin values were observed on 25 Sept, followed by a decline. The profile of liver weight over time in fed fish showed an apparent breakpoint on 11 Sept. It is possible that these changes are related. In brown trout (*Salmo trutta*), insulin injection increased plasma IGF-I (Banos *et al.* 1999), and insulin increases liver glycogen storage in many fish species (Mommsen *et*

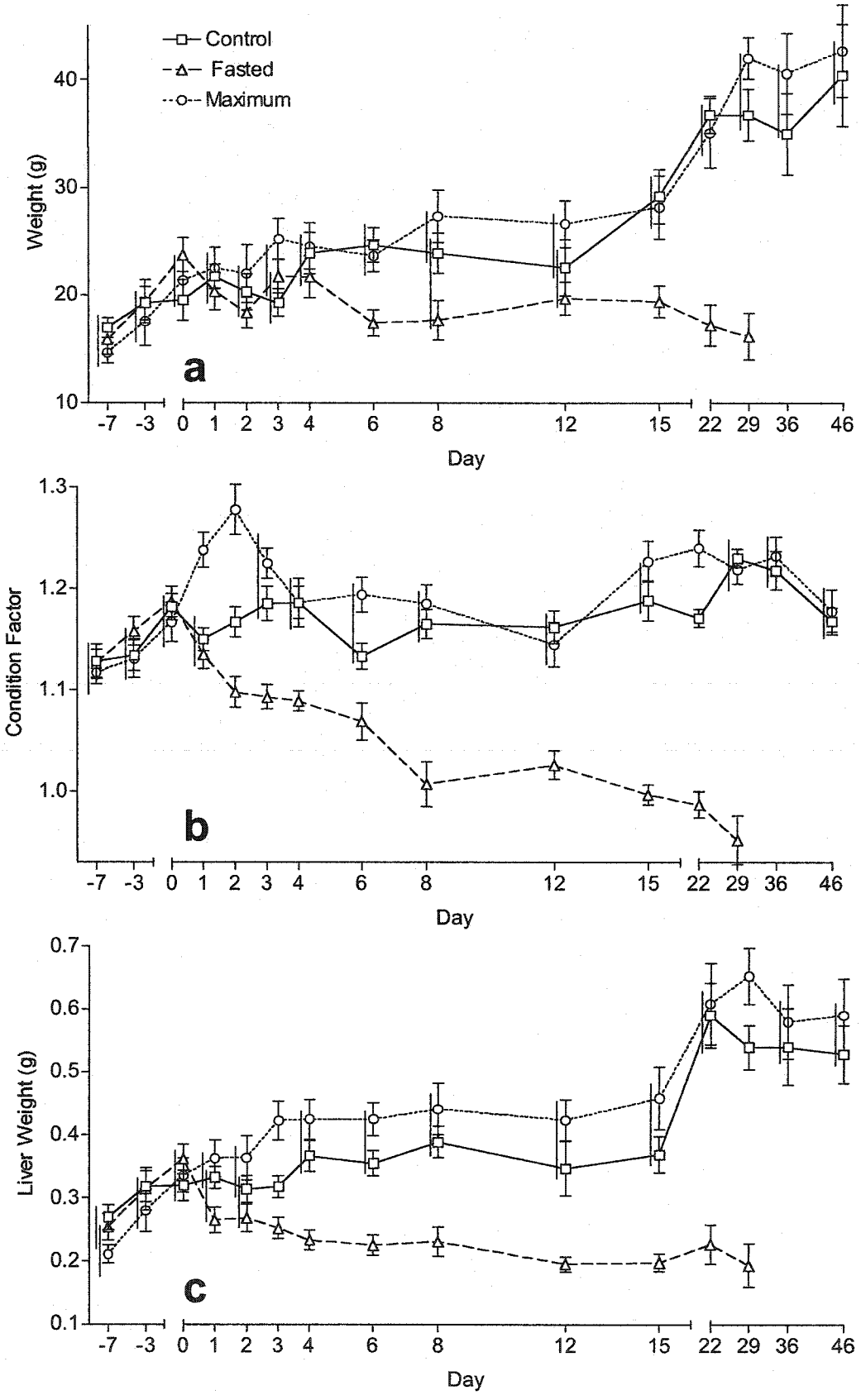
al. 1991). Metabolic changes near the autumn equinox might be part of a physiological process preparing fish for overwintering.

Plasma IGF-I was significantly correlated with body weight at all sampling points (Table 2.1). Similar relationships between body weight and plasma IGF-I were found in other studies on the same group of fish (Beckman *et al.* 2001; Pierce *et al.* 2002). These correlations can be explained in part as based on an underlying relationship between growth rate and IGF-I, since larger fish will have greater growth rates over the lifetime of the fish. Where individual fish growth rates and IGF-I levels have been measured, they have been found to be significantly positively correlated over growth periods from 1 - 3 weeks (Pierce *et al.* 2001; Beckman *et al.* 2003b; Uchida *et al.* 2003). However, in the present study the correlation between body weight and plasma IGF-I consistently held even within Fasted fish, except for a single time point (day 6, $p = 0.068$). Therefore, there may be an additional relationship between body size and plasma IGF-I level independent of growth rate, which could relate to the residual 40% of plasma IGF-I not regulated by nutritional status. Further studies are required to resolve this issue.

Some of the results of the current study support the model of nutritional regulation of the salmon growth axis presented in the introduction, whereas the model must be modified to account for other results. The correlations found between liver IGF-I gene expression and plasma IGF-I support liver IGF-I production as a main point of regulation of the growth axis. The coordinated declines in plasma IGF-I, plasma 41-kDa IGFBP, and liver IGF-I gene expression, in spite of increasing GH, suggests that the

development of liver GH resistance is a key event in the growth axis response to fasting. Determining how nutritional status regulates hepatocyte GH responsiveness is a research priority in understanding how the growth axis responds to nutritional conditions. The strong increase in GH after IGF-I had reached its nadir is consistent with regulation of GH at this stage by relaxation of negative feedback from IGF-I. However, the earlier increases in GH are not consistent with the model, and other mechanisms must be added to the model to account for these increases.

Figure 2.1. Response of weight (a), condition factor (b), and liver weight (c) to fasting and increased ration over time. Error bars represent standard error of the mean. Vertical lines to the left of symbols indicate differences among treatment groups at each point, with points connected by a line not significantly different. Differences over time within each treatment are listed sequentially in the following, with points sharing a letter not significantly different. (a) Control: **-7 to 4** a, **6** ab, **8 to 12** a, **15** ac, **22 to 29** cd, **36** cbd, **46** d; Fasted: **-7** a, **-3** ab, **0** b, **1 to 29** ab; Maximum: **-7** a, **-3 to 2** ab, **3 to 6** abc, **8 to 12** bc, **15** bcd, **22** ce, **29** e, **36** de, **46** e. (b) Control: **-7 to –3** a, **0** abc, **1** ab, **2 to 4** abc, **6** a, **8 to 22** abc, **29** c, **36** bc, **46** abc; Fasted: **-7** def, **-3** ef, **0** f, **1** def, **2** de, **3 to 4** cde, **6** bcd, **8** ab, **12** abc, **15 to 29** a; Maximum: **-7** a, **-3** ab, **0** a-d, **1** de, **2** e, **3** cde, **4 to 8** a-e, **12** abc, **15** cde, **22** de, **29** b-e, **36** cde, **46** a-d. (c) Control: **-7 to 6** a, **8** ab, **12 to 15** a, **22 to 36** c, **46** bc; Fasted: **-7** ab, **-3** bc, **0** c, **1 to 8** ab, **12 to 15** a, **22 to 29** ab; Maximum: **-7** a, **-3 to 2** ab, **3** b, **4** bc, **6** bcd, **8 to 12** bc, **15** bcd, **22** cd, **29** d, **36 to 46** cd.


Control
Fasted
Maximum
Weight (g)
40
30
20
10
a
-7 -3 0 1 2 3 4 6 8 12 15 22 29 36 46
Day
Condition Factor
1.3
1.2
1.1
1.0
b
-7 -3 0 1 2 3 4 6 8 12 15 22 29 36 46
Day
Liver Weight (g)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
c
-7 -3 0 1 2 3 4 6 8 12 15 22 29 36 46
Day

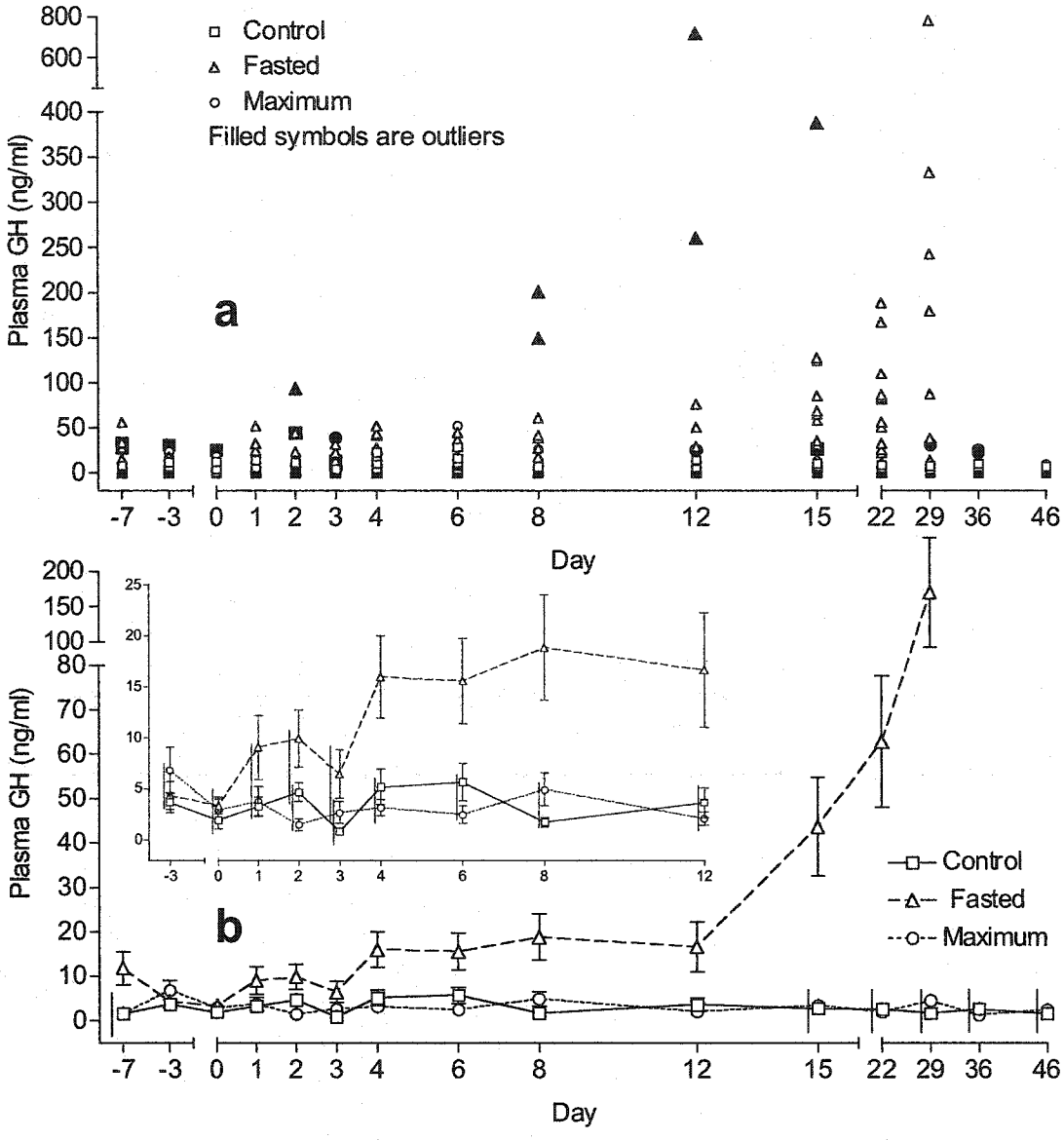
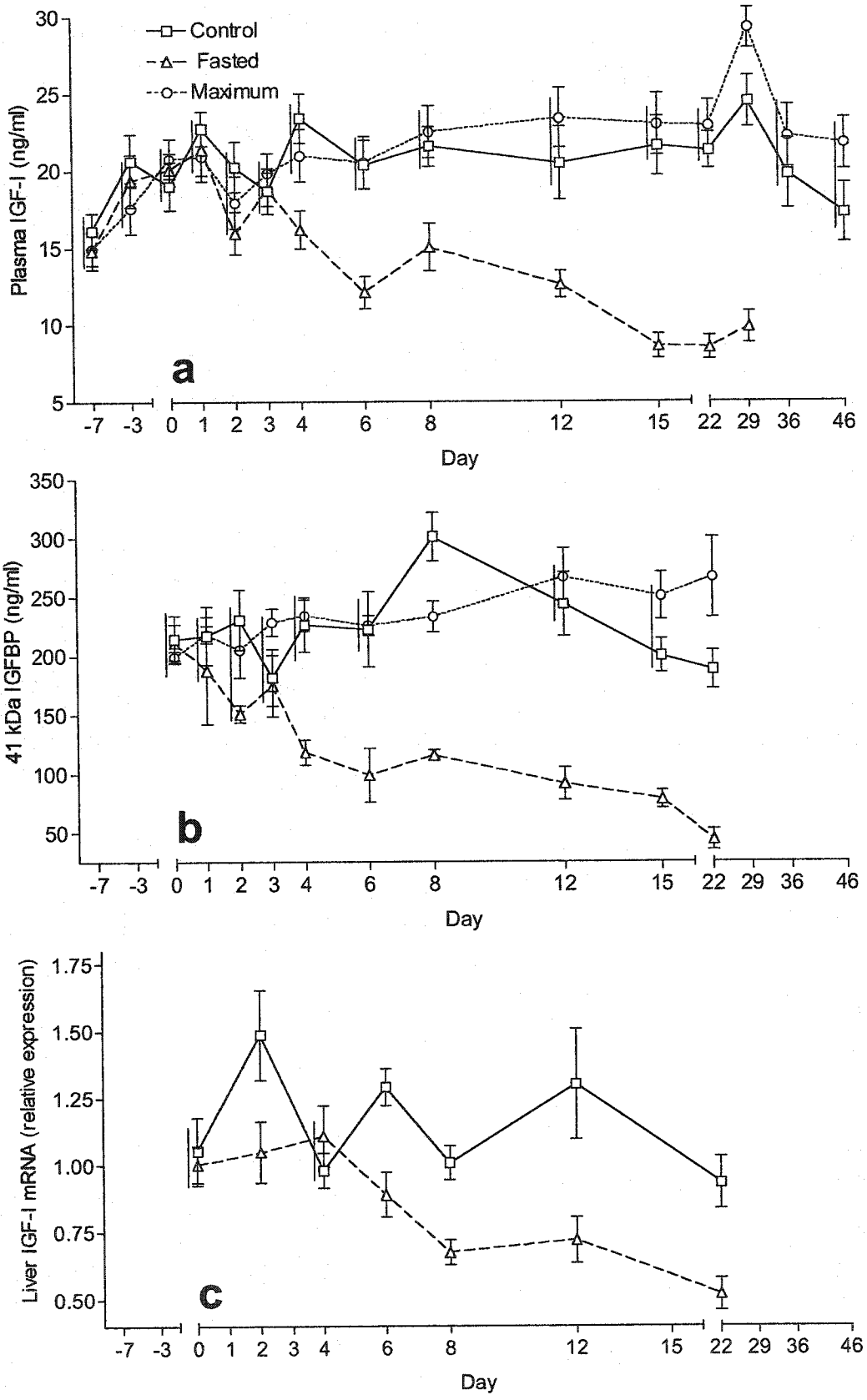


Figure 2.2. Response of individual (a) and treatment average (b) GH levels to fasting and increased ration over time. In (b), outliers were excluded. Inset shows early data with reduced y-axis range. Error bars and significances are shown as in Figure 2.1. A comparison of the combined Control and Maximum treatments against Fasted gave similar results, except Fasted GH was significantly elevated versus combined fed from day 1 onward. Control and Maximum: no significant changes over time; Fasted: **-7** ab, **-3 to 1** a, **2** ab, **3** a, **4 to 6** ab, **8** abc, **12** ab, **15** bc, **22** cd, **29** d.

Figure 2.3. Response plasma total IGF-I (a), plasma 41-kDa IGFBP (b), and liver IGF-I mRNA levels (c) to fasting and increased ration over time. Error bars and significances are shown as in Figure 2.1. (a) Control: **-7** a, **-3 to 22** ab, **29** b, **36 to 46** ab; Fasted: **-7** bc, **-3 to 0** cd, **1** d, **2** bcd, **3** cd, **4** bcd, **6** ab, **8** bc, **12** ab, **15 to 22** a, **29** ab; Maximum: **-7** a, **-3 to 4** ab, **6** abc, **8 to 22** bc, **29** c, **36 to 46** abc. (b) 41-kDa IGFBP was measured in pooled plasma samples; n = 3 to 4 per treatment per time point. Control: **0 to 2** ab, **3** a, **4 to 6** ab, **8** b, **12 to 22** ab; Fasted: **0** a, **1** ab, **2** a-d, **3** abc, **4 to 8** b-e, **12** cde, **15** de, **22** e; Maximum: no significant changes over time. (c) Liver IGF-I mRNA was measured in four separate TaqMan assays (days 0 and 22, days 2 and 4, days 6 and 8, day 12; Control and Fasted samples from each time point measured in the same assay). Changes in IGF-I gene expression over time were not analyzed statistically due to uncertainties in the comparison of results from different assays.


Control
Fasted
Maximum
Plasma IGF-I (ng/ml)
30
25
20
15
10
5
a
-7 -3 0 1 2 3 4 6 8 12 15 22 29 36 46
Day
41 kDa IGFBP (ng/ml)
350
300
250
200
150
100
50
b
-7 -3 0 1 2 3 4 6 8 12 15 22 29 36 46
Day
Liver IGF-I mRNA (relative expression)
1.75
1.50
1.25
1.00
0.75
0.50
c
-7 -3 0 1 2 3 4 6 8 12 15 22 29 36 46
Day

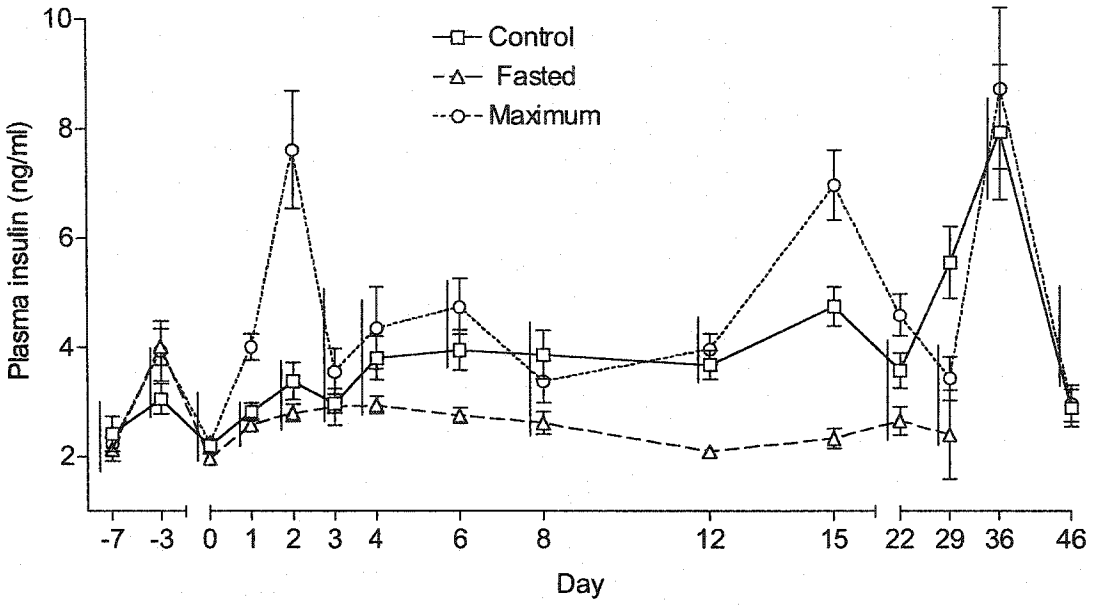


Figure 2.4. Response of plasma insulin to fasting and increased ration over time. Error bars and significances are shown as in Figure 2.1. Control: **-7 to –3** ab, **0** a, **1 to 12** ab, **15** b, **22** ab, **29** c, **36** d, **46** ab; Fasted: **-7** a, **-3** b, **0 to 2** a, **3** ab, **4 to 29** a; Maximum: **-7 to 1** a, **2** b, **3 to 12** a, **15** b, **22 to 29** a, **36** b, **46** a.

Table 2.1. Correlations between selected variables.

Correlation	Day	n	p	r	slope
Plasma IGF-I (y)	-7	59	<0.0001	0.535	0.525
vs.	-3	46	<0.0001	0.753	0.679
Body Weight (x)	0	51	<0.0001	0.563	0.410
	1	58	<0.0001	0.526	0.484
	2	52	<0.0001	0.621	0.525
	3	54	<0.0001	0.509	0.399
	4	49	<0.0001	0.628	0.538
	6	46	<0.0001	0.650	0.684
	8	52	<0.0001	0.700	0.489
	12	45	<0.0001	0.804	0.773
	15	47	<0.0001	0.730	0.653
	22	45	<0.0001	0.733	0.464
	29	41	<0.0001	0.864	0.616
	36	27	<0.0001	0.801	0.444
	46	28	<0.0001	0.627	0.325
Plasma IGF-I	0	30	0.0020	0.540	7.165
vs.	2	36	<0.0001	0.634	6.502
Liver IGF-I mRNA	6	32	0.0003	0.594	11.133
	8	32	<0.0001	0.656	15.476
	12	28	0.0019	0.560	6.635
	22	26	<0.0001	0.735	14.794
Square Root plasma GH	1	58	0.0017	0.402	0.100
vs.	15	45	0.0023	-0.444	-0.142
Plasma IGF-I	22	45	<0.0001	-0.616	-0.268
	29	38	0.0036	-0.460	-0.283
Square Root plasma GH	8	30	0.0312	-0.394	-0.055
vs.	12	27	0.0498	-0.381	-0.100
Liver IGF-I mRNA					
Plasma Insulin	4	45	0.0482	0.296	0.873
vs.	12	38	0.0046	0.450	2.831
Plasma IGF-I	15	44	<0.0001	0.618	2.210
	22	43	0.0199	0.354	1.732
	29	39	0.0293	0.349	1.095
	36	23	0.0018	0.614	1.083
Plasma Insulin	0	41	0.0270	-0.345	-0.134
vs.	2	45	0.0060	-0.403	-0.853
Square Root plasma GH	12	35	0.0211	-0.388	-0.237
	15	43	0.0059	-0.413	-0.351
	22	43	0.0179	-0.359	-0.164

Correlations are for all treatment groups combined. All significant correlations ($p < 0.05$) between the listed variables are shown.

Chapter 3: A quantitative real-time RT-PCR assay for salmon IGF-I mRNA, and its application in the study of GH regulation of IGF-I gene expression in primary culture of salmon hepatocytes.

INTRODUCTION

In fishes, as in other vertebrates, somatic growth is regulated by the growth axis. Pituitary growth hormone (GH) stimulates the liver and other tissues to produce the mitogenic protein hormone insulin-like growth factor-I (IGF-I). IGF-I mediates many of the growth promoting effects of GH. Both GH and IGF-I act through membrane bound receptors, the GH receptor (GHR) and IGF type I receptor (IGF-IR). IGF-I circulates bound to a number of IGF binding proteins (IGFBPs), which slow the clearance of IGF-I, modify the effects of IGF-I on target tissues, and exert IGF-I independent effects (Rajaram *et al.* 1997; Lee *et al.* 2002). The principal components of the growth axis have been found in fishes, and the overall operation of the axis is similar between fishes and other vertebrates (Duan 1998; Kelley *et al.* 2000; Moriyama *et al.* 2000; Perez-Sanchez 2000). In salmonids, in addition to their role in growth regulation, GH and IGF-I are also involved in smoltification, the life stage transition in which fish move from fresh water to the ocean, and in reproductive maturation (Dickhoff *et al.* 1997; Okuzawa 2002).

Liver production of IGF-I may be a crucial point of regulation in the growth axis. Most circulating IGF-I is produced by hepatocytes. However, under catabolic conditions the liver becomes resistant to the effects of GH, resulting in reduced IGF-I production and decreased plasma IGF-I in spite of increases in GH (Duan *et al.* 1995; Perez-Sanchez *et al.* 1995; Kelley *et al.* 2000; Moriyama *et al.* 2000). Hepatic GH resistance is found in catabolic mammals, where it can be due to both reduction in hepatocyte GHR number,

and post-receptor downregulation of GH signaling (Thissen *et al.* 1999). Reductions in liver membrane GH binding have been documented in stunted coho salmon (Gray *et al.* 1992), hypophysectomized long jawed mudsuckers (*Gillichthys mirabilis*) (Gray *et al.* 1991), and fasted gilthead sea bream (*Sparus aurata*) (Perez-Sanchez *et al.* 1995). In rats, pigs, and chickens, studies using primary hepatocyte culture have shown that pancreatic, stress, and thyroid hormones regulate both basal and GH stimulated hepatocyte IGF-I gene expression (Houston *et al.* 1991; Brameld *et al.* 1995; Thissen *et al.* 1999). Little is known about the endocrine mechanisms leading to hepatocyte GH resistance in fish, but there are indications that similar mechanisms exist (Duan *et al.* 1992; Schmid *et al.* 2003).

We wanted to use primary culture of salmon hepatocytes to study mechanisms regulating liver IGF-I production in salmon. However, we were unable to reliably measure IGF-I protein released into culture medium with a radioimmunoassay (RIA; Shimizu *et al.* 2000). Medium IGF-I levels were below the detection limit of the RIA, and medium contained high levels of a 22 kilodalton IGFBP which was not completely removed by acid ethanol extraction and which interfered with the RIA (A. Pierce and M. Shimizu, unpublished data). As an alternative, we developed an assay for hepatocyte IGF-I gene expression.

In the present study, we report the development of a sensitive, high throughput TaqMan real-time quantitative RT-PCR (qPCR) assay for salmon IGF-I mRNA. The development of the assay included sequencing a new reference gene, discovering and eliminating an assay-specific bias across the PCR plate, and establishing a method for

calculating relative expression levels. The new assay was valid for measurement of IGF-I gene expression *in vivo* as well as in primary hepatocyte culture. We assessed the tissue distribution of IGF-I mRNA, and examined changes in IGF-I gene expression in hepatocyte culture over time, in response to a range of medium GH concentrations, and in response to medium IGF-I protein.

MATERIALS AND METHODS

Animals

Coho salmon (*Oncorhynchus kisutch*) were raised in 1.3 m diameter cylindrical tanks with recirculated fresh water at 11-12 °C under simulated natural photoperiod at the Northwest Fisheries Science Center (Seattle, WA, U.S.A.). Fish were fed BioOregon-Grower pellets at the rations specified (see experiments). Two year-old coho salmon were used for tissue distribution and hepatocyte culture experiments, and 1 year-old coho salmon for comparison between TaqMan and RNase protection assays.

Acidic Ribosomal Phosphoprotein P0 (ARP) sequencing

Total RNA from coho salmon liver was reverse transcribed as described for TaqMan assays (see later). Cloning primers were designed based on conserved regions of the zebrafish (*Dania rerio*) and catfish (*Ictalurus punctatus*) ARP sequences: forward TGGAAGCACTGCAAAGATGC and reverse ATGGTTCCTCTGGAGATCTTGG. 50 µl PCR reactions were performed using 5 µl cDNA template, 100 nM forward and reverse primers, 2.5 U/µl Promega Taq polymerase, 200 µM dNTPs, and 1.5 mM $MgCl_2$; cycling conditions Taq activation 95 °C for 5 min followed by 36 cycles of 94 °C for 45

s, 55 °C for 45 s, and 72 °C for 1 min 30 s, with a final 72 °C extension for 5 min. PCR products were sequenced in both directions with cloning primers. Unincorporated nucleotides and primers were removed from the cloning PCR reaction by filtration through a Nanosep membrane, and the cleaned up PCR product was used as template for a BigDye Terminator cycle sequencing reaction. Unincorporated dyes were removed from the BigDye reaction by chromatography with Sephadex G100 in Centrisep columns, and sequencing was performed on an Applied Biosystems, Inc. (ABI) 3100 Genetic Analyser.

TaqMan assays

Assay design. Assays for salmon IGF-I and ARP were designed with ABI's Primer Express program (Table 3.1). Primer and probe sequences were designed to avoid differences between salmonid species. One primer in each assay was placed across a cDNA intron/intron boundary to reduce signal from genomic DNA. Gene specific fluorogenic probes were purchased from ABI. We used ABI's pre-developed 18S ribosomal RNA assay (primer and probe sequences proprietary).

RNA processing. After removing culture medium and adding Tri-Reagent (MRC, Cincinnati, OH), cell culture plates were frozen at –80 °C and stored for up to 1 month. Plates were thawed, wells scraped, and RNA isolated following the MRC protocol, with bromochloropropane as the phase separation reagent. Two 75% ethanol washes were performed. Yields were 10-20 μg RNA/well. RNA was quantified and purity assessed by spectrophotometry; 260:280 ratios were 1.8 to 2.0. Visualization on 1% agarose gels showed that RNA was not degraded. RNA was diluted to 100 ng/μl.

Reverse Transcription (RT). First strand cDNA was synthesized in 15 µl RT reactions with 3 µl RNA template, 2.5 U/µl SuperScript II reverse transcriptase (Invitrogen), 5 µM random hexamer primers, 500 µM dNTPs, 0.4 U/µl RNase inhibitor (ABI), 10 mM DTT, and 1X RT buffer. RT reactions were set up on ice and loaded with a multichannel pipette to avoid loading order biases. RT reactions were performed in 96-well plates, with cycling conditions of 25 °C for 10 min, 48 °C for 60 min, and 95 °C for 5 min.

TaqMan PCR. TaqMan PCR was performed on an ABI 7700 Sequence Detector, using the standard cycling conditions recommended by the manufacturer (50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min). Wells contained 25 µl PCR mixture, made from ABI Universal PCR Master Mix (0.01 U/µl AmpErase uracil N-glycosylase, 0.025 U/µl AmpliTaq Gold DNA polymerase, passive reference dye I, 200 µM dNTPs including dUTP, 3.5 mM $MgCl_2$), with 200 nM probe, 900 nM forward and reverse primers, and 2 µl (18s and ARP assays) or 4 µl (IGF-I assays) cDNA template. For each assay on each plate, a 5-fold serial dilution (12 wells) of sample cDNA was run to assess PCR efficiency. Assays were not multiplexed. TaqMan PCRs for target and reference genes were run from the same RT reaction.

Data analysis. Data from TaqMan PCR runs were collected with ABI's Sequence Detector program. Fluorescence signal baseline and threshold were set manually for each run, generating a threshold cycle (C_T) for each sample. Quantification of relative expression levels with the $\Delta\Delta C_T$ method was unsuccessful, because assays did not consistently pass the required validation experiment (ABI User Bulletin 2; Livak *et al.*

2001). This occurred for IGF-I and other target genes, using both 18S and ARP as reference genes. Therefore, an "efficiency corrected relative expression" method was used for calculating relative expression (Equation 3.1; Pfaffl 2001; Liu *et al.* 2002). PCR efficiencies were calculated from the slope of the relationship log input cDNA versus C_T for the serial dilution of a sample: $E = 10^{-1/slope} - 1$. A sample was arbitrarily selected as a calibrator. Relative expression was calculated from the C_Ts for IGF-I and ARP from a

$$\text{Equation 3.1 : relative expression} = \frac{\left[\frac{(E_{ARP}+1)^{C_T^{ARP}}}{(E_{IGF}+1)^{C_T^{IGF}}}\right]_{sample}}{\left[\frac{(E_{ARP}+1)^{C_T^{ARP}}}{(E_{IGF}+1)^{C_T^{IGF}}}\right]_{calibrator}}$$

given sample, and the PCR efficiencies of the IGF-I (E_{IGF}) and ARP (E_{ARP}) amplifications.

TaqMan assay assessment. No template controls were used to confirm that reagents were not contaminated. Reverse transcriptase was omitted from RT reactions to test for interference from residual genomic DNA in RNA preparations. Assays were used over a C_T range where the relationship log input cDNA versus C_T for a serially diluted sample was linear with a high r^2. Potential effects of experimental treatments on ARP were checked by comparing ARP C_Ts between treatments with equal amounts of RNA loaded. Variability in RT and PCR reactions in the IGF-I assay was assessed by performing multiple RT reactions from the same sample, and then multiple PCR reactions from each RT.

Comparison to ribonuclease protection assay (RPA). To compare the TaqMan assay with another method of measuring mRNA, IGF-I mRNA levels were measured with both the TaqMan assay and a solution hybridization RPA (Duan *et al.* 1993a). Liver mRNA levels were measured in samples from an experiment in which 60-75g coho salmon were fed (1.5% body weight per day) or fasted for 6 weeks (D. Larsen and B. Beckman, unpublished data). Samples from fed and fasted fish were taken 3 and 6 weeks after beginning fasting. Total nucleic acid (TNA) was prepared from samples, and RNA quantitated with the RPA and expressed relative to DNA content. Before running TNA samples in the TaqMan assay, DNA was removed by DNase digestion. TNA samples containing 0.5 μg DNA were treated with 0.5 U DNase I (Invitrogen) in 10 μl reactions. Samples were incubated for 15 min at 25 °C, and then DNase digestion was stopped by adding 1 μl EDTA (25 mM) and incubating for an additional 10 min at 65 °C.

Tissue distribution of IGF-I mRNA. Tissues were collected from six coho salmon (3 males and 3 females). Gill arch, gonad, heart, head and posterior kidney, liver, muscle, pituitary, and spleen were dissected out and snap frozen in liquid nitrogen. RNA was isolated as described, except tissues were homogenized in Tri-reagent prior to phase separation. Tissue mRNA levels were normalized to the average muscle mRNA level.

Hepatocyte culture

Isolation. Hepatocytes were isolated following the procedure of Mommsen et al. (1994b). Animals were anesthetized with buffered MS-222, placed into a sterile hood, and viscera exposed. The liver was perfused *in situ* in the normal direction of blood flow through a fine polyethylene cannula at a flow rate of 2 ml/min. All solutions were sterile

filtered and contained 1% antibiotic/antimycotic (Gibco). The liver was first perfused for 20 to 30 min with calcium free modified Hanks' buffered saline solution (HBSS). Perfusion was continued with HBSS containing collagenase (Sigma type IV, 15-20 mg/100 ml HBSS, made fresh) for an additional 15 to 25 min, until digestion was complete. The liver was then removed from the fish, chopped fine, and filtered through coarse (252 μm) and fine (73 μm) plankton netting with ~50 ml calcium free HBSS. Cells were collected by low speed centrifugation (70 g for 2 min), and washed three times in HBSS containing calcium and supplemented with 2% BSA and amino acids (1x Gibco MEM essential and non-essential amino acid solutions), followed by sedimentation on ice for at least 1 h. Cells were then resuspended in culture medium, counted, and assessed for viability by trypan blue exclusion. Cells from isolations showing acceptable cell numbers, appearance, and viability were pooled and plated. Isolated hepatocytes typically showed trypan blue exclusion > 95% and yields 10-20 x 10^6 cells/g liver.

Culture. Cells were cultured on Falcon Primaria 24-well plates (0.5 ml medium per well) at 15°C, in modified RPMI 1640 medium (Gibco), under plain air. We replaced the bicarbonate buffer in RPMI with a HEPES buffering system (20 mM HEPES, 5 mM $NaHCO_3$), to better match the composition of teleost plasma (Mommsen *et al.* 1994a). The medium was supplemented with 1% antibiotic/antimycotic. Hepatocytes were plated at a density of ~4 x 10^6 cells/well in plain medium and allowed to adhere for 24 h, and then medium was gently changed to test medium. Cell cultures were stopped by placing culture plates on ice, removing the medium, and adding Tri-Reagent (0.5 ml) to wells.

Experiments. Fish used for hepatocyte culture were fed 0.6% body weight per day. Food was withheld for 24 h before sampling. Experiments took place from Sept through Dec. For each experiment, hepatocytes were pooled from two immature male fish (143 ± 9 g; mean ± standard error). Hormones used were native coho salmon GH purified by HPLC (Rand-Weaver *et al.* 1992a), recombinant salmon IGF-I (GroPep Inc.), and triiodothyronine (T_3), bovine insulin, and water soluble dexamethasone (Sigma). In the time course experiment, separate culture plates were used for each time point, with five to six wells per treatment per time point. Treatments were plain medium, 5 nM GH, and 5 nM GH plus metabolic hormones (1 μM insulin, 100 nM T_3, and 0.1 nM dexamethasone). In the GH concentration/response experiment, two culture plates each were used for separate hepatocyte pools from fed and fasted fish, with five to six wells per GH concentration. Fasted fish had been fasted for 16 d. Treatments were plain medium and 7 GH concentrations from 0.05 to 500 nM. In the IGF-I protein effect on IGF-I mRNA experiment, a single culture plate with four wells per treatment was used, and treatments were plain medium, 5 nM IGF-I, 5 nM GH, and 5 nM IGF-I with 5 nM GH. Three separate TaqMan assays (i.e. one PCR plate each for IGF-I and ARP) were run for the three cell culture experiments.

Statistical analysis

Results were considered statistically significant at $p < 0.05$. Linear regression by least squares and Pearson's correlation coefficient (r^2) were used to investigate relationships between repeated TaqMan assays and between the RPA and the TaqMan assays. A linear correction factor was applied to remove small culture plate effects in the

GH concentration/response experiment. ANOVA followed by the Bonferroni/Dunn test was used to examine tissue gene expression levels and treatment effects in hepatocyte culture.

RESULTS

ARP sequence

PCR for coho salmon liver ARP cDNA gave a single product, which was sequenced (Figure 3.1). Sequence from coho salmon pituitary template from a different individual was identical to that obtained from liver template (data not shown). The predicted amino acid sequence for coho salmon ARP was 93-96% identical to other vertebrates (Figure 3.1 legend). Sequence from chinook salmon (*O. tshawytscha*) liver template differed only by two synonymous single base pair changes (data not shown). Salmon ARP sequences have been deposited in GenBank (accession numbers AY255630, AY255631).

TaqMan assays

No amplification was observed in PCR reactions without cDNA template. Omission of reverse transcriptase from RT reactions resulted in an increase in C_Ts of at least 10 cycles in all assays, indicating that signal from residual DNA in RNA preparations was unimportant. Serial dilutions of samples gave linear log input cDNA vs. C_T plots with $r^2 > 0.99$. PCR efficiencies varied between assays (Table 3.1), and also between runs.

The B Domain IGF-I TaqMan assay (Table 3.1) showed a systematic bias across the PCR plate when identical cDNA template was loaded into an internal row, with higher C_Ts near the center of the plate (Figure 3.2). This bias was quite repeatable, did not occur in other assays, did not depend on species, tissue source, RNA isolation method, or order of loading the PCR plate, and increased in magnitude when an alternative reverse primer was used or the annealing temperature was reduced to 58 °C (data not shown). When an alternative IGF-I assay was designed (Leader assay; Table 3.1), the bias was not found. All further gene expression data was generated with the Leader assay.

Initial trials used 18S ribosomal RNA as a reference gene. ABI's pre-developed 18S assay worked in salmon. However, the large difference in mRNA level between IGF-I and 18S necessitated dilution of cDNA before assaying for 18S, and high concentrations of 18S template appeared to inhibit the RT reaction (data not shown). Therefore, ARP was adopted as a reference gene. Under our experimental and assay conditions, ARP mRNA levels typically resulted in C_Ts of 19 to 21, with IGF-I C_Ts of 21 to 28. 18S C_Ts were ~13.5 cycles lower than ARP. When equal amounts of RNA were loaded into the assay, ARP C_Ts did not vary between treatments or over time in the hepatocyte culture experiments described.

Intraassay variation in IGF-I relative expression was 5.0% (coefficient of variation or CV; based on 9 repeats in 3 assays). Different calibrator samples were used in the different TaqMan assays, precluding calculation of interassay variation. IGF-I relative expression levels measured in repeated runs of the same samples were highly

correlated ($r^2 = 0.954$, $n = 21$, $p < 0.0001$). However, the slope of the regression line differed substantially from 1 ($y = 0.577x + 0.081$). This indicates that the relative magnitude of differences between samples is reliably reproduced by the assay, but the absolute magnitude of the differences may vary between runs. At the level of output C_T for the IGF-I TaqMan assay, variation in the RT reaction (2.5% CV; based on 12 RT reactions) was greater than that in the PCR reaction (0.7% CV; based on 36 PCR reactions). Variation in C_T underestimates variation in relative expression; nonetheless, this shows that there is greater variability in the RT reaction than the PCR reaction in the IGF-I assay.

IGF-I mRNA levels in liver samples measured with the TaqMan assay correlated highly ($r^2 = 0.805$, $n = 19$, $p < 0.0001$) with mRNA levels measured with an RPA (Figure 3.3), indicating that the TaqMan assay is valid for use *in vivo* under our experimental conditions. Based on C_Ts, ARP template loaded varied ~5 fold. Thus, the correlation shows that normalization using ARP effectively controls for RNA loading.

IGF-I mRNA levels measured with the new assay were significantly higher in the liver than in all other tissues assessed (Figure 3.4). Levels in gill, heart, and muscle were lowest, while levels in gonad, head and posterior kidney, pituitary and spleen were somewhat higher; however, the only significant difference in non-liver tissues was between heart and spleen.

Effects of GH and IGF-I in hepatocyte culture

Hepatocytes formed an adherent layer with cellular aggregates over the incubation times used. The flattened and spread morphology found in salmon hepatocytes under

longer incubation times was not seen (Duan *et al.* 1993b). IGF-I gene expression declined in cultured hepatocytes in plain medium from 3 to 48 h (Figure 3.5A); the steepest decline occurred between 3 and 6 h. Addition of 5 nM GH increased IGF-I gene expression significantly over plain medium levels at all time points. In the 5 nM GH treatment, IGF-I gene expression first increased nonsignificantly from the 3- to the 6-h timepoint, and then declined significantly from 6 to 48 h. IGF-I gene expression levels in the GH plus metabolic hormones treatment were significantly lower than in the GH treatment and higher than in plain medium from 6 to 24 h. This treatment was not significantly different from controls at 3 h, or from the GH treatment at 48 h. RNA yield per well declined to 64% of initial values over the culture period, with the bulk of the decline occurring from 24 to 48 h and no difference between treatment groups. An incubation period of 18 h was selected for further studies. This incubation period was chosen in order to avoid periods when IGF-I gene expression levels might be changing rapidly or differentially between treatments in culture, and to give cells ample time in which to modify receptor and second messenger levels potentially involved in GH response.

In hepatocytes from both fed and fasted fish, GH stimulated IGF-I gene expression in a concentration dependent manner (Figure 3.5B). In cells from fed fish, concentrations of GH less than and including 0.25 nM were ineffective, 0.5 nM GH stimulated IGF-I gene expression slightly but significantly, a steep increase occurred from 0.5 to 2.5 nM, maximal simulation occurred from 5 to 50 nM, and IGF-I gene expression declined significantly from maximal at 500 nM. In hepatocytes from fasted

fish, concentrations of GH less than and including 0.5 nM were ineffective, concentrations from 2.5 to 50 nM resulted in maximal IGF-I gene expression, and 500 nM GH reduced IGF-I gene expression to basal level.

Recombinant salmon IGF-I protein (5 nM) added to culture medium did not affect basal or GH stimulated IGF-I mRNA levels in hepatocytes (Figure 3.6).

DISCUSSION

The most significant difficulty encountered in the development of the TaqMan assay for IGF-I was the bias across the PCR plate shown in Figure 3.2. The ~1 C_T amplitude of the bias could obscure two-fold changes in gene expression. Fortunately, the bias was specific to the IGF-I B domain assay, and was not found when the IGF-I assay was redesigned (Leader assay). We recommend that TaqMan assays be checked for PCR plate bias. We are not aware of any published descriptions of similar problems with plate based qPCR machines. Positional biases have been found in the carousel based LightCycler, which were attributed to minor temperate differences (Wilhelm *et al.* 2000; Zuna *et al.* 2002). We do not know why our bias occurred. Given its regularity, however, it may be due to the interaction of a particular TaqMan PCR reaction with a small physical gradient across the PCR plate, likely a small temperature or time at temperature gradient.

Gene expression levels measured in qPCR assays may be expressed in terms of the amount of RNA loaded into the assay, or normalized to the mRNA level of an invariant reference gene (Freeman *et al.* 1999; Bustin 2000). Normalization with a reference gene corrects for variation in RT efficiency, since presumably variation in RT

efficiency will affect target and reference genes equally. This may be a significant advantage, since variation in RT efficiency has been stated to be the greatest source of variation in quantitative RT-PCR (Freeman *et al.* 1999). Consistent with this, we found greater variation in the RT step than the PCR step in our IGF-I assay. The disadvantage of reference genes is that one must know or assume that reference gene expression does not vary between experimental treatments (Bustin 2000; Bustin 2002). Ribosomal RNA transcripts such as 18S may be unlikely to vary between treatments, but the very high level of gene expression of 18S makes it difficult to use as a reference gene (Bustin 2000). We sequenced part of the cDNA encoding the ribosomal structural protein ARP for use as a reference gene. ARP mRNA levels are much closer to those of our target genes than 18S, ARP has not varied between experimental treatments in cell culture or *in vivo* experiments using liver, pituitary, gonad, or kidney template (J. Dickey and A. Pierce, unpublished data), and tissue relative gene expression levels of IGF-I normalized to ARP fit expectations (Figure 3.4).

Relative expression levels of a target gene may be normalized to a reference gene by use of the $\Delta\Delta C_T$ method (ABI User Bulletin 2; Livak *et al.* 2001). This method assumes that $E_{target} = E_{reference} = 1$. However, the failure of our IGF-I and other assays to pass the required validation experiment shows that the PCR efficiencies of target and reference genes differ. Indeed, PCR efficiencies of different amplicons in qPCR assays may often differ (Giulietti *et al.* 2001; Liu *et al.* 2002). The efficiency corrected relative expression method we used (Equation 1; Pfaffl 2001; Liu *et al.* 2002), is a more general version of the $\Delta\Delta C_T$ method, which does not assume $E_{target} = E_{reference} = 1$, but which does

require that the PCR efficiencies for target and reference are known. Since we found that PCR efficiency in each assay varied from one run of the assay to the next, we measured PCR efficiency for each run with a serial dilution of sample cDNA. Our method is very similar to the use of a standard curve on each plate.

The clear biological effects seen in hepatocyte culture show that the new assay is a valid method for measurement of IGF-I gene expression, as does the tissue distribution of IGF-I mRNA and the high correlation found between mRNA levels measured with the TaqMan assay and RPA. The use of gene-specific primers and probe should yield highly specific assays. The most significant remaining drawback of our and other qPCR assays is the difficulty of comparing gene expression levels between runs. Whenever possible, it is desirable to keep all samples to be compared within one TaqMan assay.

IGF-I mRNA was much higher in the liver than in other tissues, confirming the RPA results of Duan et al. (1993a), and consistent with the liver as the source of endocrine IGF-I in salmon as in other vertebrates (Duan 1998; Thissen *et al.* 1999; Kelley *et al.* 2000; Moriyama *et al.* 2000; Perez-Sanchez 2000). Levels were lower, but measurable in other tissues, consistent with potential local IGF-I effects.

Primary hepatocyte culture is a valuable tool in the study of fish metabolism, toxicology, and endocrinology. We used a hepatocyte culture system with no serum or coating on culture plates. Cells were given 24 h to adhere to plates before treatments were begun. IGF-I mRNA declined over time in hepatocyte culture (Figure 3.5A). Similar declines occurred in plain medium, GH, and GH plus metabolic hormone treatments, suggesting that the decline cannot be attributed to the lack of any of these

factors in the medium. At 48 h, all treatments had decreased to 15-30% of the level at 3 h. ARP gene expression did not change over time in culture, showing that the decrease in IGF-I gene expression was specific. In the only comparable fish study of which we are aware, Schmid et al. (2000) showed similar declines in basal and GH stimulated IGF-I gene expression in primary cultured tilapia (*Oreochromis mossambicus*) hepatocytes over a 42-h incubation period, with no decline in their actin reference signal. The reasons for the decline in IGF-I gene expression over time are not known. However, stimulation of IGF-I gene expression by GH (3 to 48 h), and modulation of the GH effect by metabolic hormones (3 to 24 h) were consistently observed in spite of the decline, suggesting that incubation times of 3 to 24 h are feasible for GH response and metabolic hormone studies, and those of 12 to 24 h appear best.

The GH plus metabolic hormone treatment was intended to maximize IGF-I mRNA response, based on reported enhancement of basal and/or GH stimulated IGF-I gene expression by insulin, T_3, and low levels of glucocorticoids in other vertebrate classes (Houston *et al.* 1991; Brameld *et al.* 1995; Thissen *et al.* 1999). In fish hepatocyte culture, insulin has been reported to increase the IGF-I gene expression response to GH, and T_3 to stimulate IGF-I gene expression independently of GH (Duan *et al.* 1992; Schmid *et al.* 2003). However, in the present study, the combination of metabolic hormones unexpectedly inhibited the IGF-I response to GH at all time points up to 48 h. Further work is required to elucidate the reasons for this inhibition.

IGF-I protein did not change basal or GH stimulated IGF-I mRNA levels in hepatocyte culture (Figure 3.6). The lack of effect on basal gene expression confirms

results in tilapia (Schmid *et al.* 2000). Our results are consistent with the low level of IGF-IR gene expression in fish liver (Maures *et al.* 2002a; Nakao *et al.* 2002). The 5 nM (37.5 ng/ml) IGF-I level used is higher than most salmon plasma levels in fresh water. Measurement of IGF-I protein levels in hepatocyte culture medium has proved difficult, but we believe it is unlikely they are higher than plasma levels, and therefore, our results show that endogenous IGF-I is unlikely to affect the results of hepatocyte culture experiments. However, it remains possible that an effect exists which is obscured by the presence of IGFBPs in the medium.

The response of IGF-I gene expression to concentration of GH was studied in hepatocytes from fed fish, and from fish fasted for 16 d (Figure 3.5B). The threshold for response was 0.5 nM GH in cells from fed fish and 2.5 nM GH in cells from fasted fish. Duan *et al.* (1993a) found a response at 0.5 nM GH in cultured coho salmon hepatocytes from fed fish, while Shamblott et al. (1995) showed an increase at 0.25 nM GH in hepatocytes from rainbow trout (*O. mykiss*) fasted 2 weeks. In tilapia hepatocytes, 1 nM GH significantly increased IGF-I gene expression (Schmid *et al.* 2000). Thus, the threshold for IGF-I gene expression response in primary cultured fish hepatocytes seems to be 0.25 to 2.5 nM GH. GH secretion in salmonids is episodic, with basal levels in fed fish quite low, typically below 0.05 nM, rising to levels of up to 0.5 nM during secretion episodes lasting several h (Gomez *et al.* 1996). Thus, basal GH levels are below the threshold for stimulation of IGF-I gene expression found in hepatocyte culture, while levels attained during GH pulses may cross the threshold. It is not known if the response of hepatocytes in culture differs from that *in vivo*. Recent work in rats has found that the

transcription factor STAT5b is activated in response to individual GH pulses (Tannenbaum *et al.* 2001), and STAT5b transduces at least part of the GH effect on IGF-I transcription (Davey *et al.* 2001), suggesting that IGF-I transcription may respond to GH pulses in rats.

IGF-I gene expression declined significantly from maximal at the highest GH concentration (500 nM) in hepatocyte pools from both fed and fasted fish, indicating that the response curve is dome shaped or biphasic. To our knowledge, this is the first biphasic IGF-I gene expression response curve reported for cultured fish hepatocytes, possibly because previous studies have not employed GH concentrations as high as 500 nM. A recent study using lamprey (*Petromyzon marinus*) liver slices showed an apparent decrease in IGF-I gene expression at high GH concentrations (Kawauchi *et al.* 2002). In transgenic tilapia, an optimal GH dose exists for overall body growth stimulation, with higher levels of transgene expression inhibiting the growth response (Hernandez *et al.* 1997). Stimulation of rainbow trout growth by a GH transgene was ineffective in a domesticated strain selected for rapid growth, suggesting possible maximization of GH signaling in this strain (Devlin *et al.* 2001). The biphasic response curve found here indicates that high GH levels downregulate GH signaling. Two known mechanisms might account for this: inhibition of receptor activation by ligand excess or downstream inhibition by induction of intracellular inhibitors. In mammals, the unusual 2 receptors: 1 ligand stoichiometry of GH's interaction with the GHR can result in inhibition of receptor activation by high GH levels (Frank 2002). The stoichiometry of GH receptor binding is not yet known in fish. In mammals, the recently discovered suppressors of

cytokine signaling (SOCS) inhibit GH signaling. This family of intracellular proteins is induced by cytokine ligands such as GH and inhibits signaling by interfering in second messenger cascades (Krebs *et al.* 2001). SOCS proteins are unexplored in fish, however, homologs exist in the fugu genome.

The response to GH was biphasic in hepatocyte pools from both fed and fasted fish, suggesting that this characteristic of GH response is found in a variety of physiological states. However, the response to GH was lower in hepatocytes from fasted fish. This suggests that the response of hepatocytes *in vitro* may differ based on the physiological condition of the source animals. Consistent with this idea, we found a 3- to 4-fold reduction in GH stimulation of IGF-I gene expression in hepatocytes from a maturing male fish which had stopped feeding as compared with two immature fish (A. Pierce, unpublished data). We cannot attribute the reduction in GH response to feeding treatment, because only one hepatocyte pool each from fed and fasted fish was tested. However, the reduced response is consistent with the hepatic GH resistance that has been found during fasting and other catabolic states *in vivo* (Gray *et al.* 1991; Gray *et al.* 1992; Duan *et al.* 1995; Perez-Sanchez *et al.* 1995). Since the change in responsiveness persists for at least 42 h after hepatocytes are removed from the source animals, it apparently does not require the presence of blood factors for its maintenance. Further studies are required to determine the endocrine and cellular mechanisms leading to changes in hepatocyte GH responsiveness.

```
.AG TCC AAC TAT TTT ATG AAA ATC ATC CAA TTG CTG GAT GAC TAT CCC AAA TGT TTC ATT
GTC GGC GCT GAC AAT GTG GGC TCC AAG CAG ATG CAG GCC ATC CGT CTG TCC TTG CGT GGG
AAG GCT GTG GTG CTC ATG GGT AAA AAC ACC ATG ATG CGC AAA GCC ATC CGC GGC CAC CTG
GAG AAC AAC CCC GCC CTG GAG AAG TTG CTG CCT CAC ATC AAA GGA AAT GTG GGC TTT GTC
TTC ACC AAG GAG GAC CTG GCT GAG ATC AGG GAC ATG CTG CTG GCC AAC AAG GTG CCA GCT
GCT GCC CGT GCC GGT GCT ATT GCC CCC TGT GAC GTG ACT GTG CCA GCC CAG AAC ACT GGG
CTG GGT CCT GAG AAG ACT TCC TTC TTC CAG GCC CTG GGC ATC ACC A..
```

Figure 3.1. Acidic ribosomal phosphoprotein P0 (ARP) cDNA sequence from coho salmon liver. Identities between predicted coho salmon amino acid sequence and other species were as follows: catfish (*Ictalurus punctatus*) 94%; zebrafish (*Dania rerio*) 93%; pufferfish (*Fugu rubripres*) 95%; clawed frog (*Xenopus laevis*) 94%; rat (*Rattus norvegicus*) 95%; chicken (*Gallus gallus*) 96%.

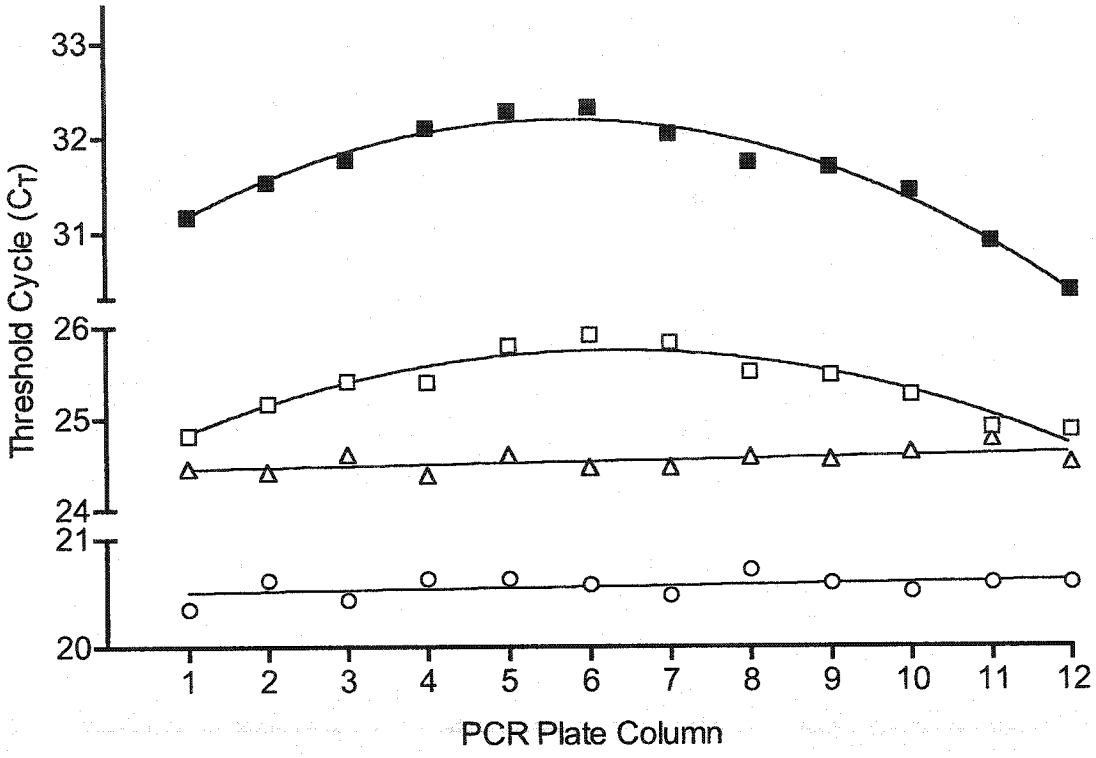


Figure 3.2. PCR plate bias in TaqMan assays. Total RNA isolated from coho salmon tissues was reverse transcribed in a single bulk reaction, and cDNA loaded into all wells of an internal row on a 96-well PCR plate. O ARP assay, liver cDNA; Δ IGF-I leader peptide assay, liver cDNA; □ IGF-I B domain assay, liver cDNA; ■ IGF-I B domain assay, pituitary cDNA.

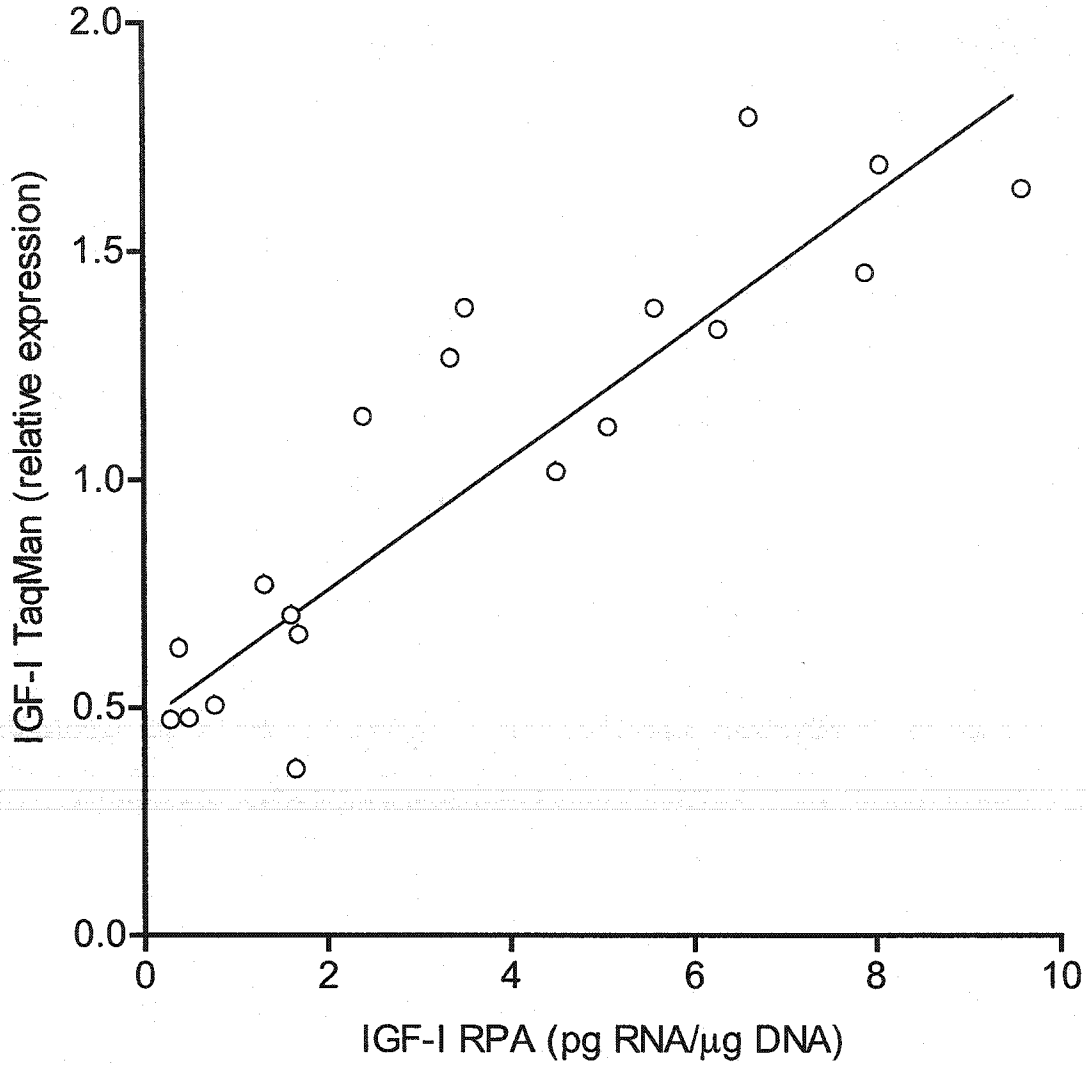


Figure 3.3. Comparison of liver IGF-I gene expression levels measured with the TaqMan RT-PCR assay and an RNase protection assay (RPA) in coho salmon. Regression r^2 = 0.805, $p < 0.0001$, n=19, $y = 0.141x + 0.519$.

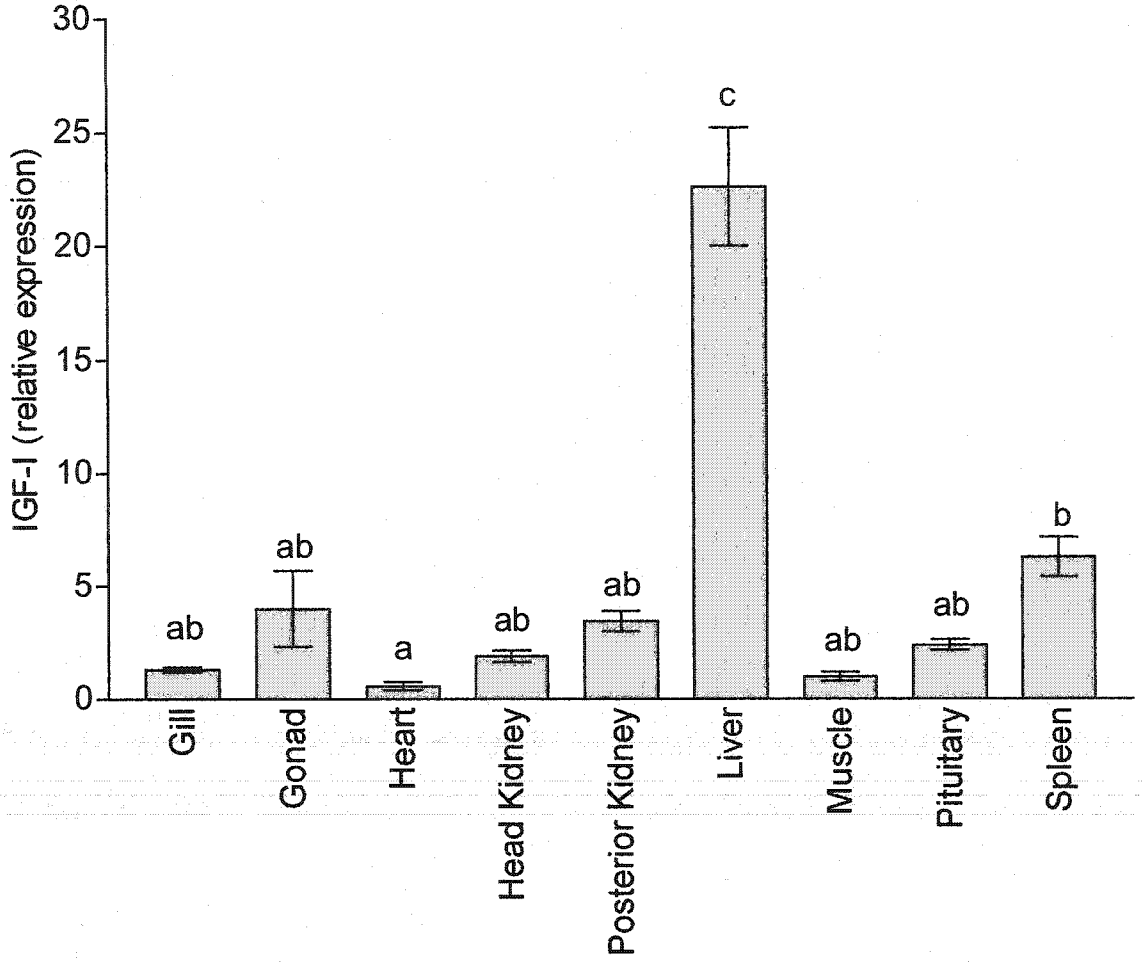
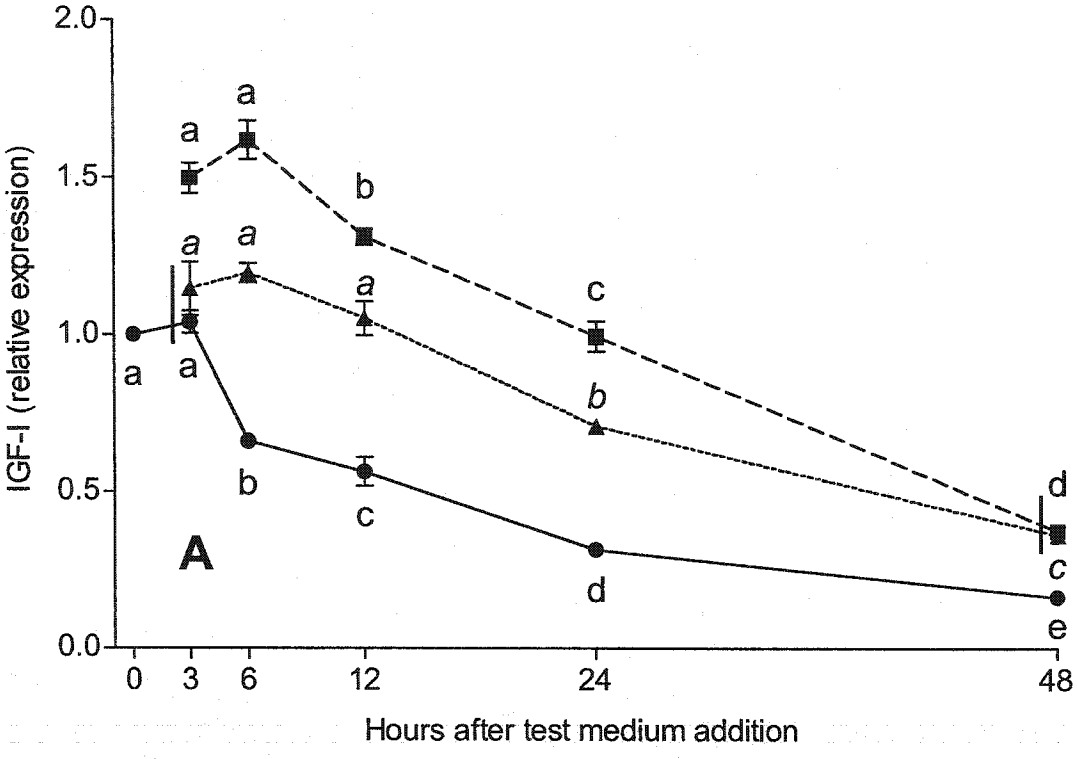
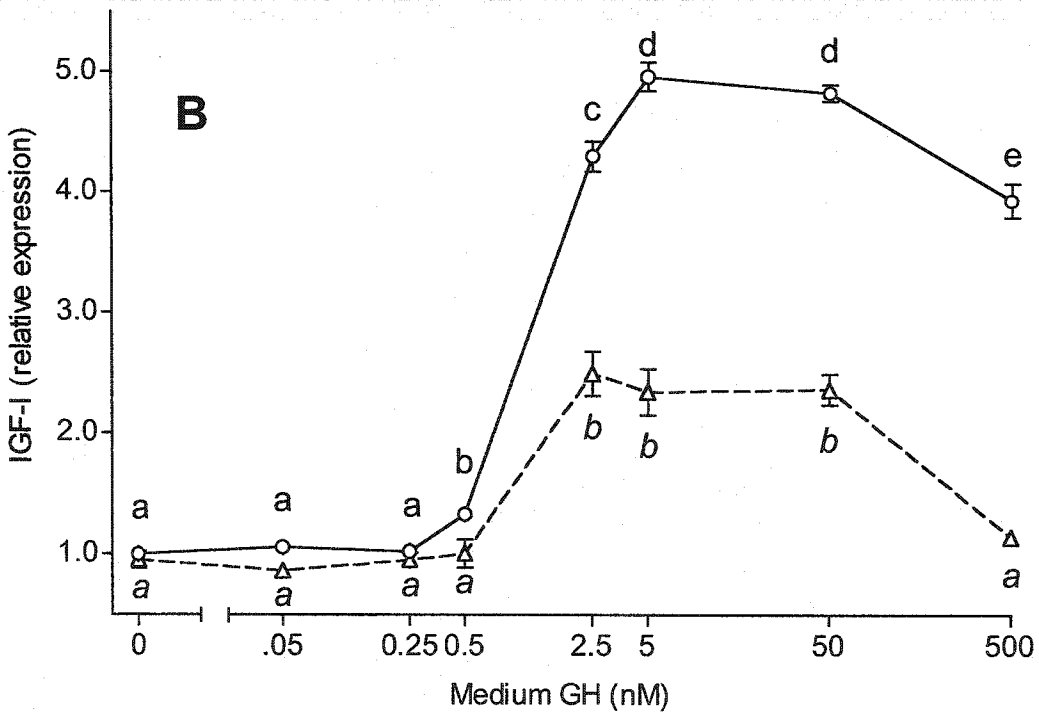


Figure 3.4. Comparison of IGF-I mRNA levels measured with the TaqMan RT-PCR assay in different tissues. IGF-I gene expression levels were normalized to ARP. Bars with different letters are significantly different ($p < 0.05$). Error bars represent standard error of the mean, $n = 6$ fish per tissue.

Figure 3.5. Effects of GH on IGF-I gene expression in primary hepatocyte culture. Hepatocytes were harvested as described in the text, plated in RPMI medium without added hormones or serum in 24-well Primaria culture plates, and incubated for 24 h at 15 °C. Medium was then changed to test medium. Letters indicate significant differences over the x-axis variable within each treatment; points with a letter in common do not differ significantly. Error bars represent standard error of the mean, n = 5 to 6 wells per treatment. (A) Time course in culture. Incubation was stopped at the indicated time; the 0 point was taken immediately prior to hormone addition. ● plain medium; ■ GH 5 nM; ▲ GH 5 nM with insulin 1 μM, T_3 100 nM, and dexamethasone 0.1 nM. A pool of hepatocytes from two fed male fish (105 and 144 g) was used. Vertical lines to the left of symbols indicate differences among treatment groups at each point; points connected by a line do not differ significantly. (B) Concentration/response relationship between medium GH and cellular IGF-I gene expression in hepatocytes from fed and fasted fish. Incubation was stopped 18 h after the addition of hormones. Pools of hepatocytes from two male fish were used: O fed fish (146 and 179 g); Δ fish fasted for 16 d (105 and 145 g).


IGF-I (relative expression)
2.0
1.5
1.0
0.5
0.0
A
0
3
6
12
24
48
Hours after test medium addition


IGF-I (relative expression)
5.0
4.0
3.0
2.0
1.0
B
0
.05
0.25
0.5
2.5
5
50
500
Medium GH (nM)

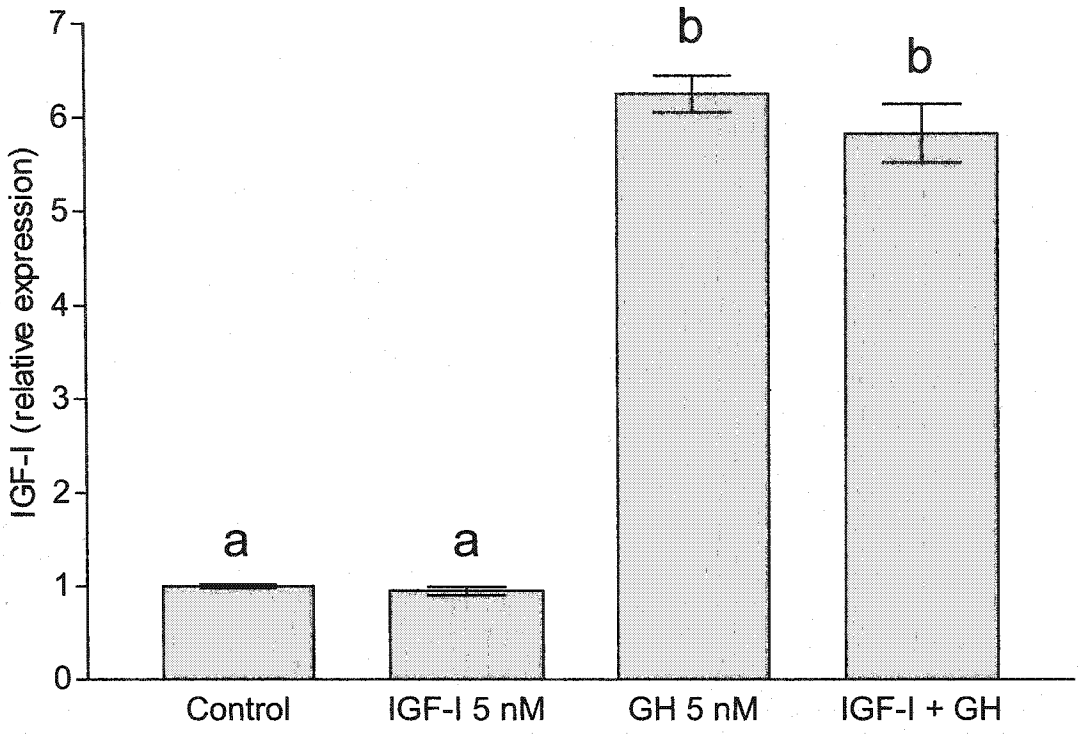


Figure 3.6. Effect of medium IGF-I on hepatocyte IGF-I gene expression in primary hepatocyte culture. Hepatocytes were harvested as described in the text, plated in RPMI medium without added hormones or serum in 24-well Primaria culture plates, and incubated for 24 h at 15 °C. Medium was then changed to test medium containing hormones, and hepatocytes incubated for 18 additional h. A pool of hepatocytes from two fed male fish (154 and 163 g) was used. Bars with different letters are significantly different ($p < 0.0001$). Error bars represent standard error of the mean, $n = 4$ wells per treatment.

Table 3.1. Primer and probe sequences for TaqMan RT-PCR assays.

Assay	Element	Sequence (5'-3')	Amplicon; PCR Efficiency
IGF-I	F primer	TGACTTCGGCGGCAACA	117 bp;
B domain	R primer	CATACCCCGTTGGTTTACTGAAATA	0.8978
	Probe	Fam-ACAAACTGCAGCGTGTCCACCAGCT-Tamra	
IGF-I	F primer	GATGTCTTCAAGAGTGCGATGTG	84 bp;
Leader	R primer	CGCCGAAGTCAGGGTTAGG	0.7022
	Probe	Fam-TCTCCTGTACCCACACCCTCTCACTGCT-Tamra	
ARP	F primer	GAAAATCATCCAATTGCTGGATG	106 bp;
	R primer	CTTCCCACGCAAGGACAGA	0.7692
	Probe	Vic-CTATCCCAAATGTTTCATTGTCGGCGC-Tamra	

The PCR efficiency of ABI's pre-developed 18S assay was 0.8735.

Chapter 4: Modulation of the effect of GH on IGF-I gene expression by metabolic hormones and amino acids in primary cultured salmon hepatocytes.

INTRODUCTION

The growth axis is the primary endocrine system that regulates body growth in vertebrates (reviewed in Rosenfeld *et al.* 1999). The principal hormones in the growth axis are pituitary growth hormone (GH) and liver-derived endocrine insulin-like growth factor-I (IGF-I). IGF-I circulates bound to a number of IGF binding proteins (IGFBPs), which modulate its growth stimulatory effects. GH stimulates the production of IGF-I by hepatocytes. During fasting, liver IGF-I gene expression and circulating levels of IGF-I decline in all vertebrate species studied, whereas plasma GH increases in all species studied except rats (Tannenbaum *et al.* 1979; Straus 1994; Thissen *et al.* 1994; Estivariz *et al.* 1997). This apparent paradox is explained by the development of hepatocyte GH resistance (Thissen *et al.* 1994; Ketelslegers *et al.* 1995; Thissen *et al.* 1999). Hepatocyte IGF-I production becomes less sensitive to stimulation by GH, which more than counteracts the increase in GH during fasting. Hepatocyte GH resistance appears to be a common outcome of catabolic states brought about by a variety of causes, and has been found in diverse species, including rats (Ross *et al.* 1995; Thissen *et al.* 1999; Beauloye *et al.* 2002; Butler *et al.* 2003).

The endocrine and cellular mechanisms that lead to hepatocyte GH resistance are not completely understood. Primary hepatocyte culture studies in mammals and birds have shown that pancreatic, stress, and thyroid hormones can both regulate IGF-I production independently of GH, and modulate the effect of GH on IGF-I production.

Insulin, thyroid hormones, and low concentrations of glucocorticoids potentiate GH-stimulated IGF-I gene expression, whereas glucagon and high concentrations of glucocorticoids inhibit basal or GH-stimulated IGF-I gene expression (Tollet *et al.* 1990; Boni-Schnetzler *et al.* 1991; Houston *et al.* 1991; Denver *et al.* 1994; Brameld *et al.* 1995; Beauloye *et al.* 1999). *In vivo* data support a physiologically significant positive role for insulin in the regulation of GH sensitivity (Griffen *et al.* 1987; Hanaire-Broutin *et al.* 1996; Butler *et al.* 2003). The growth suppression caused by high levels of glucocorticoids may be mediated by glucocorticoid-induced liver GH resistance (Luo *et al.* 1989), or by glucocorticoid induction of liver IGFBP1, a growth inhibitory IGFBP (Luo *et al.* 1990). Hepatic portal levels of nutrients such as amino acids and glucose may also directly regulate GH sensitivity and IGF-I production (Brameld *et al.* 1999; Thissen *et al.* 1999; Wheelhouse *et al.* 1999). Within the hepatocyte, changes in GH sensitivity occur at both the receptor and post-receptor levels (Thissen *et al.* 1994; Thissen *et al.* 1999; Beauloye *et al.* 2002).

The components and operation of the growth axis in teleost fishes are similar to what is found in other vertebrates (Duan 1998; Nicoll *et al.* 1999; Perez-Sanchez *et al.* 1999; Kelley *et al.* 2000; Moriyama *et al.* 2000; Mommsen 2001a). GH injection increases liver IGF-I gene expression and plasma IGF-I levels in numerous fish species (Cao *et al.* 1989; Moriyama *et al.* 1994; Perez-Sanchez *et al.* 1994a; Hashimoto *et al.* 1997; Kermouni *et al.* 1998; Shimizu *et al.* 1999a; Kajimura *et al.* 2001). During fasting in fishes, plasma IGF-I and liver IGF-I mRNA decrease in all species studied, and GH increases in most species (Sumpter *et al.* 1991; Duan *et al.* 1993c; Johnsson *et al.* 1996;

Matthews *et al.* 1997b; Moriyama *et al.* 1999; Meton *et al.* 2000; Baker *et al.* 2003; Uchida *et al.* 2003; Chapter 2). As in mammals, hepatic GH resistance occurs in a variety of catabolic states in fishes (Perez-Sanchez *et al.* 1999). Liver IGF-I gene expression and circulating IGF-I were reduced in spite of increased GH in coho salmon (*Oncorhynchus kisutch*) suffering from growth arrest due to premature transfer to saltwater, a phenomenon known as stunting (Duan *et al.* 1995). Liver membrane GH binding was reduced in stunted salmon (Gray *et al.* 1992), hypophysectomized gobys (*Gillyichthys mirabilis*) (Gray *et al.* 1991), and nutritionally restricted gilthead sea bream (*Sparus aurata*) (Perez-Sanchez *et al.* 1995). However, the endocrine mechanisms leading to GH resistance in fishes are relatively unexplored. GH increases IGF-I gene expression in primary cultured salmon and tilapia (*Oreochromis mossambicus*) hepatocytes (Duan *et al.* 1992; Duan *et al.* 1993b; Shamblott *et al.* 1995; Schmid *et al.* 2000; Pierce *et al.* 2003). Thus, regulation of both basal and GH-dependent IGF-I gene expression can be investigated *in vitro*.

To investigate endocrine and nutritional mechanisms modulating hepatocyte GH sensitivity in salmon, we performed a series of hepatocyte culture experiments. Primary hepatocytes were isolated, cultured, and exposed to pancreatic, thyroid, and glucocorticoid hormones in the presence and absence of GH. Significant modulatory effects on GH sensitivity were found for dexamethasone, insulin, and glucagon. The overall amino acid (AA) concentration in culture medium, and the effects of deletion of individual AAs were also tested. However, manipulation of medium AA concentration did not change basal or GH-stimulated hepatocyte IGF-I gene expression.

MATERIALS AND METHODS

Animals

Coho salmon were raised in 1.3 m diameter cylindrical tanks with recirculated fresh water at 11-12 °C under simulated natural photoperiod at the Northwest Fisheries Science Center (Seattle, WA, U.S.A.). Fish were fed BioOregon-Grower pellets at a ration of 0.6% body weight per day. Two year-old coho salmon were used. Experiments took place from Sept to Jan, 2002-3.

Hepatocyte culture and RNA isolation

Hepatocytes were isolated and cultured following the procedure of Mommsen and co-workers (Mommsen *et al.* 1994b), as previously described (Pierce *et al.* 2003). Fish were fasted for 1 day before isolation of hepatocytes. Cells were cultured on Falcon Primaria 24-well plates at 15 °C. For hormonal experiments, cells were cultured in modified RPMI 1640 medium with the standard amino acid (AA) concentrations (Gibco BRL, Gaithersburg, MD; buffer changed to 20 mM HEPES, 5 mM $NaHCO_3$). For AA experiments, RPMI with the same buffer modification was used, and AA concentrations were manipulated using a RPMI select-amine kit (Gibco). AA concentrations were set to approximate multiples of standard salmonid plasma AA concentrations (Table 1), and pairs of individual AAs were deleted from the medium. Constant medium osmolality was maintained by slight changes in medium NaCl concentration. Hepatocytes were plated at a density of ~4 x 10^6 cells/well in plain medium and allowed to adhere for 24 hours, and then medium was changed to test medium containing hormones. Cultures were stopped 18 hours later by adding Tri-Reagent (MRC, Cincinnati, OH) to wells. The

18 hour time point was selected on the basis of a previous time course experiment (Pierce *et al.* 2003). RNA was harvested following the MRC protocol, with bromochloropropane as the phase separation reagent and two 70% ethanol washes. RNA was quantitated by spectrophotometry (260:280 ratios 1.8 – 2.0), and diluted to 100 ng/µl.

Hormones

Native coho salmon GH (sGH) was purified by HPLC (Rand-Weaver *et al.* 1992a). Native coho salmon insulin (sIns), glucagon (sGlu), and glucagon-like peptide (sGLP) were a generous gift from Dr. Erika Plisetskaya (University of Washington, Seattle). Triiodothyronine (T_3), water-soluble dexamethasone (Dex), native bovine insulin (bIns), and native bovine glucagon (bGlu) were purchased from Sigma (St. Louis, MO).

Experiments

A standard stimulatory concentration of 5×10^{-9} M sGH was chosen based on previous sGH concentration-response studies (Pierce *et al.* 2003). The survey of hormonal effects (Figures 4.1 and 4.5) was performed on cells cultured separately from three female fish, 149.7 ± 4.1g (mean ± SEM) body weight, using one 24-well culture plate per experiment per fish. Confirmation of the insulin and glucagon effects (Figure 4.2) and the Dex concentration-response (Figure 4.3) experiments were performed on pooled cells from two male fish, 162.5 ± 16.5g body weight, using one and two culture plates, respectively. The sIns and bGlu concentration-responses (Figure 4.4) were performed on pooled cells from four male fish, 182.5 ± 5.1g body weight, using two

culture plates each. Amino acid experiments (Figure 4.6) were performed on the same pool of cells as used for the sIns and bGlu experiments.

Real-time quantitative RT-PCR (qPCR) assays

Hepatocyte IGF-I mRNA levels were quantified using a qPCR amplicon in the leader peptide region of the gene as previously described (Pierce *et al.* 2003). Growth hormone receptor (GHR) mRNA levels were quantified using a qPCR amplicon in the extracellular domain as previously described (Fukada *et al.* 2003). Gene expression data were normalized to the expression level of acidic ribosomal phosphoprotein P0 (ARP), using an efficiency corrected relative expression technique. Assays were run in 96-well format using TaqMan probes. All samples to be compared were run on the same PCR plate.

Data analysis

Gene expression levels were expressed relative to the control treatment for each fish or hepatocyte pool. In the hormone survey experiment, for each fish, the magnitude of responses was normalized to the average stimulation by GH alone found in all the fish. In the concentration-response experiments, gene expression levels were expressed relative to wells containing the control treatment for each culture plate. One- or two-way ANOVA followed by the Student-Newman-Keuls test was used to examine treatment and fish effects. Results were considered statistically significant at $p < 0.05$.

RESULTS

As expected, sGH (5×10^{-9} M) significantly increased IGF-I mRNA levels in all experiments. IGF-I mRNA increased by 3.62 ± 0.27 fold (mean ± SEM; n=12). The threshold cycle for ARP did not vary between treatments in any experiment, indicating that treatments did not affect our reference gene.

Hormonal treatment significantly affected IGF-I mRNA levels in the hormone survey experiments (Figure 4.1). The effect of fish was not significant after normalization in the T_3 and Dex experiment or the sGLP experiment (Figure 4.1A, 4.1C), but it was significant in the bIns and sGlu experiment (Figure 4.1B). Besides sGH, none of the hormones tested changed IGF-I gene expression from control levels by itself. T_3 (10^{-7} M) and sGLP (10^{-6} M) did not modify the stimulation of IGF-I mRNA levels by sGH. RNA was degraded in some wells from the sGLP experiment, resulting in a decreased number of replicates. Dex (10^{-6} M) inhibited the response to GH to 27% of the level without Dex, bIns (10^{-6} M) inhibited the response significantly in all fish to 43±7%, and sGlu (10^{-6} M) inhibited the response significantly in two of three fish to 75±11%. Inhibition of the GH response by bIns was unexpected. Therefore, the effects of bovine Ins (bIns) and salmon Glu (sGlu) were tested using alternate hormone sources (Figure 4.2). However, similar results were obtained. Salmon Ins (sIns, 10^{-6} M) inhibited the IGF-I mRNA response to 33% of the level with sGH alone, and bovine Glu (bGlu, 10^{-6} M) inhibited the response to 71% of the level with sGH alone.

The inhibition of the IGF-I mRNA response to sGH by Dex was concentration-dependent over the range of 10^{-12} M to 10^{-8} M (Figure 4.3). Dex concentrations from 10^{-8}

to 10^{-6} M inhibited the response to 14% of the level without Dex, not significantly different from the no GH treatment. Although no significant differences were found in the 10^{-8} to 10^{-6} M range by ANOVA, a continued decreasing trend in IGF-I gene expression with increasing Dex was observed.

The inhibition of the IGF-I mRNA response to sGH by sIns was concentration dependent (Figure 4.4A). Concentrations of 10^{-11} and 10^{-10} M sIns were ineffective, but concentrations from 10^{-9} to 10^{-6} M progressively inhibited the sGH response. Maximal inhibition was observed at 10^{-6} M sIns, which suppressed the sGH response to 36% of the level with sGH alone. In contrast, inhibition of the sGH response by bGlu was only observed at 10^{-6} M (Figure 4.4B), which suppressed the response to 62% of the level with sGH alone. bGlu concentrations from 10^{-11} to 10^{-7} M did not significantly attenuate the response to sGH.

GHR mRNA levels were significantly affected by hormonal treatment (Figure 4.5). bIns inhibited GHR mRNA significantly in two of three fish, to 55 ± 11% of control levels. In contrast, Dex increased GHR significantly in all fish, to 309 ± 40% of control levels. T_3 and sGlu did not significantly affect GHR mRNA.

Manipulation of AA concentrations in culture medium did not significantly affect basal or GH-stimulated IGF-I gene expression (Figure 4.6). However, stimulation of IGF-I gene expression by GH was observed, indicating that cells were alive and responsive.

DISCUSSION

GH (5 x 10^{-9} M) consistently stimulated IGF-I gene expression in cultured hepatocytes by ~3.5 fold. Similar stimulation was found in salmon hepatocyte culture in previous experiments using the same IGF-I mRNA assay (Pierce *et al.* 2003), and in experiments using an RNase protection assay (Duan *et al.* 1992). In tilapia hepatocytes, an ~3.25-fold stimulation of IGF-I gene expression over controls was found after 18 hours of incubation with 10^{-8} M GH (Schmid *et al.* 2000). Greater stimulation of IGF-I mRNA (10-20 fold) was found in other studies using salmonid hepatocytes (Duan *et al.* 1993a; Shamblott *et al.* 1995). Consistent stimulation of IGF-I gene expression by GH in fish hepatocyte culture confirms that this functional relationship in the growth axis is conserved in fishes (Duan 1998; Perez-Sanchez *et al.* 1999; Kelley *et al.* 2000; Moriyama *et al.* 2000; Mommsen 2001a). The differences in the magnitude of GH stimulation may be due to differences between culture conditions, animals, or the assay used. All fish hepatocyte culture studies have measured IGF-I mRNA levels rather than IGF-I protein secreted into the medium. It is not known how closely IGF-I gene expression reflects IGF-I protein secretion in fish hepatocyte culture. However, IGF-I is constitutively secreted (D'Ercole *et al.* 1987), and cellular IGF-I mRNA levels have been found to be highly correlated with the amount of IGF-I protein secreted into culture medium in rat hepatocyte culture (Phillips *et al.* 1991; Goya *et al.* 1999; Goya *et al.* 2001).

The synthetic glucocorticoid dexamethasone substantially inhibited the IGF-I mRNA response to GH in salmon hepatocyte culture, without affecting basal IGF-I gene expression. To our knowledge, no previous studies have examined the effects of

glucocorticoid hormones on IGF-I gene expression in fish hepatocyte culture. However, primary salmonid hepatocytes respond to glucocorticoids in terms of many other endpoints, including changes in metabolism, enzyme activities, cellular stress responses, and glucocorticoid receptor level (Mommsen *et al.* 1999; Sathiyaa *et al.* 2001; Boone *et al.* 2002; Sathiyaa *et al.* 2003). The effects of cortisol on the growth axis have recently been examined in tilapia *in vivo*. Cortisol injection decreased liver IGF-I mRNA in a dose-dependent manner and decreased plasma IGF-I without changing plasma GH, suggesting that the cortisol effects may be mediated by GH resistance (Kajimura *et al.* 2003). Our results in salmon hepatocytes support this idea. Consistent with inhibition of liver IGF-I production, cortisol injection suppresses growth in fishes (Davis *et al.* 1985; Barton *et al.* 1987), as does stress (Barton *et al.* 1991).

Cultured salmon hepatocytes were extremely sensitive to dexamethasone: significant inhibition of the GH response occurred at 10^{-12} M. Cortisol is the primary glucocorticoid hormone in fishes, and plasma cortisol levels range from approximately 10^{-8} M in unstressed to 10^{-6} M in stressed salmonids (Barton *et al.* 1991). Thus, significant inhibition of the IGF-I mRNA response to GH occurred at dexamethasone concentrations 10,000-fold lower than circulating cortisol levels. This may be due in part to the greater potency of dexamethasone. Dexamethasone binds as or more strongly than cortisol to the glucocorticoid receptor in a variety of fish species including salmon (Mommsen *et al.* 1999). Rainbow trout hepatocytes responded maximally to 10^{-9} M dexamethasone versus 10^{-8} M cortisol in potentiation of a cytochrome p450 dependent activity (Devaux *et al.* 1992). However, even allowing for higher dexamethasone

potency, the results of the present study suggest that GH-dependent liver IGF-I production may be tonically inhibited by cortisol. Further, dexamethasone inhibited the GH response substantially, to less than 30% of the response without dexamethasone at 10^{-6} M. Together, these results suggest that cortisol may be a major regulator of GH-dependent liver IGF-I production in salmon.

In contrast to the strong inhibition of the GH response by dexamethasone found in salmon hepatocytes, both positive and negative effects are found in mammalian hepatocyte culture. In pig hepatocytes, 10^{-7} M dexamethasone increased mRNA levels of the GHR, increased the IGF-I mRNA response to GH, and increased basal IGF-I mRNA levels (Brameld *et al.* 1995). In rat hepatocytes, dexamethasone stimulated basal IGF-I gene expression (maximally at 10^{-8} M), and had a biphasic effect on GHR gene expression, increasing GHR gene expression and enhancing the IGF-I response to GH at low concentrations (10^{-10} M), and decreasing GHR and the response to GH at higher concentrations (Beauloye *et al.* 1999). In the present study, dexamethasone (10^{-6} M) increased GHR gene expression, but diminished the response to GH. The increase in GHR gene expression with dexamethasone treatment was also seen in previous hepatocyte cultures (A. Pierce, unpublished data). This suggests that regulation of hepatocyte GH responsiveness by glucocorticoids occurs by a different cellular mechanism in salmon than in mammals.

Insulin inhibited the IGF-I mRNA response to GH in salmon hepatocyte culture. This surprising result was observed in five different hepatocyte preparations, from both male and female fish, and with both bovine and salmon insulin. Consistent with the

inhibition of GH response, insulin decreased GHR gene expression to approximately one-half of the level without insulin, an effect seen in previous salmon hepatocyte cultures (A. Pierce, unpublished data). Insulin had no effect on basal IGF-I gene expression, and did not change transcript levels of ARP, our housekeeping gene. The observed inhibitory effect of insulin is most likely due to action through the insulin receptor. Although insulin binds to the IGF type 1 receptor (IGF-IR) in brown trout (*Salmo trutta*), the affinity is ~200 fold lower than that of IGF-I (Moon *et al.* 1996). In other studies, we found that 5 x 10^{-9} M IGF-I added to culture medium did not inhibit the IGF-I mRNA response to GH (Pierce *et al.* 2003). Further, available information in fish suggests that IGF-IR levels are very low in fish liver (Maures *et al.* 2002a; Nakao *et al.* 2002) as in adult mammal hepatocytes (Hallak *et al.* 2002). In tilapia hepatocytes, IGF-I did not affect basal IGF-I mRNA (Schmid *et al.* 2000), and in primary eel hepatocytes, GH but not IGF-I enhanced the vitellogenin response to estradiol (Peyon *et al.* 1998). In salmonids, concentrations of insulin delivered to the liver by the hepatic portal vein are 3-5 times the level in the general circulation (Plisetskaya *et al.* 1989), and insulin levels in the peripheral circulation are 3-20 and <1-4 ng/ml, in fed and fasted fish, respectively (Mommsen *et al.* 1991; Navarro *et al.* 1995). Therefore, insulin levels experienced by the liver range from less than 0.5 x 10^{-9} M to approximately 15 x 10^{-9} M. The inhibition of GH response found in the present study was concentration dependent within this range. Metabolic responses to insulin are seen at similar concentrations in salmonid hepatocyte culture (Plisetskaya *et al.* 1984; Mommsen *et al.* 1991; Duan *et al.* 1993b; Segner *et al.* 1994). Thus, the inhibition of GH-dependent IGF-I production occurs at physiological

concentrations of insulin *in vitro*, suggesting that this effect may operate under normal physiological conditions *in vivo*.

The only previous fish hepatocyte culture study on this point found an enhancement of the response to GH by insulin after 6 hours of incubation (Duan *et al.* 1992). The previous study took place in the summer, and subsequent attempts to replicate the results were unsuccessful (C. Duan, personal communication). Salmon growth and growth endocrinology are highly seasonal (Dickhoff *et al.* 1997; Beckman *et al.* 2000). Since our experiments took place in fall and winter, it is possible that there are seasonal differences in the response of cultured salmon hepatocytes to insulin. Further work is required to exclude this possibility. In a previous time course experiment, a combination of insulin (10^{-6} M), dexamethasone (10^{-10} M), and T_3 (10^{-7} M) inhibited the response to GH over 3-24 hours of incubation but not after 48 hours (Pierce *et al.* 2003), suggesting that modulation of GH response may depend on time in culture. Injection of insulin in brown trout increased plasma IGF-I levels 3 hours later (Banos *et al.* 1999). This result may be reconciled with the present study if the *in vivo* effect is indirect, possibly through increases in circulating GH in response to insulin-induced hypoglycemia. In the isletectomized goby, an experimental model of insulin-dependent diabetes in a teleost fish, GH injection stimulated growth and cartilage sulfation activity, a measure of IGF-I bioactivity (Kelley *et al.* 1993). Thus in the goby, unlike mammals, isletectomy and the consequent removal of insulin did not cause GH resistance. In gilthead sea bream, liver IGF-I mRNA showed diurnal variation in response to feeding, decreasing, albeit nonsignificantly, after a meal and rebounding 10 hours later (Meton *et*

al. 2000). Insulin increases after feeding and then declines (Navarro *et al.* 1995); thus, these results can be interpreted as consistent with the mechanism found in the present study. Finally, studies on the fasting response in salmon have found that liver IGF-I mRNA and plasma IGF-I decrease during fasting before any change in basal insulin is seen, and indeed insulin sometimes does not decrease (Duan *et al.* 1993c; Baker *et al.* 2003; Chapter 2), implying that decreased plasma insulin cannot be primarily responsible for the development of GH resistance in fasting salmon.

The inhibition of the response to GH by insulin in salmon hepatocytes is the inverse of what is found in tetrapods. In rat hepatocytes, insulin increased the IGF-I mRNA response to GH by ~2-fold (Tollet *et al.* 1990; Boni-Schnetzler *et al.* 1991; Krishna *et al.* 1996), though there is some variation among studies about whether this is an additive or synergistic effect. Insulin also potentiated the GH response at the level of IGF-I protein secretion into medium in chicken and rat hepatocytes (Houston *et al.* 1991; Villafuerte *et al.* 1992). Insulin was required for GHR gene expression in pig hepatocytes (Brameld *et al.* 1995), and increased specific GH binding, but not GHR gene expression in rat hepatocytes (Tollet *et al.* 1990). Studies in diabetic mammals support a requirement for normal portal insulin levels to maintain liver IGF-I production, plasma IGF-I levels, and growth (Griffen *et al.* 1987; Hanaire-Broutin *et al.* 1996). Insulin infusion is sufficient to restore liver IGF-I production and plasma IGF-I levels in catabolic cows (Butler *et al.* 2003). Further, in mammals, IGF-I inhibits pancreatic beta cell insulin secretion (e.g., Zhao *et al.* 1997). Along with the stimulation of liver IGF-I production by insulin, this has led to the proposal of a physiologically significant

feedback loop between the pancreas and the liver (Zhao *et al.* 1997). As far as we are aware, no *in vivo* or *in vitro* studies in any tetrapod species have shown an inhibition of basal or GH-stimulated liver or hepatocyte IGF-I mRNA or protein by insulin. This suggests that the role of insulin in growth regulation in salmon is quite different from that in mammals and birds. Obviously, more information on the role of insulin in piscine growth regulation is needed before detailed conclusions can be drawn. Glucose homeostasis is much less important in the metabolism of fishes than in tetrapods. Consequently, the metabolic functions of insulin may be less important and the growth stimulatory functions more important in fishes than in tetrapods (Mommsen *et al.* 1991; Kelley *et al.* 1993; Plisetskaya 1998; Mommsen 2001a). The inhibition of IGF-I by insulin is consistent with a reciprocal relationship between the hormones.

No effect of T_3 (10^{-7} M) was found on basal or GH-stimulated IGF-I gene expression, and T_3 did not affect GHR gene expression. Duan (1992) found stimulation of IGF-I mRNA by 100 ng/ml T_3 in salmon hepatocyte culture, but no synergism with GH. The concentration used was higher than the present study, which, among other factors, could account for the difference in results. Schmid (2003) found a fairly modest (1.5-fold) stimulation of basal IGF-I mRNA by T_3 (10^{-7} M) in tilapia hepatocyte culture, suggesting that species differences may exist in this response between salmon and tilapia. In contrast to the lack of effect or modest effects found in fishes, T_3 strongly potentiated the response to GH in rat hepatocyte culture (Tollet *et al.* 1990), and both strongly potentiated the response to GH and induced GHR in pig hepatocyte culture (Brameld *et al.* 1995). In hypothyroid chickens, thyroid hormone replacement normalized decreases

in serum IGF-I, liver IGF-I mRNA, liver GHR mRNA, and liver GH binding (Tsukada *et al.* 1998). On the basis of these differences, one might speculate that thyroid hormones play a greater role in maintaining hepatocyte GH responsiveness in tetrapods than in salmon.

High concentrations (10^{-6} M) of glucagon inhibited the IGF-I mRNA response to GH without significantly changing GHR gene expression. Inhibition was to ~75% of the level with GH alone, and was seen with both salmon and bovine glucagon. However, in a concentration-response study, inhibition did not occur at lower concentrations of glucagon. Physiological concentrations of glucagon experienced by the liver are ~10^{-10} M, 10,000-fold lower than the concentration where an effect was seen (Plisetskaya *et al.* 1989). Primary fish hepatocytes respond metabolically to glucagon concentrations as low as 10^{-8} M (Mommsen *et al.* 1990; Plisetskaya *et al.* 1996; Mommsen 2000). Therefore, the effect of glucagon found in the present study is likely to be pharmacological. In contrast, physiological concentrations of glucagon inhibited both basal and GH-stimulated IGF-I gene expression and secretion into culture medium in rat hepatocytes (Arany *et al.* 1993; Denver *et al.* 1994). GLP is a pancreatic hormone with metabolic effects similar to glucagon on the liver in fishes, which is often more potent than glucagon (Mommsen *et al.* 1990; Plisetskaya *et al.* 1996; Mommsen 2000). The present study provides no evidence for a role for GLP in the regulation of the growth axis. However, the fact that an effect of glucagon was seen at 10^{-6} M, whereas no effect of GLP was seen at the same concentration, is consistent with the idea that these peptides have differential as well as overlapping effects in fish hepatocytes (Mommsen 2000).

It is possible that the effects of metabolic hormones on GH sensitivity found in the present study are mediated by changes in intracellular nutrient levels. Medium amino acid concentrations regulate GH-dependent IGF-I production in pig, rat, and sheep hepatocytes (Brameld *et al.* 1999; Thissen *et al.* 1999; Wheelhouse *et al.* 1999), and medium glucose concentrations regulate GH-dependent IGF-I production in rat and pig hepatocytes (Brameld *et al.* 1999; Goya *et al.* 1999). Glucocorticoids increase glycogenolysis and gluconeogenesis in cultured fish hepatocytes (Mommsen *et al.* 1999). Insulin increases glucose and amino acid uptake, protein synthesis, and glycogen synthesis, and inhibits gluconeogenesis (Plisetskaya *et al.* 1984; Mommsen *et al.* 1991; Duan *et al.* 1993b). However, glucagon and GLP also have strong metabolic effects in fish hepatocytes, increasing glycogenolysis and gluconeogenesis (Plisetskaya *et al.* 1996; Mommsen 2000), but these hormones did not modify GH sensitivity. Further, manipulation of overall medium amino acid levels or deletion of individual amino acids from the medium did not affect basal or GH-stimulated IGF-I gene expression in salmon hepatocytes (Figure 4.6). Therefore, in our opinion, the effects of dexamethasone and insulin are not likely to be mediated by changes in intracellular nutrient levels. However, further work is required to exclude this possibility.

None of the hormones tested affected IGF-I gene expression by themselves; however, strong modulatory effects on GH sensitivity were found. This striking result suggests that hepatocyte GH sensitivity is an important mechanism in the regulation of IGF-I production in salmon. As previously discussed, studies in mammalian hepatocyte culture have found basal effects of insulin, glucagon, T_3, and dexamethasone, which may

confound analysis of hormonal effects on GH sensitivity. Inclusion of insulin and dexamethasone in basal culture media have been found to be necessary to maintain cell viability and responsiveness in mammals (e.g., Brameld *et al.* 1995). These differences make primary cultured salmon hepatocytes an appealingly simple model for studying the regulation of GH sensitivity. Our results point out the necessity of measuring GH response in assessing treatment effects on hepatocyte IGF-I gene expression. Further, it is possible that there are modulatory effects that are only seen with combinations of three or more hormones.

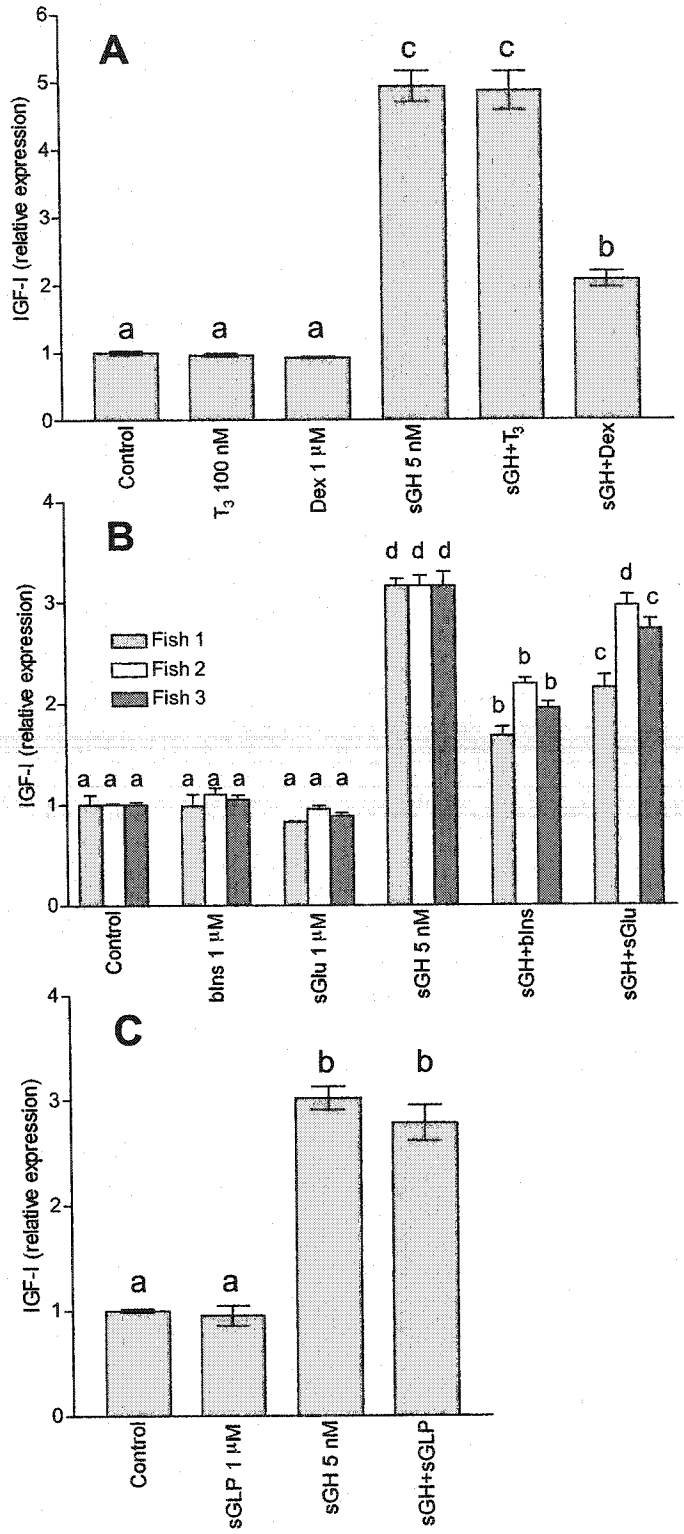
The GHR has recently been cloned in a number of fish species (Ozaki *et al.* 2000; Calduch-Giner *et al.* 2001; Lee *et al.* 2001; Calduch-Giner *et al.* 2003; Fukada *et al.* 2003; Tse *et al.* 2003). The increase in GHR mRNA with dexamethasone treatment and decrease with insulin treatment in the present study suggest that different cellular mechanisms mediate the inhibition of GH-stimulated IGF-I gene expression seen in these treatments. In some mammalian species, truncated forms of the GHR mRNA and protein exist which lack intracellular signaling domains and may downregulate GH signaling; these and other short forms of the GHR mRNA produce the GH binding protein (GHBP) in some species (Edens *et al.* 1998). A GHBP has been isolated from rainbow trout plasma (Sohm *et al.* 1998). A truncated form of the GHR mRNA without the intracellular domains required for signaling has been reported in turbot (Calduch-Giner *et al.* 2001); however, in gilthead sea bream, attempts to find alternative GHR transcripts were unsuccessful (Calduch-Giner *et al.* 2003). No information is available on alternative splicing of the GHR transcript in salmon. Our GHR mRNA assay used an amplicon in

the extracellular domain near the putative ligand binding site, which would presumably detect all transcripts (Fukada *et al.* 2003). The increase in GHR mRNA and decrease in GH sensitivity with dexamethasone treatment could be the result of the induction of a non-signaling form of the receptor. The decrease in GHR mRNA and decrease in GH sensitivity with insulin treatment could be the result of a decrease in signaling receptors. Further work is required to investigate these possibilities. The effects of insulin and dexamethasone on GH sensitivity in the present study may also occur downstream of the GHR. The signal transduction pathway from the GHR to IGF-I gene expression appears to be conserved between mammals and salmon. The salmon IGF-I promoter was activated by GH treatment in a mammalian hepatoma cell line, after reconstruction of the pathway by co-expression of the GHR (rat) the GH signal transducer STAT5 (mouse), and the liver-enriched transcription factor HNF-1α (sheep) (Meton *et al.* 1999). The suppressors of cytokine signaling (SOCS) family of proteins inhibit GH and other cytokine signaling by interfering in second messenger cascades (Krebs *et al.* 2001). In rats, fasting induces SOCS3 and causes GH resistance in the liver (Beauloye *et al.* 2002). The dexamethasone and insulin induced GH resistance in salmon hepatocytes could be caused by induction of SOCS proteins or other intracellular modulators of GH signaling.

Negative results were obtained in tests of potential effects of overall AA level on basal and GH-stimulated IGF-I gene expression (Figure 4.6A). Further, deletion of individual pairs of amino acids from culture medium did not affect GH-stimulated IGF-I gene expression in salmon hepatocytes (Figure 4.6B). In cultured rat, sheep, and pig hepatocytes, IGF-I gene expression increased with increasing medium AA concentration

(Thissen *et al.* 1994b; Brameld *et al.* 1999; Wheelhouse *et al.* 1999). Deletion of individual AAs from culture medium showed that some AAs regulate IGF-I gene expression in mammalian hepatocytes (Harp *et al.* 1991; Brameld *et al.* 1999; Stubbs *et al.* 2002). Most of the same AAs were tested in the present study. The reasons for the negative results obtained in salmon hepatocytes are not clear. It is possible that cellular regulatory processes compensated for experimental manipulations, resulting in no changes in intracellular AA concentrations and therefore, no changes in IGF-I gene expression. It is also possible that regulation of salmon hepatocyte IGF-I gene expression differs from mammals in this regard. Before conclusions can be drawn about regulation of IGF-I gene expression by AA concentration in salmon hepatocyte culture, it will be necessary to show that cells respond to variation in medium AA concentration in some way, such as changes in gene expression of some other AA responsive gene.

Figure 4.1. Effects of metabolic hormones on basal and GH-stimulated IGF-I gene expression in primary salmon hepatocyte culture. Hepatocytes were isolated, plated in plain RPMI medium, and incubated for 24 hours at 15 °C. Medium was then changed to test medium containing the indicated hormones (sGH: salmon growth hormone; T_3: triiodothyronine; Dex: dexamethasone; bIns: bovine insulin; sGlu: salmon glucagon; sGLP, salmon glucagon-like peptide) and cells incubated an additional 18 hours. Cells from three fish (female, average body weight 149.7±4.1g) were kept separately and used in independent experiments. Data were normalized so that the response of each fish to GH alone was set equal to the average response of all the fish. After normalization, the effects of fish and fish x treatment were not significant in (A) and (C), however, significant effects remained in (B); therefore, fish are presented separately in (B). (A) GH responses 5.18, 5.48, and 4.14 fold Control levels, n = 12 wells per treatment. (B) GH responses 3.19, 3.25, and 3.06 fold Control levels, n = 4 wells per treatment for each fish. (C) GH responses 3.64 and 2.41 fold Control levels, n = 5-7 wells per treatment (number lower due to RNA degradation, one fish omitted due to RNA degradation). Error bars represent standard error of the mean. Bars with different letters are significantly different ($p < 0.05$). In (B), letters refer to significant differences between treatments within each fish.


A
IGF-I (relative expression)
Control
T3 100 nM
Dex 1 µM
sGH 5 nM
sGH+T3
sGH+Dex
B
Fish 1
Fish 2
Fish 3
Control
bIns 1 µM
sGlu 1 µM
sGH 5 nM
sGH+bIns
sGH+sGlu
C
sGLP 1 µM
sGH+sGLP

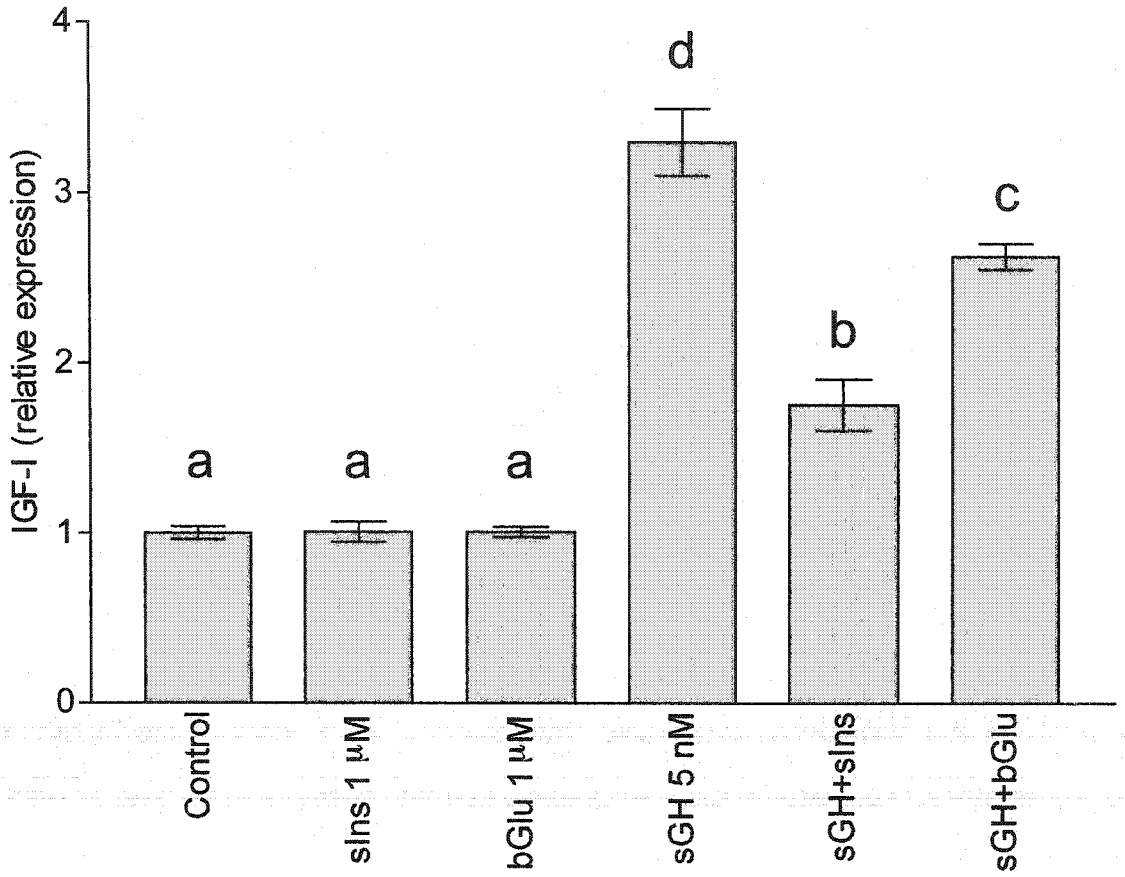


Figure 4.2. Confirmation of insulin and glucagon effects using alternative hormone sources. Cells were isolated and cultured as in Figure. 4.1 (two male fish, 162.5±16.5g). The experiment was performed as in Figure 4.1, except salmon insulin (sIns) and bovine glucagon (bGlu) were used. n = 4 wells per treatment. Error bars and significances are indicated as in previous figures.

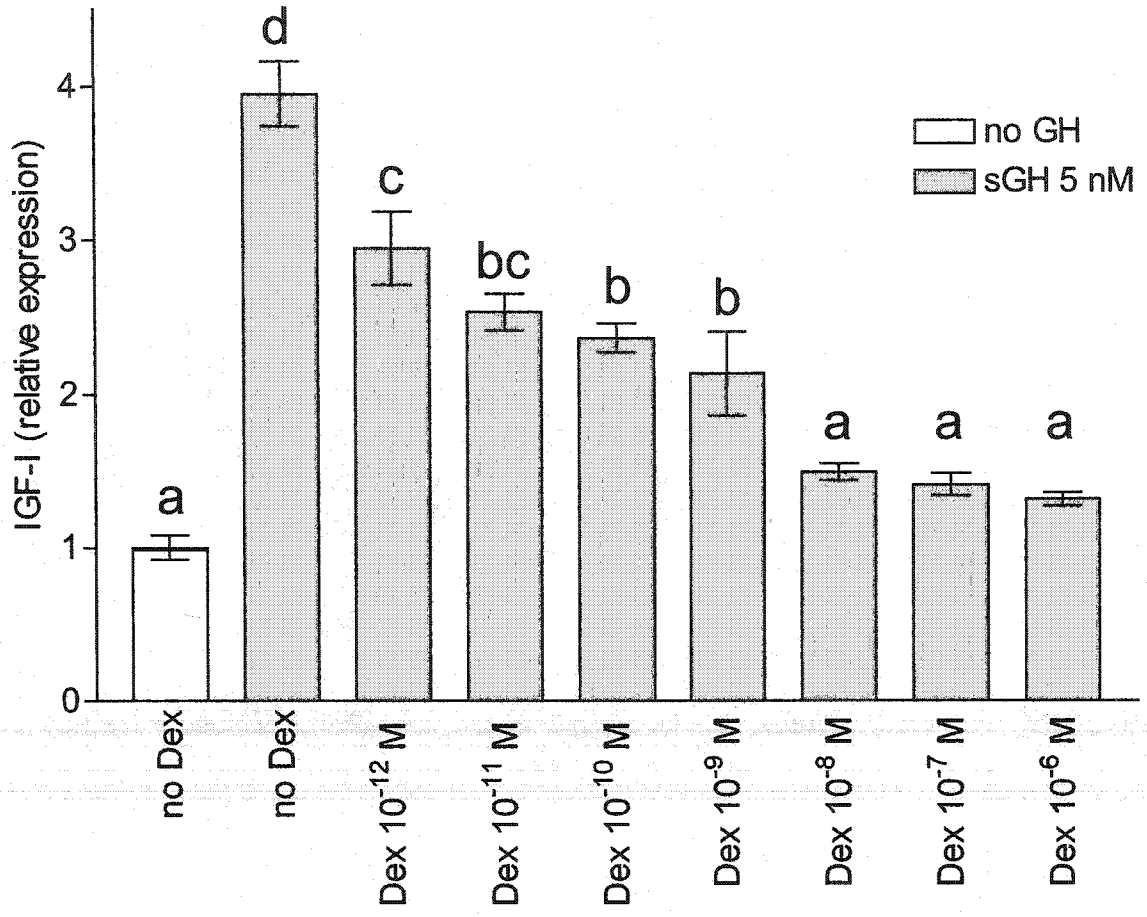


Figure 4.3. Concentration dependence of dexamethasone inhibition of the IGF-I gene expression response to GH. Cells were isolated and cultured as in Figure 4.1 (two male fish, average body weight 162.5±16.5g). n = 4–5 wells per treatment. Hormone abbreviations, error bars and significances are indicated as in previous figures.

Figure 4.4. Concentration dependence of insulin (A) and glucagon (B) inhibition of the IGF-I gene expression response to GH. Cells were isolated and cultured as in Figure 4.1 (four male fish, average body weight 182.5±5.1g). n = 6 wells per treatment. Hormone abbreviations, error bars and significances are indicated as in previous figures.

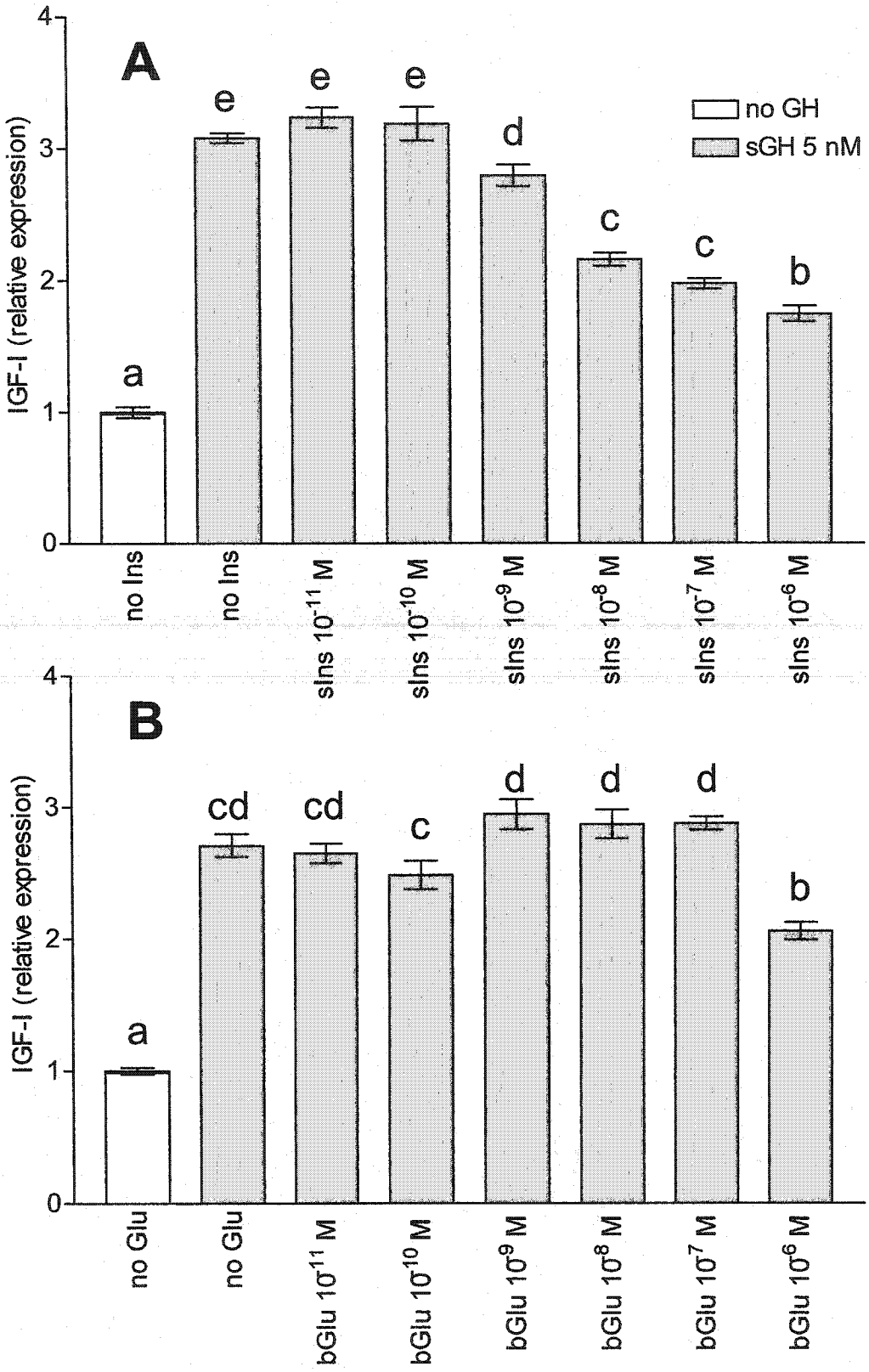
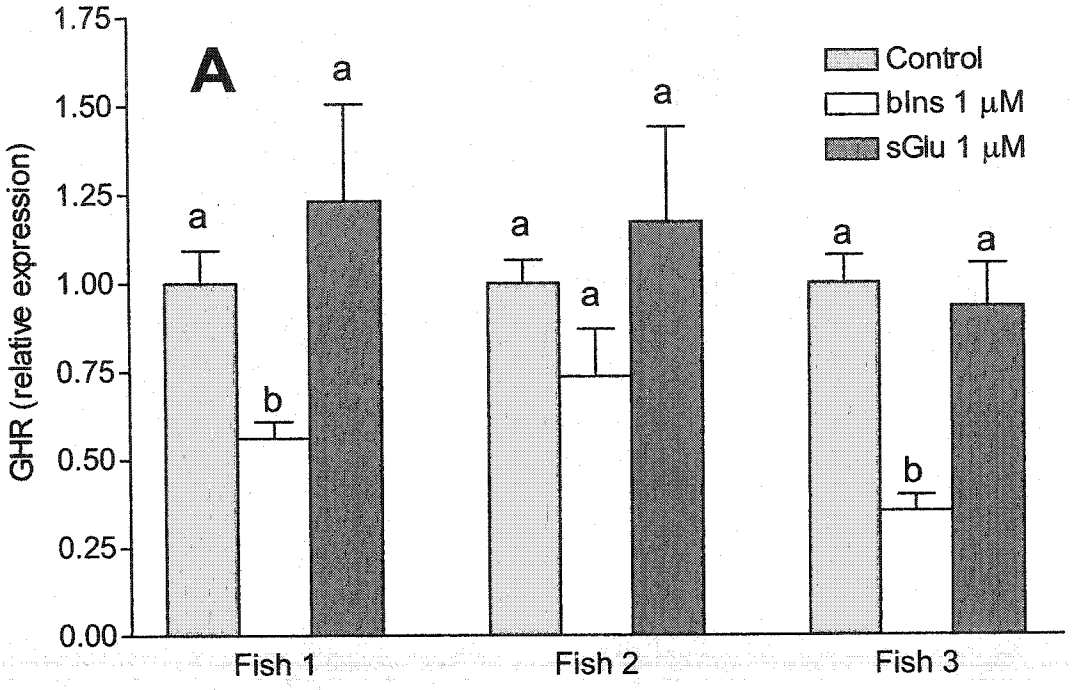

A
B
IGF-I (relative expression)
no GH
sGH 5 nM
a
e
e
e
d
c
c
b
cd
cd
c
d
d
d
b
no Ins
no Ins
sIns 10-11 M
sIns 10-10 M
sIns 10-9 M
sIns 10-8 M
sIns 10-7 M
sIns 10-6 M
no Glu
no Glu
bGlu 10-11 M
bGlu 10-10 M
bGlu 10-9 M
bGlu 10-8 M
bGlu 10-7 M
bGlu 10-6 M
0
1
2
3
4

Figure. 4.5. Effects of insulin and glucagon (A), and triiodothyronine and dexamethasone (B) on growth hormone receptor (GHR) gene expression in primary salmon hepatocyte culture. GHR mRNA was measured in selected samples from the experiment described in Figure 4.1. Hormone abbreviations, error bars and significances are indicated as in previous figures. Letters refer to significant differences between treatments within each fish.


A
Control
bIns 1 μM
sGlu 1 μM
GHR (relative expression)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
a
b
Fish 1
Fish 2
Fish 3

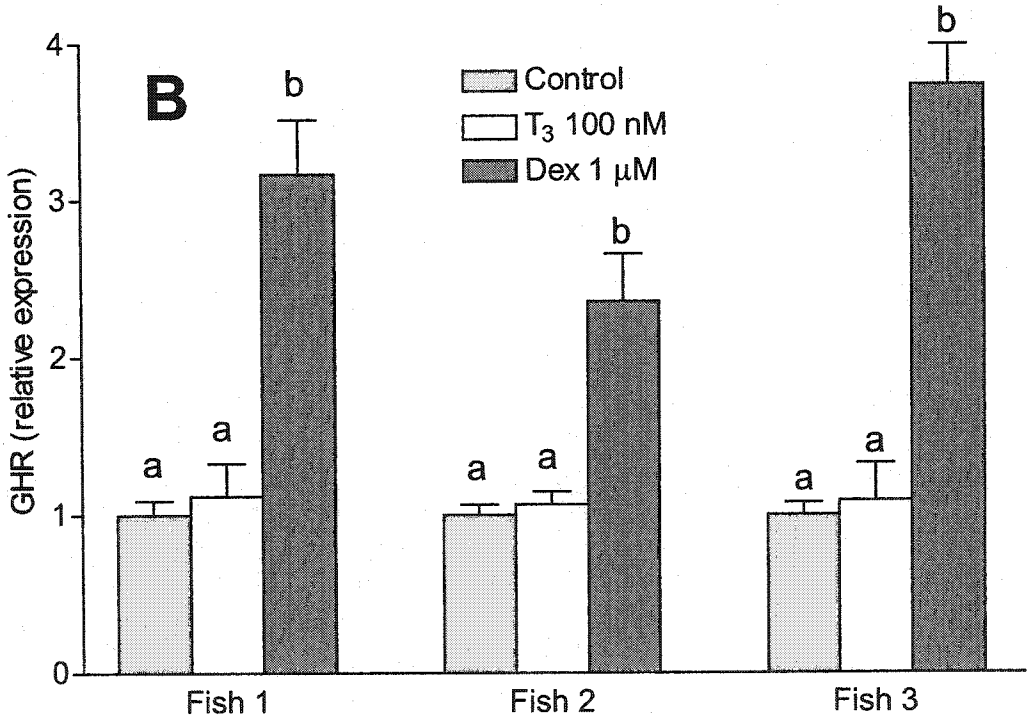
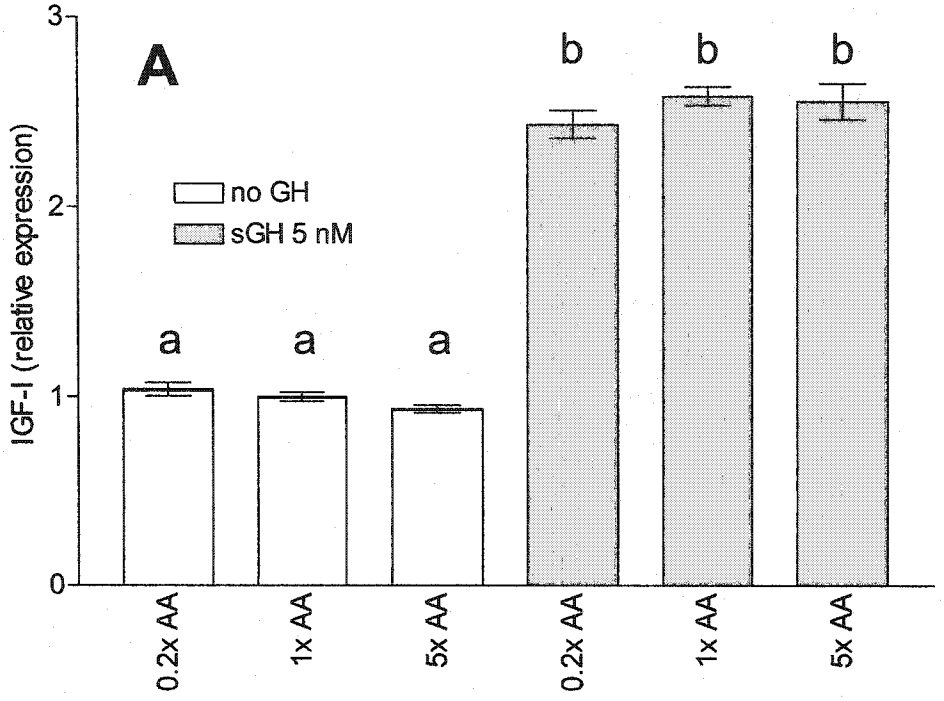

B
Control
T3 100 nM
Dex 1 μM
GHR (relative expression)
4
3
2
1
0
a
b
Fish 1
Fish 2
Fish 3

Figure 4.6. Effects of overall amino acid level (A), and deletion of specific amino acids (B) on IGF-I gene expression in primary salmon hepatocyte culture. Cells were isolated and cultured as in Figure 4.1, except the amino acid concentrations in culture medium were modified (four male fish, average body weight 182.5±5.1g). n = 7-8 wells per treatment. In (B), "All AA del" signifies that all eight of the amino acids deleted in pairs in other treatments were deleted from the medium in this treatment. Hormone abbreviations, error bars and significances are indicated as in previous figures.


A
IGF-I (relative expression)
3
2
1
0
no GH
sGH 5 nM
a
a
a
b
b
b
0.2x AA
1x AA
5x AA
0.2x AA
1x AA
5x AA

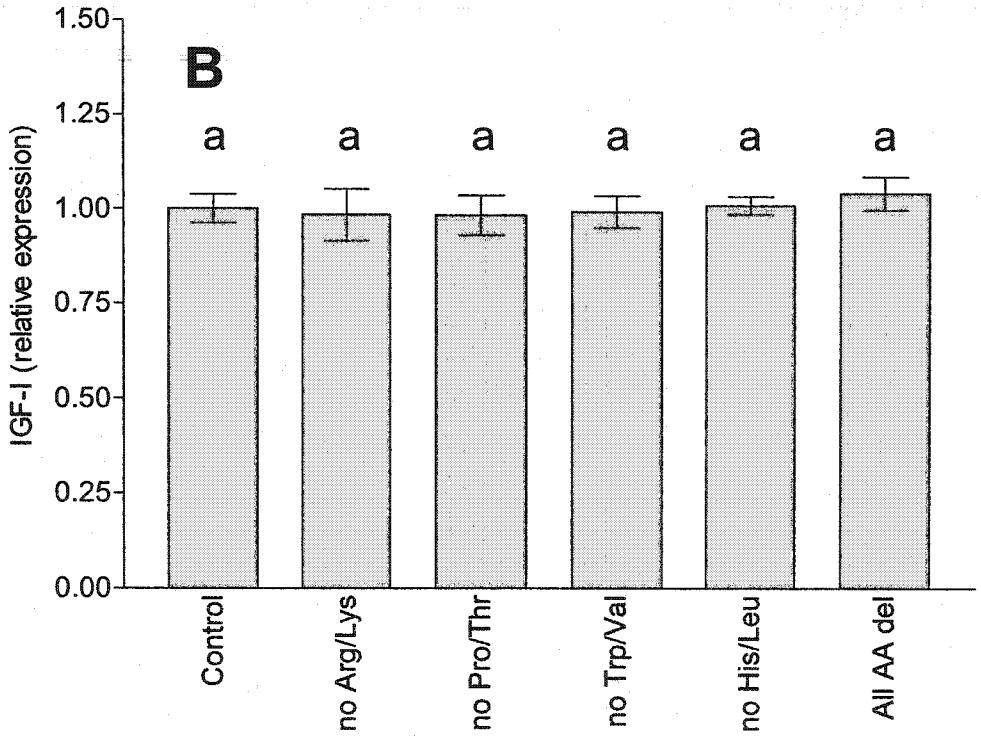

B
IGF-I (relative expression)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
a
a
a
a
a
a
Control
no Arg/Lys
no Pro/Thr
no Trp/Val
no His/Leu
All AA del

Table 4.1. Amino acid concentrations used in culture medium to approximate salmonid plasma levels.

Amino Acid	1x Salmonid plasma (μM)	Standard RPMI (μM)
Alanine (Ala)	636	
Arginine (Arg)	111	1149
Asparagine (Asn)	392	379
Aspartic acid (Asp)	73	150
Cysteine (Cys)	35	
Glutamine (Gln)	538	2055
Glutamic acid (Glu)	299	136
Glycine (Gly)	545	133
Histidine (His)	190	97
Isoleucine (Ile)	208	382
Leucine (Leu)	317	382
Lysine (Lys)	217	220
Methionine (Met)	187	101
Phenylalanine (Phe)	133	91
Proline (Pro)	295	174
Serine (Ser)	181	286
Threonine (Thr)	342	168
Tryptophan (Trp)	143	25
Tyrosine (Tyr)	138	110
Valine (Val)	508	171

Sources: (Mommsen *et al.* 1980; Walton *et al.* 1986; Tantikitti *et al.* 1995; Milligan 1997; Navarro *et al.* 1997).

Chapter 5: Summary and synthesis.

The research described herein contributes to scientific understanding of endocrine regulation of growth in salmon. While some questions have been answered, in the process, additional lines of enquiry have opened. In this section, the major questions answered as well as questions raised are discussed.

The time course experiment (Chapter 2) provides basic information on the regulation of the growth axis by nutritional status. This information contributes toward a comparative perspective on the function of the growth axis in vertebrates. Ideally, one would like to compare the response of the growth axis to fasting in representatives of the major vertebrate groups: mammals, birds, reptiles, amphibians, and fishes. Surprisingly little information is available on this topic. Our results show that the increase in plasma GH during fasting cannot be explained solely as a consequence of decreased plasma IGF-I. This raises the question of what regulates plasma GH during the early stages of fasting, and several candidates (ghrelin and IGFBP-1) are mentioned. The consistent correlations obtained between liver IGF-I gene expression and plasma IGF-I suggest that liver IGF-I production regulates plasma IGF-I level. The similarity in the response of plasma IGF-I and 41-kDa IGFBP (putative IGFBP-3) suggests that IGF-I and IGFBP-3 are co-regulated in salmon, raising the question of how IGFBP-3 is regulated. Do liver macrophages become GH resistant during fasting, or is IGFBP-3 production regulated by paracrine IGF-I from adjacent hepatocytes? Plasma IGF-I declined to a basal level of approximately 40% of fed levels during fasting, and appeared to remain at this basal

level. This raises questions regarding the source (hepatic or non-hepatic) and the function of basal IGF-I.

Fish hepatocyte culture experiments described herein and in other published work have consistently shown that GH stimulates hepatocyte IGF-I expression (Chapters 3 and 4; Duan *et al.* 1992; Duan *et al.* 1993b; Shamblott *et al.* 1995; Schmid *et al.* 2000). GH injection experiments have shown the same thing *in vivo* (Cao *et al.* 1989; Moriyama *et al.* 1994; Perez-Sanchez *et al.* 1994a; Hashimoto *et al.* 1997; Kermouni *et al.* 1998; Shimizu *et al.* 1999a; Kajimura *et al.* 2001). Thus, the decrease in IGF-I gene expression and circulating IGF-I in spite of increased plasma GH in the time course experiment shows that the liver becomes GH resistant in fasting salmon. Unfortunately, the physiological signal that induces liver GH resistance during fasting was not identified. However, based on the lack of a strong response of basal insulin to fasting, the fact that the insulin response occurred after plasma IGF-I changed in this and other experiments (Chapter 2; Duan *et al.* 1993c; Baker *et al.* 2003), and that insulin inhibited GH sensitivity in hepatocyte culture experiments (Chapter 4), we can hypothesize that insulin is not a major link between nutritional status and liver GH sensitivity in salmon. In mammals, insulin does seem to be crucial to maintaining liver GH sensitivity. In dairy cows that were in negative energy balance due to the onset of lactation, infusion of insulin (and glucose) to restore physiological levels was sufficient to restore liver GH sensitivity and plasma IGF-I levels (Butler *et al.* 2003). Therefore, there seems to be a difference between fishes and mammals in the role of insulin in the fasting response. This contributes to a substantial body of evidence that the role of insulin is quite different

in fishes versus mammals (Mommsen *et al.* 1991; Plisetskaya *et al.* 1993; Plisetskaya 1995; Mommsen 1998; Plisetskaya 1998; Mommsen 2001b; Mommsen 2001a).

The very strong inhibition of GH stimulated IGF-I gene expression by dexamethasone in salmon hepatocyte culture (Chapter 4) suggests that cortisol may play a significant role in regulation of the salmon growth axis. Salmon hepatocytes responded to dexamethasone at much lower levels than seen in mammalian hepatocytes, and even very low concentrations of dexamethasone inhibited the response to GH. In mammals, low concentrations of dexamethasone induce the GHR and enhance GH sensitivity (Beauloye *et al.* 1999). In retrospect, it would have been interesting to assess the response of plasma cortisol to fasting in the time course experiment. Cortisol may provide a physiological link between nutritional status and liver GH resistance in salmon. Further work can address this possibility. The strong effect of dexamethasone suggests that it would be interesting to examine the effects of other steroids on GH stimulated IGF-I expression. Reproductive steroids may regulate the growth axis at the level of hepatocyte IGF-I production. Modulation of hepatocyte GH sensitivity through steroid receptor pathways could provide mechanisms for effects of environmental pollutants on fish growth.

Hepatocyte culture experiments in Chapter 3 showed a biphasic response of IGF-I gene expression to stimulation by GH. High concentrations of GH resulted in a reduction in IGF-I gene expression from maximal levels, which were obtained at intermediate GH concentrations. These results are consistent with two GH receptors interacting with a single ligand, as in mammals. At high GH concentrations, receptors will be occupied,

preventing dimerization. However, this is not the only possible mechanism that could give rise to a biphasic concentration/response curve. High concentrations of GH could interact with prolactin and somatolactin receptors, or downregulate GH signaling by induction of SOCS proteins. The dimunition of response at high GH levels could account for the biphasic effect of gene dosage on growth in GH transgenic tilapia (*Oreochromis mossambicus*; Hernandez *et al.* 1997).

The apparently diminished response to GH in hepatocytes from fasted fish (Chapter 3) suggests that GH resistance caused by fasting is preserved through the process of isolation and culture of hepatocytes. This might provide an experimental model with which to address the question of how GH resistance is implemented at the cellular level. Insulin, dexamethasone, and glucagon all caused GH resistance, but GHR gene expression was suppressed by insulin, enhanced by dexamethasone, and not changed by glucagon (Chapter 4), suggesting that cellular regulation of GH sensitivity within hepatocytes is complex.

The inhibition of the IGF-I response to GH by insulin (Chapter 4) is an intriguing and unexpected result. A substantial number of hepatocyte culture and *in vivo* studies in mammals indicate that insulin enhances the hepatocyte IGF-I gene expression and protein secretion response to GH (Griffen *et al.* 1987; Tollet *et al.* 1990; Boni-Schnetzler *et al.* 1991; Villafuerte *et al.* 1992; Thissen *et al.* 1994a; Hanaire-Broutin *et al.* 1996; Krishna *et al.* 1996; Thissen *et al.* 1999; Butler *et al.* 2003). The reasons for this difference between fish and mammals are not clear, but a number of non-mutually exclusive possibilities can be mentioned. The difference could relate to seasonal growth in salmon.

During the fall and winter, when our experiments were conducted, fish might preferentially store fat, while in the summer growing season protein anabolism might be favored. Increases in insulin associated with food intake might suppress IGF-I production in winter and enhance IGF-I production in summer. This possibility can be addressed by repeating the experiment during the summer. It would be remarkable if hepatocytes could change their behavior depending on the season. Another possibility is that there could be a feedback loop between the pancreas and liver in salmon, as postulated for mammals (Zhao *et al.* 1997), but with the interactions reversed. The inhibition of IGF-I by insulin found in salmon hepatocytes might then predict that IGF-I should stimulate pancreatic beta cell insulin secretion. Finally, the inhibition of IGF-I by insulin could be a mechanism regulating total insulin/IGF-I signaling. If there is a greater overlap between the functions of insulin and IGF-I in fish than in mammals, then one might expect that total insulin/IGF-I signaling would be regulated to a greater degree.

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VITA

Andrew Lee Pierce was born in 1961 and raised in Golden, Colorado. He attended Carleton College in Northfield, Minnesota, and received his Batchelor of Arts in Philosophy in 1984. Subsequently, he worked as a computer programmer in Minneapolis and Seattle, and completed his first year of Medical School at the University of Colorado Health Sciences Center, before coming to the University of Washington School of Aquatic and Fishery Sciences. He conducted his Master's research on the endocrinology of growth in salmon under the supervision of Dr. Walton W. Dickhoff, and was awarded the Master of Science degree in 1999. He continued his studies in the same area and with the same supervisor for the Doctor of Philosophy degree, which he received in 2003.

PUBLICATIONS

Pierce, A.L., Fukada, H., & Dickhoff, W.W. Metabolic hormones modulate the effect of growth hormone on insulin-like growth factor-I expression in primary culture of salmon hepatocytes. In preparation.

Fukada, H., Ozaki, Y., Pierce, A.L., Adachi, S., Yamauchi, K., Hara, A., Swanson, P., & Dickhoff, W.W. 2003. Salmon growth hormone receptor: molecular cloning, protein expression, binding assay, and gene expression. *General and Comparative Endocrinology* Submitted Dec 2003.

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GRANTS TO WHICH I CONTRIBUTED

Biology of Salmon IGF Binding Proteins, submitted to USDA Jan 2003 by Dr. Walton W. Dickhoff. Funded grant #2003-03314.

Salmon IGF Binding Proteins, submitted to USDA Feb 2001 by Dr. Walton W. Dickhoff. Funded grant #2001-03320.

Control of IGF-I Production in Salmon, submitted to USDA Feb 2000 by Dr. Walton W. Dickhoff.

Control of IGF-I Production in Salmon, submitted to USDA Feb 1999 by Dr. Walton W . Dickhoff.

Endocrine Control of Salmonid Growth, submitted to USDA Feb 1998 by Dr. Walton W. Dickhoff.

AWARDS, FELLOWSHIPS, AND OFFICES HELD

Best Student Poster, Growth Section. International Congress on Fish Biology, Summer 2002, Vancouver, Canada.

Best Ph.D. Talk. Graduate Student Symposium, University of Washington School of Aquatic and Fishery Sciences, Fall 2001.

University of Washington School of Aquatic and Fishery Sciences funding awards:
H. Mason Keeler Endowment for Excellence 1996.
John E. Halver Fellowship 1998, 2001, 2003
Claire T. and Evelyn S. Egdvedt Fellowship 2001
John N. Cobb Scholarship in Fisheries 2002
Institute of Food Science and Technology 2003

Fisheries graduate student organization (FINS) representative. University of Washington School of Aquatic and Fishery Sciences, 1997 - 1999.

Merck Award for Academic Achievement. University of Colorado Health Sciences Center Medical School, 1995.

William Carleton Scholar, Carleton College.