

THE DISCOVERY OF A NOVEL GROWTH HORMONE RECEPTOR AND THE
NUTRITIONAL REGULATION OF THE GROWTH RELATED ACTIONS OF GROWTH
HORMONE

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ABSTRACT

The growth hormone (GH) family peptides such as GH, prolactin (PRL), and somatolactin (SL) regulate a wide array of physiological actions including but not limited to growth, metabolism, osmoregulation, and lipolysis. These actions are regulated by many factors both internal and external. I used rainbow trout (*Oncorhynchus mykiss*) as a model organism to study the effects of GH-family peptides, nutritional state, and serum on insulin-like growth factor (IGF) and growth hormone receptor (GHR) expression. Gene sequencing and phylogenetic analysis was applied to characterize a novel GHR. Real-time quantitative-PCR was used to determine IGF and GHR expression levels in liver, muscle, and adipose tissue. Western blotting and pharmacological inhibitors were used to determine signaling pathways.

A novel GHR was characterized and determined to be a type 1 GHR with a diverse distribution. It was found to have many features conserved in other GHRs including binding regions, a Y/FGFEFS motif, cysteine residues, and N-glycosylation sites. Fasting was shown to decrease GHR1 expression in the liver, adipose tissue and red muscle. GH and PRL were shown to stimulate IGF expression through the ERK, PI3K/Akt, and JAK-STAT signaling pathways. GH-stimulated IGF expression was dependent on nutritional state, as GH was only able to stimulate IGF expression in fed fish. Nutritional state has no direct effect on GH-stimulated GHR expression. Serum was determined to be the mediator of the change in GH sensitivity as pre-treatment with serum from cells of an opposite nutritional state caused cells to react like the opposite nutritional state in GH-stimulated IGF expression. These findings contribute to the understanding of the actions of GH-family peptides and the mechanisms through which GH conducts its diverse actions in times of differing nutritional availability.

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LIST OF ABBREVIATIONS

Akt.....	Protein kinase B
ANOVA	Analysis of variance
Bp.....	Base pair
BSA.....	Bovine serum albumin
cDNA	Complimentary deoxyribonucleic acid
CHO	Chinese hamster ovarian
CRH	Corticotropin-releasing hormone
c-SrC	proto-oncogene tyrosine-protein kinase
CT	Threshold cycle number
DNA.....	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ERK.....	Extracellular signal-regulated kinase
FSGD	Fish specific genome duplication
GH.....	Growth hormone
GHBP	Growth hormone binding protein
GHR	Growth hormone receptor
GHRH	Growth hormone releasing hormone
Grb	Growth factor receptor-bound protein
GRF.....	Growth hormone releasing factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IGFR	Insulin-like Growth Factor Receptor
IRS	Insulin receptor substrates

JAK.....	Janus Tyrosine Kinase
M-6-P.....	Mannose 6-phosphate
MAPK.....	Mitogen-activated protein kinase
MEK.....	MAPK kinase
mRNA.....	Messenger ribonucleic acid
Nt.....	Nucleotide
NTC.....	No template control
PACAP.....	Pituitary adenylate cyclase activating polypeptide
PCR.....	Polymerase chain reaction
PI3K.....	Phosphoinositide 3-kinase
PKC.....	Protein kinase C
PLC.....	Phospholipase C
PMSF.....	Phenylmethylsulfonyl fluoride
PRL.....	Prolactin
PRLR.....	Prolactin Receptor
PTP.....	Protein tyrosine phosphatases
QPCR.....	quantitative polymerase chain reaction
RACE.....	Rapid amplification of cDNA ends
RNA.....	Ribonucleic acid
RNase.....	Ribonuclease
RT-QPCR.....	Real-time quantitative polymerase chain reaction
SEM.....	Standard error of the mean
SL.....	Somatolactin
SLR.....	Somatolactin receptor
SOCS.....	Suppressor of cytokine signaling

SOSSon of sevenless
SSSomatostatin
STAT.....Signal transducer and activation of transcription
T₃.....Triiodothyronine
TBE.....Tris borate ethylenediaminetetraacetic acid
TRH.....Thyrotropin-releasing hormone
TSH.....Thyroid-stimulating hormone
UTR.....Untranslated region

CHAPTER 1: GENERAL INTRODUCTION

Introduction

The regulation of growth is a highly studied field. Since its first isolation (Li and Evans 1944), growth hormone (GH) has been studied extensively and reviewed thoroughly over the years. GH has been shown to have many different actions across many different organisms, not just the most commonly studied area of growth regulation. These other actions include feeding, metabolism, reproduction, osmoregulation, immune function, behavior, and lipolysis (Bjornsson et al., 2004; Forsyth and Wallis 2002; Moller and Jorgensen 2009; Norbeck et al., 2007; Norrelund 2005; Bergan et al., 2015). GH conducts these actions by binding through a growth hormone receptor (GHR) and causing a signaling cascade. The mechanisms that GH uses to perform this diverse array of actions are not fully understood. In this review, I will discuss GH and its regulation, as well as its primary actions of growth promotion.

Growth hormone

Growth hormone (GH) is a member of a greater family of somatotropin hormones including prolactin (PRL), and somatolactin (SL) (Forsyth and Wallis 2002). PRL has been found in most gnathostomes while SL has only been found in bony fishes (Kawauchi and Sower, 2006). PRL shares growth promoting actions with GH (Rynikova et al., 1988) but also has many other actions including osmoregulation (Maetz 1970), metabolism (Vilalba et al., 1991), behavior (Blum and Fiedler 1965), reproduction (Dunaif et al., 1982), and immunity (Nagy et al., 1983). SL is the newest and least studied of these hormones has actions including reproductive maturation (Johnson et al., 1997), acid–base balance (Kakizawa et al., 1996), background adaptation (Kakizawa et al., 1995), immune function (Calduch-Giner et al., 1998), energy mobilization and stress (Rand-Weaver et al., 1993), lipid metabolism and pigmentation (Zhu and

Thomas 1997; Fukamachi et al., 2009), and the regulation of chromatophores (Zhu and Thomas 1997). These hormones are all similar from an evolutionary standpoint and all also have similar type I cytokine receptors (Kossiakoff and Vos 1999; Wallis 1992). These hormones, while sharing many overlapping functions and evolutionary similarity, also have many differences to be studied including their role in growth promoting actions.

The genetic structure of GH has been conserved through vertebrate evolution. GH is a very well-studied hormone, as GH or a GH-encoding cDNA have been discovered in over 100 different species across all groups of vertebrates (Moriyama et al., 2006). Most vertebrates have a single copy of the GH-encoding genes with the exceptions of carpine ruminants (Wallis et al., 1998) and isospondylid teleosts (Yang et al., 1997) which each have two copies, and higher primates which have four copies (Chen et al., 1989). Salmonids, including our research organism rainbow trout, contain multiple copies of GH due to recent tetraploidization events (Volf, 2005). The gene that encodes GH includes five exons and four introns (Rajesh and Majumdar, 2007) for the majority of species while several groups of fish including salmoniforms, perciforms, and tetradontiforms have six exons and five introns (Moriyama et al., 2008).

Growth hormone receptor

The actions of GH are conducted through a growth hormone receptor (GHR). As mentioned earlier, GHRs belong to a class of type 1 cytokine receptor along with many other receptors including a PRL receptor, interleukin receptors, and colony-stimulating factor receptors (Cosman et al., 1990). All of these cytokines share a single transmembrane domain (Postel-Vinay and Finidori, 1995). GHRs are well studied in over 100 different species including over 25 species of fish with their GHRs identified and sequenced (Reindl et al., 2009; Ellens et al., 2013). GHR expression is the highest in liver, but GHRs are expressed in many tissues including

muscle, adipose, gill, brain, and intestines mirroring the many diverse actions of GH (Kopchick and Andry 2000; Very et al., 2005; Walock et al., 2014). Two GHRs are present in mammals due to alternative splicing of GHR mRNA (Talamantes and Ortiz, 2002). Similar findings are also present in fish with multiple GHRs being present, each encoded from its own mRNA (Perez-Sanchez et al., 2002). In rainbow trout which possess multiple GHRs, the GHRs are differentially expressed (Reindl and Sheridan, 2012). This differential expression is one possible cause of the multiple actions of GH.

GHRs from across multiple species share some general structural traits and have conserved features. In the extracellular domain GHRs contain multiple cysteine residues used in disulfide bonds that are needed for ligand binding (Fuh et al., 1990). GHRs also share another feature called the WXSXX motif in the extracellular membrane (Goffin and Kelly 1997). This WXSXX motif is believed to be very important in ligand binding (deVos et al., 1992). Across the intracellular domain GHRs also share similar features called box 1 and box 2 with the other members of the cytokine receptor family (Carter-Su et al., 1996). These areas are highly conserved and important in signal transduction as a mutation in either region leads to interruption in GHR signal transduction (Baumann et al., 1994).

GHR signaling

When GH binds to GHR, the action of GH is mediated through a number of signaling pathways. In this review, I will briefly go over some classical signaling pathways used by GHRs. When bound by GH a pair of GHR dimers undergoes a conformational change during activation (Brown et al., 2005). The most common signaling pathway of cytokine receptors is janus kinase (JAK) (Ihle et al., 1995) and specifically for GHR, JAK2. After GH binding JAK2 phosphorylates the GHR to open binding sites for other signaling elements (Kiu and Nicholson,

2012). Traditionally the next signaling elements activated after JAK2 are Signal Transducers and Activators of Transcription (STATs) specifically STAT5a and b and to a lesser extent STAT1 and STAT3 (Herrington et al., 2000). These activated STATs then dimerize with each other and move to the nucleus of the cell where they can bind to DNA to regulate gene transcription (Mohr et al., 2012). The action of STAT5 is crucial to the growth promoting action of GH such as insulin-like growth factor (IGF) 1 gene expression (Chia et al., 2006).

JAK2 can also activate other signaling elements other than STATs. JAK2 has been shown to activate the mitogen-activated protein kinase (MAPK) pathway then finally to extracellular signal-regulated kinases (ERK1/2) which can then affect gene transcription either directly or through transcription factors such as c-AMP responsive element-binding protein (Zhu et al., 2001). Additionally JAK2 also activates phosphoinositide 3-kinase (PI3K) (Moutoussamy et al., 1998). These are just a few of the signaling elements GH can activate through JAK2 (Zhu et al., 2001). In addition to the many signaling elements GH activates through JAK2 phosphorylation, GHR activation can cause the phosphorylation of proto-oncogene tyrosine-protein kinase (c-Src) which is a component of its own signaling pathways independent of JAK2 (Zhu et al., 1998). c-Src activation leads to many different growth promoting actions including cell cycle control, proliferation, and differentiation (Schlessinger, 2000).

GH signaling is also controlled by both negative regulators and cross-talk between cell signaling pathways. One group of negative GHR regulators is Suppressors of Cytokine Signaling (SOCS). SOCS is a group of negative regulators of all cytokine receptor signaling in which the action was thoroughly reviewed by Flores-Morales ,(2006). The three major ways through which SOCS work are directly blocking JAK2 activation (Yasukawa et al., 1999), binding to tyrosines

in the GHR and blocking STAT5b activation (Hansen et al., 1999), and finally degrading GHRs through increased ubiquitination (Kamura et al., 2004).

Protein tyrosine phosphatases (PTPs) are another group of negative regulators of GHR signaling. PTPs also block GHR signaling utilizing different mechanisms including PTP-1 inhibiting STAT5b (Ram and Waxman 1997) and dephosphorylating activated GHR (Gu et al., 2003).

GHR signaling cross-talks with multiple other receptor signaling systems and other related signaling pathways. Both GH and insulin regulate both growth and metabolism and share many similar signaling elements, as thoroughly reviewed by Xu and Messian (2009). Briefly, insulin binding to its receptor for a short time is also shown to activate the MAPK pathways downstream of GHR but a longer time binding has negative effects through the same pathways. Additionally, long term high dose GH treatment has inhibitory effects on insulin signaling pathways (IRS1, 2, 3) actions through PI3K. This cross talk and regulatory elements provide another possible mechanism through which GH exerts its diverse actions, especially in times of fasting as both GH and insulin are involved.

GH actions

The primary action of GH is its ability to promote growth. The understanding of how GH promotes growth has evolved from the initial discovery of a sulfation factor intermediate in the actions of GH in gill sulfur uptake 70 years ago (Salmon and Daughaday, 1957). This discovery led to the naming of the factor as a somatomedin to better describe its actions in mediating the growth (Salmon and Daughaday, 1957). This understanding was further expanded with the isolation and characterization of IGF 1 and 2 (Rinderknecht and Humbel 1978; Klapper et al., 1983). These somatomedins were first called insulin-like since they share a similar ability to

induce glucose uptake in fat and muscle cells with insulin (Randle 1954). When IGF-1 and IGF-2 were fully characterized they were found to share approximately 50% amino acid identities with insulin (Rinderknecht and Humbel 1978).

These early discoveries started the original somatomedin hypothesis of growth promotion by GH. This has evolved even further over time to include more diverse actions of GH. The full evolution of the somatomedin hypothesis has been thoroughly reviewed (Le Roith et al., 2001). GH has been shown to have different growth promoting actions across many different levels. Early GH research showed GH to increase protein synthesis in muscle (Kostyo, 1968). In addition to the cellular level, GH treatment was shown to increase protein in the whole body (Wolf et al., 1992).

IGF

Many of the actions of GH are mediated through IGFs. These are many of the most studied aspects of GH actions, including in fish, as reviewed by Wood et al., (2005). IGFs have many actions, with the primary actions relating to growth including sulfur incorporation (Salmon and Daughaday, 1957), cell proliferation (Hu et al., 2004), protein synthesis (Upton et al., 1997), smoltification (Madsen and Burns, 2003), and reproduction (Adashi, 1998). The primary location of IGF production is the liver but IGFs have been found in almost every tissue (Jones and Clemmons, 1995). These sources of IGF outside the liver are very important as they have shown the ability to compensate for liver IGF-1 production (Yakar et al., 1999).

IGF receptors

IGF receptors (IGFRs) belong to a large family of receptors that include IGFR1, insulin receptor, and the IGF-2 mannose-6-phosphate (M-6-P) receptor (Nissley and Lopaczynski, 1991). IGFRs are expressed in a large number of organs and tissues including brain, muscle,

liver, ovary, testis, intestine, and gill (Maures et al., 2002). IGFR1 has been shown to conduct the actions of both IGF-1 and IGF-2 (Le Roith et al., 2001). The insulin receptor is primarily responsible for the actions of insulin and has also been shown to be responsive to IGF-2 *in utero* (Louvi et al., 1997). While the IGFR2 receptor has no effect on IGF signaling it has been shown to lower levels of IGF-2 in fetal development (Baker et al., 1993). In salmonids including our model organism rainbow trout, multiple IGFR1s (IGFR1A and IGFR1B) have been found (Chan et al., 1997). These receptors share similar structures with IGFR1 and the insulin receptors, sharing a 60% amino acid identity (Ullrich et al., 1986).

As mentioned earlier, IGF1R elicits its response through insulin receptor substrates (IRS) proteins which interact with the IGFR1 (Butler et al., 1998). These IRS proteins can then utilize other signaling elements such as PI3-K (Backer et al., 1993), which is also utilized in GHR signal transduction. Through other downstream signaling elements, IRS proteins are also able to stimulate the MAPK signaling pathway (Ricketts et al., 1996), which is also utilized by GHR. These connections between GH, IGF-1, and insulin greatly connect the shared actions of these hormones.

IGF binding proteins

Insulin-like growth factor binding proteins (IGFBPs) are proteins that share a very high affinity for IGFs. IGFBPs also have been very well studied and reviewed (Jones and Clemmons, 1995; Wood et al., 2005). Six different IGFBPs have been identified across vertebrates (Daza et al., 2011). IGFBPs have been found in many different tissues including brain, liver, muscle, intestine, and ovary (Ferry et al., 1999) similar to IGFRs. One of the primary actions is to bind free IGFs and protect them from degradation (Le Roith et al., 2001). In mammals 75% of all free IGF-1 and IGF-2 are bound by IGFBP-3 (Baxter et al., 1989). Another action of IGFBPs is to

increase the availability of IGF to its receptors (Miyakoshi et al., 2001). IGFbps can also inhibit the actions of IGF, such as IGFbp-4 inhibiting IGF-1-induced myogenesis (Silverman et al., 1994). In addition to actions utilizing IGFs, IGFbps have been found to have actions outside of IGFs including cell motility and adhesion (Jones et al., 1993).

IGF regulation

There are many different regulators of all the components of IGF actions. These have been previously reviewed thoroughly (Reindl and Sheridan, 2012). I will briefly share some of the related regulators of IGF. The main regulator of IGF expression across all vertebrates is GH (Pawson et al., 2002) with GH stimulating IGF expression. Other hormones have also been shown to regulate IGF expression. Somatostatin (SS) has been shown to decrease IGF expression in liver (Sheridan and Hagemaster, 2010). Insulin has been shown to decrease IGF-1 expression (Pierce et al., 2005). Interestingly, insulin was also shown to increase IGF-2 expression (Pierce et al., 2010). This suggests a possible difference in the actions of IGFs in regards to insulin. Similar hormones also have actions on IGFR expression. SS decreases IGFR expression in rainbow trout hepatocytes (Very and Sheridan, 2007; Hanson et al., 2009). Insulin treatment produced contradictory results causing decreased IGFR expression in trout cardiomyocytes (Moon et al., 1996) and increased IGFR expression in the gills (Very et al., 2008) suggesting differing actions of IGFRs in different tissues. These regulators have a large amount of overlap with regards to IGF actions and, taken with IGFbps, suggest a multilevel system of regulation.

GH regulation

Circulating levels of GH are kept in balance by both the actions of secretion and clearance. GH is created and secreted from somatotroph cells located in the anterior pituitary.

GH is also produced in the placenta by mammals. The secretion of GH from the pituitary is pulsatile in both birds and mammals (Anthony et al., 1990; Hall et al., 1986). In both rats (Eden, 1979) and humans (Pincus et al., 1996) sexual differences are noted in this pulsatile pattern with males having higher peaks and lower interpeak levels and females have a less pulsatile pattern with higher interpeak levels. In rainbow trout the pulsatile pattern is seen but is also highly variable and much more complicated (Bjornsson et al., 2002). GH levels in circulation are measured in the range of 1-10 ng/ml across most species (Anthony et al., 1990; Einarsdottir et al., 2002; Norbeck et al., 2007; Fox et al., 2010; Salgin et al., 2012). While GH can be observed freely in circulation, a large amount 40-50%, is bound by growth hormone binding protein (GHBP) (Bauman et al., 1988).

GH binding proteins

GHBP is an alternate truncated form of GHR that is formed by the proteolytic cleavage of GHR in humans or by the alternative splicing of the GHR mRNA in mice (Leung et al., 2004). GHBP is found to be produced primarily in the liver (Amit et al., 2000), but is also expressed in multiple tissues outside of the liver (Barnard et al., 1994). GHBP is known to exist in three different forms: freely circulating, membrane bound, and intracellular.

GHBP plays a major role in regulating the actions of GH. Contradictory actions both promote and inhibit the activity of circulating GH. GHBP is shown to bind to circulating GH and protect it from degradation, thus, increasing its half-life (Turyn et al., 1997) and increasing the chance of GH actions due to its prolonged availability. GHBPs have also been shown to bind competitively with GHR for the binding of GH, thus, limiting GH action (Mannor et al., 1991). GHBP delivers GH to GHR to help facilitate binding (Fisker 2006). While much more is known about GHBPs in mammals where the majority of the research has been done, little is known

about GHBP in fish. In rainbow trout GHBP has been discovered (Sohm et al., 1998) but little is known about its actions.

Hormonal GH regulation

The regulation of GH is complicated. Many different factors including hormones, environmental state, and nutritional state all regulate GH. The regulation of GH starts directly with the regulation of GH by the hypothalamus. The hypothalamic control of GH is both stimulatory and inhibitory. GH releasing factors (GRFs) have been shown to stimulate the secretion of GH and have been found in all vertebrate groups (Hall et al., 1986). While GRFs have been found in every vertebrate group, they are not the same across all species (Harvey, 1993). GH-releasing hormone (GHRH) is considered to be the major GRF in mammals and higher vertebrates (Spiess et al., 1983). GHRH does stimulate GH release in fish (Peng and Peter, 1997) but with a much lower efficacy. This suggests that GHRH is not the major GRF in fish (Montero et al., 2000). The opposite holds true for major GRFs in fish, amphibians, and reptiles, such as pituitary adenylate cyclase activating polypeptide (PACAP) and corticotropin-releasing hormone (CRH), which are not active in mammals (Rousseau and Dufour, 2007). In fact, research on PACAP on the stimulation of GH release in mammals is contradictory; some research has shown PACAP to stimulate GH release (Nagy et al., 1993) whereas others have shown it to have no effect at all (Miyata et al., 1989). While GHRH and PACAP are both members of the same family (Vaudry et al., 2000), it is believed that GHRH did not become the major GRF until subsequent vertebrate evolution (Montero et al., 2000).

The major inhibitor of GH stimulation at the hypothalamic level is a family of hormones called somatostatins (Rousseau and Dufour, 2007). Somatostatins are a group of hormones with sizes ranging from 14-17 amino acids and have been found in every major group of vertebrates

(Sheridan et al., 2000). SS was first found as a 14 amino acid peptide in sheep (Brazeau et al., 1973), and this form has been found in every vertebrate group (Nelson and Sheridan, 2005). The main action of SS for which it was named is the direct inhibition of GH secretion (Brazeau et al., 1973). SS has also been shown to decrease the stimulation of GH secretion caused by other GRFs including PACAP and CRH (Lin and Peter 2001). SS has also been shown to inhibit GHRH release (West et al., 1997), itself being another major GRF. Additionally, while SS is shown to decrease GH stimulation, it does not affect GH expression (Fukata et al., 1985).

A number of other hormones also act to regulate GH expression. One such group of hormones are thyroid hormones. Thyrotropin-releasing hormone (TRH) has been shown to increase GH secretion in goldfish (Trudeau et al., 1992) as well as increasing GH protein production in goldfish pituitaries (Kagabu et al., 1998). TRH then stimulates the release of thyroid-stimulating hormone (TSH) which itself has also been shown to directly stimulate GH release (Melamed et al., 1998). An additional level of GH regulation is also found when Triiodothyronine (T_3), a thyroid hormone whose release is stimulated by TSH, has also been shown to stimulate GH synthesis (Melamed et al., 1995). This information all taken together illustrates a multi-level system of GH regulation by thyroid hormones.

GH feedback inhibition

The regulation of GH also occurs at levels beyond hypothalamic control. GH is known to be regulated by long-loop negative feedback inhibition through IGFs (Wong et al., 2006). Wherein GH stimulates IGF expression in the liver which itself later acts to decrease both GH release and GH mRNA levels (Weil et al., 1999). The inhibition of GH by IGF works through different signaling pathways. The inhibition of GH secretion is through PI3K and MAPK

signaling pathways (Fruchtman et al., 2001), while the inhibition of GH expression is through calcineurin (Huo et al., 2006).

Environmental GH regulation

Many different environmental effects also control the regulation of GH. These effects are well studied in salmonids. Photoperiod is a one well studied effector of GH expression.

Perceived photoperiod in salmon regulated endocrine cells including the somatotroph cells in the pituitary that produce GH (Komourdjian et al., 1976). This was later confirmed by experiments that showed that increased day lengths lead to an increase in circulating GH levels in salmon undergoing smoltification (Björnsson et al., 1995; McCormick et al., 1995). Smoltification is a common stage to study the effects of photoperiod in light because of the natural exposure of the salmon to longer days as early in the process of adapting to saltwater. This increase in GH is also important because GH helps shift the metabolic demands of fish towards smoltification (Winans and Nishioka 1987). Additionally, GH helps fish in early smoltification through a large number of osmoregulatory changes (Sakamoto et al., 1993). GH has also been shown to be regulated by temperature as fish raised in warmer water were shown to have higher levels of GH (Björnsson et al., 1989).

Fasting

Fasting has been well established to have a role on the regulation of GH. Fasting has actions on GH, GHRs IGFs, IGFBPs and other related hormones. I will combine the effects of nutrition together into a brief summary as this is important to my research. First fasting has been shown to increase circulating GH levels in many species (Gomez-Requeni et al., 2005; Norbeck et al., 2007; Picha et al., 2008; Reinecke, 2010). This is the first conflict because GH is most often associated with growth promoting actions, which do not occur during fasting. Fasting has

also been shown to decrease other related elements including insulin and IGF-1 (Gomez-Requeni et al., 2005; Norbeck et al., 2007) maintaining the trend of GH not promoting growth in times of fasting. Fasting has also shown to decrease GHR levels in liver (Small et al., 2006; Norbeck et al., 2007) while causing increased GHR levels in muscle (Pierce et al., 2007) and in adipose tissue (Norbeck et al., 2007), suggesting a difference in GH actions in different tissues during fasting.

Similar tissue specific and receptor differences were also observed IGFRs in regards to fasting. In cardiac muscle, fasting resulted in increased IGFR1A and IGFR1B expression and improved IGF binding (Norbeck et al., 2007), additionally, fasting had no effect on IGFR1 expression in skeletal muscle but IGFR1 expression was decreased in the gill during fasting. The effects of fasting on IGFBPs have also been studied, with fasting having different effects on different IGFBPs. IGFBP-1 has been shown to increase during fasting (Lee et al., 1997), whereas in mammals IGFBP-3 levels decrease in fasting (Clemmons and Underwood, 1991). The decrease in smaller IGFBPs, such as IGFBP-1, associated with fasting has been hypothesized as a mechanism to stop anabolic processes in times of fasting (Lee et al., 1997).

Conclusion

The GH family of hormones, GH, PRL, and SL, have been very well studied and have diverse actions in areas including growth, feeding, metabolism, reproduction, osmoregulation, immune function, behavior, stress, chromatophore regulation, pigmentation, and lipolysis. While the most studied actions of these hormones are growth related, many of them are not and only occur during times of catabolism. While well studied, all aspects of GH family hormones actions on growth are not known, additionally the full extent of how GH regulates these very different actions is not understood. Some of the opposite GH actions occur in the same tissues and it is

unknown what factors change these cells responsiveness to GH including the underlying signaling mechanisms that change to enable this switch. Many regulatory elements of the GH pathway are well studied individually but their interactions together, and upon each other, are not fully understood.

While GHRs are very well studied, the roles of individual GHR subtypes are not fully known, especially in animals that possess high numbers of GHRs. For example, a newly discovered GHR in rainbow trout has not been thoroughly examined. Many different elements of this new GHR are unknown including its characterization, function, and distribution. This new GHR will also need to be studied in the evolutionary relationship with other GHRs. The many actions on GH by fasting has been studied at multiple levels, but a fuller understanding of how nutrition can change organisms via GH is not known. Specifically, it's not fully known how cells responsiveness to GH changes under different nutritional states. Overall a better understanding of the growth-promoting actions of GH, specifically in regards to fasting is important to fully understand the diverse actions of GH.

Objectives

My aim in this dissertation work was to add to the overall understating of the growth promoting actions of GH especially as related to fasting. This primary goal will be achieved through 3 goals: (1) characterize a newly discovered GHR and test the effects of fasting on its expression, (2) study the differing effects of the growth hormone family peptides on the growth-promoting actions of IGF and GHR expression, and, (3) test the effect of fasting on the growth-promoting actions of GHIGF and GHR expression. Additionally, I investigated the effects of serum on fasting induced changes in GH sensitivity. In both the second and third goals, pharmacological inhibitors were also used to assess which signaling pathways are being utilized.

The intent of these studies is to help in understanding how GH conducts such an array of diverse actions.

References

- Adashi, E.Y. 1998. The IGF family and folliculogenesis. *J. Reprod. Immunol.* 39, 13–19.
- Amit, T., Youdim, M.B., Hochberg, Z. 2000. Does serum growth hormone (GH) binding protein reflect human GH receptor function? *J Clin Endocrinol Metab* 85:927–932.
- Anthony, N.B., Vasilatos-Younken, R., Bacon, W.L., Lilburn, M.S. 1990. Secretory pattern of growth hormone, insulin, and related metabolites in growing male turkeys: effects of overnight fasting and refeeding *Poult. Sci.*, 69:801-811.
- Backer, JM., Myers Jr MG, Shoelson SE, Chin DJ, Sun XJ, Miralpeix M, Hu P, Margolis B, Skolnik, E.Y., Schlessinger, J., White, M.F. 1992. Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO J* 11:3469–3479.
- Baker, J., Liu, J.P., Robertson, E.J., Efstratiadis, A. 1993. Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* 75:73–82.
- Barnard, R., Thordarson, G., Lopez, M.F., Yamaguchi, M., Edens, A., Cramer, S.D., Ogren, L., Talamantes, F. 1994. Expression of growth hormone-binding protein with a hydrophilic carboxyl terminus by the mouse placenta: studies in vivo and in vitro *J Endocrinol* 140 (1) 125-135.
- Baumann, G., Amburn, K., Shaw, M.A. 1988. The circulating growth hormone (GH)-binding protein complex: a major constituent of plasma GH in man. *Endocrinol.* 122:976-84.
- Baumann, G., Lowman, H.B., Mercado, M., Wells, J.A. 1994. The stoichiometry of growth hormone-binding protein complexes in human plasma: comparison with cell surface receptors. *J Clin Endocrinol Metab.* 78(5):1113–1118.

- Baxter, R.C., Martin, J.L., Beniac, V.A. 1989. High molecular weight insulin-like growth factor binding protein complex. Purification and properties of the acid-labile subunit from human serum. *J Biol Chem* 264:11843–11848.
- Bergan, H.E., Kittilson, J.D., Sheridan, M.A. 2015. Nutritional state modulates growth hormone-stimulated lipolysis *Gen. Comp. Endocrinol.*, 217–218 1-9.
- Björnsson, B. T., Johansson, V., Benedet, S., Einarsdottir, I. E., Hildahl, J., Agustsson, T., Jönsson, E. 2002. Growth Hormone Endocrinology of Salmonids: Regulatory Mechanisms and Mode of Action. *Fish Physiol. Biochem.* 27(3-4), 227–242.
- Björnsson, B.T., Johansson, V., Benedet, S., Einarsdottir, I.E., Hildahl, J., Agustsson, T., Jönsson, E. 2004. Growth hormone endocrinology of salmonids: regulatory mechanisms and mode of action. *Fish Physiol. Biochem.* 27:227-242F.
- Björnsson, B.T., Stefansson, S.O., Hansen, T. 1995. Photoperiod regulation of plasma growth hormone levels during parr-smolt transformation of Atlantic salmon: implications for hypo-osmoregulatory ability and growth. *Gen. Comp. Endocrinol.* 100: 73–82.
- Björnsson, B.T., Thorarensen, H., Hirano, T., Ogasawara, T., Kristinsson, J.B. 1989. Photoperiod and temperature affect plasma growth hormone levels, growth, condition factor and hypoosmoregulatory ability of juvenile Atlantic salmon (*Salmo salar*) during parr-smolt transformation. *Aquaculture* 82: 77–91.
- Blum, V., Fiedler, K. 1965. Hormonal control of reproductive behavior in some cichlid fish. *Gen Comp Endocrinol* 5:186–196.
- Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J., Guillemin, R. 1973. Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science (Wash DC)* 179:77–79.

- Brown, R.J., Adams, J.J., Pelekanos, R.A., Wan, Y., McKinstry, W.J., Palethorpe, K., Seeber, R.M., Monks, T.A., Eidne, K.A., Parker, M.W., Waters, M.J. 2005. Model for growth hormone receptor activation based on subunit rotation within a receptor dimer. *Nat. Struct. and Molecular Bio*, 12(9), 814–821.
- Butler, A.A., Yakar, S., Gewolb, I.H., Karas, M., Okubo, Y., LeRoith, D. 1998. Insulin-like growth factor-I receptor signal transduction: at the interface between physiology and cell biology. *Comp Biochem Physiol B Biochem Mol Biol* 121:19–26.
- Calduch-Giner, J.A., Pendon, C., Valdivia M.M., Perez-Sanchez, J. 1998. Recombinant somatotactin as a stable and bioactive protein in a cell culture bioassay: development and validation of a sensitive and reproducible radioimmunoassay. *J of Endocrinol.* 156 441–447.
- Carter-Su, C., Schwartz, J., Smit, L.J. 1996. Molecular mechanism of growth hormone action. *Annu. Rev. Physiol.* 58(1):187-207.
- Chia, D.J., Ono, M., Woelfle, J., Schlesinger-Massart, M., Jiang, H., Rotwein, P. 2006. Characterization of distinct Stat5b binding sites that mediate growth hormone-stimulated IGF-I gene transcription. *J Biol Chem* 281:3190–7.
- Chen, T.T., Agellon, L.B., Lin, C.M., Tsai, H.J., Zhang, P., González-Villasenor, L.I. 1989. Evolutionary implications of two rainbow trout growth hormone genes. *Fish Physiol. Biochem.* 7(1):381-5.
- Chowen, J.A., Frago, L.M. Argente, J., 2004. The regulation of GH secretion by sex steroids. *Europ J of Endocrinol*, 151, U95–U100.
- Clemmons, D.R., Underwood, L.E. 1991. Nutritional regulation of IGF-I and IGF binding proteins. *Ann rev of nut*, 11(1), 393-412.

- Cosman, D., Lyman, S.D., Idzerda, R.L., Beckmann, M.P., Park, L.S., Goodwin, R.G., March, C.J. 1990. A new cytokine superfamily. *Trends Biochem Sci* 15: 265–270.
- Daza, D.O., Sundström, G., Bergqvist, C.A., Duan, C., Larhammar, D. 2011. Evolution of the insulin-like growth factor binding protein (IGFBP) family. *Endocrinology* 152, 2278–2289.
- De Vos, A.M., Ultsch, M., Kossiakoff, A.A. 1992. Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science*, 255(5042), p.306+.
- Dunaif, A.E., Zimmerman, E.A., Friesen, H.G., Frantz, A.G. 1982. Intracellular localization of prolactin receptor and prolactin in the rat ovary by immunocytochemistry. *Endocrinol.* 110:1465–1471.
- Eden, S. 1979. Age- and sex-related differences in episodic growth hormone secretion in the rat. *Endocrinol.* 105:555–560.
- Einarsdottir, I.E., Sakata, S., Björnsson, B.T. 2002. Atlantic halibut growth hormone: structure and plasma levels of sexually mature males and females during photoperiod-regulated annual cycles. *Gen. Comp. Endocrinol.* 127(1), 94–104.
- Ellens, E. R., Kittilson, J. D., Hall, J. A., Sower, S. A., Sheridan, M. A. 2013. Evolutionary origin and divergence of the growth hormone receptor family: Insight from studies on sea lamprey. *Gen. Comp. Endocrinol.* 192, 222–236.
- Ferry, R.J., Cerri, R.W., Cohen, P. 1999. Insulin-like growth factor binding proteins: new proteins, new functions. *Horm Res.* 51:53–67.
- Fisker, S., 2006. Physiology and pathophysiology of growth hormone-binding protein: Methodological and clinical aspects. *Growth Hormone and IGF Research*, 16(1), 1–28.

- Flores-Morales, A., Greenhalgh, C.J., Norstedt, G., Rico-Bautista, E. 2006. Negative regulation of growth hormone receptor signalling. *Mol Endocrinol* 20: 241-53.
- Forsyth, I.A. and Wallis, M., 2002. Growth hormone and prolactin--molecular and functional evolution. *Journal of mammary gland biology and neoplasia*, 7(3), 291–312.
- Fox, B. K., Breves, J. P., Davis, L. K., Pierce, A. L., Hirano, T., Grau, E. G. 2010. Tissue-specific regulation of the growth hormone/insulin-like growth factor axis during fasting and re-feeding: Importance of muscle expression of IGF-I and IGF-II mRNA in the tilapia. *Gen. Comp. Endocrinol.* 166(3), 573–580.
- Fruchtman, S., Gift, B., Howes, B., Borski, R. 2001. Insulin-like growth factor-I augments prolactin and inhibits growth hormone release through distinct as well as overlapping cellular signaling pathways. *Comp. Biochem. Physiol.*, 129B:237-242.
- Fuh, G., Mulkerrin, M.G., Bass, S., McFarland, N., Brochier, M., Bourell, J.H., Light, D.R., Wells, J.A. 1990. The human growth hormone receptor. Secretion from *Escherichia coli* and disulfide bonding pattern of the extracellular binding domain
J. Biol. Chem., 265:3111-3115
- Fukamachi, S., Yada, T., Meyer, A., Kinoshita, M. 2009. Effects of constitutive expression of SL on skin pigmentation in medaka. *Gene* 442 81–87.
- Fukata, J., Diamond, D., Martin, J., 1985. Effects of Rat Growth-Hormone (rgh)-Releasing Factor and Somatostatin on the Release and Synthesis of Rgh in Dispersed Pituitary-Cells. *Endocrinol.*, 117(2), 457–467.
- Goffin, V., Kelly, P.A. 1997. The prolactin/growth hormone receptor family: structure/function relationships. *J Mammary Gland Biol Neoplasia* 2:7 - 17

- Gomez-Requeni, P., J. Calduch-Giner, S. Vega-Rubin de Celis, F. Medale, S.J. Kaushik, J. Pérez-Sanchez. 2005. Regulation of the somatotrophic axis by dietary factors in rainbow trout (*Oncorhynchus mykiss*), *Brit. J. Nutr.* 94:353-361.
- Gu, F., Dube, N., Kim, J.W. 2003. Protein tyrosine phosphatase 1B attenuates growth hormone-mediated JAK2-STAT signaling. *Mol Cell Biol.* 23: 3753-62.
- Hall, T.R., Harvey, S., Scanes. 1986. Control of growth-hormone secretion in the vertebrates—a comparative survey *Comp. Biochem. Physiol.*, 84A:231-253.
- Hansen, J.A., Lindberg, K., Hilton, D.J. 1999. Mechanism of inhibition of growth hormone receptor signalling by suppressor of cytokine signalling proteins. *Mol Endocrinol* 13:1832-43.
- Hanson, A., Poudyal, D., Hagemester, A., Sheridan M.A. 2009. Somatostatin inhibits insulin-like growth factor-1 receptor mRNA expression in rainbow trout gill by activating the ERK and PI3K signaling pathways *Mol. Cell. Endocrinol.*, 315:57-62
- Harvey, S. 1993. Growth hormone secretion in poikilotherms and homeotherms. *The Endocrinology of Growth, Development, and Metabolism in Vertebrates* 151-182.
- Herrington, J., Smit, L.S., Schwartz, J., Carter-Su, C. 2000. The role of STAT proteins in growth hormone signaling. *Oncogene Res* 19: 2585-97.
- Huo, J.S., McEachin, R.C., Cui, T.X., Duggal, N.K., Hai, T., States, D.J., 2006. Profiles of growth hormone (GH)-regulated genes reveal time-dependent responses and identify a mechanism for regulation of activating transcription factor 3 by GH. *J Biol Chem* 281: 4132–4141.
- Hu, S.Y., Wu, J. L., Huang, J.H. 2004. Production of tilapia insulin-like growth factor-2 in high cell density cultures of recombinant *Escherichia coli*. *J. Biotechnol.* 107, 161–171.

- Ihle, J.N., Witthuhn, B.A., Quelle, F.W. 1995. Signaling through the hematopoietic cytokine receptors. *Ann Rev Immunol* 13:369-98.
- Johnson, L.L., Norberg, B., Willis, M.L., Zebroski, H. Swanson, P. 1997. Isolation, characterization, and radioimmunoassay of Atlantic halibut somatolactin and plasma levels during stress and reproduction in flatfish. *Gen. Comp. Endocrinol* 105 194–209.
- Jones, J. I., Clemmons, D. R. 1995. Insulin-like growth factors and their binding proteins: Biological actions. *Endocr. Rev.* 16, 3–34.
- Jones, J.I., Gockerman, A., Busby, W.H., Wright, G., Clemmons, D.R. 1993. Insulin-like growth factor binding protein 1 stimulates cell migration and binds to the $\alpha 5\beta 1$ integrin by means of its Arg-Gly-Asp sequence. *Proc Natl Acad Sci USA* 90:10553–10557.
- Kagabu, Y., Mishiba, T., Okino, T., Yanagisawa, T. 1998. Effects of thyrotropin-releasing hormone and its metabolites, cyclo(His-Pro) and TRH-OH, on growth hormone and prolactin synthesis in primary cultured pituitary cells of the common carp, *Cyprinus carpio* *Gen. Comp. Endocrinol.*, 111:395-403.
- Kakizawa, S., Kaneko, T., Hasegawa, S. Hirano, T. 1995. Effects of feeding, fasting, background adaptation, acute stress, and exhaustive exercise on the plasma somatolactin concentrations in rainbow trout. *Gen. Comp. Endocrinol* 98 137–146.
- Kakizawa, S., Kaneko, T. Hirano, T. 1996. Elevation of plasma somatolactin concentrations during acidosis in rainbow trout (*Oncorhynchus mykiss*). *J of Exp Bio* 199 1043–1051.
- Kamura, T., Maenaka, K., Kotoshiba, S. 2004. VHL-box and SOCSbox domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases. *Genes Dev* 18: 3055-65.

- Kawauchi, H., S.A. Sower. 2006. The dawn and evolution of hormones in the adenohypophysis. *Gen Comp Endocrinol* 148:3–14.
- Kiu, H., Nicholson, S.E. 2012. Biology and significance of the JAK/STAT signalling pathways. *Growth Factors*, 30(2), 88–106.
- Klapper, D.G., Svoboda, M.E., Van Wyk, J.J. 1983. Sequence analysis of somatomedin-C: confirmation of identity with insulin-like growth factor I. *Endocrinol.* 112:2215–2217.
- Komourdjian, M.P., Saunders, R.L., Fenwick, J.C. 1976. Evidence for the role of growth hormone as a part of a 'light-pituitary axis' in growth and smoltification of Atlantic salmon (*Salmo salar*). *Canadian Journal of Zoology*, 54(4), 544-551.
- Kopchick, J.J., Andry, J.M. 200. Growth hormone (GH), GH receptor, and signal transduction. *Mol. Genet. Metab.* 71(1-2):293-314.
- Kossiakoff A. A. and de Vos A. 1999. Structural basis for cytokine hormone-receptor recognition and receptor activation. *Adv. Protein Chem.* 52:67–108.
- Kostyo, J.L., 1968. Rapid effects of growth hormone on amino acid transport and protein synthesis. *Ann NY Acad Sci* 148:389–407
- Lee, P.D.K., Giudice, L.C., Conover, C.A. Powell, D.R., 1997. Insulin-like growth factor binding protein-1: recent findings and new directions. *Proc. Soc. Exp. Biol. Med.*, 216 319.
- LeRoith, D., Bondy, C., Yakar, S., Liu, J.L., Butler, A. 2001. The somatomedin hypothesis: 2001. *Endocr. Rev.* 22, 53–74.
- Leung, K., Johannsson, G., Leong, G.M., Ho, K.K.Y. 2004. Estrogen regulation of growth hormone action. *Endocr. Rev.* 25, 693-721.
- Li, C. H., Evans, H.M. 1944. The isolation of pituitary growth hormone. *Science* 99, 184–186.

- Lin, X., Peter, R.E., 2001. Somatostatins and their receptors in fish. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 129(2), 543-550.
- Louvi, A., Accili, D., Efstratiadis, A. 1997. Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. *Dev Biol* 189:33–48.
- Nagy, E., Berczi, I., Friesen, H.G. 1983. Regulation of immunity in rats by lactogenic and growth hormones. *Acta Endocrinol (Copenh)* 102:351–357.
- Nissley P, Lopaczynski W 1991 Insulin-like growth factor receptors. *Growth Factors* 5:29–43.
- Madsen, S. S., Bern, H. A. 1993. In-vitro effects of insulin-like growth factor-I on gill Na⁺,K⁺ β -ATPase in coho salmon, *Oncorhynchus kisutch*. *J. Endocrinol.* 138, 23–30.
- Mannor, D.A., Winer, L.M., Shaw, M.A., Baumann G. 1991. Plasma growth hormone (GH)-binding proteins: effect on GH binding to receptors and GH action *J. Clin. Endocrinol. Metab.*, 73:30-34.
- Maetz, J. 1970. Mechanisms of salt and water transfer across membranes in teleosts in relation to the aquatic environment. *Mem Soc Endocrinol* 18:3–29.
- Maures, T., Chan, S.J., Xu, B., Sun, H., Ding, J., Duan, C. 2002. Structural, biochemical, and expression analysis of two distinct insulin-like growth factor I receptors and their ligands in zebrafish *Endocrinol.*, 143:1858-1871
- McCormick, S.D., Björnsson, B.Th., Sheridan, M.A., Eilertson, C., Carey, J.B., O'Dea, M. 1995. Increased daylength stimulates plasma growth hormone and gill Na⁺,K⁺-ATPase in Atlantic salmon (*Salmo salar*). *J. Comp. Physiol. B* 165: 245–254.
- Melamed, P., Eliahu, N., Levavi-Sivan, B., Ofir, M., Farchi-Pisanty, O., Rentier-Delrue, F., Naor, Z. 1995. Hypothalamic and thyroidal regulation of growth hormone in tilapia. *Gen. Comp. Endocrinol*, 97(1), 13-30.

- Melamed, P., Gur, G., Rosenfeld, H., Elizur, A., and Yaron, Z. 1999. Possible interactions between gonadotrophs and somatotrophs in the pituitary of tilapia: Apparent roles for insulin-like growth factor I and estradiol. *Endocrinology* 140, 1183–1191.
- Melamed, P., Rosenfeld, H., Elizur, A., Yaron, Z. 1998. Endocrine regulation of gonadotropin and growth hormone gene transcription in fish. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* 119, 325–338.
- Miyakoshi, N., Qin, X., Kasukawa, Y., Richman, C., Srivastava, A.K., Baylink, D.J., Mohan, S. 2001. Systemic administration of insulin-like growth factor (IGF)-binding protein-4 (IGFBP-4) increases bone formation parameters in mice by increasing IGF bioavailability via an IGFBP-4 protease-dependent mechanism. *Endocrinology* 142:2641–2648.
- Miyata, A., Arimura, A., Dahl, R.R., Minamino, N., Uehara, A., Jiang, L., Culler, M.D., Coy D.H. 1989. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells *Biochem. Biophys. Res. Commun.*, 164:567-574.
- Mohr, A. Chatain, N., Domszalai, T., Rinis, N., Sommerauer, M., Vogt, M., Müller-Newen, G. 2012. Dynamics and non-canonical aspects of JAK/STAT signalling. *European Journal of Cell Biology*, 91(6-7), 524–532.
- Moller, N. and Jorgensen, J.O.L., 2009. Effects of Growth Hormone on Glucose, Lipid, and Protein Metabolism in Human Subjects. *Endocrine Reviews*, 30(2), 152–177.
- Montero, M., Yon, L., Kikuyama, S., Dufour, S., Vaudry, H. 2000. Molecular evolution of the growth hormone-releasing hormone/pituitary adenylate cyclase-activating polypeptide gene family. Functional implication in the regulation of growth hormone secretion. *J of Mole Endocrinol*, 25(2), 157-168.

- Moon, T.W., Castejon, C., Banos, N., Maestro, M.A., Plisetskaya, E.M., Gutierrez, J., Navarro, I. 1996. Insulin and IGF-I binding in isolated trout cardiomyocytes
Gen. Comp. Endocrinol., 103:264-272.
- Moriyama, S., Oda, M., Takahashi, A., Sower, S. A., Kawauchi., H. 2006. Genomic structure of the sea lamprey growth hormone-encoding gene. Gen Comp Endocrinol 148:33–40.
- Moriyama, S., Oda, M., Yamazaki, T., Yamaguchi, K., Amiya, N., Takahashi, A., Kawauchi, H. 2008. Gene Structure and Functional Characterization of Growth Hormone in Dogfish, *Squalus acanthias*. Zoological Science. 25(6), 604–613.
- Moutoussamy, S., Kelly, P.A., Finidori, J. 1998. Growth-hormone-receptor and cytokine-receptor-family signaling. Eur J Biochem 255(1):1–11.
- Nelson, L.E., Sheridan, M.A. 2005. Regulation of somatostatins and their receptors in fish. Gen Comp Endocrinol. 142: 117–133.
- Nissley, S.P., Lopeszinsky, W.1991. IGF receptors. Growth Factors. 5:29–43.
- Norbeck, L.A., Kittilson, J.D. and Sheridan, M.A., 2007. Resolving the growth-promoting and metabolic effects of growth hormone: Differential regulation of GH–IGF-I system components. Gen. Comp. Endocrinol., 151(3), 332–341.
- Norrelund, H. 2005. The metabolic role of growth hormone in humans with particular reference to fasting. Growth Horm. IGF Res. 15, 95-122.
- Peng, C., Peter, R.E. 1997. Neuroendocrine regulation of growth hormone secretion and growth in fish. Zoological Studies. 36(2), 79–89.
- Perez-Sanchez, J., Calduch-Giner, J. A., Mingarro, M., Celis, S. V.R. de, Gomez-Requeni, P., Saera-Vila, A., Valdivia, M. M. 2002. Overview of Fish Growth Hormone Family. New

- Insights in Genomic Organization and Heterogeneity of Growth Hormone Receptors. *Fish Physiology and Biochemistry*. 27(3-4), 243–258.
- Pierce, A.L., Dickey, J.T., Felli, L., Swanson, P., Dickhoff, W.W. 2010. Metabolic hormones regulate basal and growth hormone-dependent igf2 mRNA level in primary cultured coho salmon hepatocytes: effects of insulin, glucagon, dexamethasone, and triiodothyronine. *J Endocrinol* 204:331–339.
- Pierce, A.L., Fox, B.K., Davis, L.K., Visitacion, N., Kitahashi, T., Hirano, T., Grau, E. G. 2007. Prolactin receptor, growth hormone receptor, and putative somatolactin receptor in Mozambique tilapia: Tissue specific expression and differential regulation by salinity and fasting. *Gen. Comp. Endocrinol.* 154(1–3), 31–40.
- Pierce, A.L., Shimizu, M., Beckman, B.R., Baker, D.M., Dickhoff, W.W. 2005. Time course of the GH/IGF axis response to fasting and increased ration in chinook salmon (*Oncorhynchus tshawytscha*). *Gen. Comp. Endocrinol.* 140(3), 192–202.
- Pincus, S.M., Gevers, E.F., Robinson, I.C., van den Berg, G., Roelfsema, F., Hartman, M.L., Veldhuis, J.D. 1996. Females secrete growth hormone with more process irregularity than males in both humans and rats. *Am J Physiol* 270:E107–115.
- Piwien-Pilipuk, G., J.S., Huo, J., Schwatz. 2002. Growth hormone signal transduction, *J. Pediatr. Endocrinol. Metab.* 15 771-786.
- Postel-Vinay, M.C., Finidori, J. 1995. Growth hormone receptor: structure and signal transduction. *Eur J Endocrinol*, 133, 654–659.
- Rajesh, R., Majumdar, K.C. 2007. A comparative account of the structure of the growth hormone encoding gene and genetic interrelationship in six species of the genus *Labeo*. *Fish Physiology and Biochemistry*, 33(4), 311–333.

- Ram, P.A., Waxman, D.J. 1997. Interaction of growth hormone-activated STATs with SH2-containing phosphotyrosine phosphatase SHP-1 and nuclear JAK2 tyrosine kinase. *J Biol Chem* 272: 17694-702.
- Randle, P.J. 1954. Plasma-insulin activity in hypopituitarism. *Lancet* 1:809–810.
- Rand-Weaver, M., Pottinger, T.G., Sumpter, J.P. 1993. Plasma somatolactin concentrations in salmonid fish are elevated by stress. *Journal of Endocrinology* 138 509–515.
- Reindl, K.M., Kittilson, J.D., Sheridan, M.A. 2009. Differential ligand binding and agonist-induced regulation characteristics of the two rainbow trout GH receptors, Ghr1 and Ghr2, in transfected cells. *J. Endocrinol.* 202(3):463-71.
- Reindl, K.M., Sheridan, M. A. 2012. Peripheral regulation of the growth hormone-insulin-like growth factor system in fish and other vertebrates *Comp. Biochem. Physiol., Part A*, 163 pp. 231–245.
- Ricketts, W.A., Rose, D.W., Shoelson, S., Olefsky, J.M. 1996. Functional roles of the Shc phosphotyrosine binding and Src homology 2 domains in insulin and epidermal growth factor signaling. *J Biol Chem* 271:26165–26169.
- Rinderknecht, E., Humbel, R.E. 1978. The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *J Biol Chem* 253:2769–2776.
- Rousseau, K., Dufour, S., 2007. Comparative aspects of GH and metabolic regulation in lower vertebrates. *Neuroendocrinology*, 86(3), 165–174.
- Rynikova, A., Koppel, J., Kuchar, S., Cikos, S., Mozes, S. 1988. Effects of ovine prolactin in infant rats. *Exp Clin Endocrinol* 92:241–244
- Sakamoto, T., McCormick, S.D., Hirano, T. 1993. Osmoregulatory actions of growth hormone and its mode of action in salmonids: a review. *Fish Physiol. Biochem.* 11: 155–164.

- Salgin, B., Marcovecchio, M. L., Hill, N., Dunger, D. B., Frystyk, J. 2012. The effect of prolonged fasting on levels of growth hormone-binding protein and free growth hormone. *Growth Hormone & IGF Research*. 22(2), 76–81.
- Salmon, W.D., Daughaday, W.H. 1957. A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. *J Lab Clin Med* 49:825–826.
- Schlessinger, J. 2000. New roles for Src kinases in control of cell survival and angiogenesis. *Cell*. 100(3):293–6.
- Sheridan, M.A., Hagemeister, A.L. 2010. Somatostatin and somatostatin receptors in fish growth. *Gen. Comp. Endocrinol.* 167(3):360-5.
- Sheridan, M.A., Kittilson, J.D., Slagter, B.J. 2000. Structure-function relationships of the signaling system for the somatostatin peptide hormone family. *Am. Zool.* 40:269-286.
- Silverman, L.A., Cheng, Z.Q., Hsiao, D., Rosenthal, S.M. 1995. Skeletal muscle cell-derived insulin-like growth factor (IGF) binding proteins inhibit IGF-I-induced myogenesis in rat L6E9 cells. *Endocrinol.* 136:720–726.
- Small, B.C., C.A., Murdock, G.C., Waldbieser, B.C., Peterson. 2006. Reduction in channel catfish hepatocyte growth hormone receptor expression in response to food deprivation and exogenous cortisol, *Domest. Anim. Endocrinol.* 31:340-356.
- Sohm, F., Manfroid, I., Pezet, A., Rentier-Delrue, F., Rand-Weaver, M., Kelly, P.A. 1998. Identification and modulation of a growth hormone-binding protein in rainbow trout (*Oncorhynchus mykiss*) plasma during seawater adaptation. *Gen. Comp. Endocrinol.* 111(2):216-24.
- Spiess, J., Rivier, J., Vale, W. 1983. Characterization of rat hypothalamic growth hormone-releasing factor. *Nature* 303:532–535.

- Talamantes, F., Ortiz, R. 2002. Structure and regulation of expression of the mouse GH receptor. *Journal of Endocrinol.*, 175(1), 55–59.
- Trudeau, V.L., Somoza, G.M., Nahorniak, C.S., Peter, R.E. 1992. Interactions of estradiol with gonadotropin-releasing hormone and thyrotropin-releasing hormone in the control of growth hormone secretion in the goldfish *Neuroendocrinology*, 56:483-490.
- Turyn, D., Dominici, F.P., Sotelo, A.I., Bartke, A. 1997. Growth hormone-binding protein enhances growth hormone activity in vivo. *Am J Physiol Endocrinol Metab.* 273:E549–E556.
- Ullrich, A., Gray, A., Tam, A.W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J., Fujita-Yamaguchi, Y. 1986. Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO J* 5:2503–2512.
- Upton, Z., Francis, G. L., Chan, S. J., Steiner, D. F., Wallace, J. C., and Ballard, F. J. 1997. Evolution of insulin-like growth factor (IGF) function: Production and characterization of recombinant hagfish IGF. *Gen. Comp. Endocrinol.* 105, 79–90.
- Vaudry, D., Gonzalez, B.J., Basille, M., Yon, L., Fournier, A., Vaudry, H. 2000. Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. *Pharmacol Rev* 52:269–324.
- Very, N.M., Kittilson, J.D., Norbeck, L.A., Sheridan, M.A. 2005. Isolation, characterization, and distribution of two cDNAs encoding for growth hormone receptor in rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem and Phys B-Biochemistry and Mole. Bio.* 140(4), 615–628.

- Very, N.M., Norbeck, N.M., Kittilson, J.D., Sheridan M.A. 2008. Regulation of insulin-like growth factor-1 receptors. 8th International Congress on the Biology of Fish, Portland, OR, July 28-August 1, 2008.
- Very, N.M., Sheridan, M.A. 2007. Somatostatin inhibits insulin-like growth factor-I receptor expression in the gill of a teleost fish (*Oncorhynchus mykiss*) FEBS lett., 581:4773-4777.
- Villalba, M., Zabala, M.T., Martinez-Serrano, A., de la Colina, R., Satrustegui, J., Garcia-Ruiz, J.P. 1991. Prolactin increases cytosolic free calcium concentration in hepatocytes of lactating rats. *Endocrinology* 129:2857–2861
- Volff, J.N., 2005. Genome evolution and biodiversity in teleost fish. *Heredity*, 94(3), 280–294.
- Wallis, M. 1992. The expanding growth hormone/prolactin family. *J. Mol. Endocrinol.* 9:185–188.
- Wallis M, Lioupis A, Wallis OC. 1998. Duplicate growth hormone genes in sheep and goat. *J Mol Endocrinol.* ;21:1–5.
- Walock, C. N., Kittilson, J. D., Sheridan, M. A. 2014. Characterization of a novel growth hormone receptor-encoding cDNA in rainbow trout and regulation of its expression by nutritional state. *Gene.* 533(1), 286–294.
- Weil, C., Carre, F., Blaise, O., Breton, B., Le Bail, P.Y. 1999. Differential effect of insulin-like growth factor I on in vitro gonadotropin (I and II) and growth hormone secretions in rainbow trout (*Oncorhynchus mykiss*) at different stages of the reproductive cycle. *Endocrinol.* 140(5):2054–6210.
- West, C. R., Gaynor, P.J., Lookingland, K.J., Tucker, H.A. 1997. Regulation of growth hormone-releasing hormone and somatostatin from perfused, bovine hypothalamic slices

1. alpha(2)-adrenergic receptor regulation. *Domestic Animal Endocrinol.*, 14(5), 334–348.
- Winans, G.A., Nishioka, R.S. 1987. A multivariate description of change in body shape of coho salmon (*Oncorhynchus kisutch*) during smoltification. *Aquaculture* 66: 235–246.
- Wood, A.W., Duan, C., Bern, H.A. 2005. Insulin-like growth factor signaling in fish. *Int. Rev. Cytol.* 243, 215–285.
- Wolf, R.F., Heslin, M.J., Newman, E., Pearlstone, D.B., Gonenne, A., Brennan, M.F. 1992. Growth hormone and insulin combine to improve whole-body and skeletal muscle protein kinetics. *Surgery* 112: 284–291
- Wong, A.O.L., Zhou, H., Jiang, Y., Ko, W.K.W. 2006. Feedback regulation of growth hormone synthesis and secretion in fish and the emerging concept of intrapituitary feedback loop. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology.* 144(3), 284–305.
- Xu J., Messina J.L. 2009. Crosstalk between growth hormone and insulin signalling. *Vitam Horm* 80:125-53.
- Yakar, S., Liu, J. L., Stannard, B., Butler, A., Accili, D., Sauer, B., LeRoith, D. 1999. Normal growth and development in the absence of hepatic insulin-like growth factor I. *Proc. Natl. Acad. Sci. USA* 96, 7324–7329.
- Yasukawa H., Misawa H., Sakamoto H. 1999. The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop. *EMBO J* 18:1309-20.
- Yang, B. Y., Chan, K. M., Lin, C-M. and Chen, T.T. 1997. Characterization of Rainbow trout (*Oncorhynchus mykiss*) growth hormone I gene and the promoter region of growth hormone 2 gene. *Arch. Biochem. Biophys.* 340: 359-368.

- Zhu, Y. Thomas, P. 1997. Effects of somatolactin on melanosome aggregation in the melanophores of red drum (*Sciaenops ocellatus*) scales. *Gen. Comp. Endocrinol.* 105.
- Zhu, T., Goh, E.L., Lobie, P.E. 1998. Growth hormone stimulates the tyrosine phosphorylation and association of p125 focal adhesion kinase (FAK) with JAK2. Fak is not required for stat-mediated transcription. *J. Biol. Chem.* 273:10682–10689.
- Zhu, T., Goh, E.L.K., Graichen, R., Ling, L., Lobie, P.E. 2001. Signal transduction via the growth hormone receptor. *Cellular Signalling*, 13(9), 599–616.

**CHAPTER 2: CHARACTERIZATION OF A NOVEL GROWTH HORMONE
RECEPTOR-ENCODING CDNA IN RAINBOW TROUT AND REGULATION OF ITS
EXPRESSION BY NUTRITIONAL STATE¹.**

Abstract

To clarify the divergence of the growth hormone receptor (GHR) family, we characterized a novel GHR from a teleost fish (rainbow trout). A 2357-nt cDNA was isolated and found to contain a single initiation site 71 nt from the most 5' end, and open reading frame of 1971 nt encoding a 657-amino acid protein, and a single polyadenylation site 229 nt from the poly-A tail. Based on structural analysis, the protein was identified as type 1 GHR (GHR1). The new GHR1 shares 42% and 43% amino acid identity, respectively, with GHR2a and GHR2b, the two type 2 GHRs isolated from trout previously. GHR1 mRNA was found in a wide array of tissues with the highest expression in liver and white muscle. Fasting animals for 4 weeks reduced steady state levels of GHR1 in liver, adipose, and red muscle. These findings help clarify the divergence and nomenclature of GHRs and provide insight in function of duplicated GHR types.

Introduction

Growth hormone (GH) plays important roles in the growth, metabolism, reproduction, immune function, osmoregulation, and other physiological functions of vertebrate animals (Bjornsson, 1997; Bjornsson et al., 2002; Norrelund, 2005; Moller and Jorgensen, 2009). The actions of GH are transduced through the GH receptor (GH), a member of the class-I cytokine receptor superfamily that consist of an extracellular binding domain containing a conserved

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Y/FGEFS motif, a single transmembrane domain, and an intracellular domain that links to several cellular effector pathways, including JAK-STAT, ERK, and PI3K-Akt (Kopchick and Andry, 2000; Forsyth and Wallis, 2002; Waters et al., 2006).

The GHR family arose through a series of genome duplication events (1R-3R) during the course of vertebrate evolution (Liongue and Ward, 2007; Ellens and Sheridan, 2013; Ellens et al., 2013). The first two events are believed to have occurred early in chordate evolution, with the 2R event perhaps taking place before the cyclostome-gnathostome split (Van de Peer et al., 2009). The 3R event, also known as the fish-specific genome duplication (FSGD) event occurred in the actinopterygian lineage (ray-finned fishes) after divergence from the sarcopterygian lineage (lobe-finned fishes; includes the common ancestor of the tetrapods) (Meyer and Van de Peer, 2005). The FSGD likely explains the existence of multiple GHRs that derive from distinct mRNAs in boney fish (teleosts), whereas tetrapods possess a single GHR gene (Ellens and Sheridan, 2013). Several groups of teleosts, including the salmonids underwent a subsequent independent duplication event (4R) (Meyer and Van de Peer, 2005). It is not surprising, then, that the divergence of GHRs and their nomenclature are somewhat confused (Fukamachi and Meyer, 2007; Ellens and Sheridan, 2013; Ellens et al., 2013). For example, the terms “GHR1” and “GHR2” were first used for naming GHR subtypes in tetraploid salmonids (cf. Very et al., 2005); this scheme continued for other groups of teleosts (cf. Ellens and Sheridan, 2013).

The aim of this study was to clarify the divergence and nomenclature of GHRs. We used rainbow trout to further study the polygenic origins of GHRs and to provide insight into the functional significance of duplicated GHR subtypes.

Materials and methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*) were selected because 1) as teleosts they occupy a pivotal point in vertebrate evolution (Van de Peer et al., 2009; Ellens and Sheridan, 2013b), they underwent a tetraploidization event subsequent to the FSGD (Meyer and Van de Peer, 2005), we previously isolated two GHR paralogs from them (Very et al., 2005). Juvenile fish of both sexes were obtained from Dakota Trout Ranch near Carrington, ND and transported to North Dakota State University where they were maintained in 800 L circular tanks supplied with recirculated (10% replacement volume per day) dechlorinated municipal water at 14 °C under a 12L:12D photoperiod. Fish were fed to satiety twice daily with AquaMax™ Grower (PMI Nutrition International, Brentwood, MO, USA), except 36–48 h before experimental manipulations. Animals were acclimated to laboratory conditions for at least 4 weeks prior to experiments. All procedures performed were in accordance with the *Guide for Care and Use of Laboratory Animals* (National Research Council, Washington, DC) and approved by the North Dakota State University Institutional Animal Care and Use Committee.

For the nutritional state experiments, fish were assigned randomly to one of six treatment groups (initial, fed continuously for 2 weeks, fasted for 2 weeks, fed continuously for 4 weeks, fasted for 4 weeks, and fasted for 2 weeks then refed for 2 weeks) in 100-L circular tanks (approximately 18–24 fish per tank) with a flow-through water supply at 14 °C under a 12:12 L:D photoperiod. Fish were allowed to acclimate for 2 days in their experimental tank prior to beginning their respective nutritional regime. For those animals receiving food, feeding was suspended 24–36 h before sampling. At the time of sampling, fish were anaesthetized in 0.05% (v/v) 2-phenoxyethanol, measured (body weight, length, and liver weight), bled via the severed

caudal vessels, and euthanized by transection of the spinal cord. Samples of liver, mesenteric adipose tissue, white skeletal muscle, and red skeletal muscle were removed, frozen on dry ice, and stored at -80°C for later analyses.

RNA extraction

Total RNA was extracted using TRI-Reagent® as specified by the manufacturer (Molecular Research Center, Cincinnati, OH, USA). Isolated RNA was dissolved in approximately 100 μL RNase-free deionized water. Total RNA was quantified by ultraviolet (A260) spectrophotometry and diluted to 100 $\text{ng}/\mu\text{L}$ in RNase-free deionized water. RNA samples were then stored at -90°C until further analysis.

Oligonucleotide primers and probes

Gene-specific primers used for isolation of cDNAs were custom synthesized by Sigma-Genosys (The Woodlands, TX, USA, USA). Additional primers for reverse transcription were provided in SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) and AffinityScript™ QPCR cDNA Synthesis Kit (Agilent Technologies Santa Clara, CA, USA). Gene-specific oligonucleotide primers and probes used for real-time PCR of GHR and β -actin were designed based upon our determined and known sequences (GenBank accession no. AF157514), respectively, using ABI Primer Express® Version 2 software and custom synthesized by Applied Biosystems (Life Technologies Applied Biosystems Carlsbad, CA, USA). The probes were minor-groove binding probes labeled with either FAM (GHR1) or VIC (β -actin probe). Primers and probes were used for reverse transcription and PCR without further purification.

Isolation and sequence analysis of growth hormone receptor cDNA

A two-phase approach was adopted for the isolation of selected cDNAs using and 3'-rapid amplification of cDNA ends (3'-RACE)-PCR (Phase 1) and RT-PCR (Phase 2). 3'-RACE PCR was performed using a SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA, USA) following the manufacturer's protocol. RNA from rainbow trout liver was reverse transcribed into cDNA containing the sequence tags necessary for SMART™ chemistry. The PCR was carried out using a 3'RACE gene-specific primer (Table 1) designed from partial GHR sequences in GenBank (accession nos. AF438178 and DQ452378). After an initial denaturation at 94 °C for 5 min, a 35-cycle PCR was performed with each cycle consisting of denaturation (94 °C for 30 sec), annealing (65 °C for 30 sec), and extension (72 °C for 1 min) phases. In the last cycle, the extension time was increased to 10 min. The PCR product was identified by electrophoresis on an agarose gel containing 1% of each OmniPur (EMD chemicals, Gibbstown, NJ, USA) and NuSieve GTG agarose (BioWhittaker Molecular Applications, Rockland, ME, USA) in 1× Tris–borate–EDTA (TBE) buffer followed by ethidium bromide staining. The resulting PCR product was cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). Purified plasmids (75 fmol) were sequenced using the CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's protocol.

Phase 2, RNA was reverse transcribed as described earlier and PCR was carried out using primers designed from our 3'RACE product and partial GHR sequences in GenBank (accession nos. AF438178 and DQ452378)(Table 1). After an initial denaturation at 94 °C for 5 min, a 35-cycle PCR was performed with each cycle consisting of denaturation (94 °C for 1 min), annealing (65 °C for 30 sec), and extension (72 °C for 2 min) phases. In the last cycle, the

extension time was increased to 10 min. The resulting PCR product was visualized, cloned into the pGEM-T Easy Vector, and sequenced as described previously.

Table 1

Primers and probes used for sequence analysis and for real-time quantitative PCR.

Target	Description	Sequence
GHR1	Phase I sequence analysis 3'RACE gene-specific primer	5'-TCCTGCCAACCCAACAAAGT-3'
GHR1	Phase II sequence analysis Forward primer Reverse primer	5'-GAAGTCGATACCCCTCGCGCAT-3' 5'-TGGGCAGTGTAGTTTTTCTTAAGG-3'
GHR1	QPCR analysis Probe Forward primer Reverse primer	5' ^{FAM} -TGCGTGTGCACTGTG- ^{MGBNFQ3} , 5'-TCAGACAGGAGAGGCGTACGA-3' 5'-CCAAAGTTATTGAAGGCCCTCAT-3'
β -actin	QPCR analysis Probe Forward primer Reverse primer	5' ^{VIC} -TGCTTGCTGATCCACAT- ^{MGBNFQ3} , 5'-GGCTTCTCTCTCCACCTTCCA-3' 5'-AGGGACCAGACTCGTCGTACTC -3'

Abbreviations: GHR, growth hormone receptor; QPCR, Real-time quantitative PCR.

Real-time PCR assay for growth hormone receptor mRNA

Preparation of cDNA standards

A cDNA standard for GHR1 was synthesized by PCR. Approximately 1 μ g of the full GHR cDNA product was used as template for PCR using forward and reverse gene-specific primers (Table 1) under the same conditions as described above. The resulting PCR product was visualized, cloned into the pGEM-T Easy Vector, and the sequences verified as described previously.

Real-time reverse transcription PCR

From 200 ng total RNA, endogenous poly(A)+ RNA was reverse transcribed in a 5 μ L reaction using a SMART™ RACE cDNA Amplification Kit containing a RNase H+ reverse transcriptase and a blend of oligo(dT) and random hexamer primers according to the manufacturer's instructions. Reactions without reverse transcriptase were included as negative controls; no amplification was detected in negative controls.

mRNA levels of GHR1 were determined by real-time RT-PCR using Stratagene Brilliant II QPCR mastermix and a STRATAGENE MX3000P® Detection System (Stratagene, La Jolla, CA, USA). Real-time PCRs were carried out for samples, standards, and no-template controls in a 10 μ L reaction, containing 2 μ L (20 ng) cDNA, 1 μ L each forward primer GHR1 (600 nM); β -actin; (900 nM), reverse primer GHR1 (600 nM); β -actin (900 nM), and probe GHR1; (150 nM); β -actin (150 nM) (Table 1) at optimized concentrations for the mRNA species to be measured, and 5 μ L TaqMan Universal PCR Master Mix. Cycling parameters for real-time PCR were as follows: 95 °C for 10 min, and 40 cycles of 95 °C for 30, 62 °C for 15 s, and 72°C for 30 s. PCR efficiency for GHR1 was 99.8%. To verify the specificity of the assays, cross hybridization was assessed by substituting alternate primer/probe sets in TaqMan reactions for each standard. No amplification was observed with mismatched primer/probe sets.

Copy number calculations were based on threshold cycle number (CT). The CT for each sample was determined by the MX3000P™ real time analysis detection software after manually setting the threshold. Sample copy number was determined by relating CT to a gene-specific standard curve, followed by normalization to β -actin. No difference ($P > 0.05$) was observed in β -actin expression among the various treatment groups. No-template control samples reached a maximum ΔR_n of 0.03 at 40 cycles. Therefore, copy numbers of mRNA were considered non-

significant if CT exceeded 44 cycles; this value corresponds to a detection limit of less than 100 mRNA copies.

Data analysis

The nucleotide and associated protein sequences were aligned and analyzed with GeneTool and PepTool sequence analysis programs, respectively (BioTools Inc., Edmonton, Alberta, Canada). Clustal X (default parameters, except corrected for multiple substitutions) was used in conjunction with the neighbor-joining method to generate the phylogenetic tree; the tree was visualized with TreeView and rooted to the lamprey GHR/PRLR. Only completely overlapping sequences containing 200+ amino acids were used in the analysis. Quantitative data are expressed as means \pm SEM. Statistical differences were estimated by ANOVA followed by Duncan's multiple range test; a probability level of 0.05 was used to indicate significance. All statistics were performed using SigmaStat (SPSS, Chicago, IL, USA).

Results

Characterization of a new GHR1 mRNA

Phase 1 3'-RACE PCR yielded a cDNA fragment approximately 1100 nt in length (Fig. 1). Sequence analysis of the fragment indicated the successful isolation of a novel GHR. Based upon the 3'-RACE product, a second set of gene-specific primers was designed. Phase 2 RT-PCR yielded a cDNA fragment approximately 2000 nt in length (Fig. 1). Assembly of the fragments revealed a 2357-nt full-length cDNA containing a 70-nt 5'-untranslated region (UTR), a 1971-nt coding sequence of 1971bps, and a 316-nt 3'-UTR (Fig. 2).

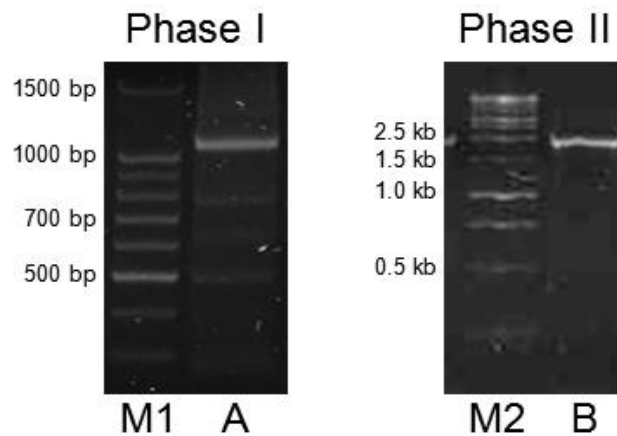


Fig. 1. PCR products resulting from 3'-RACE- (Phase 1) and reverse transcription-PCR (Phase 2) with growth hormone receptor gene-specific primers. PCR reactions were conducted as described in the Materials and Methods section. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining and UV transillumination. M1, 100bp molecular weight marker; M2 1kb molecular weight marker; lane A, PCR product resulting from 3'-RACE PCR; lane B, PCR product resulting from RT-PCR.

Based upon comparison with known GHR sequences (cf. Ellens et al., 2013), we identified the novel GHR as a teleost type 1 GHR (GenBank accession no. JQ408978). The rainbow trout GHR 1 cDNA encodes a 657-amino acid protein that shares 42% and 43 %, respectively, amino acid identity with the two GHRs characterized from rainbow trout previously (Very et al., 2005). As a result, we were able to design real-time PCR primers that were able to detect only rainbow trout GHR1 mRNA; the primer set (Table 1) did not amplify either of the two other rainbow trout GHRs (Fig. 3).


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gaagtcgatacccctcgcgcatTTTTccagctgacatctttgtgtagcaaaggaaagctccccctggc -1
atg gct gct cca tcc ttc ctc ctc ctc ttc ccc ctg ggt ttg ctg tct gct gtg 54
M A A P S F L L L F P L G L L S A V 18
aga tca gcc tcg ctg atg gac cct ggc tct atg acg tca tca gat ccc tcc gtc 108
R S A S L M D P G S M T S S D P S V 36
cag gct cct cat ttg act ggg tgt aag tcc agg gaa cag gag acg ttt agg tgt 162
Q A P H L T G C K S R E Q E T F R C 54
tgg tgg agt ccc gga agc ttc cag aat ctc acg gaa ccc gga gca ctg caa atc 216
W W S P G S F Q N L T E P G A L Q I 72
cag tac tgg aag aag aat gac ttg acg aag gaa tgg aag gag tgc cct gac tac 270
Q Y W K K N D L T K E W K E C P D Y 90
tcc tcc tct gtg aag aac gag tgt ttc ttt aat aag aac aac aca gtt atc tgg 324
S S S V K N E C F F N K N N T V I W 108
atc aaa tac tgt gtg cgg ctc cac tct gaa agc cag aac aaa acc tac gat acc 378
I K Y C V R L H S E S Q N K T Y D T 126
ttg tgc ttc gaa ttg cag gac att gtg cac cct gac ccg cca gtg gct ctg aat 432
L C F E L Q D I V H P D P P V A L N 144
tgg acg ctg ctg aac atc agc cgg tcg ggc ctg aac tat gac atc atg gcg agc 486
W T L L N I S R S G L N Y D I M A S 162
tgg gaa ccc ccg ccc tcg gcc gac gta tcc gtt gga tgg ttg aca ctg gtg tat 540
W E P P P S A D V S V G W L T L V Y 180
gaa gtc cag tac agg aga agg aac agc tca cac tgg aaa gtg ttg gag cat gag 594
E V Q Y R R R N S S H W K V L E H E 198
ttt ggc acc cag cag tct atc tac ggc ctt cag aca gga gag gcg tac gag gtg 648
F G T Q Q S I Y G L Q T G E A Y E V 216
cgt gtg cac tgt gca atg agg gcc ttc aat aac ttt ggg gag ttc agt gat gtc 702
R V H C A M R A F N N F G E F S D V 234
atc ttt gtc cac gtg cca gag att ccc aat aaa gag tca acg ttc cct gtg act 756
I F V H V P E I P N K E S T F P V T 252
ctg gtc ctg atc ttc ggg gct gtg ggc gtg gcc att ctc ctc atg ctc atc atc 810
L V L I F G A V G V A I L L M L I I 270
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K I K G I D P A L L K K G K L D E L 306
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N F I L S G A G M G A L H S D P P D 324

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Fig. 2. Nucleotide and deduced amino acid sequences of rainbow trout growth hormone receptor 1 (GHR1). Nucleotides are numbered 5' to 3' and the amino acid residues (denoted using single-letter abbreviations) are numbered beginning at the initiation methionine. Selected regions are denoted as follows: signal peptide (underlined), conserved cysteine residues in the extracellular domain (circled), potential N-glycosylation sites (boxed in squares), site B (underlined with dashes), FGEFS motif (bold), transmembrane domain (dashed box), Box 1 and Box 2 (boxed in rectangles), tyrosine residues in the intracellular domain (diamonds), and stop codon (*).

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ctg tac cag gac gtg ccc tgg gtg gag ttc atc gag ctg gac gca gac gag cct 1026
L  $\diamond$  Q D V P W V E F I E L D A D E P 342
gag cct ggg gag aag gac aac cag agc tca gac tca cag aga ctg ctg ggt cac 1080
E P G E K D N Q S S D S Q R L L G H 360
aac aac cac cac aca aac cat ggc tgc ttt cat ggc ctc agt atc cct gag gat 1134
N N H H T N H G C F H G L S I P E D 378
gac tct ggc cgg gcc agc tcc tat gat cca gag ctg cct gac cag gag acc ctg 1188
D S G R A S S  $\diamond$  D P E L P D Q E T L 396
atg tta atg gct gcc ctc ctg tcc tgc caa ccc aac aaa gtt gag cca tgc ttg 1242
M L M A A L L S C Q P N K V E P C L 414
ggg aac tgt agc cac tca agc acc cca tcc cta gat gtg ctg gag gtc cca tgc 1296
G N C S H S S T P S L D V L E V P C 432
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P G L Q A P D L P P E G G E R H L V 450
cag acc cag ctg gga ggc cct cag agc tgg gtt aac atg gac ttc tat gcc cag 1404
Q T Q L G G P Q S W V N M D F  $\diamond$  A Q 468
gtc agc gat gtg acg ccc acc gga ggg gtg gtg ctc tcg cca ggc cag cag gtc 1458
V S D V T P T G G V V L S P G Q Q V 486
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G A P E N T P T T E K Y K K G K M E 504
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G G E E E S E E E E D R R T N K L K 522
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F Q L L V V A P E A V G  $\diamond$  T T E S S 540
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S R Q M S T P D P S S P G E V  $\diamond$  H T 558
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F P P P S A E S K P H Q E A  $\diamond$  L P A 576
acc acc ctc cta ggg gac tac cag tcc ccc tac atc ctt cct gac tcc ccc cct 1782
T T L L G D  $\diamond$  Q S P  $\diamond$  I L P D S P P 594
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A Q F L P P V S D Y T V V Q D V D F 612
cag cac agc ctt ctc ctc aac ccc cct tcc ccc cag agg tcc cct aac tgt tcc 1890
Q H S L L L N P P S P Q R S P N C S 630
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P Q P P S K P L P V M P I G  $\diamond$  L T P 648
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D L L G N L S P * 657
gaggttgaggccacttgtcatgaagtcaataaacatcacagacagccttgttctacttgatcaatgctgg 2077
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taaaattggcctttctggtttgacaaggcttaaagtaatatgtaaaaaaaaaaaaaaaaaaaaaaaaaaaa 2287

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Fig. 2. Nucleotide and deduced amino acid sequences of rainbow trout growth hormone receptor 1 (GHR1) (continued). Nucleotides are numbered 5' to 3' and the amino acid residues (denoted using single-letter abbreviations) are numbered beginning at the initiation methionine. Selected regions are denoted as follows: signal peptide (underlined), conserved cysteine residues in the extracellular domain (circled), potential N-glycosylation sites (boxed in squares), site B (underlined with dashes), FGEFS motif (bold), transmembrane domain (dashed box), Box 1 and Box 2 (boxed in rectangles), tyrosine residues in the intracellular domain (diamonds), and stop codon (*).

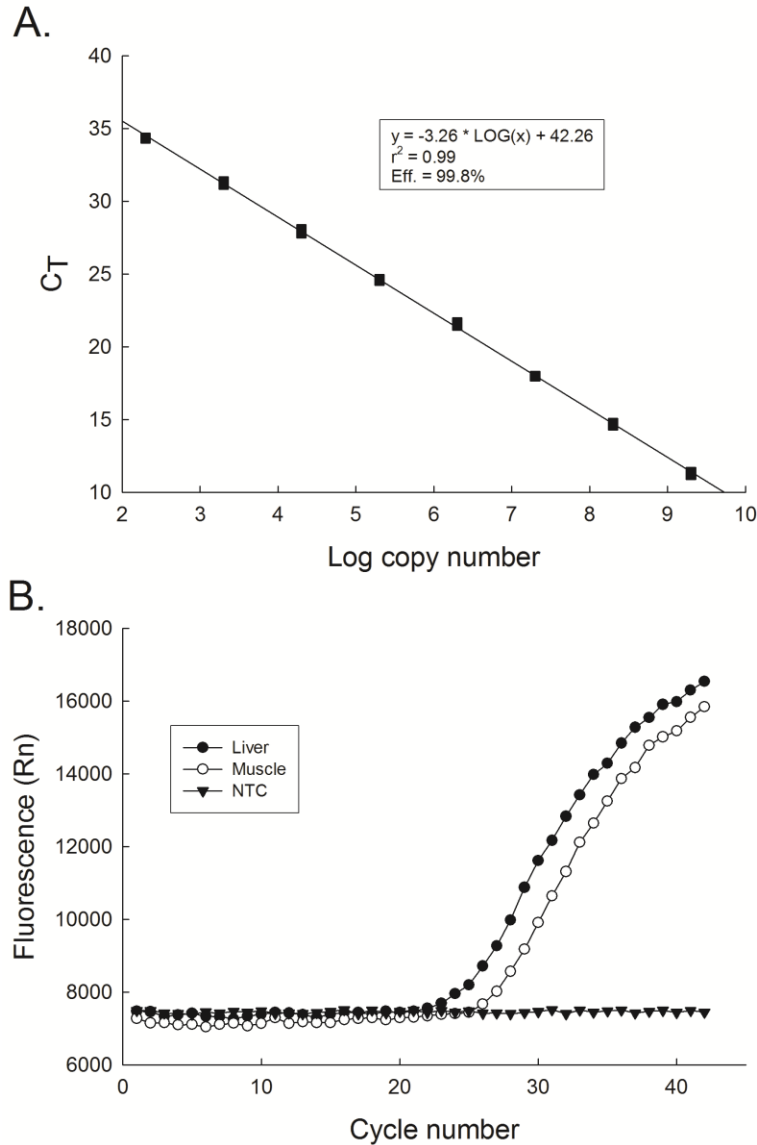


Fig. 3. Quantitative real-time PCR of trout growth hormone receptor 1 (GHR1) mRNA. Sample copy number was determined by relating CT to a standard curve (A) comprised of serial dilutions of known amounts of each GHR1 cDNA, then normalized to β -actin levels. Representative amplification plot of GHR1 mRNAs by real-time RT-PCR (B). Abbreviation: NTC, no-template control.

Differential expression of GHR1 mRNA among tissues

RNA from various tissues was isolated and reverse transcribed and the cDNA encoding GHR1 was quantified using real-time PCR. Although GHR1 mRNA was detected in every tissue examined, the abundance of GHR1 mRNA varied among tissues (Fig. 4). The highest

levels of GHR1 mRNA were found in liver and white muscle. Intermediate expression of GHR1 mRNA was found in brain, endocrine pancreas, gill, and adipose tissue. The lowest levels of GHR mRNA were detected in spleen.

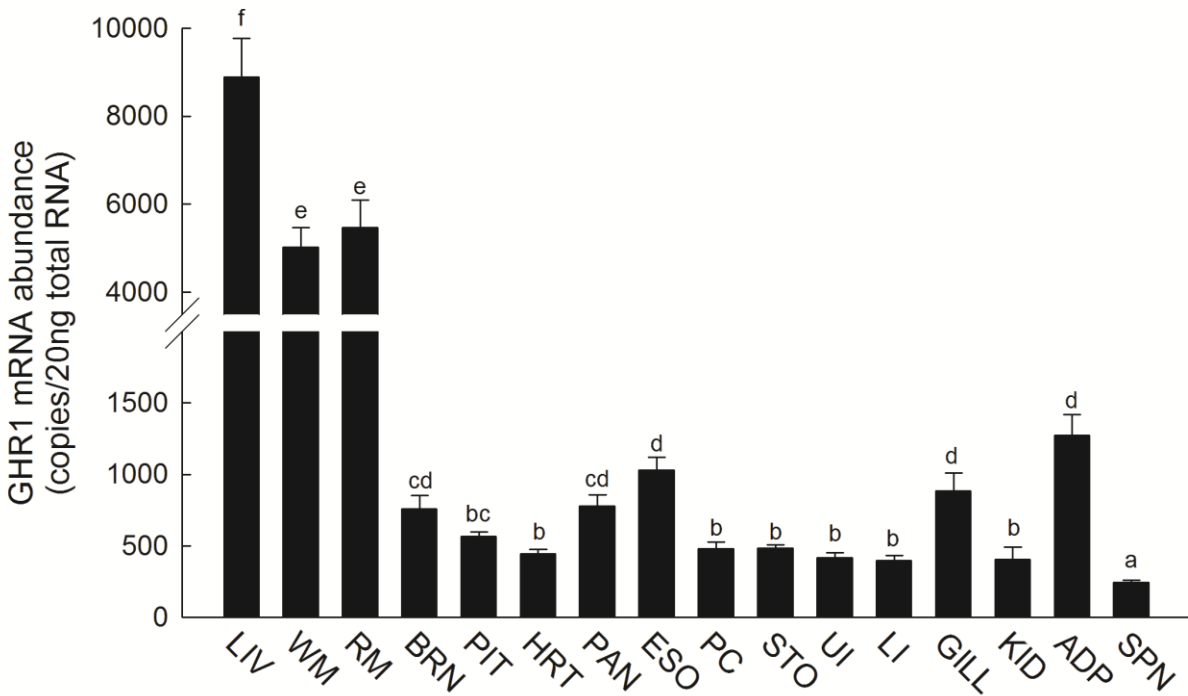


Fig. 4. Abundance of growth hormone receptor 1 (GHR1) mRNA in various tissues of rainbow trout. mRNAs were quantified by real-time RT-PCR. Data are represented by \pm SEM (n=8). Groups with different letters are significantly ($P < 0.05$) different from each. Abbreviations: LIV, liver; WM, white muscle; BRN, brain; PIT, pituitary; HRT, heart; PAN, pancreas; ESO, esophagus; PC, pyloric caeca; STO, stomach; UI, upper intestine; LI, lower intestine; GILL, gill filament; KID, kidney; ADP, adipose; and SPN, spleen.

Effects of nutritional state on GHR1 mRNA expression

The pattern of GHR1 mRNA expression was modulated by nutritional state (Fig. 5). Short-term (2 weeks) decreased levels of GHR1 mRNA in adipose tissue and red muscle. Long-term (4 week) fasting resulted in reduced expression of GHR1 mRNA in liver as well as continued fasting-associated diminution of GHR1 mRNA levels in adipose tissue and red muscle. Refeeding restored GHR1 expression in liver and adipose tissue to levels observed in

continuously fed animal; refeeding also increased levels of GHR1 mRNA in red muscle above those seen in fasted fish, but not significantly. Nutritional state did not have a significant effect on GHR1 expression in white muscle (Fig. 5).

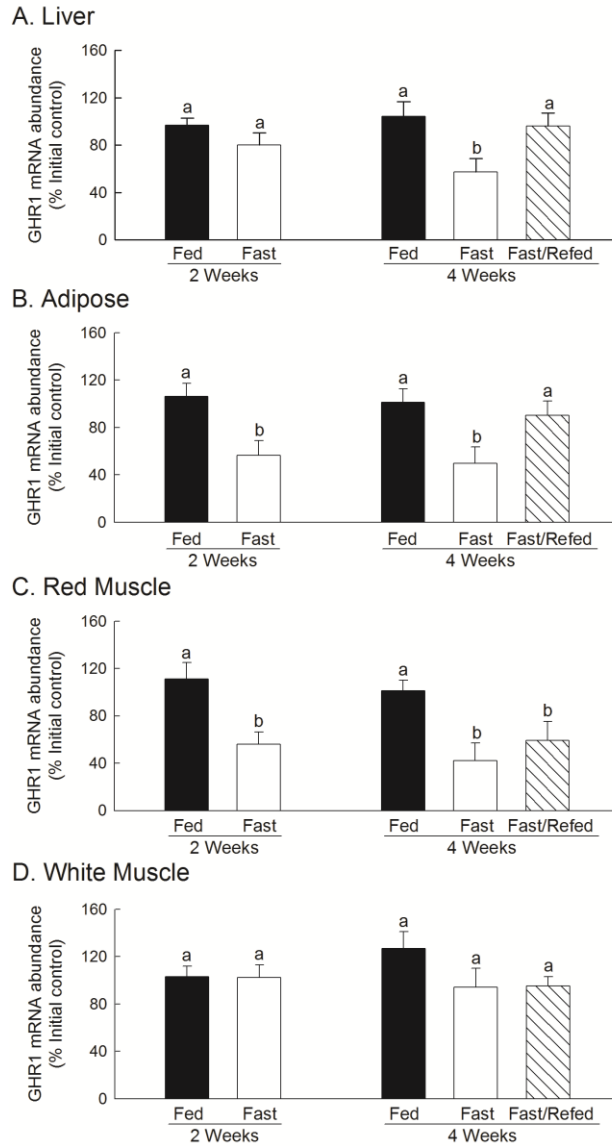


Fig. 5. Effects of nutritional state on the expression of growth hormone receptor 1 (GHR1) in (A) liver, (B) mesenteric adipose tissue, (C) red muscle, and (D) white muscle of rainbow trout. Data presented as % change from fed controls and expressed as means \pm SEM (n=8). Groups with different letters are significantly ($P < 0.05$) different from each other.

Discussion

In this study, we characterized a cDNA encoding a novel GHR in rainbow trout. This new GHR helps clarify the divergence of GHRs in vertebrates, and based on the pattern of divergence, we identified it as a teleost type 1 GHR (GHR1). The GHR1 mRNA is widely yet differentially expressed among tissues in rainbow trout, consistent with the pleiotropic actions of GH (Bjornsson, 1997; Bjornsson et al., 2002; Norrelund, 2005; Moller and Jorgensen, 2009). In general, steady state levels of trout GHR1 mRNA were significantly lower than those of trout GHR2 mRNAs (Very et al 2005). The predicted rainbow trout GHR1 protein possesses structural features conserved in vertebrate GHRs: an extracellular domain containing hormone binding regions, a Y/FGESF motif, and conserved cysteine residues and N-glycosylation sites; a single transmembrane domain; and an intracellular domain containing conserved phosphorylation sites for linkage to effector pathways.

Until now, the origins of the multiple GHRs in teleosts and their names were confusing, and it was unclear whether a particular GHR form in teleosts was a result of the FSGD event (3R) or a more recent tetraploidization (4R). We conducted a new phylogenetic analysis comparing the novel trout GHR with all other known GHRs from teleosts and selected other vertebrates (Fig. 6). The pattern that emerges is consistent with known vertebrate evolution and indicates that gnathostome GHRs arose from a common GHR/prolactin (PRL) receptor gene in agnathostomes (Ellens et al., 2013). Subsequently, the sarcopterygian lineage (lobe-finned fishes; includes the common ancestor of the tetrapods) diverged from the actinopterygian lineage (ray-finned fishes). Although the precise timing of the FSGD event is not certain, it appears to be after the divergence of chondrostei (represented by sturgeon) from the common ancestor of teleosts (Fukamachi and Meyer, 2007). Within the teleosts there are two distinct GHR clades.

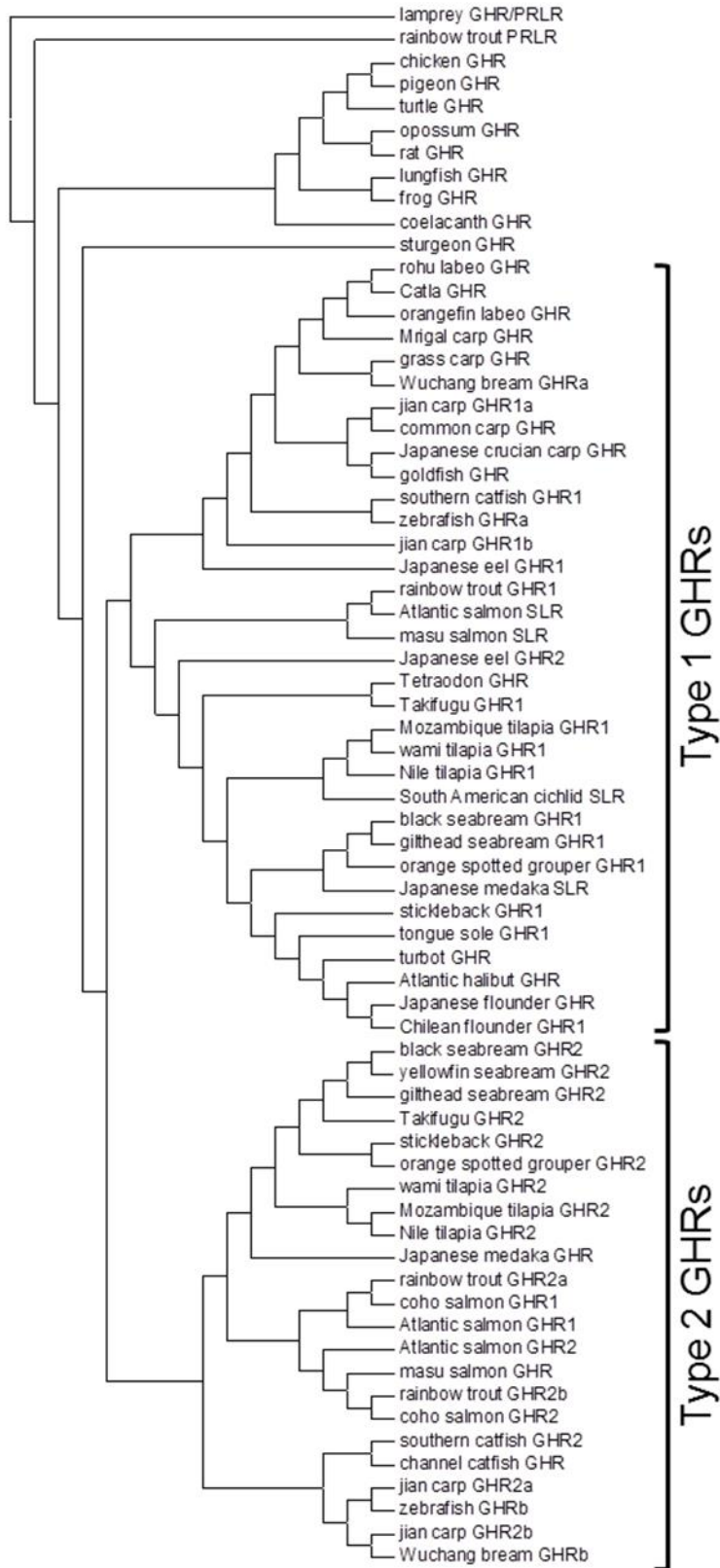


Fig. 6. Phylogenetic tree of the known growth hormone receptors (GHR) of fish and selected other vertebrates. The tree was based on the alignment of amino acid sequences using the N–J bootstrap method in Clustal X and considered only completely overlapping segments greater than 300 nt in length. The tree was rooted using the lamprey GHR/PRLR receptor as an out group and was visualized with TreeView. The branch lengths represent amino acid substitutions per site from a common ancestor and are proportional to the estimated time since divergence occurred. The nomenclature for a particular receptor reflects that given by the authors originally or that which appears in databases; if the sequence was not annotated or the receptor type/subtype was not specified, the designation on the tree is ours and was chosen for consistency with the phylogenetic analysis and our proposed nomenclature. Sequences were obtained from either this study, GenBank, (accession numbers in parentheses) or Ensembl (protein ID numbers in parentheses) as follows: Atlantic halibut GHR (DQ062814), Atlantic salmon GHR1 (NM001123576), Atlantic salmon GHR2 (NM001123594), Atlantic salmon SLR (NM001141617), black seabream GHR1 (AF502071), black seabream GHR2 (AY662334), Catla GHR (AY691178), Channel catfish GHR (DQ103502), chicken GHR (NM_001001293), Chilean flounder GHR1 (EU004149), Coelacanth GHR (ENSLACG00000005546), coho salmon GHR1 (AF403539), coho salmon GHR2 (AF403540), common carp GHR (AY741100), frog GHR (AF193799), gilthead seabream GHR1 (AF438176), gilthead seabream GHR2 (AY573601), goldfish GHR (AF293417), grass carp GHR (AY283778), Japanese crucian carp GHR (ADZ13485), Japanese eel GHR1 (AB180476), Japanese eel GHR2 (AB180477), Japanese flounder GHR (AB058418), Japanese medaka GHR (NM_001122905), Japanese medaka SLR (NP_001098560), jian carp GHR1a (ADC35573), jian carp GHR1b (ADC35574), jian carp GHR2a (ADC35576), jian carp GHR2b (ADC35577), lamprey GHR/PRLR (KF034534), lungfish GHR (EF158850), masu salmon GHR (AB071216), masu salmon SLR (AB121047), Mozambique tilapia GHR1 (AB115179), Mozambique tilapia GHR2 (EF452496), Mrigal carp GHR (AY691179), Nile tilapia GHR1 (AY973232), Nile tilapia GHR2 (AY973233), opossum GHR (NM001032976), orange spotted grouper GHR1 (EF052273), orange spotted grouper GHR2 (EF052274), orangefin labeo GHR (EU147276), pigeon GHR (D84308), rainbow trout GHR1 (JQ408978), rainbow trout GHR2a (NM001124535), rainbow trout GHR2b (NM001124731), rainbow trout PRLR (AF229197), rat GHR (NM017094), rohu labeo GHR (AY691177), South American cichlid SLR (FJ208943), southern catfish GHR1 (AY336104), southern catfish GHR2 (AY973231), stickleback GHR1 (ENSGACT00000009099), stickleback GHR2 (ENSGACT00000023732), sturgeon GHR (EF158851), Takifugu GHR1 (BAK86396), Takifugu GHR2 (BAK86397), Tetraodon GHR (ENSTNIP00000004152), tongue sole GHR1 (FJ608664), turbot GHR (AF352396), turtle GHR (AF211173), wami tilapia GHR1 (EF371466), wami tilapia GHR2 (EF371467), Wuchang bream GHRa (AFC38427), Wuchang bream GHRb (AFC38428), yellowfin seabream GHR2 (AEW29012), zebrafish GHRa (EU649774), zebrafish GHRb (EU649775).

GHRs in one of the clades resemble those of sturgeon and the sarcopterygians and appear to be their homologs (type 1; Fig 6), whereas the other clade contains GHRs that appear to be fish-specific paralogs (type 2; Fig. 6) (Fukamachi and Meyer, 2007; Ellens et al., 2013). Several

groups of teleosts, including the salmonids underwent a subsequent independent duplication event (4R) (Meyer and Van de Peer, 2005).

Unfortunately, the naming of GHRs occurred before this pattern was clear and the terms “GHR1” and “GHR2” were applied inconsistently. It is now imperative to revise the nomenclature to be consistent with the evolutionary origins of the GHR family. Accordingly, Ellens et al. (2013) proposed a nomenclature system in which different numbers be used to designate genes derived from the FSGD event and that different letters be used to designate paralogs derived from a subsequent round of duplication. In applying this naming scheme to our current analysis the type 1 GHR and type 2 GHRs which emerged during the FSGD event should be termed GHR1s and GHR2s, respectively. In some groups, subsequent duplication events gave rise to receptor subtypes. In the salmonids, for example, despite exhaustive screening only a single GHR1 could be isolated, suggested that its paralog was lost; however, in most species so far examined both type 2 GHR subtypes have been characterized. Applying the nomenclature system of Ellens et al. (2013), these subtypes should be designated GHR2a and GHR2b. This will require revision of existing names (as well already have done for rainbow trout; otherwise names originally given by authors appear in Fig. 6), to reduce confusion (e.g. salmonid “GHR1” and “GHR2” both being within the type 2 clade). In order to better represent the evolution of GHR family members, Ellens et al. (2013) also urged abandoning the term “somatolactin receptor (SLR)” to designate members of the teleost type 1 GHR clade.

Rainbow trout GHR1 contains several features, including a Y/FGEFS motif, conserved cysteines, potential N-linked glycosylate sites, and hormone binding regions, that are conserved among vertebrate GHRs and have been recognized as important in receptor functionality. The Y/FGEFS motif is common to GHRs and replaces the WSXWS motif common to all cytokine

receptors (Langenheim et al., 2006). Consistent with the pattern observed so far, this motif in rainbow trout GHR1 appears as FGEFS, which is common for most teleost GHR1s and GHRs. (Ellens and Sheridan, 2013). However, some species display variability between paralogs; in Japanese eel, the first position in GHR1 is phenylalanine whereas in GHR2 the first position is alanine (cf. Ellens and Sheridan, 2013). The motif appears to be important for maintaining structural integrity of the extracellular domain and signal propagation. Mutations of this motif in humans results in Larone syndrome, a condition of growth hormone insensitivity due to loss of GHR function (cf. Langenheim et al., 2006). Cysteine residues are involved in disulfide bonds that affect the conformation of the receptor and impact receptor dimerization, ligand binding, and signal transduction (Ellens and Sheridan, 2013). The presence of 7 conserved cysteines in rainbow trout GHR1 contribute to our understanding of GHR divergence. The pattern emerging is that 7 conserved cysteines is the ancestral condition based on sequence information from the lamprey (Ellens et al., 2013). With the advent of teleosts and the FSGD event, type 1 GHRs retained the ancestral conditions with some slight variability in the last position, whereas type 2 GHRs have between 4 and 5 Cys residues (Ellens et al., 2013).

Also conserved in the extracellular domain are potential N-glycosylation sites which may be involved in cell surface targeting. Five potential sites are present in trout GHR1, which also appear in rainbow trout GHR2s; two of these, corresponding to positions 149 and 188 of trout GHR1, are conserved in all vertebrates (cf. Ellens and Sheridan, 2013). A third site, corresponding to position 63 of trout GHR1 also is conserved in all fish as well as in frog, turtle, and some birds, but is absent in mammals (cf. Ellens and Sheridan, 2013). There are four regions on GHRs that appear important for hormone binding. The nature of these regions in trout GHR1 are similar to those of other teleost type 1 GHRs, which vary from the corresponding

regions in type 2 GHRs (Ellens et al, 2013). Collectively, the structural features of teleost GHR1s and GHR2s results in differences in the conformation of the receptors (Ellens et al., 2013) that may underlie the observed difference in ligand binding features (Reindl et al., 2009).

Phosphorylation sites on the intracellular domain mediate signal transduction to a variety of effector pathways, including JAK-STAT, ERK, and PI3K-Akt (Kopchick and Andry, 2000; Forsyth and Wallis, 2002; Waters et al., 2006). Although the number of tyrosine residues on trout GHR1 and GHR2s are similar, their relative positions vary (cf. Fig 2 and Very et al., 2005). Because such differences between the two trout type 2 GHRs result in differential activation of signal cascades (Kittilson et al., 2011), it is not unreasonable that similar differences between type 1 GHRs and type 2 GHRs would result in differential activation of signaling pathways (Jiao et al., 2006; Chen et al., 2011)

Nutritional state regulated expression of GHR1 mRNAs. This was evidenced by reduced steady-state levels of GHR1 in all of the tissues of rainbow trout examined, including liver, adipose, and red and white muscle. Similar reductions in hepatic GHR1 expression have been observed in black sea bream (Deng et al., 2004) and hybrid striped bass (Picha et al., 2008). Expression of GHR2 mRNAs in liver also have been reported in several species of fish, including rainbow trout, masu salmon, and catfish (Fukada et al., 2004; Small et al., 2006; Norbeck et al., 2007; Peterson et al., 2009). Together, these findings indicate that fasting regulates hepatic GHR1s and GHR2s in a similar manner; such widespread diminution of GHR expression would explain reduced hepatic sensitivity to GH as well as reduced hepatic IGF-1 production and growth retardation associated with food deprivation (Uchida et al., 2003; Chauvigne et al., 2003; Deng et al., 2004; Fox et al., 2006; Pedroso et al., 2006; Small et al., 2006; Norbeck et al., 2007). Interestingly, the pattern of GHR expression differs in among

tissues. In adipose tissue, GHR1 and GHR2b expression is reduced, as shown in this study, whereas GHR2a expression is increased (Norbeck et al., 2007). In muscle, levels of GHR1 decrease in hybrid striped bass increase (Picha et al., 2008) similar to GHR1 in muscle in observed in trout (this study); by contrast, levels of GHR2 increased in muscle of hybrid striped bass and tilapia (Picha et al., 2008; Fox et al., 2010). Such differential regulation of GHRs suggested a means by which some actions of GH (e.g., growth promotion; cf. Reindl and Sheridan 2012) are reduced whereas other actions (e.g., protein sparing, lipolysis), which would be adaptive during periods of food deprivation (cf. Norbeck et al., 2007). The activation of different of GH-mediated responses is possible through differential linkage of GHR subtypes to cellular effector pathways. For example, trout GHR2a preferentially activates STAT, whereas GHR2b preferentially activated ERK and Akt (Kittilson et al., 2011).

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References

- Björnsson, B. T., 1997. The biology of salmon growth hormone from daylight to dominance. *Fish Physiol. Biochem.* 17, 9-24.
- Björnsson, B.T., Johansson, V., Benedet, S., Einarsdottir, I.E., Hildahl, J., Agustsson, T., 2002. Growth hormone endocrinology of salmonids: regulatory mechanisms and mode of action. *Fish Physiol. Biochem.* 27(3), 227-42.

- Chen, M., Huang, X., Yuen, D.S.H., Cheng, C.H.K., 2011. A study on the functional interaction between the GH/PRL family of polypeptides with their receptors in zebrafish: Evidence against GHR1 being the receptor for somatolactin. *Mol. Cell. Endocrinol.* 337, 114-21.
- Deng, L., Zhang, W.M., Lin, H.R., Cheng, C.H.K. 2004. Effects of food deprivation on expression of growth hormone receptor and proximate composition in liver of black seabream *Acanthopagrus schlegeli*. *Comp. Biochem. Physiol. B.* 137, 421-432.
- Ellens, E.R., Kittilson J.D., Hall, J.A., Sower, S.A., Sheridan M.A., 2013. Evolutionary origin and divergence of the growth hormone receptor family: Insight from studies on sea lamprey. *Gen. Comp. Endocrinol.*, In Press.
- Forsyth I.A., Wallis, M., 2002. Growth hormone and prolactin—molecular and functional evolution. *J. Mammary Gland Biol. Neoplasia*, 7, 291–312.
- Fox, B.K., Breves, J.P., Davis, L.K., Pierce, A.L., Tetsuya, H., Grau, E.K., 2010. Tissue specific regulation of the growth hormone/insulin-like growth factor axis during fasting and re-feeding: Importance of muscle expression of IGF-I and IGF-II mRNA in the tilapia. *Gen. Comp. Endocrinol.* 166, 573-580.
- Fox, B.K., Riley, L.G., Hirano, T., Grau, E.G. 2006. Effects of fasting on growth hormone, growth hormone receptors, and insulin-like growth factor-I axis in seawater-acclimated tilapia, *Oreochromis mossambicus*. *Gen. Comp. Endocrinol.*, in press.
- Fukada, H., Ozaki, Y., Pierce, A.L., Adachi, S., Yamauchi, K., Hara, A., Swanson, P., Dickhoff, W.W. 2004. Salmon growth hormone receptors: molecular cloning, ligand specificity, and response to fasting. *Gen. Comp. Endocrinol.* 139, 61-71.

- Fukamachi, S., Meyer, A., 2007. Evolution of receptors for growth hormone and somatolactin in fish and land vertebrates: lessons from the lungfish and sturgeon orthologues. *J. Mol. Evol.* 65(4), 359-72.
- Jiao, B., Huang, X., Chan, C.B., Zhang, L., Wang, D., Cheng, C.H.K., 2006. The co-existence of two growth hormone receptors in teleost fish and their differential signal transduction, tissue distribution and hormonal regulation of expression in seabream. *J. Mol. Endocrinol.* 36(1), 23-40.
- Jönsson E., Björnsson B.T., 2002. Physiological functions of growth hormone in fish with special reference to its influence on behavior. *Fish Sci.* 68, 742–774.
- Kelly, P.A., Djiane, J., Postel-Vinay, M.C., Edery, M., The prolactin/growth hormone receptor family. *Endocr. Rev.* 12, 235-251.
- Kittilson, J.D., Jones, E., Sheridan, M.A., 2011. The ERK, Akt, and STAT5 are differentially activated by the two growth hormone receptor subtypes of a teleost fish (*Oncorhynchus mykiss*). *Frontiers in Experimental Endocrinology.* 2, 1-7.
- Kopchick, J.J., Andry, J.M., 2000. Growth Hormone (GH), GH receptor and signal transduction. *Mol. Genet. Metab.* 71, 293-314.
- Langenheim, J.F., Tan, D., Walker, A.M., Chen, W.Y., 2006. Two wrongs can make a right: dimers of prolactin and growth hormone receptor antagonists behave as agonists. *Mol. Endocrinol.* 20(3), 661-74.
- Liongue, C., Ward, A., 2007. Evolution of Class I cytokine receptors. *BMC Evol. Biol.* 7(1), 120-3.
- Meyer, A., Van de Peer, Y., 2005. From 2R to 3R: evidence for a fish-specific genome duplication (FSGD). *Bioessays.* 27(9), 937-45.

- Møller, N., Jørgensen, J.O.L., 2009. Effects of growth hormone on glucose, lipid, and protein metabolism in human subjects. *Endocr. Rev.* 30(2), 152-77.
- Nørrelund, H., 2005. The metabolic role of growth hormone in humans with particular reference to fasting. *Growth Horm. IGF Res.* 15(2), 95-122.
- Norbeck, L.A., Kittilson, J.D., Sheridan, M.A., 2007. Resolving the growth-promoting and metabolic effect of growth hormone: differential regulation of GH-IGF-I system components. *Gen. Comp. Endocrinol.* 151, 332-341.
- Pedroso, F.L., de Jesus-Ayson, E.G.T. Cortado, H.H., Hyodo, S., Ayson, F.G. 2006. Changes in mRNA expression of grouper (*Epinephelus coioides*) growth hormone and insulin-like growth factor-I in response to nutritional status. *Gen. Comp. Endocrinol.* 145, 237-246.
- Peterson, B.C., Bilodeau-Bourgeois, A.L., Small, B.C., 2009. Response of the somatotrophic axis to alterations in feed intake of channel catfish (*Ictalurus punctatus*). *Comp. Biochem. Physiol. A*, 153, 457–463.
- Picha, M.E., Turan, M.J., Tipsmark, C.K., Borski, R.J., 2008. Regulation of endocrine and paracrine actions of Igfs and Gh receptor during compensatory growth in hybrid striped bass (*Monroe chrysops x Monroe saxatallis*). *J. Endocrinol.* 199, 81-94.
- Reindl, K.M., Kittilson, J.D., Sheridan, M.A., 2009. Differential ligand binding and agonist-induced regulation characteristics of the two rainbow trout GH receptors, Ghr1 and Ghr2, in transfected cells. *J. Endocrinol.* 202(3), 463-71.
- Reindl, K.M., Sheridan, M.A. 2012. Peripheral regulation of the growth hormone-insulin-like growth factor system in fish and other vertebrates. *Comp. Biochem. Physiol. A*, 163, 231-245.

- Small, B.C., Murdock, C.A., Waldbieser, G.C., Peterson, B.C., 2006. Reduction in channel catfish hepatocyte growth hormone receptor expression in response to food deprivation and exogenous cortisol. *Domest. Anim. Endocrinol.* 31, 340-356.
- Sohm, F., Manffroid, I., Pezet, A., Rentier-Delrue, F., Rand-Weaver, M., Kelly, P.A., Postel-Vinnay, M.C., De Luze, A., Edery, M., 1998. Identification and modulation of a growth hormone-binding protein in rainbow trout (*Oncorhynchus mykiss*) plasma during seawater adaptation. *Gen. Comp. Endocrinol.* 111, 216-224.
- Uchida, K., Moriyama, S., Breves, J.P., Fox, B.K., Pierce, A.L., Hirano, T., Grau, E.G., 2009. Isolation and cDNA cloning of somatolactin in Mozambique tilapia (*Oreochromis mossambicus*). *Gen. Comp. Endocrinol.* 161, 162-170.
- Van de Peer, Y., Maere, S., Meyer, A., 2009. The evolutionary significance of ancient genome duplications. *Nature Reviews Genetics.* 10(10), 725-32.
- Very, N.M., Kittilson, J.D., Norbeck, L.A., Sheridan, M.A., 2005. Isolation, characterization, and distribution of two cDNAs encoding for growth hormone receptor in rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. B-Biochem. Mol. Biol.* 140, 615-28.
- Waters, M.J., Hoang, H.N., Fairlie, D.P., Pelekanos R.A., Brown R.J. 2006. New insights into growth hormone action (2006) 36, 1-7.

CHAPTER 3: DIFFERENTIAL EFFECTS OF GROWTH HORMONE-FAMILY PEPTIDES ON THE EXPRESSION OF IGF-1 AND -2 MRNAS

Abstract

Growth hormone (GH) and other hormones in the growth hormone family are responsible for a multitude of actions in organisms including feeding, metabolism, reproduction, osmoregulation, immune function and behavior. In this study, we used juvenile (postembryonic) rainbow trout to examine the influence of GH family peptides on the expression of IGF-1 and IGF-2 mRNAs and to assess the mechanism(s) through which they exert their actions. Fish were implanted with mini osmotic pumps containing GH or saline for 21 days. Growth hormone was found to significantly increase food conversion and growth in both relative length and relative weight. Growth hormone significantly increased mRNA levels of IGF-1 and IGF-2 in both liver and muscle compared to levels observed in saline-implanted fish. Western blot analysis showed that both GH and prolactin (PRL) caused an increase of phosphorylation of JAK, STAT, ERK, and AKT. The direct effects of GH, PRL, and somatolactin (SL) were assessed on isolated hepatocytes incubated in vitro. Growth hormone stimulated expression of IGF-1 and IGF-2 mRNAs in a concentration- and time-related manner. Growth hormone was more efficacious in stimulating expression of IGF-2 than IGF-1 expression. The ERK pathway inhibitor, U0126, and the PI3K/Akt pathway inhibitor, LY294002, blocked GH-stimulated IGF-1 and IGF-2 expression. Prolactin had slight but significant effects on the expression of IGF-1 and IGF-2. Prolactin-stimulated expression of IGF mRNAs also was blocked by U0126 and LY294002. Somatolactin had no effect on the expression of either IGF-1 or IGF-2 mRNA. These findings indicate that GH stimulates IGF-2 expression to a greater extent than IGF-1 expression and PRL stimulates both IGFs equally, both of these facts support a role of IGF-2 in postembryonic

growth of fish. These findings also indicate that GH- and PRL- stimulated IGF-1 and IGF-2 expression involves activation of the ERK, PI3K/Akt, and JAK2-STAT5 signaling pathways. All of this information taken together helps understand the complex nature of organismal growth as well as providing a possible mechanism for the myriad of actions of GH family hormones.

Introduction

Growth hormone (GH), prolactin (PRL), and somatolactin (SL) all belong to the larger somatotropin family of hormones and all share similar structure among fishes (Forsyth and Wallis 2002). These hormones also share similar type I cytokine receptors with a single membrane-spanning domain (Kossiakoff and Vos 1999; Wallis 1992). While these hormones have many similarities they have been shown to have both similar and unique effects. Growth hormone has been shown to regulate many processes in vertebrates including feeding, metabolism, reproduction, osmoregulation, immune function and behavior. The most studied actions of GH involve the promotion of organismal growth (Bjornsson et al., 2004; Forsyth and Wallis 2002; Moller and Jorgensen 2009; Norbeck et al., 2007; Norrelund 2005). Prolactin has shown to have a wide array of functions including osmoregulation (Maetz 1970), growth (Rynikova et al., 1988), metabolism (Vilalba et al., 1991), behavior (Blum and Fiedler 1965), reproduction (Dunaif et al., 1982), and immunity (Nagy et al., 1983). The most recently discovered of these hormones is SL. The main physiological function of SL is still debated but SLs are involved in reproductive maturation (Johnson et al., 1997), acid–base balance (Kakizawa et al., 1996), background adaptation (Kakizawa et al., 1995), immune function (Calduch-Giner et al., 1998), energy mobilization and stress (Rand-Weaver et al., 1993), lipid metabolism and pigmentation (Zhu and Thomas 1997; Fukamachi et al., 2009), and the regulation of chromatophores (Zhu and Thomas 1997). While all the hormones in this family share many

functions, this study will focus on these hormones control of growth through insulin-like growth factors 1 and 2 (IGF-1 and IGF-2).

The actions of GH are initiated by binding to GHRs present on the plasma membrane of target cells. GHRs belong to the class I cytokine receptor family, which consists of a single transmembrane protein containing an extracellular ligand binding domain and an intracellular signal transduction domain (Argetsinger and Carter-Su 1996). PRL and SL also work through similar mechanisms since they also have similar evolutionarily conserved receptors (Kelly et al., 1991). GHRs have been characterized from over 100 species, including some 25 species of fish. Fish possess multiple GHRs that appear to have derived from several genome duplication events over the course of their evolution (Fukamachi and Meyer 2007; Reindl et al., 2009).

While GHRs are most abundant in the liver of mammals and fish, they are expressed widely in many tissues, observations that are consistent with the pleiotropic actions of GH (Kopchick and Andry 2000; Very et al., 2005; Walock et al., 2014). PRLR also share similar diverse distributions also mirroring its pleiotropic actions (Nagano and Kelly 1994).

In mammals, GH binding to dimerized GHRs is followed by recruitment of Janus kinase 2 (JAK2) to the receptor complex and the rapid phosphorylation of GHR and JAK2; signal is propagated by activation of several other proteins and pathways, including transducer and activator of transcription 5 (STAT5) as well as the extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt) pathways (Pawlik-Pilipuk et al., 2002). Many of these similar signaling elements are also found to be activated by PRLR including JAK2 (Campbell et al., 1994) and STAT5 (Pezet et al., 1997). The GH-IGF-1 system has been well studied in mammals and fish, and the main elements appear to be highly conserved (Bulter and LeRoith 2001; Reinecke et al., 2005). Circulating GH stimulates the synthesis and

secretion of IGF-1 from the liver, and IGF-1, in turn, stimulates cell growth and differentiation in a variety of target tissues via distinct IGF-1 receptors (IGFR1) (Laviola et al., 2007; Wood et al., 2005). GH has also been shown to act in stimulating IGF-2 secretion from the liver (Shamblott et al., 1995; Pierce et al., 2010). The availability and actions of GH and IGF-1 are influenced by GH binding proteins and IGF binding protein (IGFBPs), respectively (Bauman et al., 1988; Duan and Xu 2005). The GH-IGF-1 system of fish is particularly complex because of the existence of multiple isoforms of GHs, GHRs, IGFs, IGFBPs, and IGFRs (Reinecke et al., 2005).

IGF-1 and IGF-2 are mitogenic peptides that control vertebrate growth (Humbel 1990; Jones and Clemmons 1995; Reinecke and Collet 1998). Both IGFs bind a common receptor IGFR1 and both have their activities modulated by multiple IGFBPs. In addition, local production of IGF-1 is important, and GH and IGFBPs have direct, non-IGF-1 dependent effects (Bulter and LeRoith 2001; Duan and Xu 2005; Wood et al., 2005). Insulin-like growth factor-1 from the liver is the primary mediator of GH-dependent growth. Insulin-like growth factor-2 is highly expressed during embryogenesis and stimulates embryonic growth (Jones and Clemmons 1995; Reinecke and Collet 1998; Wood et al., 2005; White et al., 2009). During this time IGF-1 expression is low and increases later in post-natal growth. Studies have shown that IGF-2 is not strongly stimulated by GH in postnatal animals (Humbel 1990; Holly 1998). However, recent studies have shown in many species including salmonids, sea bream, carp, catfish, eels, rabbitfish, sea bass, hybrid striped bass, tilapia, and dogfish, that, in fish, GH stimulates liver IGF-2 expression, unlike in mammals (Shamblott et al., 1995; Tse et al., 2002; Peterson et al., 2004; Carnevali et al., 2005; Ayson et al., 2007; Terova et al., 2007; Moriyama et al., 2008; Picha et al., 2008; Eppler et al., 2010). This suggests that IGF-2 may also be a major mediator of postnatal growth in fish as opposed to the current understanding of mammalian growth. Despite

this research, more work is needed to fully understand GH, PRL, and SL in their relationship to growth. In this study, we used rainbow trout as a model to examine the mechanism of GH, PRL, and SL action on IGF-1 and IGF-2 expression in fish. Specifically we tested the hypothesis that the ERK, PI3K/AKT, and JAK2-STAT5 signal pathways mediate GH and PRL stimulated expression of IGF-1 and IGF-2.

Materials and methods

Experimental animals and conditions

Juvenile rainbow trout of both sexes (ca. 1 year of age) were obtained from Dakota Trout Ranch (Carrington, ND). The animals were transported to North Dakota State University, where they were maintained in well-aerated, 800-L circular tanks supplied with recirculated (10% make-up volume per day) dechlorinated municipal water at 14°C under a 12:12 hour light: dark photoperiod. Fish were fed twice daily to satiety with AquaMax Grower (PMI Nutrition International, Brentwood, MO, USA) semi-floating trout grower, except 24–36 h prior to commencing experiments in order to prevent any postprandial responses that feeding may cause. Animals were acclimated to laboratory conditions for at least 4 weeks prior to experimentation.

Implantation experiment

The effects of growth hormone (GH) on the patterns of IGF-1 and IGF-2 mRNA expression were evaluated by implanting fish with Alzet® mini-osmotic pumps (Alza; Palo Alto, CA) containing either 0.75% (w/v) NaCl (control) or 200 ng/ml ovine growth hormone (obtained from NIH). Mini-pump flow rate was established to be 0.135 $\mu\text{l h}^{-1}$, which at 14°C should provide sustained release for 29 days. Fish were first anesthetized with 0.05% (v/v) 2-phenoxyethanol, and their body length and mass were determined. Mini-pumps were inserted into the peritoneal cavity through a 1.0-cm incision that was made 0.5 cm right of the ventral

midline and 2.0 cm in front of the pelvic fins. The incision was closed with two stitches and antibiotic ointment (Neosporin®) was applied topically to the incision. Fish were placed into 100-L tanks (one tank for each treatment group) under the same conditions as acclimation, except that each tank was treated with 250 mg erythromycin with no water turnover for 30 min while supplemented with pure oxygen and feeding was suspended.

Seventy-two hours after tagging and transfer, fish were fed twice daily at a ration of 2% of their initial body mass per feeding and continued for the duration of the experiment, except 24h prior to sampling. One hour after feeding excess food was collected and dried in an oven until moisture was removed, and weighed in order to calculate food intake (7% increase to dried food was added to adjust for moisture content). Fish were anesthetized by immersion in 0.05% (vol/vol) 2-phenoxyethanol and euthanized by transection of the spinal cord. Skeletal muscle and liver tissues were collected 21 days after implantation and tissues were immediately frozen on dry ice for later analyses.

Hepatocyte isolation

Fish were anesthetized by immersion in 0.05% (vol/vol) 2-phenoxyethanol and euthanized by transection of the spinal cord. Hepatocytes were isolated by the in situ perfusion method of (Mommensen et al., 1994). The isolated cells were allowed to recover in incubation medium [in mM: 136.9 NaCl, 5.4 KCl, 0.81 MgSO₄, 0.44 KH₂PO₄, 0.33 Na₂HPO₄, 10 HEPES, 5 NaHCO₃, and 1.5 CaCl₂, pH 7.6, with 2% (wt/vol) defatted BSA, 3 mM glucose, 2 ml Gibco MEM amino acid mix (50×)/100 ml, and 1 ml Gibco nonessential amino acid mix (100×)/100 ml] for 3 h at 14°C with gyratory shaking (100 rpm) under 100% O₂. The viability of the cells was assessed by trypan blue dye exclusion and ranged between 93–97% for all experiments. After the recovery period, hepatocytes were collected by centrifugation (500 g for 3 min at 14°C)

and resuspended in incubation medium to a final concentration of 2×10^6 cells/ml, and aliquotted into 24-well plates (2×10^6 cells/well). Cells were incubated in medium alone (control) or in medium containing the designated hormone treatment under the same conditions as those used for recovery (14°C with gyratory shaking at 100 rpm under 100% O_2) for various times as specified in the figure legends. In combination experiments involving inhibitors, the specific inhibitor was added 2 h prior to hormone treatment at concentrations shown maximally effective by the manufacturer and/or which were used previously (Reindl et al., 2011). Incubations were stopped by centrifugation (500 g for 3 min at 14°C); cell pellets were either prepared immediately for Western blot analysis or resuspended in 0.5 ml Tri Reagent (Molecular Research Center, Cincinnati, OH), frozen on dry ice, and stored at -80°C for later extraction of RNA. The hepatocytes used in replicates for a given experiment were obtained by pooling hepatocytes from individual fish and dividing into replicates.

RNA extraction and analysis

Total RNA was extracted using TRI-Reagent® (Molecular Research Center, Inc., Cincinnati, OH, USA) as specified by the manufacturer's protocol. Each RNA pellet was redissolved in 35–200 μl RNase-free deionized water and quantified by NanoDrop1000 spectrophotometry (A260) (Thermo Scientific, West Palm Beach, FL, USA). RNA samples were then stored at -80°C until further analysis.

mRNA was reverse transcribed in 5 μl reactions using 150 ng total RNA and AffinityScript QPCR cDNA Synthesis kit reagents (Master Mix, random primers, oligo-dT primers, and reverse transcriptase with block) according to the manufacturer's protocol (Stratagene, La Jolla, CA, USA). Reactions without reverse transcriptase were included as

negative controls to exclude the possibility of contamination with genomic DNA; no amplification was detected in negative controls.

Levels of IGF-1 and IGF-2 mRNAs were determined by quantitative real-time PCR as described previously (Malkuch et al., 2008). Briefly, real-time reactions were carried out for samples, standards, and no-template controls in multiplex reactions with IGF-1 or IGF-2 and β -actin. cDNA standards were generated using IGF-1 and IGF-2 gene-specific primers, the PCR products were cloned into the pGEM-T Easy Vector, and the sequences were verified. Reactions contained 2 μ l cDNA from the reverse transcription reactions, 5 μ l Brilliant® II QPCR Master Mix (Stratagene), 1 μ l of each 150 nM gene-specific probes [IGF1: 5'-FAM-AAAGCCTCTCTCTCCA-MGBNFQ -3' (150 nM); IGF2: 5'-FAM-AGATCATTTCCCATGGTGC-MGBNFQ -3' (150 nM); β -actin: 5'-VIC-TGCTTGCTGATCCACAT-MGBNFQ -3' (150 nM)], 0.5 μ l of gene-specific forward [IGF1: 5'-GTGGACACGCTGCAGTTTGT-3' (600 nM); IGF2: 5'-ACGTGTCGGCCACCTCTCTA-3' (600 nM); β -actin: 5'-GGCTTCTCTCTCCACCTTCCA-3' (900 nM)] and reverse primers [IGF1: 5'-CATACCCCGTTGGTTTACTGAAA-3' (600 nM); IGF2: 5'-TGGGACATCCTGTTTGATTGTG-3' (600 nM); β -actin: 5'-AGGGACCAGACTGTCGTA ACTC-3' (900 nM)], and 0.15 μ l reference dye (Stratagene, Agilent Technologies). Cycling parameters were set as follows: 95 °C for 10 min and 45 cycles of 95 °C for 30 s and 58 °C for 1 min. Cross reaction was assessed by substituting alternate primer/probe sets in assays for each standard; no amplification was observed under these conditions. Sample copy number was calculated from the threshold cycle number (CT) and relating CT to a gene-specific standard curve, followed by normalization to β -actin.

Western blotting

Tissues were homogenized in 300 µl 1x cell lysis buffer (Cell Signaling Technology, Beverly, MA) with 1 mM PMSF, 1x protease inhibitor (Calbiochem, San Diego, CA, USA), and 1x phosphatase inhibitor (G-Biosciences, St. Louis, MO, USA). The homogenate was incubated on ice for 5 min then centrifuged at 16,000g for 10 min at 4 °C. The protein concentration of the supernatant was determined by the Bio-Rad (Hercules, CA, USA) dye-binding method for microplates. Proteins (typically 50 µg) and molecular weight marker (Cell Signaling Technology, Beverly, MA; Catalog No. 7727) were separated by SDS–PAGE (7.5% running gel) and transferred to 0.45 µm nitrocellulose (Bio-Rad Laboratories) for western blot analysis as described by Reindl et al. (2011). Membranes were washed and visualized with chemiluminescence according to the manufacturer's instructions (GE Healthcare, Buckinghamshire, UK); chemiluminescence was detected directly and the bands quantified with a FluorChem FC2 imager (Alpha Innotech Corp, San Leandro, CA, USA). The abundance of phosphorylated ERK 1/2, Akt, JAK2, and STAT5 was normalized to total ERK 1/2, Akt, JAK2, STAT5, and β-actin, respectively. All antisera were obtained from Cell Signaling Technology (Beverly, MA); antisera for ERK 1/2, Akt, JAK2, and STAT5 were validated for the detection of signaling elements in rainbow trout previously (Reindl et al., 2011).

Data analysis

Statistical differences were estimated by one-way or two-way ANOVA, as appropriate. In all cases, main effects were significant, and no significant interactions were observed between main effects in two-way ANOVAs. Pairwise comparisons of simple effects were assessed by Duncan's multiple range test; statistical notations on the faces of the figures reflect such comparisons. A probability level of 0.05 was used to indicate significance. All statistics were

performed using SigmaStat version 1.0 (SPSS, Chicago, IL), and graphs were constructed with SigmaPlot version 8.0 (SPSS). Quantitative data are expressed as means \pm SEM.

Results

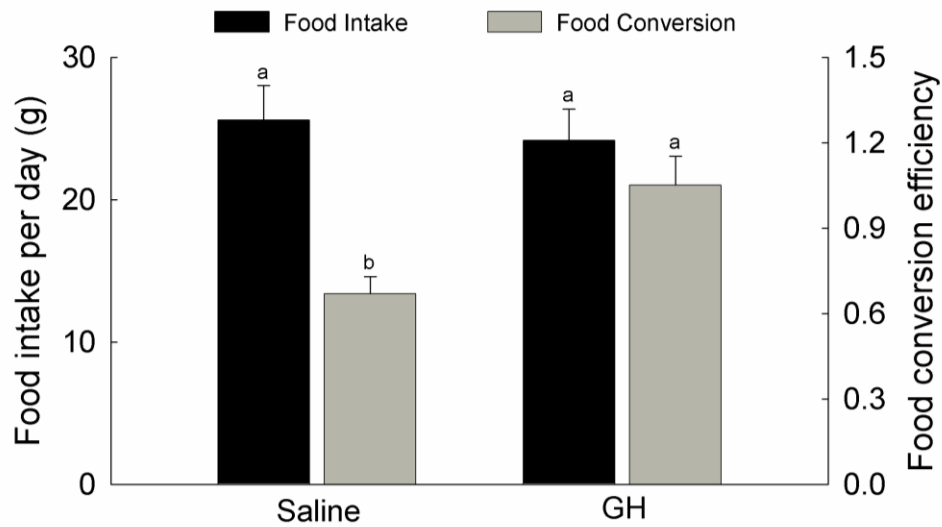
***In vivo* effects of GH on food intake, food conversion, and growth**

Although food intake was similar between saline- and GH-implanted fish, food conversion was 37% higher in GH treated animals compared to controls (Fig. 7). Growth Hormone implantation resulted in significant growth, in which the most pronounced effect was on body length. Relative growth in mass was increased by 42%, whereas relative growth in length was increased by 140% in GH treated fish compared to controls (Fig.7).

***In vivo* effects of GH on IGF expression**

GH implantation increased steady-state levels of mRNAs encoding both IGF-1 and IGF-2 in liver and skeletal muscle, with IGF-1 levels being stimulated to 133% and 169% of controls in skeletal muscle and liver accordingly and IGF-2 levels being stimulated to 171% and 226% of controls in skeletal muscle and liver accordingly (Fig. 8). Growth hormone stimulated IGF-2 mRNA expression to a greater extent than IGF-1 mRNA expression in both skeletal muscle and liver. Liver was also more sensitive to GH stimulation showing higher stimulated expression levels of both IGF-1 and IGF-2 (Fig. 8).

A. Food conversion



B. Relative growth

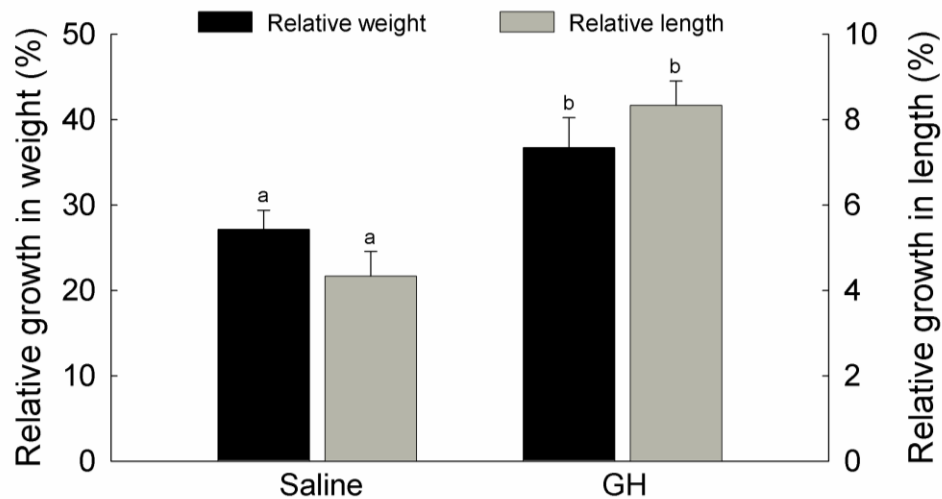
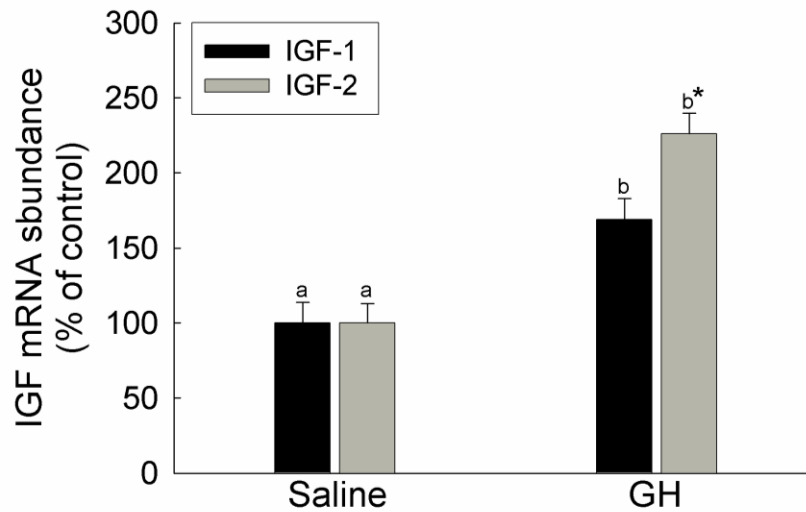


Fig. 7. Average daily food intake and food conversion ratio (A) and changes in relative growth via both relative length and weight of rainbow trout implanted with ovine growth hormone (GH) or 0.75% saline over a 21-day trial (B). Food conversion efficiency was calculated as (body wet mass gain) / (dry weight food intake per fish). Relative weight and length were calculated as $(100 * [\text{final body mass (or length)} - \text{initial body mass (or length)}]) / [\text{initial body mass (or length)}]$. Data are presented as % control from controls and expressed as means \pm SEM (n=6). For a given treatment, groups with different letters are significantly ($P < 0.05$) different.

A. Liver



B. Muscle

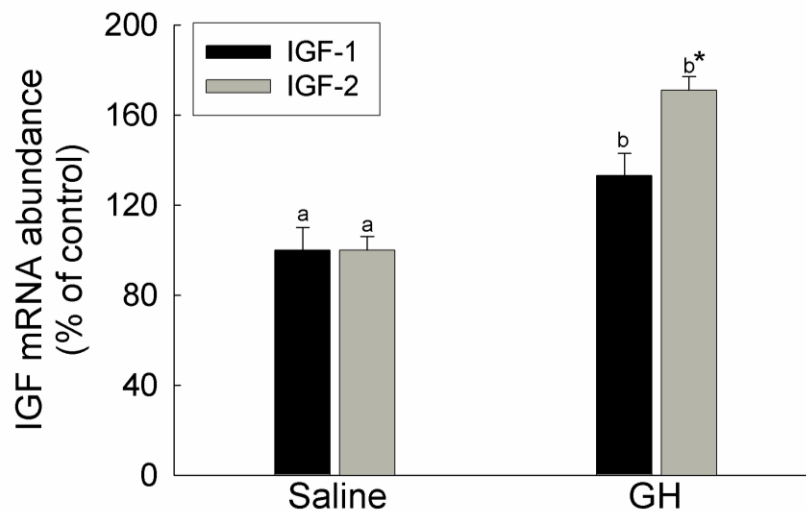


Fig. 8. Changes in the expression of insulin-like growth factor subtype (IGF-1 and IGF-2) mRNA in the liver (A) and muscle (B) of rainbow trout associated with GH implantation. Data are presented as % control from controls and expressed as means \pm SEM (n=6). For a particular tissue, letters denote significant ($P < 0.05$) differences for a given subtype across treatments; * denotes a difference in subtypes within a given treatment.

***In vitro* of GH and PRL on JAK, STAT, ERK and AKT phosphorylation**

The roles of GH and PRL on JAK, STAT, ERK, and AKT phosphorylation were examined *in vitro* using isolated hepatocytes. The activation of these cell signaling pathways was

studied on lysates from hepatocytes probed with phosphospecific and control antibodies. Treatment of hepatocytes in vitro with GH activated JAK, STAT, ERK, and AKT (Fig. 9). GH activated JAK most effectively to 193% of control. GH activation of STAT, ERK, and AKT were 146%, 187%, and 162% respectively (Fig. 9). Treatment of hepatocytes in vitro with PRL also activated JAK, STAT, ERK, and AKT (Fig. 10). PRL activated ERK most effectively to 168% of controls. PRL activation of JAK, STAT, and AKT were 147%, 134%, and 156% respectively (Fig. 10).

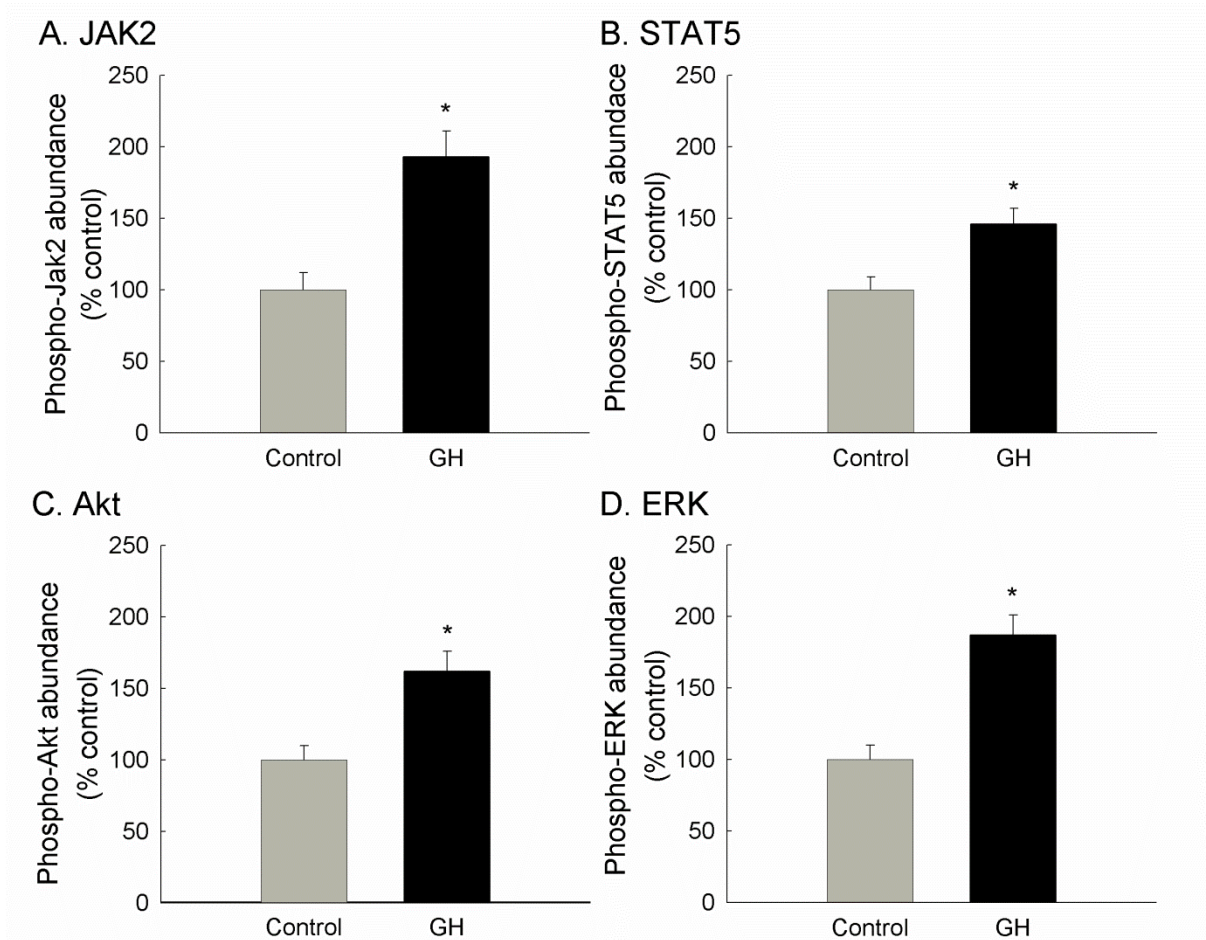


Fig. 9. Effects of growth hormone (GH) on the abundance of phosphorylated Janus kinase 2 (JAK2), signal transducer and activator of transcription 5 (STAT5), protein kinase B (Akt), and extracellular signal-regulated kinase (ERK) in isolated hepatocytes of rainbow trout. A, B, C, D: phosphorylation of JAK2, STAT5, Akt, and ERK respectively, in cells incubated with 100 ng/ml GH for 30 min (control is 0 ng/ml GH). Cell lysates were separated by SDS-PAGE followed by Western immunoblotting, and the blots were quantified with a FluorChem imager. The abundance of phosphorylated JAK2, STAT5, Akt, and ERK was normalized to total JAK2, STAT5, Akt, and ERK respectively. Data are presented as means + SEM (n=6). Groups with different letters are significantly ($p < 0.05$) different.

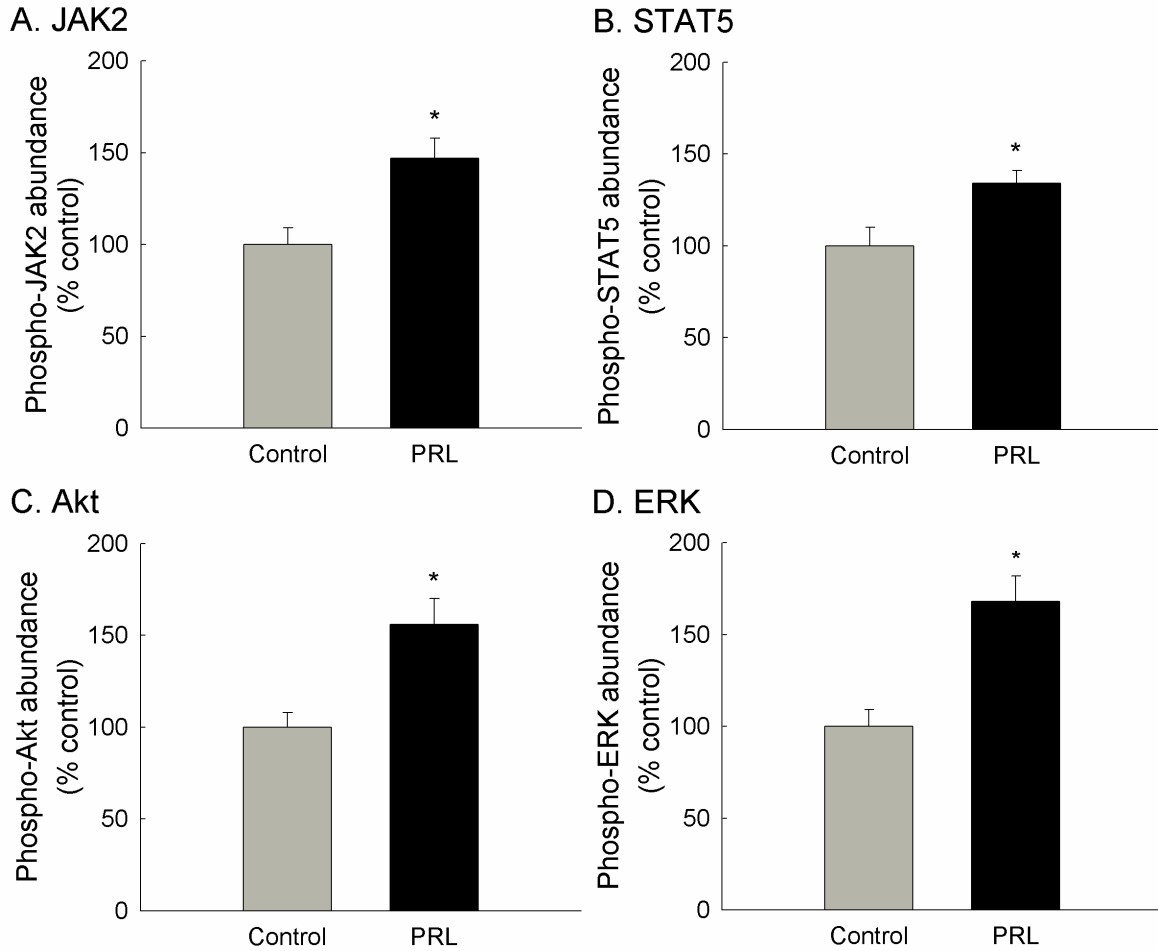


Fig. 10. Effects of prolactin (PRL) on the abundance of phosphorylated Janus kinase 2 (JAK2), signal transducer and activator of transcription 5 (STAT5), protein kinase B (Akt), and extracellular signal-regulated kinase (ERK) in isolated hepatocytes of rainbow trout. A, B, C, D: phosphorylation of JAK2, STAT5, Akt, and ERK respectively, in cells incubated with 100 ng/ml PRL for 30 min (control is 0 ng/ml PRL). Cell lysates were separated by SDS-PAGE followed by Western immunoblotting, and the blots were quantified with a FluorChem imager. The abundance of phosphorylated JAK2, STAT5, Akt, and ERK was normalized to total JAK2, STAT5, Akt, and ERK respectively. Data are presented as means + SEM (n=6). Groups with different letters are significantly ($p < 0.05$) different.

***In vitro* effects of GH, PRL, and SL on IGF expression**

The roles of GH, PRL, and SL on IGF expression were examined *in vitro* using isolated hepatocytes. Growth hormone directly stimulated the expression of IGF-1 and IGF-2 mRNAs in isolated hepatocytes in a time-dependent and concentration-related manner. Maximum

stimulation occurred after 6 h treatment with IGF-1 expression at 178% and IGF-2 at 243% of controls (Fig. 11). Growth hormone was significantly more efficacious at stimulating IGF-2 over IGF-1 (Fig. 11). Prolactin had a similar expression pattern to GH, stimulating IGF-1 and IGF-2 mRNAs in isolated hepatocytes in a time- and concentration-dependent manner. Maximum stimulation occurred after 6 h treatment with IGF-1 expression at 139% and IGF-2 at 146% of controls (Fig. 11). Unlike GH, PRL stimulated both IGF-1 and IGF-2 to similar levels (Fig. 11). Somatolactin treatment had no effect on either IGF-1 or IGF-2 mRNA expression (Fig. 11).

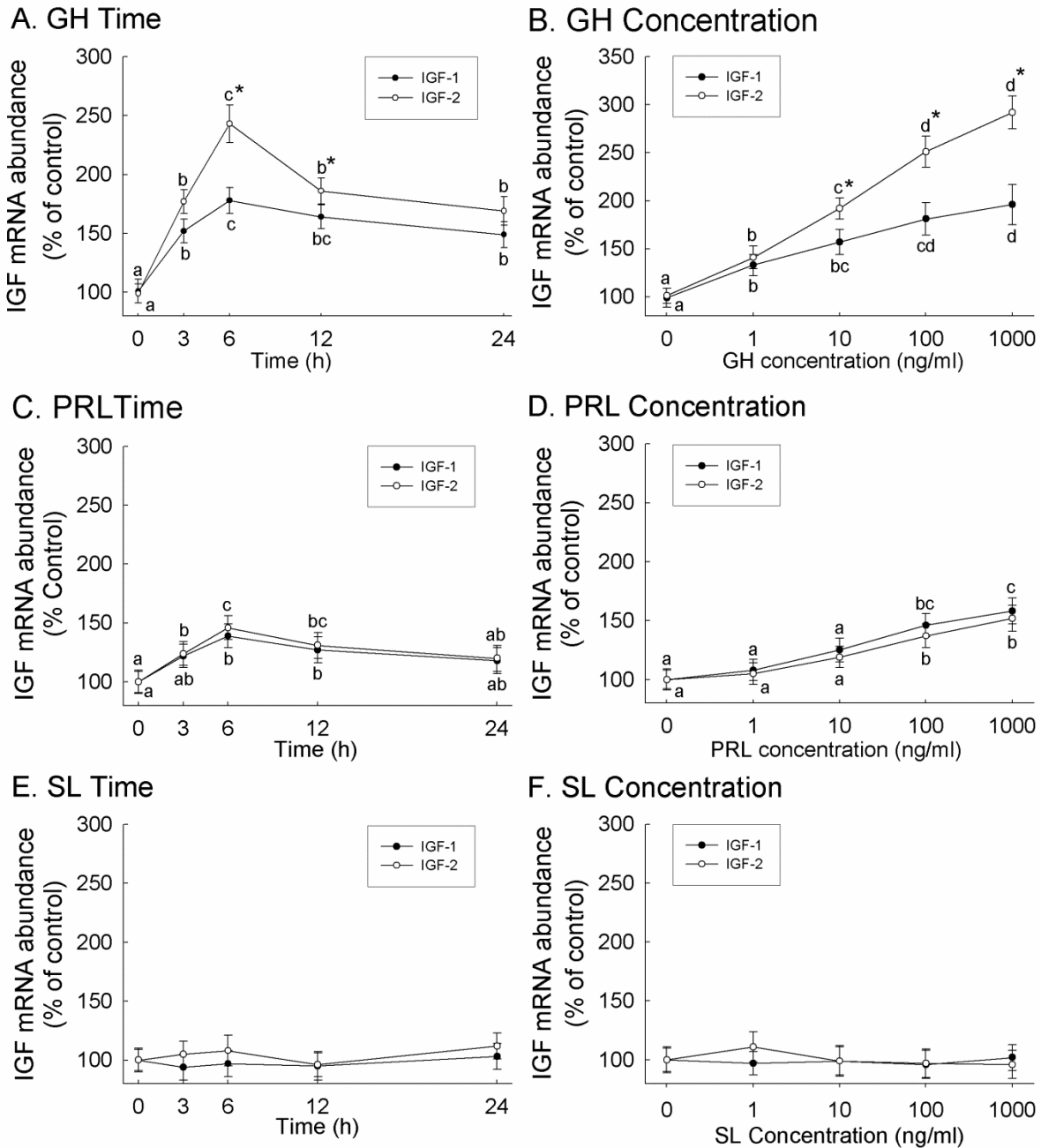


Fig. 11. Effects of growth hormone (GH), prolactin (PRL), and somatotactin (SL) on the expression of insulin-like growth factor subtype (IGF-1 and IGF-2) mRNA in isolated hepatocytes of rainbow trout. A, C, E: time dependent IGF-1 and IGF-2 mRNA expression in cells incubated for varying times with 100 ng/ml GH, PRL, or, SL respectively. B, D, F: dose dependent IGF-1 and IGF-2 mRNA expression in cells incubated for 6 hours in varying concentrations of GH, PRL or, SL respectively. Data are presented as % control from controls and expressed as means \pm SEM (n=6). For a particular hormone, letters denote significant (P<0.05) differences for a given subtype across treatments; * denotes a difference in subtypes within a given treatment.

Linkage of the ERK, PI3K/AKT, and JAK/STAT signaling pathways to GH-stimulated IGF-1 and IGF-2 expression

The roles of the ERK, PI3K/AKT, and JAK/STAT signaling pathways on GH-, PRL-, and SL-stimulated IGF-1 and IGF-2 expression in isolated hepatocytes was investigated using inhibitors specific to these pathways. Initial experiments examined the effects of inhibitors alone and in combination with GH, PRL, and SL. As previously observed (Fig. 11), GH and PRL increased steady-state levels of IGF-1 and IGF-2 mRNA in isolated hepatocytes incubated in vitro while SL still had no effect on IGF expression. Incubation with any inhibitor alone had no effect on IGF-1 and IGF-2 mRNA expression. Blockade of the ERK pathway with the MEK inhibitor U0126 (U) partially inhibited GH and PRL-stimulated IGF-1 and IGF-2 expression (Fig. 12). Blockade of the PI3K/AKT pathway with a PI3K inhibitor LY294002 (LY) or AKT inhibitor 1L6-hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecyl-sn-glycerocarbonate (CARB) partially inhibited GH- and PRL-stimulated IGF-1 and IGF-2 expression; for GH-stimulated IGF expression, the effect of PI3K inhibition was greater than AKT inhibition (Fig. 12). Inhibition of STAT5 with N'-((4-oxo-4H-chromen-3-yl)methylene)nicotinohydrazide (NICO) partially blocked GH- and PRL- stimulated IGF-1 and IGF-2 expression (Fig. 12) to levels similar to ERK and AKT inhibition. Inhibition of JAK with 1,2,3,4,5,6-hexabromocyclohexane (HEX) abolished GH- and PRL-stimulated IGF-1 and IGF-2 expression to control levels (Fig. 12).

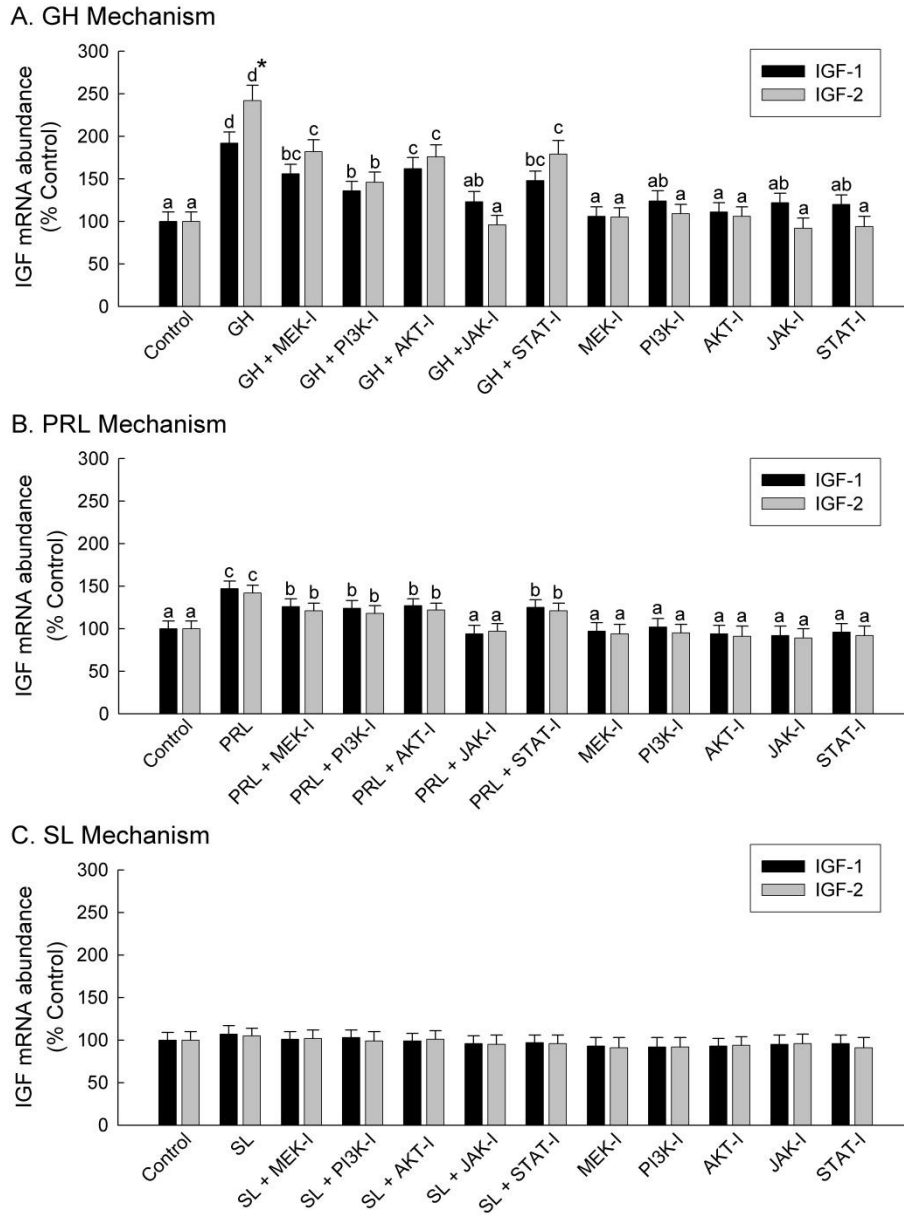


Fig. 12. Effects of signaling element blockades on growth hormone (GH), Prolactin (PRL), and somatotactin (SL)-stimulated expression of insulin-like growth factor subtype (IGF-1 and IGF-2) mRNA in isolated hepatocytes of rainbow trout. Hepatocytes were preincubated for 2 h with or without specific inhibitors for the following signaling elements: MEK (10 μ M U0126=MEK-I), PI3K (10 μ M LY294002=PI3K-I), Akt (10 μ M Carb=Akt-I.), JAK2 (10 μ M Hex=JAK-I), STAT5 (10 μ M NICO=STAT-I); and in the absence or presence of GH(A), PRL(B) or, SL(C) at 100 ng/ml for 6 h (control is 0 ng/ml GH, PRL or, SL); after which time, cells were treated with 100 ng/ml GH, PRL or, SL for 6 h (control is 0 ng/ml GH, PRL or, SL). Data are presented as % control from controls and expressed as means \pm SEM (n=6). For a given hormone, groups with different letters are significantly ($p < 0.05$) different across treatments; * denotes a difference in subtypes within a given treatment.

Discussion

The results of this study indicate that GH and PRL stimulate IGF-1 and IGF-2 expression. This expression is mediated through the ERK, PI3K/Akt, and JAK2-STAT5 signaling pathways. These findings support our starting hypothesis that the ERK, PI3K/AKT, and JAK2-STAT5 signal pathways mediate GH and PRL stimulated expression of IGF-1 and IGF-2. These findings provide new insight into the mechanisms that underlie organismal growth as well as coordinating GH and PRLs multitude of actions.

GH was previously shown to stimulate hepatic IGF-1 expression and to increase levels of IGF-1 in the plasma of several species of fish, including carp, coho salmon, rainbow trout, and tilapia (Very et al., 2008; Wood et al., 2005). GH has also been shown to increase IGF-1 expression in rainbow trout hepatocytes (Reindl et al., 2011) and increase IGF-2 expression in multiple species of fish including salmonids (Shamblot et al., 1995; Pierce et al., 2010). The present results confirm the effects of GH on IGF-1 and IGF-2 expression in isolated trout hepatocytes incubated in vitro and in vivo in both liver and muscle, and support our starting hypothesis. PRL has also been shown to increase IGF-1 levels in rats (Hill et al., 1977) and IGF-2 levels in bats (Viengchareun et al., 2008). Our results also confirm the effects of PRL on IGF-1 and IGF-2 expression in isolated trout hepatocytes incubated in vitro, which supports our starting hypothesis. SL was shown to have no effects on either IGF-1 or IGF-2 expression supporting our original hypothesis that SL would have no effect on IGF-1 and IGF-2 expression. This is supported by the fact that SL is less involved in growth regulation than the other somatotropins. These findings confirm the mechanisms through which GH and PRL exerts their growth-promoting actions and provide insight into the signaling pathways that may underlie the many other actions of GH and PRL in fish.

The role of the ERK pathway in GH and PRL-stimulated IGF-1 and IGF-2 production was supported by several observations. GH and PRL directly induced the phosphorylation of ERK1/2 in trout hepatocytes. Blockade of the ERK pathway with U0126 also partially inhibited hepatic expression of IGF-1 and IGF-2. These findings are consistent with those in mammals in which GH-induced activation of the ERK pathway and the activation of IGF-1 transcription (Argetsinger and Carter-Su 1996; Kopchick and Andry 2000; Piwien-Pilipuk et al., 2002), as well as with PRL stimulated ERK phosphorylation in human endometrium natural killer cells (Gubbay et al., 2002). Because U0126 only partially blocked GH-induced IGF-1 production in trout hepatocytes, mechanisms other than the ERK pathway could be operating to influence the growth-promoting actions of GH.

The role of the PI3K/Akt pathway in GH and PRL-stimulated IGF-1 and IGF-2 production also was supported by several observations. GH and PRL directly induced the phosphorylation of Akt in trout hepatocytes. Blockade of the PI3K/Akt pathway with the selective PI3K inhibitor LY294002 partially inhibited hepatic expression of IGF-1 and IGF-2. For GH-stimulated IGF expression, the effect of PI3K inhibition was greater than Akt inhibition, suggesting the involvement of a downstream target of PI3K other than Akt. Collectively, these findings are consistent with those in mammals in which GH-induced activation of the PI3K/Akt pathway and the activation of IGF-1 transcription (Argetsinger and Carter-Su 1996; Kopchick and Andry 2000; Piwien-Pilipuk et al., 2002) as well with PRL stimulation of PI3K/Akt pathway in human breast cancer lines (Richert et al., 2001). As was the case with ERK blockade of GH- and PRL-induced IGF-1 and IGF-2 production in trout hepatocytes, PI3K/Akt blockade was not complete, which suggested that yet other pathways could be operating to influence the growth-promoting actions of GH.

The role of the JAK-STAT pathway in GH-stimulated IGF-1 production also was supported by several observations. GH and PRL directly induced the phosphorylation of JAK2 and STAT5 in trout hepatocytes. Blockade of the JAK-STAT pathway with JAK2 specific inhibitor Hex completely inhibited hepatic expression of IGF-1 and IGF-2. That GH-stimulated IGF-1 production was dependent, at least in part, on STAT5 was confirmed by the observation that the STAT5-specific inhibitor NICO partially blocked GH- and PRL-stimulated IGF-1 and IGF-2 expression. Together, these findings are consistent with those in mammals that demonstrated the role of JAK2 in the activation of STAT5 and the subsequent role of STAT5 in stimulating IGF-1 transcription (Argetsinger and Carter-Su 1996; Kopchick and Andry 2000; Piwien-Pilipuk et al., 2002) as well as with PRL stimulation of JAK2 and STAT5 (Campbell et al., 1994; Pezet et al., 1997). By observing and combining the roles of each signaling pathway studied, we were able to come up with a model summarizing the mechanisms behind the actions of GH and PRL (Fig. 13). Rainbow trout are known to possess multiple differentially regulated GHR receptors (Very et al., 2005; Walock et al., 2014) as well as a PRLR (Rouzic et al., 2001). For this study individual GHRs or GHR/PRLR were not studied for their relationship to downstream signaling, so we can't assume which receptor is being bound. Knowing that GHRs have differential expression and are differentially regulated (Very et al., 2005; Walock et al., 2014) would make isolating the binding and possible differential effects of each individual receptor type an interesting possible avenue of future studies.

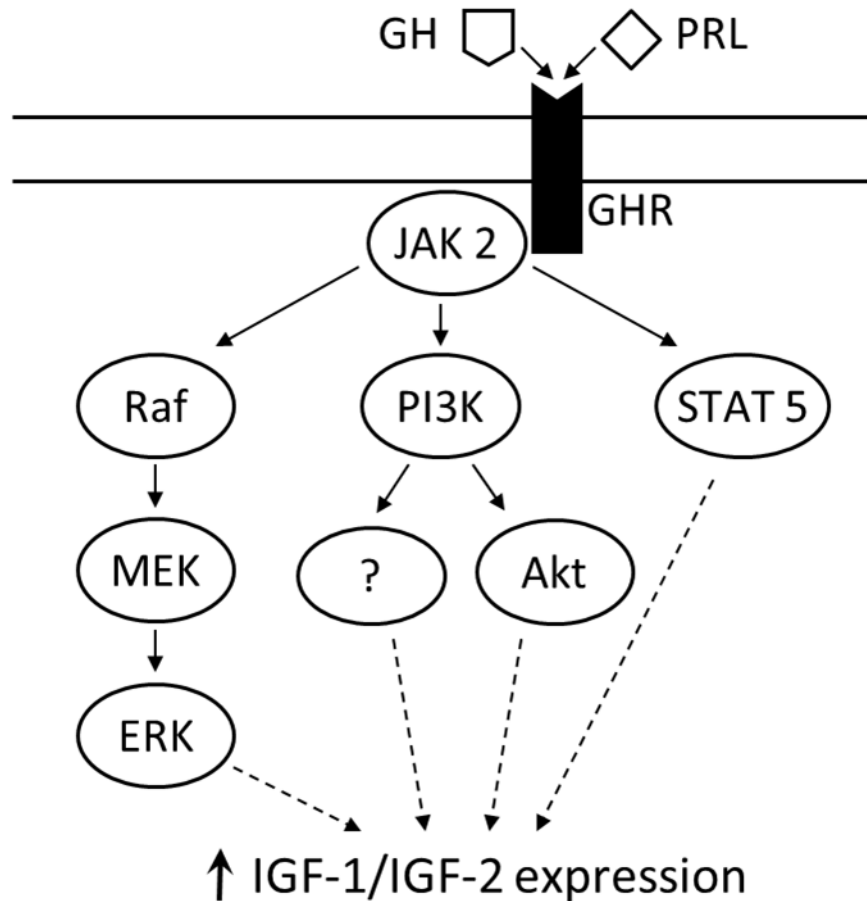


Fig. 13. Model of signaling events associated with GH- and PRL-stimulated IGF-1 and IGF-2 expression in hepatocytes of rainbow trout. JAK2 is activated following binding of GH and PRL to the GH receptor (GHR). The activation of JAK2 is important for the continuation of downstream signaling through ERK, PI3K/Akt, and STAT5, all of which mediate the actions of GH and PRL on IGF-1 and IGF-2 expression.

In summary we found that somatotropin family hormones are able to stimulate growth via both IGF-1 and IGF-2. GH was able to stimulate IGF-1 and IGF-2 expression both *in vivo* in liver and muscle and well as in *in vitro* in isolated hepatocytes. PRL was able to stimulate IGF-1 and IGF-2 expression *in vitro* in isolated hepatocytes, while SL was found to have no effect on IGF-1 or IGF-2 expression. Specifically, GH and PRL stimulated IGF-1 and IGF-2 expression in a time and dose dependent manner. This effect was found to be elicited through the ERK, PI3K/Akt, and JAK2-STAT5 signaling pathways. The fact that the GH and PRL stimulated IGF

expression is mediated through multiple signaling pathways illustrates the possibility of multiple ways to differentially regulate GH and PRLs many actions including feeding, metabolism, reproduction, osmoregulation, immune function and behavior, organismal growth (Bjornsson et al., 2004; Forsyth and Wallis 2002; Moller and Jorgensen 2009; Norbeck et al., 2007; Norrelund 2005; Maetz 1970; Rynikova et al., 1988; Vilalba et al., 1991; Blum and Fiedler 1965; Dunaif et al., 1982; Nagy et al., 1983). These multiple signaling pathways also give possible ways for the cells in an organism to adjust to changes in its environment.

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References

- Argetsinger, L.S., Carter-Su, C. 1996. Mechanism of signaling by growth hormone receptor. *Physiol Rev* 76: 1089–1107.
- Ayson, F.G., de Jesus-Ayson E.G., Takemura, A. 2007. mRNA expression patterns for GH, PRL, SL, IGF-I and IGF-II during altered feeding status in rabbitfish, *Siganus guttatus*. *Gen. Comp. Endocrinol.* 150 196–204.
- Baumann, G., Amburn, K., Shaw, M.A. 1998. The circulating growth hormone binding protein complex: a major constituent of plasma growth hormone in man. *Endocrinol.* 122: 976–984.

- Bjornsson, B.Th., Johansson, V., Benedet, S., Einarsdottir, I.E., Hildahl, J., Agustsson, T., Jonsson, E. 2004. Growth hormone endocrinology of salmonids: regulatory mechanisms and mode of action. *Fish Physiol Biochem* 27: 227–242.
- Blum, V., Fiedler, K. 1965. Hormonal control of reproductive behavior in some cichlid fish. *Gen Comp Endocrinol* 5:186–196.
- Butler, A.A., LeRoith, D.L. 2001. Control of growth by the somatotrophic axis: growth hormone and the insulin-like growth factors have related and independent roles. *Ann Rev Physiol* 63: 141–164.
- Calduch-Giner, J.A., Pendon, C., Valdivia M.M., Perez-Sanchez, J. 1998. Recombinant somatolactin as a stable and bioactive protein in a cell culture bioassay: development and validation of a sensitive and reproducible radioimmunoassay. *Journal of Endocrinol.* 156 441–447.
- Campbell, G.S., Argetsinger, L.S., Ihle, J.N., Kelly, P.A., Rillema, J.A., Carter-Su, C. 1994. Activation of JAK2 tyrosine kinase by prolactin receptors in Nb2 cells and mouse mammary gland explants. *Proc Natl Acad Sci USA* 91:5232–5236.
- Carnevali, O., Cardinali, M., Maradonna, F., Parisi, M., Olivotto, I., Polzonetti-Magni, A.M., Mosconi, G., Funkenstein, B. 2005. Hormonal regulation of hepatic IGF-I and IGF-II gene expression in the marine teleost *Sparus aurata*. *Molecular Reproduction and Development* 71 12–18.
- Duan, C., Xu, Q. 2005. Roles of insulin-like growth factor (IGF) binding proteins in regulating IGF actions. *Gen Comp Endocrinol* 142: 44–52.

- Dunaif, A.E., Zimmerman, E.A., Friesen, H.G., Frantz, A.G. 1982. Intracellular localization of prolactin receptor and prolactin in the rat ovary by immunocytochemistry. *Endocrinol.* 110:1465–1471.
- Eppler, E., Berishvili, G., Mazel, P., Caelers, A., Hwang, G., Maclean, N., Reinecke, M. 2010. Distinct organ-specific up- and down-regulation of IGF-I and IGF-II mRNA in various organs of a GH-overexpressing transgenic Nile tilapia. *Transgenic Research* 19 231–240.
- Forsyth, I. A., Wallis, M. 2002. Growth hormone and prolactin molecular and functional evolution. *J Mammary Gland Biol Neoplasia* 7: 291–312.
- Fukamachi, S., Meyer, A. 2007. Evolution of receptors for growth hormone and somatolactin in fish and land vertebrates: lessons from the lungfish and sturgeon orthologues. *J Mol Evol* 65: 359–372.
- Fukamachi, S., Yada, T., Meyer, A., Kinoshita, M. 2009. Effects of constitutive expression of SL on skin pigmentation in medaka. *Gene* 442 81–87.
- Gubbay, O., Critchley, H.O.D., Bowen, J.M., King, A., Jabbour, H.N. 2002. Prolactin induces ERK phosphorylation in epithelial and CD56(+) natural killer cells of the human endometrium. *J Clin Endocrinol Metab* 87:2329–2335.
- Holly, J.M.P. 1998. Editorial: the IGF-II enigma. *Growth Hormone and IGF Research* 8 183–184.
- Hill, D.J., Francis, M.J., Milner, R.D. 1977. Action of rat prolactin on plasma somatomedin levels in the rat and on somatomedin release from perfused rat liver. *J Endocrinol* 75:137–143.
- Humbel, R.E. 1990. Insulin-like growth factors I and II. *European Journal of Biochemistry* 190 445–462.

- Johnson, L.L., Norberg, B., Willis, M.L., Zebroski, H. Swanson, P. 1997. Isolation, characterization, and radioimmunoassay of Atlantic halibut somatolactin and plasma levels during stress and reproduction in flatfish. *Gen. Comp. Endocrinol.* 105 194–209.
- Jones, J.I. Clemmons, D.R. 1995. Insulin-like growth factors and their binding proteins: biological actions. *Endocrine Reviews* 16 3–34.
- Kakizawa, S., Kaneko, T., Hasegawa, S. Hirano, T. 1995. Effects of feeding, fasting, background adaptation, acute stress, and exhaustive exercise on the plasma somatolactin concentrations in rainbow trout. *Gen. Comp. Endocrinol.* 98 137–146.
- Kakizawa, S., Kaneko, T. Hirano, T. 1996. Elevation of plasma somatolactin concentrations during acidosis in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Bio.* 199 1043–1051.
- Kelly, P.A., J. Djiane, M.C. Postel-Vinay, M. Edery. 1991. The prolactin/growth hormone receptor family. *Endocrinol. Rev.* 12:235-251.
- Kopchick, J.J., Andry, J.M. 2000. Growth hormone (GH), GH receptor and signal transduction. *Mol Genet Metab* 71: 293–314.
- Kossiakoff A. A. and de Vos A. 1999. Structural basis for cytokine hormone-receptor recognition and receptor activation. *Adv. Protein Chem.* 52:67–108.
- Laviola, L., Natalicchio, A., Giorgino, F. 2007. The IGF1 signaling pathway. *Curr Pharm Des* 13: 663–669.
- Malkuch, H., Walock, C., Kittilson, J.D., Raine, J., Sheridan, M.A. 2008. Differential expression of preprosomatostatin- and somatostatin receptor-encoding mRNAs in association with the growth hormone-insulin-like growth factor system during embryonic development of rainbow trout (*Oncorhynchus mykiss*). *Gen Comp Endocrinol* 159: 136–142.

- Moller, N., Jorgensen, J.O.L. 2009. Effects of growth hormone on glucose, lipid, and protein metabolism in human subjects. *Endocr Rev* 30: 152–177.
- Mommsen, T.P., Moon, T.W., Walsh, P.J. 1994. Hepatocytes: isolation, maintenance, and utilization. In: *Biochem Mol Bio Fish*, edited by Hochachka PW, Mommsen TP. New York: Elsevier Science, p. 355-373.
- Moriyama, S., Yamaguchi, K., Takasawa, T., Chiba, H., Kawauchi, H. 2008. Identification of two insulin-like growth factor IIs in the Japanese eel, *Anguilla japonica*: cloning, tissue distribution, and expression after growth hormone treatment and seawater acclimation. *Comp. Biochem. Phys.* 149B 47–57.
- Nagano, M., Kelly, P.A. 1994. Tissue distribution and regulation of rat prolactin receptor gene expression: quantitative analysis by polymerase chain reaction. *J Biol Chem* 269:13337–13345.
- Nagy, E., Berczi, I., Friesen, H.G. 1983. Regulation of immunity in rats by lactogenic and growth hormones. *Acta Endocrinol (Copenh)* 102:351–357.
- Norbeck L.A., Kittilson J.D., Sheridan M.A. 2007. Resolving the growth-promoting and metabolic effects of growth hormone: differential regulation of GH-IGF-I system components. *Gen Comp Endocrinol* 151: 332–341.
- Norrelund, H. 2005. The metabolic role of growth hormone in humans with particular reference to fasting. *Growth Horm IGF Res* 15: 95–122.
- Peterson, B.C., Waldbieser, G.C., Bilodeau, L. 2004. IGF-I and IGF-II mRNA expression in slow and fast growing families of USDA103 channel catfish (*Ictalurus punctatus*). *Comp. Biochem. and Phys.* 139A 317–323.

- Pezet, A., Ferrag, F., Kelly, P.A., Edery, M. 1997. Tyrosine docking sites of the rat prolactin receptor required for association and activation of Stat5. *J Biol Chem* 272:25043–25050.
- Picha, M.E., Turano, M.J., Tipsmark, C.K., Borski, R.J. 2008. Regulation of endocrine and paracrine sources of Igfs and GH receptor during compensatory growth in hybrid striped bass (*Morone chrysops* × *Morone saxatilis*). *J. Endocrinol.* 199 81–94.
- Pierce, A., Dickey, J., Felli, L., Dickhoff, W., 2010 Metabolic hormones regulated basal and growth hormone-dependent igf2 mRNA level in primary cultured coho salmon hepatocytes: effects of insulin, glucagon, dexamethasone, and triiodothyronine. *J. Endocrinol.* 204:331-9
- Piwien-Pilipuk, G., Huo, J.S., Schwartz, J. 2002. Growth hormone signal transduction. *J Pediatr Endocrinol Metab* 15: 771–786.
- Rand-Weaver, M., Pottinger, T.G., Sumpter, J.P. 1993. Plasma somatolactin concentrations in salmonid fish are elevated by stress. *J. Endocrinol.* 138 509–515.
- Reindl, K.M., Kittilson, J.D., Sheridan, M.A. 2009. Differential ligand binding and agonist-induced regulation characteristics of the two rainbow trout GH receptors, GHR1 and GHR2, in transfected cells. *J Endocrinol* 202: 463–471.
- Reindl, K.M., Kittilson, J.D., Bergan, H.E., Sheridan M.A. 2011. Growth hormone-stimulated insulin-like growth factor-1 expression in rainbow trout (*Oncorhynchus mykiss*) hepatocytes is mediated by ERK, PI3K-AKT, and JAK-STAT. *Am. J. Physiol.*, 301: R236–R243.
- Reinecke, M., Bjornsson, B.Th., Dickhoff, W.W., McCormick, S.D., Navarro, I., Power, D.M., Gutierrez, J. 2005. Growth hormone and insulin-like growth factors in fish: where we are and where to go. *Gen Comp Endocrinol* 142: 20–24.

- Reinecke, M., Collet, C. 1998. The phylogeny of the insulin-like growth factors. *International Review of Cytology* 183 1–94.
- Richert, M.M., Decker, K., Anderson, S.M., 2001. Mechanisms underlying constitutive activation of Akt in breast cancer cell lines. p 551. Program of the 83rd Annual Meeting of The Endocrine Society; Denver, CO. P3–478.
- Rouzic, P. L., Olivier, S., Jeanne, G., Françoise, R., Olivier, J., Michel, T., Farzad, P. 2001. Evidence of Rainbow Trout Prolactin Interaction with Its Receptor through Unstable Homodimerisation. *Mole Cell Endocrinol.* 172, no. 1–2: 105–13.
- Rynikova, A., Koppel, J., Kuchar, S., Cikos, S., Mozes, S. 1988. Effects of ovine prolactin in infant rats. *Exp Clin Endocrinol* 92:241–244
- Shamblott, M., Cheng, C. Bolt, D., Chen, T.T. 1995. Appearance of insulin-like growth factor mRNA in the liver and pyloric ceca of a teleost in response to exogenous growth hormone. *Proc. Natl. Acad. Sci. USA* 92:6943-6946.
- Terova, G., Rimoldi, S., Chini, V., Gornati, R., Bernardini, G., Saroglia M. 2007. Cloning and expression analysis of insulin-like growth factor I and II in liver and muscle of sea bass (*Dicentrarchus labrax*, L.) during long-term fasting and refeeding. *Journal of Fish Biology* 70 219–233.
- Tse, M.C., Vong, Q.P., Cheng, C.H., Chan, K.M. 2002. PCR-cloning and gene expression studies in common carp (*Cyprinus carpio*) insulin-like growth factor-II. *Biochimica et Biophysica Acta* 1575 63–74.
- Very, N.M., Kittilson, J.D., Klein, S., Sheridan, M.A. 2008. Somatostatin inhibits basal and growth hormone-stimulated insulin-like growth factor-I production. *Mol. Cell. Endocrinol.* 281: 19-26.

- Very, N.M., Kittilson, J.D., Norbeck, L.A., Sheridan M.A. 2005. Isolation, characterization, and distribution of two cDNAs encoding for growth hormone receptor in rainbow trout (*Oncorhynchus mykiss*) *Comp. Biochem. Physiol. B*, 140 pp. 615–628.
- Viengchareun, S., Serval, N., Feve, B., Freemark, M., Lombes, M., Binart, N. 2008. Prolactin receptor signaling is essential for perinatal brown adipocyte function: a role for insulin-like growth factor-2. *PLoS ONE* 3:e1535.
- Villalba, M., Zabala, M.T., Martinez-Serrano, A., de la Colina, R., Satrustegui, J., Garcia-Ruiz, J.P. 1991. Prolactin increases cytosolic free calcium concentration in hepatocytes of lactating rats. *Endocrinol* 129:2857–2861
- Wallis, M. 1992. The expanding growth hormone/prolactin family. *J. Mol. Endocrinol.* 9:185–188.
- Walock, C.N., Kittilson, J.D., Sheridan M.A. 2014. Characterization of a novel growth hormone receptor-encoding cDNA in rainbow trout and regulation of its expression by nutritional state. *Gene* 533:286–294
- White, Y.A., Kyle, J.T., Wood, A.W. 2009. Targeted gene knockdown in zebrafish reveals distinct intraembryonic functions for insulin-like growth factor II signaling. *Endocrinol* 150 4366–4375.
- Wood, A.W., Duan, C., Bern, H.A. 2005. Insulin-like growth factor signaling in fish. *Int Rev Cytol* 243: 215–285.
- Zhu, Y. Thomas, P. 1997. Effects of somatolactin on melanosome aggregation in the melanophores of red drum (*Sciaenops ocellatus*) scales. *General and Gen. Comp. Endocrinol* 105.

CHAPTER 4: THE ROLE OF NUTRITIONAL STATE AND SERUM TREATMENT IN GROWTH HORMONE MEDIATED INSULIN-LIKE GROWTH FACTOR AND GROWTH HORMONE RECEPTOR EXPRESSION

Abstract

Growth hormone (GH) regulates a wide array of actions including growth, metabolism, and lipolysis. These disparate actions often take place in the same tissues but are associated with different nutritional availability such as growth in times of plenty and lipolysis in times of fasting. In this study, we isolated hepatocytes from both fed and fasted rainbow trout (*Oncorhynchus mykiss*) to test the role of nutritional state on the actions of GH including insulin-like growth factor (IGF) and growth hormone receptor (GHR) expression and which signaling pathways GH is working through. Additionally we tested the role of serum in changing cells responsiveness to GH by pretreating these cells with 10% serum from blood of fish either fed or fasted. Our hypothesis is that the growth promoting actions of GH, including increased IGF expression, will only be observed in fed fish and that blood serum will mediate this change in cell responsiveness. As expected, GH-stimulated IGF expression was only observed in cells from fed fish and not those from fasted fish. This action was found to be through the ERK, JAK/STAT, and PI3K/Akt signaling pathways because inhibiting those pathways blocked GH-stimulated IGF expression. Similar results were not observed with GHR expression; nutritional state was observed to have minimal effect on GH-stimulated GHR expression. Serum was found to change cells responsiveness to GH in regards to IGF but not GHR expression. While GH was unable to stimulate IGF expression in fasted cells, pretreatment of these fasted cells with serum from fed cells allowed these cells to change responsiveness to GH, showing an increase in GH-

stimulated IGF expression as seen in fed cells. These findings support the role of nutritional state and more specifically serum in regulating the disparate actions of GH.

Introduction

Growth hormone (GH) is a widely studied hormone with a wide array of actions including but not limited to growth, feeding, metabolism, reproduction, osmoregulation, immune function and behavior (Bjornsson et al., 2004; Forsyth and Wallis 2002; Moller and Jorgensen 2009; Norbeck et al., 2007; Norrelund 2005). Growth hormone is a part of a wider somatotropin family of hormones including Somatolactin (SL) and Prolactin (PRL) (Kossiakoff and Vos 1999; Wallis 1992). The main growth regulation action of GH occurs primarily through the GH insulin-like growth factor (IGF) pathway. In short, GH released from the pituitary circulates and stimulates IGF production primarily in the liver and muscle which then goes on to promote organismal growth (LeRoith et al., 2001). This GH-stimulated IGF expression is done through the JAK–STAT, PI3K–Akt, and ERK signaling pathways (Reindl et al., 2011). While growth is primarily associated with times of feeding and nutritional availability, many other actions of GH are not; more are associated with fasting such as lipolysis (Sheridan 1994; Bergan et al., 2013). These disparate actions suggest that cells are modulated to perform both actions.

Fasting is known to play a large role in many different aspects of the GH IGF pathways. During times of fasting, rainbow trout first demonstrate decreases in weight and condition (Norbeck et al., 2007; Sheridan and Mommsen, 1991). This decrease in growth is observed despite the fact that fasting causes an increase in GH plasma levels in many species (Gomez-Requeni et al., 2005; Norbeck et al., 2007; Picha et al., 2008; Reinecke, 2010). Fasting also leads to other changes in plasma including decreased levels of insulin and IGF-1 (Gomez-Requeni et al., 2005; Norbeck et al., 2007). Fasting has also been shown to decrease hepatic growth

hormone receptor (GHR) levels (Small et al., 2006; Norbeck et al., 2007) while causing increase in GHR levels in muscle (Pierce et al., 2007) and in adipose tissue (Norbeck et al., 2007). These changes taken together suggest that the programming and sensitivity of cells to GH changes in different nutritional states and depend on the type of cell.

While much is known about the actions of GH and their signaling pathways (Waters et al., 2006), much more is still unknown about the growth promoting actions of GH in differing nutritional states and how this differs from the lipolytic actions of GH (Bergan et al., 2015). In this study, we used hepatocytes isolated from continuously fed and 4 week fasted rainbow trout (*Oncorhynchus mykiss*) as a model to study the growth-related actions of GH including IGF expression and GHR expression. In addition, serum from each of these groups of fish was isolated and used to treat cells to see if the serum might be the mechanism that accounts for the disparate actions of GH across different nutritional states. My hypothesis is that the growth promoting actions of GH, including increased IGF-1 and IGF-2 expression will only be observed in fish fed continuously and that the nutritional state of the blood serum will program cells to respond to GH by either allowing or inhibiting these growth promoting actions.

Materials and methods

Materials

All chemicals and reagents used were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise. Salmonid GH was generously provided by Prof. Akiyoshi Takahashi and Dr. Shunsuke Moriyama (Kitasato University, Japan).

Experimental animals and conditions

Juvenile rainbow trout of both sexes (*ca.* 1 year of age) were obtained from Dakota Trout Ranch (Carrington, ND). The animals were transported to North Dakota State University, where

they were maintained in well-aerated, 800-L circular tanks supplied with recirculated (10% make-up volume per day) dechlorinated municipal water at 14°C under a 12:12 hour light:dark photoperiod. Fish were fed twice daily to satiety with AquaMax Grower (PMI Nutrition International, Brentwood, MO, USA) semi-floating trout grower, until commencing experiments where fish were fasted continuously for 4 weeks or fed for 4 weeks until 2 hours before experimental manipulations. Animals were acclimated to laboratory conditions for at least 4 weeks prior to experimentation. All procedures were performed in accordance with the *Guide for Care and Use of Laboratory Animals* (National Research Council, Washington, DC) and were approved by the North Dakota State University Institutional Animal Care and Use Committee.

Hepatocyte isolation and serum collection

At the time of sampling, fish were anaesthetized in 0.05% (v/v) 2-phenoxyethanol, measured (body weight and fork length) and euthanized by transection of the spinal cord. In some fish, whole blood was collected by caudal venipuncture (Galt et al., 2014), incubated at 4°C for 1 hour to allow clotting, then centrifuged (11,000 g, 3 min) to secure only the serum and stored at -80° C for later analysis. In other fish, hepatocytes were isolated by *in situ* perfusion (Mommsen et al., 1994). The isolated cells were incubated in recovery medium [in mM: 137.8 NaCl, 5.4 KCl, 0.80 MgSO₄, 0.4 KH₂PO₄, 0.34 Na₂HPO₄, 4.2 NaHCO₃, and 10 HEPES, 0.65 glucose, pH 7.6, with 2% defatted BSA, 2 ml MEM amino acid mix (50X)/100 ml, and 1 ml nonessential amino acid mix (100X)/100 ml] for 2 h at 14°C with gyratory shaking (100 rpm under 100% O₂). The viability of the cells was assessed by trypan blue dye exclusion and ranged between 93-97% for all experiments. After the recovery period, hepatocytes were collected by centrifugation (550 g for 8-10 min) and resuspended in incubation media (recovery media with

1.5 mM CaCl₂) to a final concentration of 2.3 x10⁶ cells/ml and aliquoted into 24-well plates (2.3 x10⁶ cells/well).

Previous nutritional state experiment

Previous nutritional state experiments were designed and executed with (Bergan et al., 2015). In this experiment, cells were incubated in medium alone (control) or in medium with GH as specified in the figure legends under the same conditions as those used for recovery (14°C with gyratory shaking at 100 rpm under 100% O₂). In combination experiments involving pathway inhibition, inhibitors were added 2 h prior to GH treatment at concentrations specifically recommended by the manufacturer and/or used by us previously (Reindl et al., 2011) as follows: 20 µM LY294002, 10 µM U0126, 50 µM Hex, 10 µM chelerythrine chloride, and 10 µM U73122 . After incubation, cells were pelleted (1,000 x g for 4 min) and the supernatant medium was removed. Cells were washed with 0.5 ml phosphate-buffered saline. Samples were immediately frozen on dry ice then stored at -80°C until further analysis.

Cross nutritional state experiment

Cross nutritional state experiments were designed and executed with (Bergan-Roller et al., 2017). For this experiment, cells were incubated in serum from fed or fasted fish with or without GH under the same conditions as those used for recovery (14°C with gyratory shaking at 100 rpm under 100% O₂). Concentrations of 10% serum and 100 ng/ml of GH were used. Serum treatments lasted for 6 h for mRNA expression with or without GH. After incubation, cells were pelleted (1,000 x g for 4 min) and the supernatant medium was removed. Cells were washed with 0.5 ml phosphate-buffered saline. Samples were immediately frozen on dry ice then stored at -80°C until further analysis.

Somatostatin experiments

In this experiment, cells were incubated in medium alone (control) or in medium with SS as specified in the figure legends under the same conditions as those used for recovery (14°C with gyratory shaking at 100 rpm under 100% O₂). In experiments involving pathway inhibition, inhibitors were added 2 h prior to GH and/or SS (100 ng/ml of GH and SS) treatment at concentrations specifically recommended by the manufacturer and used by us previously (Very et al., 2008). After incubation, cells were pelleted (1,000 x g for 4 min) and the supernatant medium was removed. Cells were washed with 0.5 ml phosphate-buffered saline. Samples were immediately frozen on dry ice then stored at -80°C until further analysis.

RNA extraction and analysis

Total RNA was extracted using TRI-Reagent® (Molecular Research Center, Inc., Cincinnati, OH, USA) as specified by the manufacturer's protocol. Each RNA pellet was redissolved in 35–200 µl RNase-free deionized water and quantified by NanoDrop1000 spectrophotometry (A260) (Thermo Scientific, West Palm Beach, FL, USA). RNA samples were then stored at -80 °C until further analysis.

mRNA was reverse transcribed in 5 µl reactions using 150 ng total RNA and AffinityScript QPCR cDNA Synthesis kit reagents (Master Mix, random primers, oligo-dT primers, and reverse transcriptase with block) according to the manufacturer's protocol (Stratagene, La Jolla, CA, USA). Reactions without reverse transcriptase were included as negative controls to exclude the possibility of contamination with genomic DNA; no amplification was detected in negative controls.

Levels of IGF1 and IGF2 mRNAs were determined by quantitative real-time PCR as described previously (Malkuch et al., 2008). Levels of GHR1, GHR2a, and GHR2b were also

determined by quantitative real-time PCR as described by Very et al. (2005) and Walock et al. (2014). Briefly, real-time reactions were carried out for samples, standards, and no-template controls in multiplex reactions with the gene of interest and β -actin. cDNA standards were generated using gene-specific primers; the PCR products were cloned into the pGEM-T Easy Vector, and the sequences were verified. Cross reaction was assessed by substituting alternate primer/probe sets in assays for each standard; no amplification was observed under these conditions. Sample copy number was calculated from the threshold cycle number (CT) and relating CT to a gene-specific standard curve, followed by normalization to β -actin.

Data analysis

Statistical differences were estimated by one-way or two-way ANOVA, as appropriate. In all cases, main effects were significant, and no significant interactions were observed between main effects in two-way ANOVAs. In groups with non-normal distribution, data were log transformed to attain normal distribution. Pairwise comparison of simple effects was assessed by Duncan's multiple range test or by Tukey's range test; statistical notations on the faces of the figures reflect such comparisons. A probability level of 0.05 was used to indicate significance. Statistics were performed using SigmaStat v. 1.0 (SPSS, Chicago, IL, USA) or JMP v.11 (SAS institute Cary, NC, USA) and graphs were constructed with SigmaPlot version 8.0 (SPSS). Quantitative data are expressed as means \pm SEM.

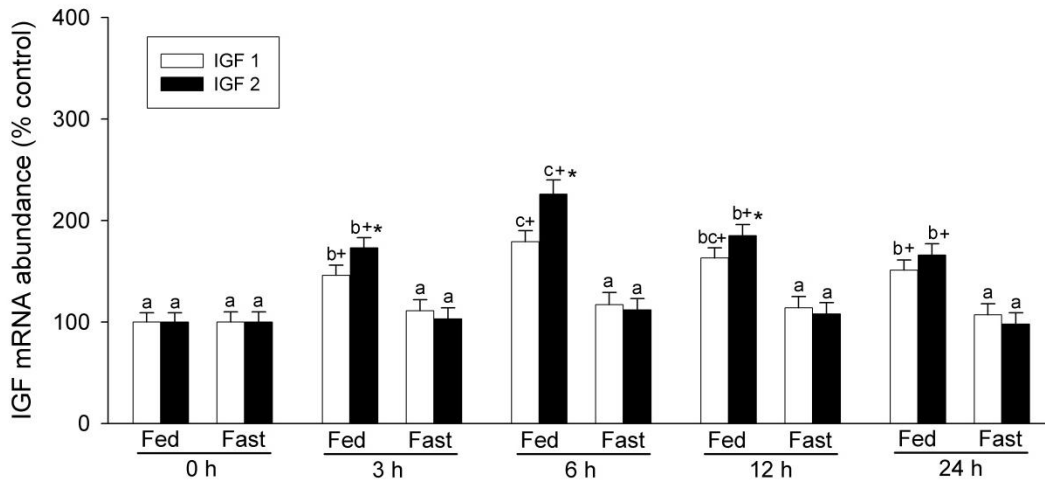
Results

The effect of previous nutritional state on GH-stimulated IGF-1 and IGF-2 expression

The roles of nutritional state on GH-stimulated IGF expression were examined in vitro using isolated hepatocytes from fish either fed continuously or fasted prior to experimentation. GH stimulated the expression of IGF-1 and IGF-2 in fed fish in a time- and concentration-

dependent manner (Fig. 14). Maximal stimulation occurred 6 h treatment for both IGF-1 and IGF-2. After 6 h, expression levels decreased continuously through the full 24 h. In concentration studies, higher concentrations of GH show greater ability to stimulate IGF expression with maximal expression occurring at 100ng/mL GH and staying high at 1000ng/mL. GH was more efficacious in stimulating IGF-2 than IGF-1 showing expression levels higher relative to controls. GH was unable to stimulate IGF-1 and IGF-2 expression in hepatocytes from fasted fish.

A. GH time



B. GH concentration

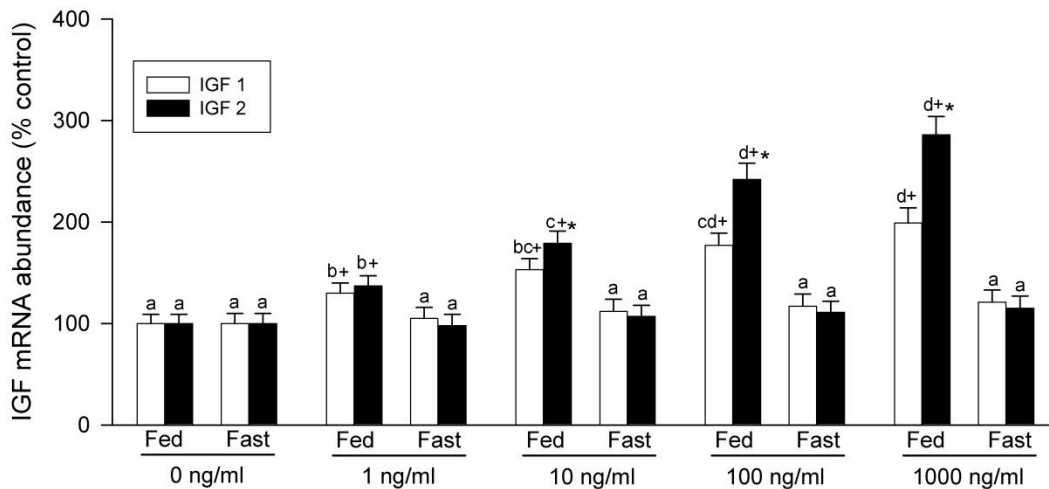


Fig. 14. Growth hormone (GH)-stimulated expression of insulin-like growth factor (IGF) 1 and IGF-2 mRNAs in hepatocytes isolated from rainbow trout fed continuously or fasted for 4 weeks. A: time dependent IGF-1 and IGF-2 mRNA expression in cells incubated for varying times with 100 ng/ml GH. B: dose dependent IGF-1 and IGF-2 mRNA expression in cells incubated for 6 hours in varying concentrations of GH. Data are presented as % control and expressed as means \pm SEM (n=6). Letters denote significant ($P < 0.05$) differences for a given IGF subtype across treatments; * denotes a difference in IGF subtypes within a given treatment.; + indicates significant difference between cells from different nutritional states for a given IGF isoform treated with GH for a given time or at a given concentration.

The effect of previous nutritional state on the linkage of cell signaling elements to GH-stimulated IGF-1 and IGF-2 expression

The linkage of specific cell signaling pathways to GH-stimulated IGF-1 and IGF-2 expression in isolated hepatocytes from fish both previously fasted and fed was studied using pharmacological inhibitors previously shown effective in rainbow trout. As observed previously, GH (100 ng/ml) stimulated IGF-1 and IGF-2 expression in fed cells with GH being more efficacious in stimulating IGF-2 expression (Fig. 15). GH was unable to stimulate IGF expression in fasted cells. Pretreatment of hepatocytes with the JAK2 inhibitor, hex, completely abolished GH-stimulated IGF expression in fed cells. The MEK inhibitor, U0126, and the PI3K inhibitor, LY294002, both partially blocked GH-stimulated IGF expression in fed cells. However, the PKC inhibitor, chelerythrine chloride, and the PLC inhibitor, U73122, had no effect on GH-stimulated IGF expression in fed cells. Signaling pathway inhibitors had no effect on GH-stimulated IGF mRNA expression in fasted fish.

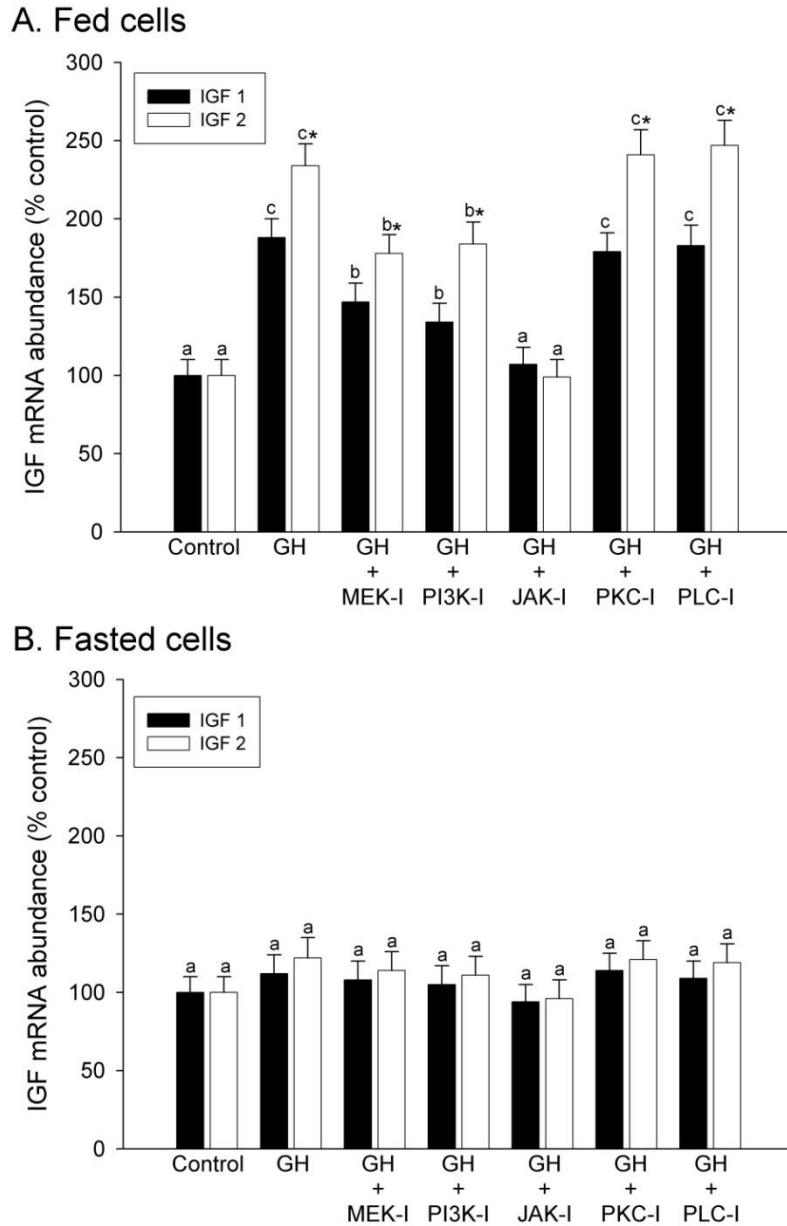


Fig. 15. The effects of signaling element inhibition on growth hormone (GH)-stimulated expression of insulin-like growth factor (IGF) 1 and IGF-2 mRNAs in hepatocytes isolated from rainbow trout fed continuously (A) or fasted for 4 weeks (B). Cells were pretreated for 2 h with or without specific inhibitors for the following signaling elements: MEK (10 μ M U0126), PI3K (10 μ M LY294002), JAK (10 μ M Hex.), PKC (10 μ M chelerythrine chloride), PLC (10 μ M U73122); after which time, cells were treated with 100 ng/ml GH for 6 h. Data are presented as % control and expressed as means \pm SEM (n=6). For a given IGF isoform, groups with different letters are significantly ($p < 0.05$) different. Letters denote significant ($P < 0.05$) differences for a given IGF subtype across treatments. * denotes a difference in IGF subtypes within a given treatment.

The role of blood serum in reprogramming nutritionally regulated GH-stimulated IGF-1 and IGF-2 expression

In an attempt to reprogram cells, and specifically their response to GH, we took hepatocytes from each nutritional state (e.g., fed) and treated them with serum from their opposite nutritional state (e.g., fasted) as well as with serum from their native nutritional state. First we isolated hepatocytes from fish fasted four weeks, and pretreated them with serum isolated from other fish fasted 4 weeks or continuously fed. In fasted cells, pretreatment with serum from fasted cells had no effect on either IGF-1 or IGF-2 expression (Fig. 16). GH was also unable to stimulate either IGF-1 or IGF-2 expression in these same cells as expected. In fasted cells, pretreatment with serum from fed cells caused a slight but non-significant increase in IGF-1 expression but no effect on IGF-2 expression. Thus, pretreating fasted cells with fed serum suggested that these cells were reprogrammed to behave like fed cells showing a significant increase in GH-stimulated IGF-1 and IGF-2 expression.

Isolated hepatocytes were also obtained from continuously fed fish and were pretreated with serum from other fish fasted for 4 weeks or from continuously fed fish. In fed cells, pretreatment with fed serum showed a slight but non-significant decrease in IGF-1 expression (Fig. 16). These results were similar to those observed in fed cells treated with fasted serum, showing a similar non-significant decrease in IGF-1 expression. Treating these cells with GH caused a further slight but non-significant decrease in IGF-1 expression. This decrease was significantly different from no-serum controls.

Serum pretreatment had different results on IGF-2 expression. In fed cells, pretreatment with serum from fed cells had no effect on IGF-2 expression. In fed cells pretreated with fed serum, GH was able to slightly but non-significantly increase IGF-2 expression. The combined

effect of fed serum and GH caused significant increase in IGF-2 expression compared to no-serum controls. Pretreatment of fed cells with fasted serum caused a slight but non-significant increase in IGF-2 expression. In fed cells pretreated with fasted serum, GH surprisingly decreased IGF-2 expression back to levels seen in fed cells with no serum.

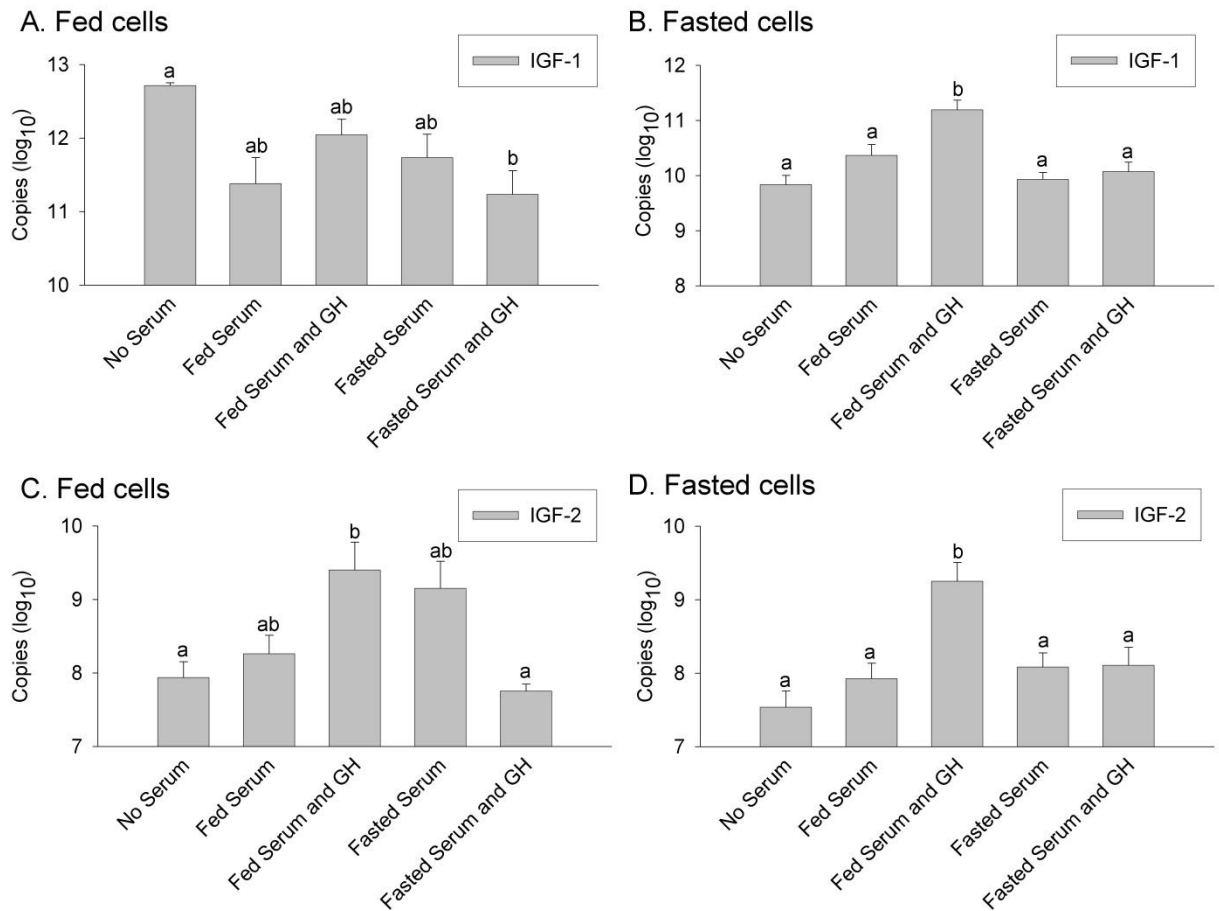


Fig. 16. The effects of serum on growth hormone (GH)-stimulated expression of insulin-like growth factor (IGF) 1 (A and B) and IGF-2 (C and D) mRNAs in hepatocytes isolated from rainbow trout fed continuously (A and C) or fasted for 4 weeks (B and D). Cells were treated for 24 hours with 10% serum harvested from the blood plasma of fed or fasted fish then treated with or without 100 ng/ml of GH for 6 hours. Data are presented as log₁₀ copies and expressed as means \pm SEM (n=6). Letters denote significant ($p < 0.05$) difference within a nutritional state and IGF subtype.

The effect of somatostatin on IGF-2 expression

The role of SS on IGF-2 expression was examined in vitro using isolated hepatocytes from rainbow trout. SS (100 ng/ml) was shown to inhibit IGF-2 expression in a time dependent manner (Fig. 17). Slight but non-significant inhibition was first observed at 6 h. Maximal inhibition of IGF-2 expression was observed at 12 h and the inhibition started to decrease at 24 h. SS inhibition of IGF-2 expression was not found to be significant in a concentration response study.

The linkage of cell signaling elements to SS inhibition of GH-stimulated IGF-2 expression

The linkage of specific cell signaling pathways to the SS inhibition of GH-stimulated IGF-2 expression in isolated hepatocytes was studied using pharmacological inhibitors. GH was first shown to slightly but non significantly increase IGF-2 expression. In this study, the AKT inhibitor, carb, was shown to decrease GH-stimulated IGF-2 expression in combination with SS more than SS on its own. The MEK inhibitor, U0126, with GH was shown to have a combined inhibition slightly but not significantly greater than just SS alone but significantly decreased from GH treated cells (Fig. 17). AKT inhibition was shown to have a greater effect on GH-stimulated IGF-2 expression than MEK inhibition.

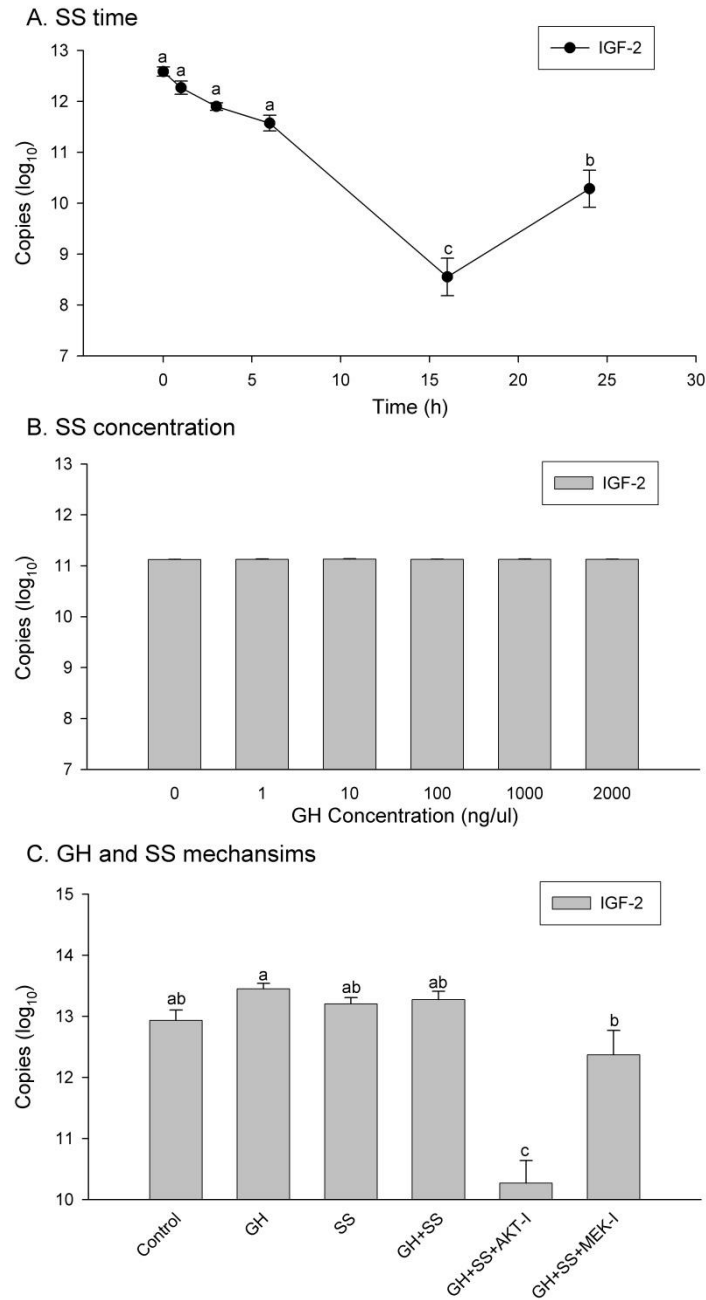


Fig. 17. The effects of somatostatin (SS) on insulin like growth factor (IGF) 2 expression and the role of SS and signaling element inhibition on growth hormone stimulated IGF-2 mRNA expression in hepatocytes isolated from rainbow trout. A: time dependent IGF-2 mRNA expression in cells incubated for varying times with 100 ng/ml SS. B: dose dependent IGF-2 mRNA expression in cells incubated for 6 hours in varying concentrations of SS. C: Hepatocytes were preincubated for 2 h with specific inhibitors for the following signaling elements: MEK (10 μ M U0126=MEK-I) and Akt (10 μ M Carb=Akt-I.) Cells were treated with combinations of 100 ng/ml GH and or SS for 6 h. Data are presented as log₁₀ copies and expressed as means \pm SEM (n=10). Letters denote significant ($p < 0.05$) differences between treatments.

The effect of previous nutritional state on GH-stimulated GHR1, GHR2a, and GHR2b expression

The role of nutritional state on GH-stimulated GHR expression was examined *in vitro* using isolated hepatocytes from fish either fed continuously or fasted prior to experimentation (Fig. 18 and 19). GH treatment was shown to have a positive effect on GHR1 expression in both fed and fasted fish. GH treatment at doses 10ng/ml and 1000ng/ml caused a significant and equal increase in GHR1 expression in fed fish, while 100ng/ml showed slight but non-significant increase (Fig. 18). Fasted fish only saw a slight but non-significant increase in GHR1 expression at the 100 ng/ml GH dose. However this increase was significantly different from 1ng/ml treatments. GH treatment at differing doses was shown to have no effect on either GHR2a or GHR2b in either fed or fasted fish.

The relationship with time based treatments of GH had a more complicated relationship with GHR expression. GH was shown to cause a decrease in GHR1 in a time dependent manner in both fed and fasted fish. In both fed and fasted fish this decrease is seen at 12 and 24 hours. As previously observed, GH treatments had no effect on GHR2a in either fed or fasted fish. However, GH treatment caused a slight but non-significant decrease in GHR2b expression occurring at 12 h and continuing through 24 h in fed fish while fasted fish first saw a slight but non-significant decrease at 12 h and significant decrease at 24 h (Fig. 19).

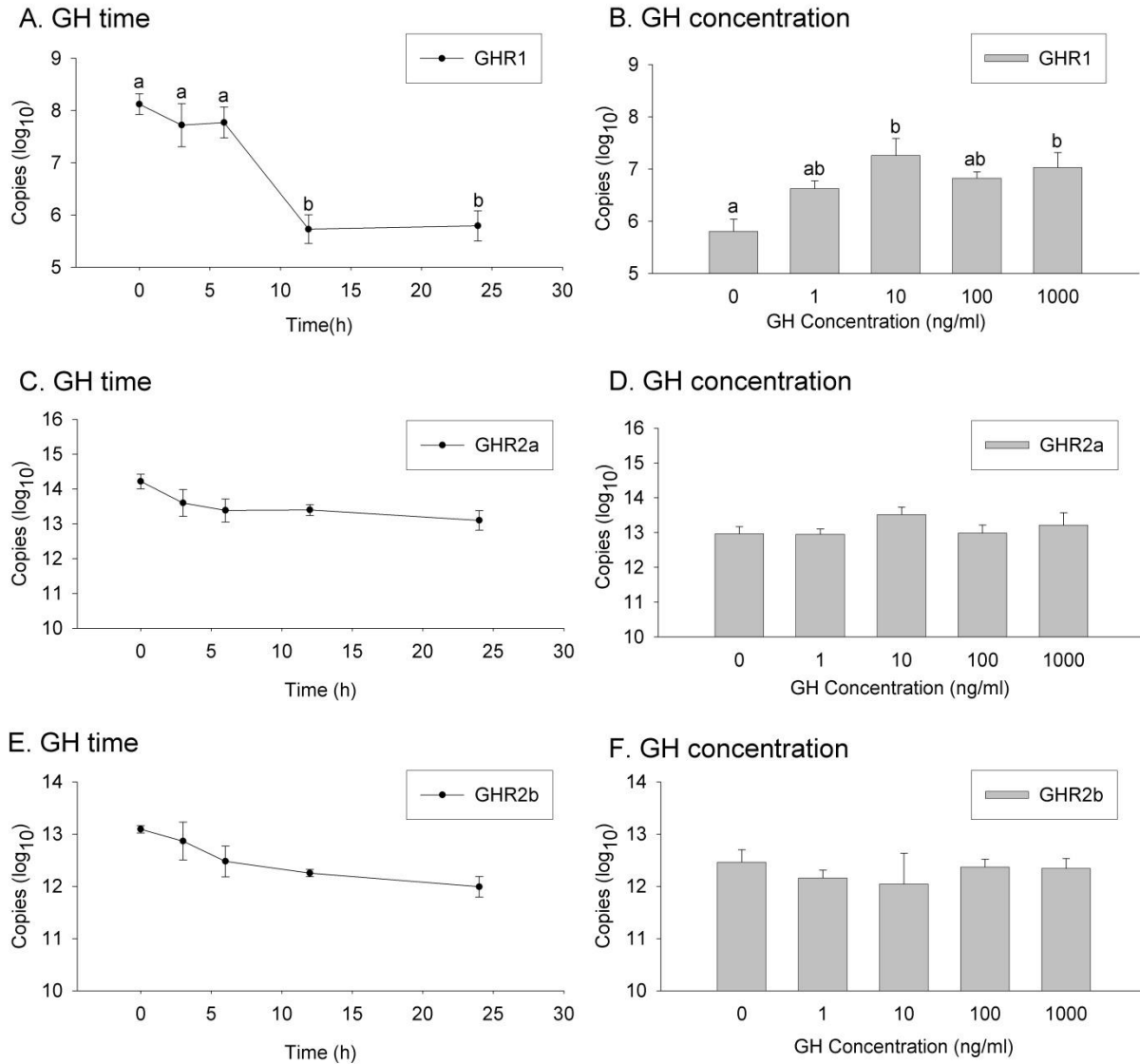


Fig. 18. Growth hormone (GH)-stimulated expression of growth hormone receptor (GHR) 1, GHR2a, and GHR2b mRNAs in hepatocytes isolated from rainbow trout fed continuously. A, C, and E: time dependent GHR1 (A and B), GHR2a, (C and D) and GHR2b (E and F) mRNA expression in cells incubated for varying times with 100 ng/ml GH. B, D, and F: dose dependent GHR1, GHR2a, and GHR2b mRNA expression in cells incubated for 6 hours in varying concentrations of GH. Data are presented as log₁₀ copies and expressed as means \pm SEM (n=6). Letters denote significant ($p < 0.05$) difference within a given treatment and GHR subtype.

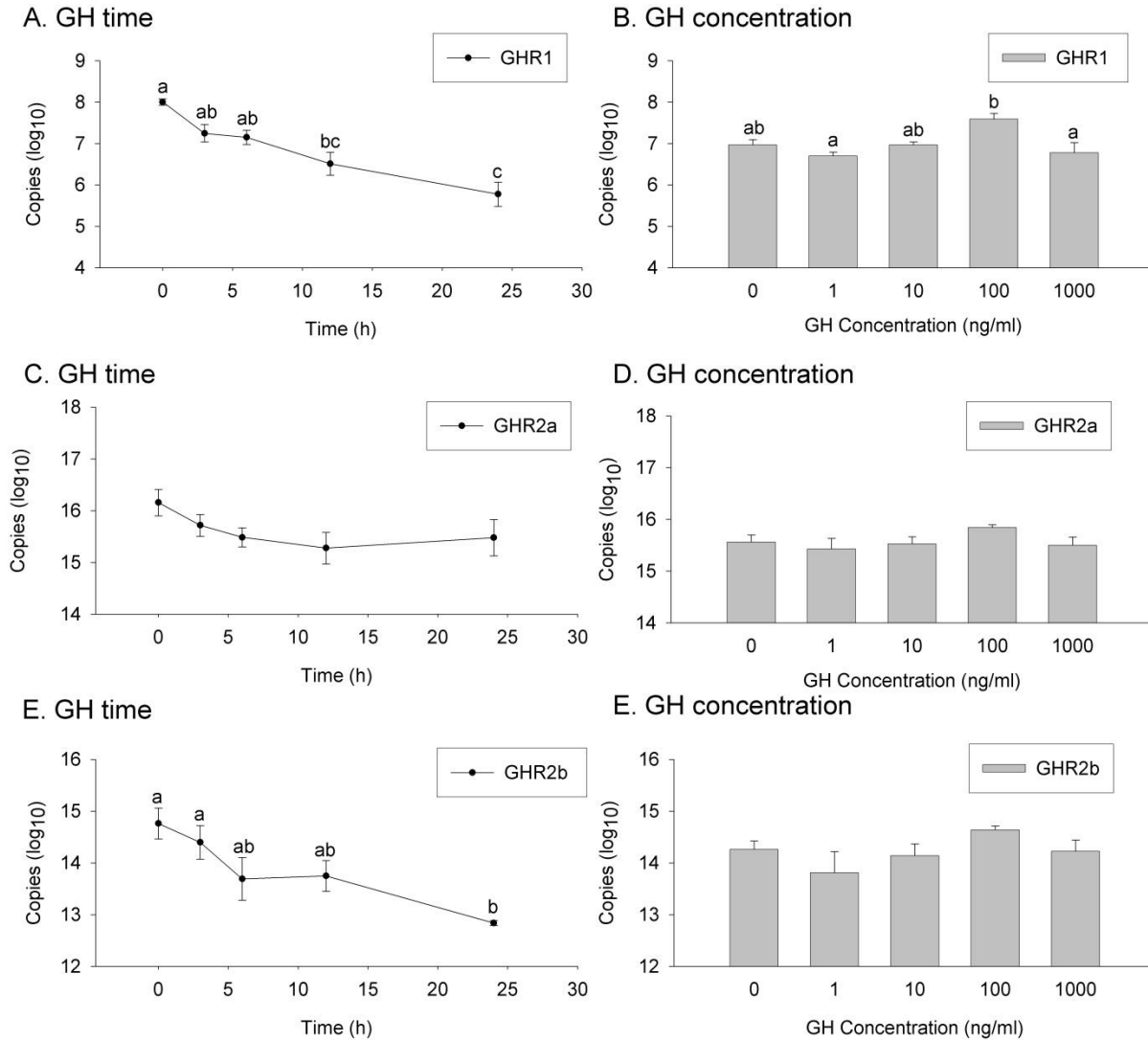


Fig. 19. Growth hormone (GH)-stimulated expression of growth hormone receptor (GHR) 1, GHR2a, and GHR2b mRNAs in hepatocytes isolated from rainbow trout fasted for 4 weeks. A, C, and E: time dependent GHR1 (A and B), GHR2a, (C and D) and GHR2b (E and F) mRNA expression in cells incubated for varying times with 100 ng/ml GH. B, D, and F: dose dependent GHR1, GHR2a, and GHR2b mRNA expression in cells incubated for 6 hours in varying concentrations of GH. Data are presented as log₁₀ copies and expressed as means \pm SEM (n=6). Letters denote significant ($p < 0.05$) difference within a given treatment and GHR subtype.

The effect of previous nutritional state on the linkage of cell signaling elements to GH-stimulated GHR1, GHR2a, and GHR2b expression

The linkage of specific cell signaling pathways to GH-stimulated GHR1, GHR2a, and GHR2b expression in isolated hepatocytes from fish both previously fasted and fed was studied

using pharmacological inhibitors previously shown effective in rainbow trout. GH was shown to have no effect on GHR1 expression in fed fish unlike our earlier observations. As seen before, GH treatment had no effect on GHR2a or GHR2b expression. JAK inhibition caused a significant decrease in GH-stimulated GHR1 expression in fed fish, this difference was significant compared to all groups and other inhibitors. Signaling pathway inhibitors had a very complicated relationship with GH-stimulated GHR2a expression. While no inhibitor alone caused a significant change from GH controls, PLC inhibition both caused a slight but non-significant decrease in GH-stimulated GHR2a expression. In fed fish, MEK inhibition caused a significant increase in GH-stimulated GHR2a expression compared to PLC inhibition. In fed fish, all pharmacological inhibitors had no effect on the GH-stimulated expression of GHR2b (Fig. 20).

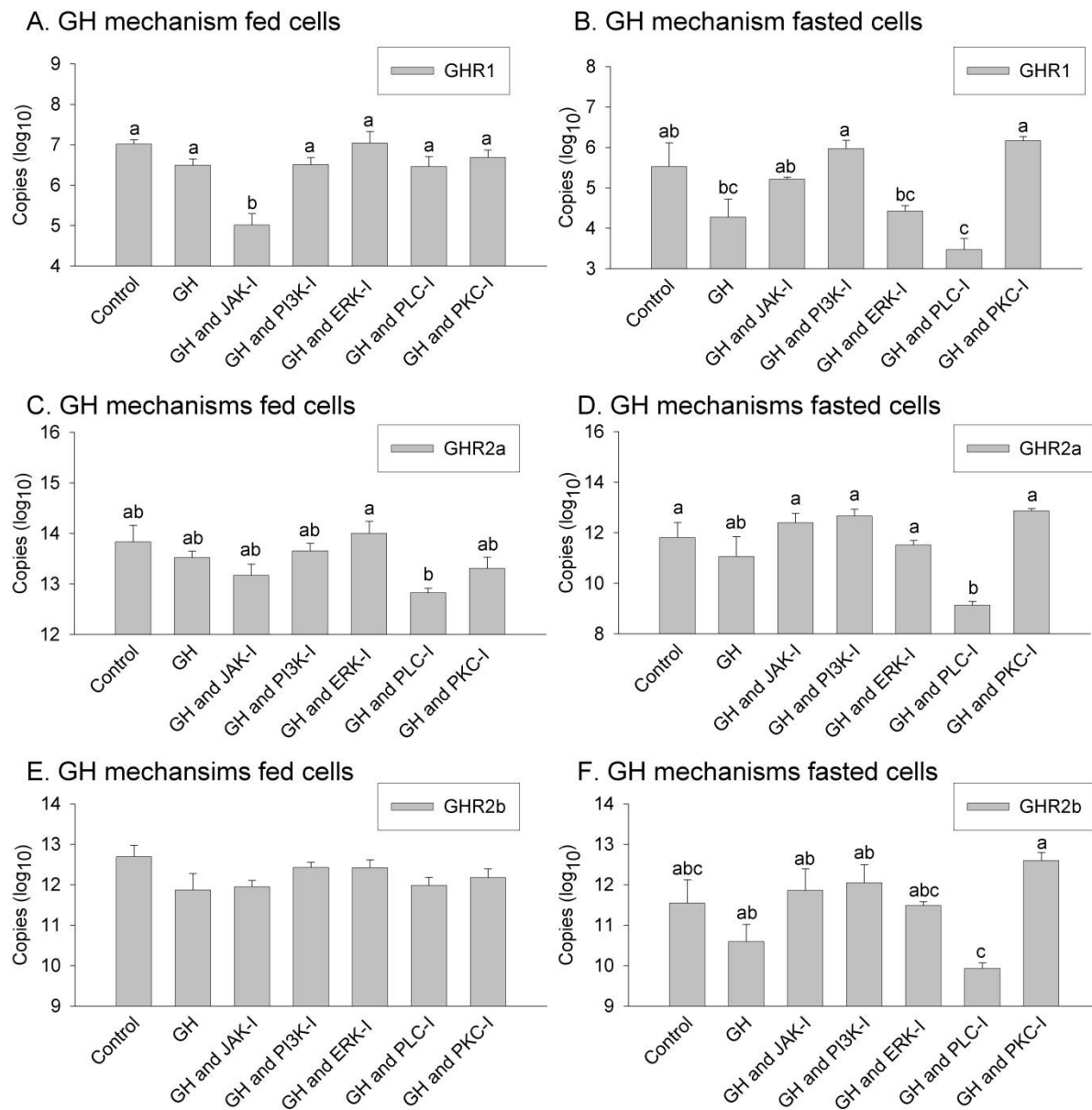


Fig. 20. The effects of signaling element inhibition on growth hormone (GH)-stimulated expression of growth hormone receptor (GHR) 1 (A and B), GHR2a, (C and D) and GHR2b (E and F) mRNAs in hepatocytes isolated from rainbow trout fed continuously (A, C, and E) or fasted for 4 weeks (B, D, and F). Cells were pretreated for 2 h with or without specific inhibitors for the following signaling elements: MEK (10 μ M U0126), PI3K (10 μ M LY294002), JAK (10 μ M Hex.), PKC (10 μ M chelerythrine chloride), PLC (10 μ M U73122); after which time, cells were treated with 100 ng/ml GH for 6 h. Data are presented as log₁₀ copies and expressed as means \pm SEM (n=6). Letters denote significant ($p < 0.05$) difference within a nutritional state and GHR subtype.

Fasted fish were shown to have a complicated relationship with pharmacological inhibitors and their effects on GHR expression. GH was also shown to cause a slight but non-significant decrease in GHR1 expression similar to what we observed in time studies but opposite of what we saw in dose studies. GH was shown to have no effect on GHR2a in fasted fish (Fig. 20). GH was also shown to cause a slight but non-significant decrease in GHR2b expression. In fasted fish, PLC inhibition was shown to cause a significant decrease in GH-stimulated GHR2a expression when compared to all groups except GH treatment similar to what was seen in fed fish. JAK, PI3K, and PKC inhibition all caused a slight but non-significant increase in GH-stimulated GHR2a expression, while MEK inhibition had no effect on GH-stimulated GHR2a expression. These pharmacological inhibitors had a similar effect on GH-stimulated GHR2b expression compared to GH-stimulated GHR2a expression. While not significantly different from GH controls, PLC inhibition caused a significant decrease in GH-stimulated GHR2b expression compared to PKC, PI3K, and JAK inhibition. Just like with GH-stimulated GHR2a expression, JAK and PI3K inhibition all caused a slight but non-significant increase in GH-stimulated GHR2b expression, while PKC caused a significant increase in GH-stimulated GHR2b expression compared to GH controls. MEK had no effect on GH-stimulated GHR2b expression. GH-stimulated GHR1 expression was also similarly effected by pharmacological inhibitors in fasted fish. PKC and PI3K inhibition, along with GH, counteracted the decrease in GHR1 expression caused by GH alone and brought GHR1 expression levels back towards control levels. JAK and MEK had no significant effect on GH-stimulated GHR1 expression compared to GH controls. PLC caused significantly lower levels of GH-stimulated GHR1 expression from all groups except GH and MEK continuing the trend we see when PLC and GH cause the lowest levels of GH-stimulated GHR expression.

The role of blood serum in reprogramming nutritionally regulated GH-stimulated GHR1, GHR2a, and GHR2b expression

In an attempt to reprogram cells, and specifically their response to GH, we took hepatocytes from each nutritional state (e.g., fed) and treated them with serum from their opposite nutritional state (e.g., fasted) as well as with serum from their native nutritional state. In fed fish the serum pre-treatment had very minimal effect on GHR expression (Fig. 21). Pre-treatment of fed cells with fed and fasted serum caused a slight but non-significant increase in GHR1 expression. In fed cells pre-treated with fed serum GH was able to further increase GHR1 expression to levels significantly higher than no serum treated cells. In fed cells treated with fasted plasma GH slightly but non-significantly decreased GHR1 expression. Pre-treatment with either serum had no effect on either GHR2a or GHR2b mRNA expression. In addition, GH had no effect on either GHR2a or GHR2b mRNA expression in cells from fed fish with either serum treatment.

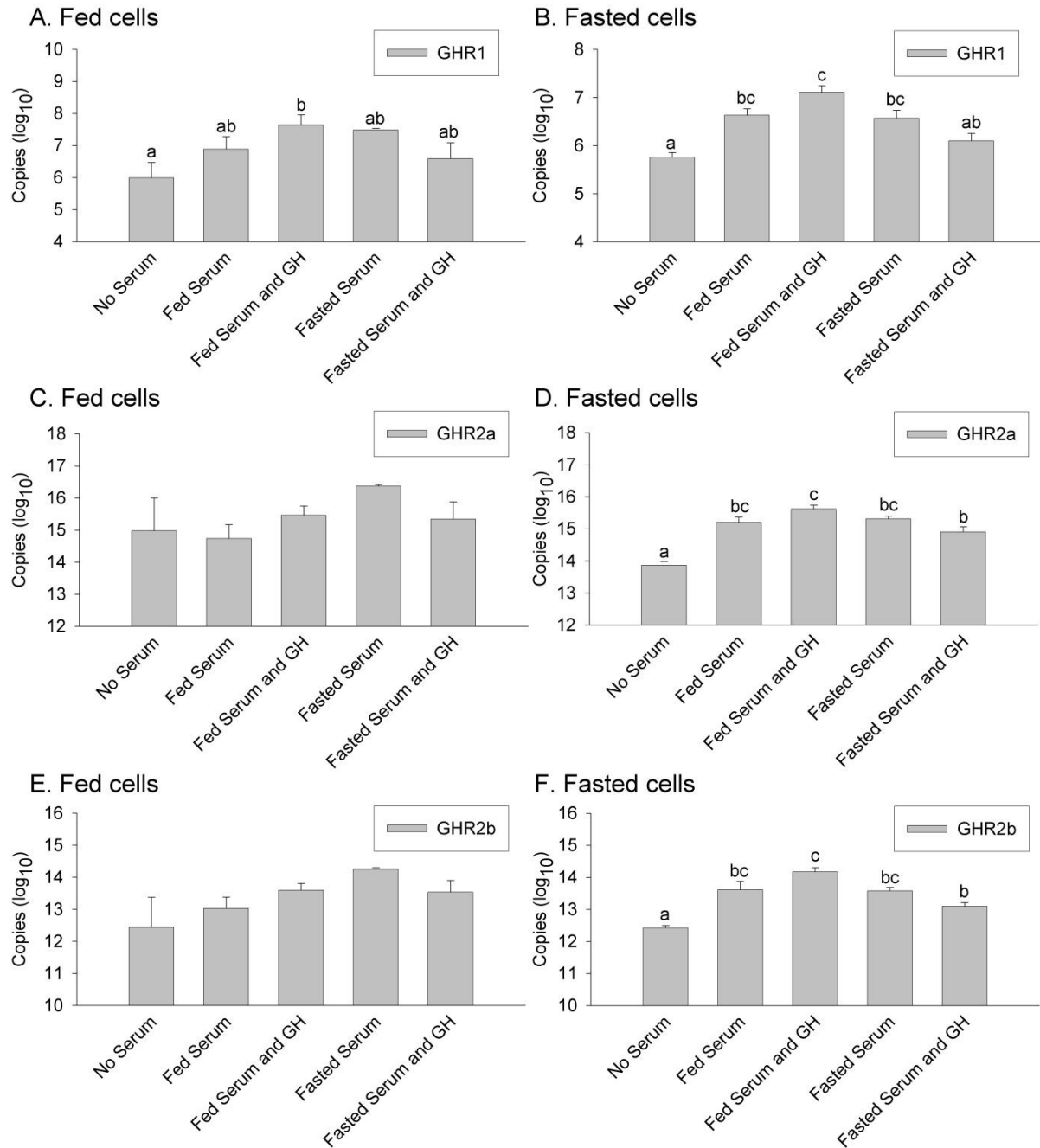


Fig. 21. The effects of serum on growth hormone (GH)-stimulated expression of growth hormone receptor (GHR) 1 (A and B), GHR2a, (C and D) and GHR2b (E and F) mRNAs in hepatocytes isolated from rainbow trout fed continuously (A, C, and E) or fasted for 4 weeks (B, D, and F). Cells were treated for 24 hours with 10% serum harvested from the blood plasma of fed or fasted fish then treated with or without 100 ng/ml of GH for 6 hours. Data are presented as log₁₀ copies and expressed as means ± SEM (n=6). Letters denote significant (p < 0.05) difference within a nutritional state and GHR subtype.

In hepatocytes from fasted fish, pre-treatment with either serum from fed fish or fasted fish increased GHR1, GHR2a, and GHR2b mRNA expression (Fig. 21). For each GHR, mRNA expression was increased to the same level with serum from fasted and fed fish. In fasted cells pre-treated with serum from fed fish, the addition treatment with GH caused a slight but non-significant increase in GHR1, GHR2a, and GHR2b expression. In fasted cells pre-treated with serum from fasted cells, the additional treatment of GH caused a slight but non-significant decrease in GHR1, GHR2a, and GHR2b. While both the GH-stimulated increase in GHR expression in fasted cells treated with fed serum and the GH-stimulated decrease in GHR expression in fasted cells treated with fasted serum were not significant, they were significantly different from each other in all three GHRs.

Discussion

GH-stimulated IGF expression is dependent on nutritional state. GH was only able to stimulate IGF-1 and IGF-2 mRNA expression in hepatocytes from fed cells as opposed to hepatocytes isolated from fasted fish. This is consistent with other research showing GH promoting IGF-1 expression in rainbow trout hepatocytes (Reindl et al., 2011) and IGF-2 expression in multiple species of fish (Shamblot et al., 1995; Pierce et al., 2010). This helps explain the fact that fasting causes a decrease in plasma IGF-1 levels (Gomez-Requeni et al., 2005; Norbeck et al., 2007). GH-stimulated hormone sensitive lipase (HSL) expression is dependent on nutritional state, with GH causing increased HSL expression only in fasted fish but not in fed fish (Bergan et al., 2015). The increase in HSL expression can be assumed to also lead to an increase in lipolysis. HSL was chosen as a target to measure lipolysis because HSL and adipose triacylglyceride lipase account for 90% of lipolysis through the hydrolysis of fatty acids off the glycerol backbone of the triacylglycerides (Watt and Spriet, 2010; Jaworski et al., 2007).

This is consistent in the literature finding GH-stimulated lipolysis in the livers of fish (Bergan et al., 2013; O'Connor et al., 1993). These two facts taken together illustrate that GH has multiple different roles in the same cells dependent on nutritional state in both growth and lipolysis. This also explains the increase in GH in fasting fish (Gomez-Requeni et al., 2005; Norbeck et al., 2007; Picha et al., 2008; Reinecke, 2010) despite that fact that GH is not active in increasing IGF expression.

Somatostatin plays a role in the regulation of growth via IGF-2 expression. This conclusion was supported through the observation that SS decreased IGF-2 expression in a time dependent manner. While not significant in a concentration related manner this is possible since the largest effect on IGF-2 was observed at the later time point of 12 h in the dose study was conducted at 6 h. This is similar to what was previously seen in the literature with SS causing decreased IGF-1 expression in rainbow trout (Very et al., 2008) and similar to the observation of SS decreasing IGF-2 expression in orange-spotted grouper (Wang et al., 2016). AKT and MEK inhibition was shown to decrease GH-stimulated IGF-2 expression in combination with SS to below base levels. Little can be inferred from this inhibition study, but it shows that the AKT and MEK pathways are active in GH-stimulated IGF-2 expression, as shown by other research that GH-stimulated IGF expression works through the AKT and MEK pathways (Reindl et al., 2011). This study would benefit from being repeated with larger sample sizes and more inhibitors to increase statistical power and more fully elucidate the pathways these processes work through. These observations together help to further establish the role of IGF-2 in post embryonic growth in teleost.

GH-stimulated GHR expression is not dependent on nutritional state. This conclusion is supported by the observations that the nutritional state of the fish caused no change in how the

hepatocytes reacted to GH relative to GHR1, GHR2a, or GHR2b mRNA expression. While the role of GH has been well studied on GHR expression, the results are conflicting. In rainbow trout GH treatment was shown to increase GHR expression (Very and Sheridan 2007), while in black seabream GH was shown to have no effect on GHR expression (Jiao et al., 2006) and rats have shown that GH treatment decreases GHR expression (Maiter et al., 1988). Our observation that GH caused an increase in GHR1 expression in both fed and fasted fish is similar to the effects observed in rainbow trout while our observation that GH had no effect on GHR2a or GHR2b expression is more similar to the results seen in black seabream.

In time-course studies we saw different results that in the longest GH treatment of 12 and 24 h GH caused a decrease in all GHRs. This suggests a feedback mechanism of long term GH treatment causing decreased GHR receptors for the GH to work upon in longer treatments.

Despite the fact that we found nutritional state to have no effect on how cells react to GH, the role of nutritional state on GHRs in the liver has been well studied. Fasting has previously been shown to decrease GHR expression in the hepatocytes of many fish including rainbow trout (Norbeck et al., 2007), masu salmon (Fukada et al., 2004), black seabream (Deng et al., 2004), cat fish (Small et al., 2006), and striped sea bass (Picha et al., 2008) while fasting was shown to have no effect on GHR expression in tilapia (Pierce et al., 2007). This information taken together suggests that while both nutritional state and GH are important in regulating GHR, nutritional state is not important in how cells react to GH in regards to GHR expression.

JAK/STAT and PI3K/Akt are the pathways through which nutritionally dependent GH-stimulated IGF expression occurs. This conclusion is backed by several observations. JAK2 inhibition completely abolished GH-stimulated IGF expression in fed cells, and MEK and PI3K inhibition both partially blocked GH-stimulated IGF expression in fed cells. This is similar to

what we observed in our earlier studies as well as the literature showing that GH works through the JAK/STAT and PI3K/Akt pathways (Reindl et al., 2011). The second observation that protein kinase C (PKC) and phospholipase c (PLC) inhibition had no effect on GH-stimulated IGF expression in fed cells also supported this initial conclusion as these two inhibitors are used to inhibit lipolysis and had no effect on IGF expression. The fact that PKC and PLC inhibition completely block GH-stimulated lipolysis and GH-stimulated HSL expression while JAK/STAT and PI3K/Akt inhibition had no effect (Bergan et al., 2013), further establishes our conclusion that nutritional state switches the cells responsiveness to GH. This information also illustrates that different responses to GH growth vs lipolysis are controlled by different signaling pathways.

As observed previously, the relationship between nutritional state and GH-stimulated GHR expression is very complicated. Since GH stimulation in general has a small effect on GHR expression, it was difficult to find many patterns in the data regarding nutritional state and pathway inhibition. Inhibiting signal pathways in general had a much lesser effect in fed fish compared to fasted fish, with the only significant effect being the difference between slight negative effects of PLC inhibition compared to the slight increase in GH-stimulated GHR2a expression caused by MEK inhibition. The only other difference in fed fish was that JAK inhibition caused significant decrease in GH-stimulated GHR1 expression compared to every other treatment. This is similar to our observation that in fed fish the inhibition of JAK led to the complete blockage of GH-stimulated IGF expression. This observation is consistent with the literature that JAK activation is the first step in GH action as observed in both fish (Reindl et al., 2011) and in mammals (Waters et al., 2006; Piwien-Pilipuk et al., 2002). This suggests that JAK activation is important in regulating its own future actions by inhibiting GHR1 expression in fed fish.

The relationship between inhibitors and GH-stimulated GHR expression in fasted fish was even more complicated. Compared to fed fish, more inhibitors had an effect on GH-stimulated GHR expression. In fasted fish, several general trends were observed. The first being that PLC inhibition caused a decrease in GH-stimulated GHR expression for every GHR. A similar effect was only noticed on GHR2a expression in fed fish. This inhibition was also more pronounced in fasted fish. Another trend found is that PKC inhibition caused a slight increase in GH-stimulated GHR expression with every GHR. This was different from what was seen in fed fish, as in every group PKC inhibition as found to have no effect. Other research has similarly shown that both PKC and PLC inhibition completely abolished GH-stimulated HSL expression and lipolysis, observed as glycerol release, that was only present in fasted fish (Bergan et al., 2015). Although no clear effect of GH-stimulated GHR expression was observed in the present study, our results support the proposed mechanism that nutritional state is changing how cells react to GH. In a fed state, cells respond to GH through the JAK/STAT and PI3K/Akt pathways while in fasted fish GH works through PLC and PKC pathways.

The ability of cells to respond differentially to GH in different nutritional states is regulated by serum. Fasted cells that are pre-treated with serum from fed cells react like fed cells when treated with GH. Normally in fasted cells, GH treatment has no effect on IGF-1 or IGF-2 expression, but when treated with serum from fed fish GH treatment caused an increase in both IGF-1 and IGF-2 expression as seen in fed fish. This is also similar to results seen in the literature for GH-stimulated IGF-1 (Reindl et al., 2011) and IGF-2 expression in multiple species of fish (Shamblot et al., 1995; Pierce et al., 2010). Similar reprogramming was also observed in fed cells. In fed cells pretreatment of cells with fasted serum caused GH to cause significant decreases in both IGF-1 and IGF-2 expression compared to no serum controls. This is similar to

the previous observation that GH has no effect on IGF expression in fasted cells, but this goes one step further showing the levels of IGF-1 and IGF-2 decreasing from the higher levels they would have been at in times of feeding (Gomez-Requeni et al., 2005; Norbeck et al., 2007). This suggests that the serum is again changing how the cells react to GH. These observations along with the fact that a similar effect was also observed by us that serum was able to reprogram fed cells to react like fasted cells when treated with GH in regards to HSL expression and lipolysis (Bergan-Roller et al., 2017) form a strong hypothesis that serum contains one or more factors that allow cells to differentially react to GH in different nutritional states.

Serum was also found to have a role in IGF-1 and IGF-2 expression. In fasted cells treatment with fed serum caused a slight but not significant increase in IGF-1 expression. This is probably from other elements already in the serum such as GH that would naturally cause an increase in IGF-1 as seen in literature (Reindl et al., 2011). Interestingly in fed cells treatment with fasted serum caused a slight but not significant decrease in both IGF-1 and IGF-2 expression. This is similar to what is seen in fasting organisms having decreased levels of IGF-1 (Gomez-Requeni et al., 2005; Norbeck et al., 2007) suggesting that the fasted serum is causing the fed cells to react as if they were fasted. Further studies should be done on individual components of the serum such as insulin, IGF, and perhaps others to see what roles they play on the reprogramming of cells in response to GH in regards to IGF and GHR expression similar to what was done in (Bergan-Roller et al., 2017) in regards to lipolysis and HSL expression.

Serum plays a role in regulating GHR expression in fasted fish. This conclusion is supported by several observations. First in fasted cells treatment with either serum caused an increase in GHR1, GHR2a, and GHR2b expression. This suggests that something in the serum is causing an increase in GHR expression. As mentioned earlier, this would be a good avenue for

further studies to find which components in the serum cause this increase. The serum also reprogramed how the cells would respond to GH. In fasted cells, after pretreatment with fed serum, GH caused an additional increase in GHR1, GHR2a, and GHR2b, expression levels, but in similar cells treatment with fasted serum caused the cell to react to GH by causing decreased levels of GHR1, GHR2a, and GHR2b expression. Although we observed that nutritional state didn't cause any changes in cells response to GH in regards to GHR expression, some component in the serum that changed in response to the change in nutritional state did cause the cells to react differently to GH. This is another area for future research. These effects of serum on GHR expression and reprogramming cells in regards to response to GH are only observed in fasted fish, whereas in fed fish, serum has very little effect on either GHR expression or GH-stimulated GHR expression. This suggests that the fasted cells are more sensitive to the serum as a result of food deprivation.

In summary we found that nutritional state modulates the growth-promoting actions of GH but not the expression of GHRs. The growth promoting action of GH that we studied in this experiment was the expression of IGF encoding mRNAs. During feeding, GH activates the JAK-STAT, PI3K/Akt, ERK pathways resulting in the increased expression of IGFs. During periods of fasting, on the other hand, GH activates a different complement of signal pathways (PLC/PKC) that do not promote growth. This suggests that some mechanism is switching how these cells react under the different nutritional states. We proposed that serum was the mediator of this change in regards to IGF expression. When treated with serum from the opposite nutritional state cells would act according to the nutritional state of the serum they were treated with as opposed to their native nutritional state. The expression of GHRs was not as clearly regulated by nutritional state or serum. While nutritional state didn't cause a direct change in

how cells reacted to GH in regards to GHR expression, serum treatment did change how cells react to GH. These findings help provide insight into the diverse actions of GH in different nutritional states. We suggest that individual elements in the serum are responsible for this switching of cell responsiveness. Further studies should be conducted to determine what elements of the serum are active in causing this switch. In addition, further studies could be done on the different GHRs to determine which GHRs are selectively active during different nutritional states to see if this is helping to promote the switch between the growth promoting and lipolytic actions of GH.

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References

- Bergan, H.E., Kittilson, J.D., Sheridan, M.A. 2013. PKC and ERK mediate GH-stimulated lipolysis. *J. Mol. Endocrinol.* 51, 213-224.
- Bergan, H.E., Kittilson, J.D., Sheridan, M.A. 2015. Nutritional state modulates growth hormone-stimulated lipolysis *Gen. Comp. Endocrinol.*, 217–218 1-9.
- Bergan-Roller, H.E., Ickstadt, A.T., Kittilson, J.D., Sheridan, M.A. 2017. Insulin and insulin-like growth factor-1 modulate the lipolytic action of growth hormone by altering signal pathway linkages. *Gen. Comp. Endocrinol*, 248, 40-48.

- Bjornsson, B.T., Johansson, V., Benedet, S., Einarsdottir, I.E., Hildahl, J., Agustsson, T., Jonsson, E. 2004. Growth hormone endocrinology of salmonids: regulatory mechanisms and mode of action. *Fish Physiol. Biochem.* 27:227-242F.
- Deng, L., Zhang, W. M., Lin, H.R., & Cheng, C.H. 2004. Effects of food deprivation on expression of growth hormone receptor and proximate composition in liver of black seabream *Acanthopagrus schlegeli*. *Comparative Biochemistry and Physiology Part B: Biochem Mole Biol*, 137(4), 421-432.
- Forsyth, I.A. and Wallis, M., 2002. Growth hormone and prolactin--molecular and functional evolution. *Journal of mammary gland biology and neoplasia*, 7(3), 291–312.
- Fukada, H., Ozaki, Y., Pierce, A.L., Adachi, S., Yamauchi, K., Hara, A., Swanson, P., Dickhoff, W.W., 2004. Salmon growth hormone receptor: molecular cloning, ligand specificity, and response to fasting. *Gen. Comp. Endocrinol*, 139(1):61-71.
- Galt, N.J., Froehlich, J.M., Meyer, B.M., Barrows, F.T., Biga, P.R. 2014. High-fat diet reduces local myostatin-1 paralog expression and alters skeletal muscle lipid content in rainbow trout, *Oncorhynchus mykiss*. *Fish Phys Biochem*, 40(3), 875–886.
- Gomez-Requeni, P., J. Calduch-Giner, S. Vega-Rubin de Celis, F. Medale, S.J. Kaushik, J. Pérez-Sanchez. 2005. Regulation of the somatotrophic axis by dietary factors in rainbow trout (*Oncorhynchus mykiss*), *Brit. J. Nutr.* 94:353-361.
- Jaworski K., Sarkadi-Nagy E., Duncan R. E., Ahmadian M., Sul H. S. 2007. Regulation of triglyceride metabolism. IV. Hormonal regulation of lipolysis in adipose tissue. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 293(1), G1–G4.
- Jiao, B., Huang, X., Chan, C.B., Zhang, L., Wang, D., Cheng, C. H. 2006. The co-existence of two growth hormone receptors in teleost fish and their differential signal transduction,

- tissue distribution and hormonal regulation of expression in seabream. *J Mole Endocrinol*, 36(1), 23-40.
- Kossiakoff A. A. and de Vos A. 1999. Structural basis for cytokine hormone-receptor recognition and receptor activation. *Adv. Protein Chem.* 52:67–108.
- LeRoith, D., Bondy, C., Yakar, S., Liu, J.L., Butler, A. 2001. The somatomedin hypothesis: 2001. *Endocr. Rev.* 22, 53–74.
- Maiter, D., Underwood, L.E., Maes, M., Ketelslegers, J.M. 1988. Acute down-regulation of the somatogenic receptors in rat liver by a single injection of growth hormone. *Endocrinology*, 122(4), 1291-1296.
- Malkuch, H., Walock, C., Kittilson, J.D., Raine, J.C., Sheridan, M.A. 2008. Differential expression of preprosomatostatin- and somatostatin receptor-encoding mRNAs in association with the growth hormone-insulin-like growth factor system during embryonic development of rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* 159(2-3):136-42.
- Moller, N. and Jorgensen, J.O.L., 2009. Effects of Growth Hormone on Glucose, Lipid, and Protein Metabolism in Human Subjects. *Endocrine Reviews*, 30(2), 152–177.
- Norbeck, L.A., Kittilson, J.D. and Sheridan, M.A., 2007. Resolving the growth-promoting and metabolic effects of growth hormone: Differential regulation of GH–IGF-I system components. *Gen. Comp. Endocrinol*, 151(3), 332–341.
- Norrelund, H. 2005. The metabolic role of growth hormone in humans with particular reference to fasting. *Growth Horm. IGF Res.* 15, 95-122.
- O'Connor, P.K., Reich, B., Sheridan, M.A. 1993. Growth hormone stimulates hepatic lipid mobilization in rainbow trout, *Oncorhynchus mykiss*. *J Comp Physiol B* 163: 427-431.

- Picha, M.E., Turano, M.J., Tipsmark, C.K., Borski, R.J., 2008. Regulation of endocrine and paracrine sources of igfs and gh receptor during compensatory growth in hybrid striped bass (*Morone chrysops*×*Morone saxatilis*). *J. Endocrinol.* 199, 81–94.
- Pierce, A.L., Dickey, J.T., Felli, L., Swanson, P., Dickhoff, W.W. 2010. Metabolic hormones regulate basal and growth hormone-dependent igf2 mrna level in primary cultured coho salmon hepatocytes: effects of insulin, glucagon, dexamethasone, and triiodothyronine. *J. Endocrinol* 204:331–339.
- Pierce, A.L., Fox, B.K., Davis, L.K., Visitacion, N., Kitahashi, T., Hirano, T., Grau, E.G. 2007. Prolactin receptor, growth hormone receptor, and putative somatolactin receptor in Mozambique tilapia: Tissue specific expression and differential regulation by salinity and fasting. *Gen. Comp. Endocrinol.* 154(1–3), 31–40.
- Piwien-Pilipuk, G., J.S., Huo, J., Schwatz. 2002. Growth hormone signal transduction, *J. Pediatr. Endocrinol. Metab.* 15 771-786.
- Reindl, K.M., Kittilson, J.D., Bergan, H.E., Sheridan M.A. 2011. Growth hormone-stimulated insulin-like growth factor-1 expression in rainbow trout (*Oncorhynchus mykiss*) hepatocytes is mediated by ERK, PI3K-AKT, and JAK-STAT *Am. J. Physiol.*, 301: R236–R243.
- Reinecke, M. 2010. Influences of the environment on the endocrine and paracrine fish growth hormone–insulin-like growth factor-I system. *J. Fish Biol.* 76(6):1233-54.
- Shamblott, M., Cheng, C. Bolt, D., Chen, T.T. 1995. Appearance of insulin-like growth factor mRNA in the liver and pyloric ceca of a teleost in response to exogenous growth hormone. *Proc. Natl. Acad. Sci. USA* 92:6943-6946.

- Sheridan, M., 1994. Regulation of Lipid-Metabolism in Poikilothermic Vertebrates. *Comparative Biochemistry and Physiology B-Biochemistry and Molecular Biology*, 107(4), 495–508.
- Sheridan, M.A., Mommsen, T.P. 1991. Effects of nutritional state on in vivo lipid and carbohydrate metabolism of coho salmon, *Oncorhynchus kisutch*. *Gen. Comp. Endocrinol.* 81, 473-483.
- Small, B.C., C.A., Murdock, G.C., Waldbieser, B.C., Peterson. 2006. Reduction in channel catfish hepatocyte growth hormone receptor expression in response to food deprivation and exogenous cortisol, *Domest. Anim. Endocrinol.* 31:340-356.
- Very, N.M., Kittilson, J.D., Klein, S.E., Sheridan, M.A. 2008. Somatostatin inhibits basal and growth hormone-stimulated hepatic insulin-like growth factor-I production. *Mole cell endocrinol*, 281(1), 19-26.
- Very, N.M., Kittilson, J.D., Norbeck, L.A., Sheridan, M.A. 2005. Isolation, characterization, and distribution of two cDNAs encoding for growth hormone receptor in rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology B-Biochemistry and Molecular Biology*, 140(4), 615–628.
- Very, N.M., Sheridan, M.A. 2007. Somatostatin regulates hepatic growth hormone sensitivity by internalizing growth hormone receptors and by decreasing transcription of growth hormone receptor mRNAs. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 292(5), R1956-R1962.
- Wallis, M. 1992. The expanding growth hormone/prolactin family. *J. Mol. Endocrinol.* 9:185–188.

- Walock, C.N., Kittilson, J.D., Sheridan, M.A. 2014. Characterization of a novel growth hormone receptor-encoding cDNA in rainbow trout and regulation of its expression by nutritional state. *Gene*. 533(1), 286–294.
- Wang, B., Jia, J., Yang, G., Qin, J., Zhang, C., Zhang, Q., Sun, C., Li, W. 2016. In vitro effects of somatostatin on the growth hormone-insulin-like growth factor axis in orange-spotted grouper (*Epinephelus coioides*). *Gen. Comp. Endocrinol*, 237:1-9.
- Waters, M.J., Hoang, H.N., Fairlie, D.P., Pelekanos, R.A., Brown, R.J. 2006. New insights into growth hormone action. *J Mol Endocrinol*, 36, 1-7.
- Watt, M.J. and Spriet, L.L., 2010. Triacylglycerol lipases and metabolic control: implications for health and disease. *American Journal of Physiology-Endocrinology and Metabolism*, 299(2), E162–E168.

CHAPTER 5: GENERAL CONCLUSIONS

Growth hormone family peptides such as GH, PRL, and SL regulate a wide array of physiological actions including but not limited to growth, feeding, metabolism, reproduction, osmoregulation, immune function, behavior, stress, chromatophore regulation, pigmentation, and lipolysis. This wide array of actions is regulated on many different levels and by a multitude of factors both internal and external. Even though the most studied action of GH is growth promotion, it is not fully understood. Through this research, my goal was to contribute to the understanding of the actions of GH family peptides and determine more of the underlining mechanisms through which GH conducts its diverse actions in times of differing nutritional availability in rainbow trout (*Oncorhynchus mykiss*). This was done by characterizing a novel GHR and determining how nutritional state regulates its expression. Next, the effects of GH family peptides on the growth promoting actions of GH as observed by IGF-1 and IGF-2 expression and which signaling pathways these actions utilize were demonstrated. Finally, the ability of nutritional state to change the actions of GH *in vitro* and the role of serum as a mediator of this change in cells sensitivity to GH was studied.

A new GHR was discovered and characterized in rainbow trout. The discovery of this new GHR helps clarify the evolution and naming of vertebrate GHRs. Phylogenetic analysis identified this GHR as a type 1 GHR, and suggested using the naming change suggested by Ellens et al., (2013). GHR1 mRNA was differently expressed among all tissues examined, with the highest levels observed in liver and white muscle. This is consistent with the multitude of diverse actions in GH in many different tissues. The expression levels of this new GHR were found to be much lower than with other trout GHRs (cf. Very et al., 2005). This new GHR shared many conserved features among other GHRs. In the extracellular domain this consisted of

hormone binding regions, a Y/FGEFS motif, cysteine residues, and potential N-glycosylation sites. This receptor also contained a single transmembrane domain as with all GHRs.

Additionally the intercellular domain contains conserved phosphorylation sites used in linking to cell signaling pathways.

The expression of GHR1 is changed by nutritional state. Short term fasting (2 week) was shown to decrease GHR1 mRNA expression in both adipose and red muscle. Similar decreases were observed in liver after long term fasting (4 weeks). Feeding brought decreased GHR1 expression back up to levels seen in fed fish in both liver and adipose tissue. Similar effects were observed after re-feeding in white muscle, but not statistically significant. Fasting decreased GHR expression was similar to results seen in other species of fish (Deng et al., 2004; Picha et al., 2008; Fukada et al., 2004; Small et al., 2006; Norbeck et al., 2007; Peterson et al., 2009). Some differential expression of different GHRs in regards to fasting is also present. GH has been shown to increase GHR2a expression in adipose tissue (Norbeck et al., 2007) as well as increase GHR2 expression in muscle of striped bass and tilapia (Picha et al., 2008; Fox et al., 2010). This differential regulation of GHRs is a possible mechanism for reduced growth promoting aspects of GH in times of fasting while other actions that are needed in fasting, e.g., lipolysis, flourish.

Somatotropin family hormones stimulate growth through IGF-1 and IGF-2. GH implantation caused increased growth in rainbow trout via increased food conversion as observed in both increases in length and mass. *In vivo* GH increased both IGF-1 and IGF-2 mRNA expression. Similar results were also observed *in vitro* as GH was shown to increase both IGF-1 and IGF-2 expression in a time and dose dependent manner in hepatocytes. PRL also increased both IGF-1 and IGF-2 expression in a time and dose dependent manner *in vitro*. SL did not cause any change in either IGF-1 or IGF-2 expression. Our results are similar to other research

revealing that GH has been shown to increase IGF-1 expression in rainbow trout hepatocytes (Reindl et al., 2011), as well as IGF-2 expression in other fish (Shamblot et al., 1995; Pierce et al., 2010). Previous research has also show the ability of PRL to increase IGF-1 levels in rats (Hill et al., 1977) and IGF-2 levels in bats (Viengchareun et al., 2008). The fact that SL was unable to stimulate IGF illustrates that SL is less involved in growth regulation.

GH- and PRL-stimulated IGF-1 and IGF-2 expression is mediated through common signaling pathways including ERK, PI3K/Akt, and JAK-STAT. The first demonstrated pathway for GH and PRL actions was ERK. GH and PRL directly induce the phosphorylation of ERK1/2. Also, blocking the ERK pathway with a MEK inhibitor partially inhibited GH- and PRL-stimulated IGF expression. Next, GH and PRL were shown to act through the PI3K/Akt pathway. GH and PRL directly induced the phosphorylation of Akt. This conclusion was further strengthened through both PI3K and Akt inhibition causing the inhibition of GH- and PRL-stimulated IGF expression. Finally, GH and PRL were found to work through the JAK-STAT pathway. GH and PRL directly induced the phosphorylation of JAK2 and STAT5. Furthermore, JAK2 inhibition completely inhibited GH- and PRL-stimulated IGF expression. However, STAT5 inhibition partially blocked GH- and PRL-stimulated IGF expression. The pathways we found GH to work through are the same as those found in the literature to be activated by GH and during IGF-1 expression (Argetsinger and Carter-Su 1996; Kopchick and Andry 2000; Piwien-Pilipuk et al., 2002). PRL has also been shown to activate the pathways of ERK (Gubbay et al., 2002), PI3K/Akt (Richert et al., 2001), and JAK2-STAT5 (Campbell et al., 1994; Pezet et al., 1997). Taken together this information helps further clarify the GH and PRL signaling transduction as well as illustrating possible mechanisms that GH can utilize to conduct its many diverse actions.

SS regulates growth through IGF-2 expression. This is supported by our observation that SS caused decreased IGF-2 expression. The role of SS is well studied in regards to IGF-1 but regulation through IGF-2 is less studied. Our observation is similar to others who demonstrated the ability of SS to decrease both IGF-1 (Very et al., 2008) and IGF-2 expressions (Wang et al., 2016). We also demonstrated that AKT and MEK would decrease GH-stimulated IGF-2 expression in combination with SS to below basal levels. This helps to further establish the role of SS in regulating growth through IGF-2 in addition to IGF-1 and also confirms that GH-stimulated IGF-2 expression is mediated through pathways similar to those previously observed by us and other studies (Reindl et al., 2011). These findings together with the ability of GH and PRL to stimulate IGF-2 expression help to further establish the role of IGF-2 in post embryonic growth in teleost fish as opposed to its initial proposed role of only promoting embryonic growth.

Nutritional state regulates the actions of GH. GH-stimulated IGF expression is regulated by nutritional state. GH-stimulated IGF-1 and IGF-2 mRNA expression was only observed in cells from fed fish and not those from fasted fish. This is consistent with literature showing GH stimulation of IGF-1 (Reindl et al., 2011) and IGF-2 expression (Shamblot et al., 1995; Pierce et al., 2010). The fact that GH did not illicit a response in fasting fish is supported by the observation that fasting causes a decrease in plasma IGF-1 levels despite increased GH levels (Gomez-Requeni et al., 2005; Norbeck et al., 2007), suggesting the increased GH is working on other actions in times of fasting. However, GH-stimulated GHR expression does not depend on nutritional state, as the changing nutritional state did not affect how cells react to GH in regards to GHR1, GHR2a, or GHR2b mRNA expression. While nutritional state had no role in GH-stimulated GHR expression, we nonetheless found some effect of GH on GHR expression. GH

caused an increase in GHR1 expression but had no effect on GHR2a or GHR2b. The effect of long term (12 and 24 hour) treatment with GH was shown to decrease all GHRs suggesting a long term feedback mechanism. The effects of GH on GHR expression have been very conflicting as GH has been shown to increase GHR expression, (Very and Sheridan 2007), have no effect on GHR expression (Jiao et al., 2006), and decreases GHR expression (Maiter et al., 1988). Our differing results on the effects of GH on GHR expression by GHR subtype mirrors the conflicting results illustrated in the literature.

Nutritional-dependent GH-stimulated IGF expression is mediated through the ERK, JAK/STAT, and PI3K/Akt signaling pathways. This is supported by the observations that JAK2 inhibition completely inhibited GH-stimulated IGF expression, and MEK and PI3K inhibition both partially blocked GH-stimulated IGF expression. These are the same pathways we observed GH to work through relative to IGF expression as well as having been seen in other studies (Reindl et al., 2011). The use of lipase-related inhibitors PKC and PLC had no effect on GH-stimulated IGF expression in fed cells. Taken together with the fact that PKC and PLC inhibition inhibited lipolysis while JAK/STAT and PI3K/AKT inhibition had no effect on lipolysis (Bergan et al., 2013), confirms that nutritional state changes the responsiveness to cells to GH and this change is mediated via alternative signaling pathways.

As observed earlier, nutritional state has no effect on GH-stimulated GHR expression and GH also had little effect on GHR expression. These facts limit what can be learned through the use of cell signaling inhibitors. The only real conclusion in fed fish is that JAK inhibition caused a significant decrease in GH-stimulated GHR1 expression compared to every other treatment. This is similar to the fact the JAK activation is the first step in GH cell signaling (Waters et al., 2006; Piwien-Pilipuk et al., 2002). Additionally in fasted fish, PKC inhibition caused a slight

increase in GH-stimulated GHR expression. This is similar to other observations that PKC inhibition is active in GH-stimulated HSL expression and lipolysis only in fasted fish (Bergan et al., 2015). While nutrition state had no effect on GH-stimulated GHR expression, these observations help further the understanding of the multiple signaling pathways utilized by GH in different nutritional states.

The differential response of cells to GH is mediated by serum. This conclusion is supported by 2 observations. The pre-treatment of fasted cells with serum from fed cells causes those fasted cells to act like fed cells. Just like observed earlier, GH had no effect on IGF expression in fasted cells, but after pre-treatment with serum from fed cells, GH treatment caused an increase in IGF-1 and IGF-2 expression as similar to what we see in fed fish. Similar reprogramming was observed in fed cells. Pre-treatment of these fed cells with fasted serum caused GH to significantly decrease IGF-1 and IGF-2 expression. This is similar to our observation that in fed cells GH had no effect on IGF expression, but this also goes further by actively decreasing IGF from their higher levels normally seen in feeding to lower levels as observed in fasting (Gomez-Requeni et al., 2005; Norbeck et al., 2007). These two observations along with the previous studies from our lab showing similar results of serum making fed cells act like fasted cells when treated with GH with HSL expression and lipolysis (Bergan-Roller et al., 2017) provide a strong basis for the conclusion that serum is the factor that regulates the different actions of GH under different nutritional states. Serum treatment also changed how cells react to GH relative to GHR expression. In fasted cells, pre-treatment with fed serum plus GH treatment caused an increase in the expression of all GHRs but, pre-treatment with serum from fasted cells plus GH caused a decrease in the expression of all GHRs. Despite the fact that nutritional state had no effect on GH-stimulated GHR expression, some component in serums

from different cells did change how the cells respond to GH. These actions were only limited to fasted cells. Fed cells serum treatment had no effect on GH-stimulated GHR expression, suggesting that fasted cells are more sensitive to the particular factors in serum due to the lack of food.

Serum treatment also affects both IGF and GHR expression directly. First we observed serum to have an effect on IGF expression. In fasted cells, treatment with serum from fed cells caused a slight increase in IGF-1 expression. This increase is probably caused by high GH levels in fasted fish that would cause an increase in IGF expression (Reindl et al., 2011). Interestingly in fed cells, treatment with serum from fasted cells caused a slight decrease in IGF-1 and IGF-2 expression similar to the observation that fasted organisms naturally have lower level of IGF-1 (Gomez-Requeni et al., 2005; Norbeck et al., 2007). This suggests that something present in or absent from fasted serum is causing these fed cells to act as if they were fasted. The effects of serum were also observed on GHR expression. In fasted cells, treatment with any serum caused an increase in all the expression of all GHRs suggesting that some common element in both serums is causing this increase. No effects were observed with either serum treatment on fed cells. As with GH-stimulated GHR expression, this may suggest that fasted cells are more sensitive to serum.

Future studies

The ability of GH to perform its vast array of actions may be mediated through different GHR receptor subtypes. It is known that fish possess multiple GHRs (Perez-Sanchez et al., 2002; Walock et al., 2014) including a newly discovered GHR that was first characterized by our group. While some work has been done on the different GHR subtypes (Reindl and Sheridan, 2012), further work needs to be done to fully investigate the roles of the different GHR subtypes.

This is even more important with the discovery of our new GHR in rainbow trout, which has not yet been studied extensively. This characterization could be achieved by creating cell lines that only express one GHR subtype. These individual receptors may play different roles in the diverse actions of GH in different nutritional states. For example one GHR may be responsible for the growth promoting actions of GH such as IGF production during times of feeding while another may be more active in catabolic actions such as lipolysis in times of fasting. We also know that the GHRs in rainbow trout are differentially distributed (Walock et al., 2014; Very et al., 2005), providing further reason to believe the GHRs have unique roles in different tissues.

While we did find serum to play a role in regulating cells responsiveness to GH, we don't fully know what components of the serum are responsible. Serum should be further characterized by first conducting an analysis of serums from both fed and fasted fish to see what components are different between them. Then the differences could be tested by assessing what effects the individual components might have on changing cells responsiveness. Possible initial candidates for differences in the serum would be insulin and IGF, as nutritional state changes is known to change levels of these (Gomez-Requeni et al., 2005; Norbeck et al., 2007) and both are important factors in the regulation of GH. The effects of these components on lipolysis were tested in collaborative work by our lab (Bergan-Roller et al., 2017). Further insights could be found by testing these same components on growth-promoting actions such as IGF expression and GHR expression. Taken together these studies will help more fully understand the diverse actions of GH.

The actions of fasting and serum on cell responsiveness to GH also should be tested in multiple different tissues. As mentioned earlier, GHRs are differentially expressed and are present in every tissue we tested. This suggests many potential targets in which for fasting and

serum experiments, such as in muscle, gill and adipose tissues. Muscle would be of interest due to its large role in organismal growth. Gill and adipose tissue on the other hand would provide interesting potential studies with their roles in osmoregulation and lipolysis respectively, which are both promoted by GH.

It is known that the actions of GH are regulated on many different levels. Additional research studying different endpoints further downstream of IGF, including IGFBPs and IGFRs would allow deeper understanding of the multiple actions of GH. Nutritional state effects both IGFBP (Clemmons and Underwood, 1991; Lee et al., 1997) and IGFR levels (Norbeck et al., 2007) but it is not known how these factors respond to GH in both fed and fasted fish. If differences were found we could study the effects of serum and different serum components such as insulin and IGF in this switch. In addition to known genes, RNA sequencing could be used to generate more potential targets that are differential expressed during fasting. Obviously much work is still needed to fully understand even the full growth promoting actions of GH and their changes due to nutritional state, as well as the complex multilevel regulation of GH actions.

References

- Argetsinger, L.S., Carter-Su, C. 1996. Mechanism of signaling by growth hormone receptor. *Physiol Rev* 76: 1089–1107.
- Bergan, H.E., Kittilson, J.D., Sheridan, M.A. 2013. PKC and ERK mediate GH-stimulated lipolysis. *J. Mol. Endocrinol.* 51, 213-224.
- Bergan, H.E., Kittilson, J.D., Sheridan, M.A. 2015. Nutritional state modulates growth hormone-stimulated lipolysis *Gen. Comp. Endocrinol.*, 217–218 1-9.

- Bergan-Roller, H.E., Ickstadt, A.T., Kittilson, J.D., Sheridan, M.A. 2017. Insulin and insulin-like growth factor-1 modulate the lipolytic action of growth hormone by altering signal pathway linkages. *Gen. Comp. Endocrinol*, 248, 40-48.
- Campbell, G.S., Argetsinger, L.S., Ihle, J.N., Kelly, P.A., Rillema, J.A., Carter-Su, C. 1994. Activation of JAK2 tyrosine kinase by prolactin receptors in Nb2 cells and mouse mammary gland explants. *Proc Natl Acad Sci USA* 91:5232–5236.
- Clemmons, D.R., Underwood, L.E. 1991. Nutritional regulation of IGF-I and IGF binding proteins. *Ann rev nutr*, 11(1), 393-412.
- Deng, L., Zhang, W. M., Lin, H.R., & Cheng, C.H. 2004. Effects of food deprivation on expression of growth hormone receptor and proximate composition in liver of black seabream *Acanthopagrus schlegeli*. *Comp Biochem and Physiol Part B: Biochem and Mole Biol*, 137(4), 421-432.
- Ellens, E.R., Kittilson, J.D., Hall, J.A., Sower, S.A., Sheridan, M.A. 2013. Evolutionary origin and divergence of the growth hormone receptor family: Insight from studies on sea lamprey. *Gen. Comp. Endocrinol.* 192, 222–236.
- Fox, B. K., Breves, J. P., Davis, L. K., Pierce, A. L., Hirano, T., Grau, E. G. 2010. Tissue-specific regulation of the growth hormone/insulin-like growth factor axis during fasting and re-feeding: Importance of muscle expression of IGF-I and IGF-II mRNA in the tilapia. *Gen. Comp. Endocrinol.* 166(3), 573–580.
- Fukada, H., Ozaki, Y., Pierce, A.L., Adachi, S., Yamauchi, K., Hara, A., Swanson, P., Dickhoff, W.W., 2004. Salmon growth hormone receptor: molecular cloning, ligand specificity, and response to fasting. *Gen. Comp. Endocrinol*, 139(1):61-71.

- Gomez-Requeni, P., J. Calduch-Giner, S. Vega-Rubin de Celis, F. Medale, S.J. Kaushik, J. Pérez-Sanchez. 2005. Regulation of the somatotrophic axis by dietary factors in rainbow trout (*Oncorhynchus mykiss*), *Brit. J. Nutr.* 94:353-361.
- Gubbay, O., Critchley, H.O.D., Bowen, J.M., King, A., Jabbour, H.N. 2002. Prolactin induces ERK phosphorylation in epithelial and CD56(+) natural killer cells of the human endometrium. *J Clin Endocrinol Metab* 87:2329–2335.
- Hill, D.J., Francis, M.J., Milner, R.D. 1977. Action of rat prolactin on plasma somatomedin levels in the rat and on somatomedin release from perfused rat liver. *J Endocrinol* 75:137–143.
- Jiao, B., Huang, X., Chan, C.B., Zhang, L., Wang, D., Cheng, C.H. 2006. The co-existence of two growth hormone receptors in teleost fish and their differential signal transduction, tissue distribution and hormonal regulation of expression in seabream. *J Mole Endocrinol*, 36(1), 23-40.
- Kopchick, J.J., Andry, J.M. 2000. Growth hormone (GH), GH receptor and signal transduction. *Mol Genet Metab* 71: 293–314.
- Lee, P.D.K., Giudice, L.C., Conover, C.A., Powell, D.R. 1997. Insulin-like growth factor binding protein-1: recent findings and new directions. *Proc. Soc. Exp. Biol. Med.*, 216 319.
- Maiter, D., Underwood, L.E., Maes, M., Ketelslegers, J.M. 1988. Acute down-regulation of the somatogenic receptors in rat liver by a single injection of growth hormone. *Endocrinol*, 122(4), 1291-1296.

- Norbeck, L.A., Kittilson, J.D. and Sheridan, M.A., 2007. Resolving the growth-promoting and metabolic effects of growth hormone: Differential regulation of GH–IGF-I system components. *Gen. Comp. Endocrinol*, 151(3), 332–341.
- Perez-Sanchez, J., Calduch-Giner, J.A., Mingarro, M., Celis, S. V.R. de, Gomez-Requeni, P., Saera-Vila, A., Valdivia, M.M. 2002. Overview of Fish Growth Hormone Family. *New Insights in Genomic Organization and Heterogeneity of Growth Hormone Receptors. Fish Phys Biochem.* 27(3-4), 243–258.
- Peterson, B.C., Bilodeau-Bourgeois, A.L., Small, B.C., 2009. Response of the somatotrophic axis to alterations in feed intake of channel catfish (*Ictalurus punctatus*). *Comp. Biochem. Physiol. A*, 153, 457–463.
- Pezet, A., Ferrag, F., Kelly, P.A., Edery, M.1997. Tyrosine docking sites of the rat prolactin receptor required for association and activation of Stat5. *J Biol Chem* 272:25043–25050.
- Picha, M.E., Turano, M.J., Tipsmark, C.K., Borski, R.J., 2008. Regulation of endocrine and paracrine sources of IGFs and GH receptor during compensatory growth in hybrid striped bass (*Morone chrysops*×*Morone saxatilis*). *J. Endocrinol.* 199, 81–94.
- Pierce, A.L., Dickey, J.T., Felli, L., Swanson, P., Dickhoff, W.W. 2010. Metabolic hormones regulate basal and growth hormone-dependent IGF-2 mRNA level in primary cultured coho salmon hepatocytes: effects of insulin, glucagon, dexamethasone, and triiodothyronine. *J Endocrinol* 204:331–339.
- Piwien-Pilipuk, G., J.S., Huo, J., Schwatz. 2002. Growth hormone signal transduction, *J. Pediatr. Endocrinol. Metab.* 15 771-786.
- Reindl, K.M., Kittilson, J.D., Bergan, H.E., Sheridan M.A. 2011. Growth hormone-stimulated insulin-like growth factor-1 expression in rainbow trout (*Oncorhynchus mykiss*)

- hepatocytes is mediated by ERK, PI3K-AKT, and JAK-STAT Am. J. Physiol., 301: R236–R243.
- Reindl, K.M., Sheridan, M.A. 2012. Peripheral regulation of the growth hormone-insulin-like growth factor system in fish and other vertebrates Comp. Biochem. Physiol., Part A, 163 pp. 231–245.
- Richert, M.M., Decker, K., Anderson, S.M., 2001. Mechanisms underlying constitutive activation of Akt in breast cancer cell lines. p 551. Program of the 83rd Annual Meeting of The Endocrine Society; Denver, CO. P3–478.
- Shamblott, M., Cheng, C. Bolt, D., Chen, T.T. 1995. Appearance of insulin-like growth factor mRNA in the liver and pyloric ceca of a teleost in response to exogenous growth hormone. Proc. Natl. Acad. Sci. USA 92:6943-6946.
- Small, B.C., C.A., Murdock, G.C., Waldbieser, B.C., Peterson. 2006. Reduction in channel catfish hepatocyte growth hormone receptor expression in response to food deprivation and exogenous cortisol, Domest. Anim. Endocrinol. 31:340-356.
- Very, N.M., Kittilson, J.D., Klein, S.E., Sheridan, M.A. 2008. Somatostatin inhibits basal and growth hormone-stimulated hepatic insulin-like growth factor-I production. Mole cell endocrinol. 281(1), 19-26.
- Very, N.M., Kittilson, J.D., Norbeck, L.A., Sheridan, M.A. 2005. Isolation, characterization, and distribution of two cDNAs encoding for growth hormone receptor in rainbow trout (*Oncorhynchus mykiss*).Comp Biochem Physiol B-Biochem Mole Bio. 140(4), 615–628.
- Very, N.M., Sheridan, M.A. 2007. Somatostatin regulates hepatic growth hormone sensitivity by internalizing growth hormone receptors and by decreasing transcription of growth

- hormone receptor mRNAs. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 292(5), R1956-R1962.
- Viengchareun, S., Servel, N., Feve, B., Freemark, M., Lombes, M., Binart, N. 2008. Prolactin receptor signaling is essential for perinatal brown adipocyte function: a role for insulin-like growth factor-2. *PLoS ONE* 3:e1535.
- Walock, C.N., Kittilson, J.D., Sheridan, M.A. 2014. Characterization of a novel growth hormone receptor-encoding cDNA in rainbow trout and regulation of its expression by nutritional state. *Gene*. 533(1), 286–294.
- Wang, B., Jia, J., Yang, G., Qin, J., Zhang, C., Zhang, Q., Sun, C., Li, W. 2016. In vitro effects of somatostatin on the growth hormone-insulin-like growth factor axis in orange-spotted grouper (*Epinephelus coioides*). *Gen. Comp. Endocrinol*, 237:1-9.
- Waters, M.J., Hoang, H.N., Fairlie, D.P., Pelekanos, R.A., Brown, R.J. 2006. New insights into growth hormone action. *J Mole Endocrinol*. 36, 1-7.