1 **Leptin stimulates hepatic growth hormone receptor and insulin-like growth factor gene**
2 **expression in a teleost fish, *the hybrid striped bass***

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ABSTRACT

Leptin is an anorexigenic peptide hormone that circulates as an indicator of adiposity in mammals, and functions to maintain energy homeostasis by balancing feeding and energy expenditure. In fish, leptin tends to be predominantly expressed in the liver, another important energy storing tissue, rather than in fat depots as it is in mammals. The liver also produces the majority of circulating insulin-like growth factors (IGFs), which comprise the mitogenic component of the growth hormone (GH)-IGF endocrine growth axis. Based on similar regulatory patterns of leptin and IGFs that we have documented in previous studies on hybrid striped bass (HSB: *Morone saxatilis* × *M. chrysops*), and considering the co-localization of these peptides in the liver, we hypothesized that leptin might regulate the endocrine growth axis in a manner that helps coordinate somatic growth with energy availability. Using a HSB hepatocyte culture system to simulate autocrine or paracrine exposure that might occur within the liver, this study examines the potential for leptin to modulate metabolism and growth through regulation of IGF gene expression directly, or indirectly through the regulation of GH receptors (GHR), which mediate GH-induced IGF expression. First, we verified that GH (50 nM) has a classical stimulatory effect on IGF-1 and additionally show it stimulates IGF-2 transcription in hepatocytes. Leptin (5 and/or 50 nM) directly stimulated *in vitro* GHR2 gene expression within 8 hrs of exposure, and both GHR1 and GHR2 as well as IGF-1 and IGF-2 gene expression after 24 hrs. Cells were then co-incubated with submaximal concentrations of leptin and GH (25 nM each) to test if they had a synergistic effect on IGF gene expression, possibly through increased GH sensitivity following GHR upregulation by leptin. In combination, however, the treatments only had an additive effect on stimulating IGF-1 mRNA despite their capacity to increase GHR mRNA abundance. This suggests that leptin's stimulatory effect on GHRs may be limited to

enhancing transcription or mRNA stability rather than inducing full translation of functional receptors, at least within a 24-h time frame. Finally, leptin was injected IP (100 ng/g and 1 ug/g BW) to test the *in vivo* regulation of hepatic IGF-1 and GHR1 gene expression. The 100 ng/g BW leptin dose significantly upregulated *in vivo* IGF-1 mRNA levels relative to controls after 24 hrs of fasting, but neither dosage was effective at regulating GHR1 gene expression. These studies suggest that stimulation of growth axis component transcripts by leptin may be an important mechanism for coordinating somatic growth with nutritional state in these and perhaps other fish or vertebrates, and represent the first evidence of leptin regulating GHRs in vertebrates.

Keywords: Leptin, growth hormone receptor, GHR, insulin-like growth factor, IGF, growth, teleost, fish, hepatocyte

I. Introduction

The growth hormone (GH) / insulin-like growth factor (IGF) axis is central to regulating somatic growth in vertebrates (Duan, 1998; Perez-Sanchez and LeBail, 1999; Reinecke, 2006). In this system, circulating pituitary GH stimulates production of IGF-1, a potent mitogen that is largely responsible for somatic growth, and IGF-2, which is less studied but appears to elicit similar actions in teleosts (Chen et al., 2000; Gabillard et al., 2006; Peterson et al., 2004; Terova et al., 2007). The systemic or endocrine source of IGFs is thought to be primarily derived from the liver (Duan, 1998; Reinecke and Collet, 1998; Terova et al., 2007). Stimulation of IGF production by GH is mediated by GH receptors (GHR), two distinct forms of which have been identified in fish (Jiao et al., 2006), although it is not clear to what extent the individual receptor types mediate the growth promoting versus metabolic effects of GH (Kittilson et al., 2011; Pierce et al., 2007). Increased expression of hepatic GHRs elevates ligand sensitivity to GH-induced IGF production during anabolic states, whereas the liver is desensitized to GH during catabolic states due to downregulation of GHRs (Picha et al., 2008; Saera-Vila et al., 2005). Hence, declines in hepatic GHR expression lead to reduced IGF-1 production and somatic growth, this despite an elevation in systemic GH that occurs during fasting-induced catabolism (Norbeck et al., 2007; Picha et al., 2009; Picha et al., 2008; Won and Borski, 2013). The endocrine mechanisms that mediate changes in the GH-IGF growth axis under differential metabolic or nutritional states are not fully understood at present.

Leptin is an anorexigenic peptide hormone that circulates as an endocrine indicator of adiposity in mammals (Ahima and Flier, 2000). Extensive mammalian and clinical research shows that circulating leptin levels communicate the status of endogenous energy reserves to the brain and periphery, and function to balance appetite and energy expenditure based on fat

deposition. Positive energy states are accordingly characterized by elevated plasma leptin levels, decreased energy intake and increased energy utilization (Arora and Arora, 2008). Aside from having a similar function to mammals in suppressing appetite (de Pedro et al., 2006; Douros et al., 2014; Murashita et al., 2008; Won et al., 2012), relatively little is known about leptin actions in lower vertebrates, including fish (Londrville et al., 2014).

In many fish, leptin is produced predominantly by the liver (Dours et al., 2014; Gorissen et al., 2009; Huising et al., 2006; Kurokawa and Murashita, 2009; Rønnestad et al., 2010), which serves as an energy storage site for lipids and glycogen, although it can be found in other tissues as well. Its regulation by nutritional state varies depending on species (Ahima, 2008; Copeland et al., 2011; Dours et al., 2014). Impeding a consensus on the functional role of leptin in fish, genome duplication events specific to certain lineages have resulted in multiple leptin paralogs that may be in various stages of neofunctionalization, while some species appear to have only a single leptin gene (Angotzi et al., 2013; Londrville et al., 2014). Regulation of leptin in response to nutritional state can vary between species, tissue and leptin type, **albeit evidence suggests it tends to increase with fasting in most teleost studied to date**. Among several salmonids, gene expression of one or both A-type leptins (A1 and A2) rise in the liver during feed-restriction (Frøiland et al., 2012; Fuentes et al., 2012; Jorgensen et al., 2013; Rønnestad et al., 2010; Trombley et al., 2012), possibly contributing to a rise in circulating leptin during fasting (Kling et al., 2009; Trombley et al., 2012). Hepatic leptin-like immunoreactivity in European sea bass (Gambardella et al., 2012) and circulating hormone in flounder (Fuentes et al., 2012) also increased during fasting. Conversely, A1-type leptin gene expression in Atlantic salmon was upregulated in the visceral fat of fish fed to satiety compared to feed-restricted fish (Rønnestad et al., 2010). Fasting also decreased hepatic gene expression of leptin-b relative to

fed controls in *danio* (Gorissen et al., 2009). The endocrine growth axis of the hybrid striped bass (HSB: *Morone saxatilis* × *M. chrysops*), a commercially valuable finfish cultivar that was used in this study, has been extensively characterized under different metabolic states, making it a practical subject for inquiries into regulation by leptin. In previous studies, we show that hepatic mRNA levels of leptin A (*lepa*) declined with fasting and increased during refeeding (Won et al., 2012), similar to the expression patterns of IGF-1, IGF-2, GHR1 and GHR2 in the liver (Picha et al., 2014; Picha et al., 2009; Picha et al., 2008).

The concordant regulation of leptin, GHR and IGF expression by nutritional state in HSB, along with their colocation in the liver, suggests a possible regulatory relationship between leptin and the endocrine growth axis. In cases where leptin levels are reflective of energy state in an adipostatic manner, as in mammals, leptin might reasonably serve as an endocrine signal for opportunistic physiological processes that require surplus energy. For example, leptin levels function as a permissive factor for reproduction (Casanueva and Dieguez, 1999; Roa et al., 2010), and could ostensibly serve a similar role in regulating somatic growth. Chronic leptin treatment increased circulating IGF-1 in leptin-deficient women and in mice *in vivo*, the latter accompanied by increased muscle mass and bone formation (Bartell et al., 2011a; Chan et al., 2008b) suggesting that leptin may act permissively on tissue growth by mediating IGF-1 production. Leptin's actions may occur, at least in part, in the liver, insofar as leptin was shown to increase IGF-1 mRNA levels in isolated porcine hepatocytes (Ajuwon et al., 2003). Whether leptin regulates IGF-1 production directly, or through alterations in GH sensitivity or GHR expression in these cases is unknown. A potential role for leptin in modulating GHRs or IGFs in teleost is yet to be established. The aim of this study was to examine if leptin might act alone or

in synergy with GH to regulate hepatic GHR1, GHR2, IGF-1 and IGF-2 gene expression in HSB, a perciform fish representative of advanced teleosts.

2. Materials and methods

2.1 Animals

Phase I HSB were obtained from Pungo Fisheries (Pinetown, NC) and housed in fresh water recirculating aquaculture systems (hardness = 200 mg/L; alkalinity = 250 mg/L; temperature = 22° C; photoperiod = 12L:12D) at North Carolina State University (NCSU; Raleigh, NC). For two weeks prior to experiments, fish were fed a maintenance diet of 1.5% body weight / day (Ziegler Silver 5mm pellets, Gardners, PA; 40% protein, 10% lipid), which is half of the typical commercial feeding rate. This regimen was chosen in order to maintain a mild anabolic state and intermediate GHR and IGF gene expression levels compared to those elicited under higher ration levels or fasting (Picha et al., 2008). **Protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee.**

2.2 Hepatocyte cultures

In vitro experiments on HSB hepatocytes were conducted in three separate trials to test the regulatory effects of GH and leptin on GHR and IGF gene expression. **Experiments were repeated with similar results.** In the first experiment, cells were incubated in growth medium containing 5 or 50 nM bovine GH (National Hormone and Peptide Program, Torrance, CA) to validate that cells were responsive to IGF-1 stimulation and were hence functioning appropriately. This study also evaluated if GH might regulate IGF-2 synthesis in *Morone*. In the

second experiment, hepatocytes were incubated with 5 and 50 nM human recombinant leptin (National Hormone and Peptide Program, Torrance, CA) for 8 or 24 hrs to test for effects on hepatic GHR1, GHR2, IGF-1 and IGF-2 gene expression. A third culture experiment was conducted to evaluate if leptin and GH might have a synergistic effect in regulating IGF gene expression. Leptin and GH were administered at half the highest concentration used in previous experiments (25 nM each) to avoid inducing a maximal threshold of IGF gene expression by the combined hormonal treatments, which could mask potential synergistic or additive effects.

Livers from fish (170-200g) were used for each *in vitro* experiment in order to yield enough cells for up to six replicates per group (N = 4-6 wells). Hepatocytes were harvested as described by Mommsen (Mommsen et al., 1994). In short, after fish were lethally dosed with buffered MS222, the posterior intestinal vein was cannulated and the liver flushed of blood with calcium-free Hank's buffered salt solution (HBSS) for 5 minutes using a peristaltic pump (2 mL/min), then digested *in situ* with HBSS containing type IV collagenase (Sigma, St. Louis, MO) for 10-15 minutes. The bulbus of the heart was nicked to alleviate fluid backpressure as solutions were pumped through the vasculature. After partial digestion, the liver was removed, finely chopped with a razor blade in a glass petri dish and slowly agitated in collagenase solution for 10 minutes at room temperature. The digested liver was then mechanically pushed through a 260 micron mesh filter to remove structural tissue, allowed to drip through a second 60 micron mesh, and collected in a beaker on ice to slow further digestion. The harvested cells were washed, allowed to recover on ice for one hour, then re-suspended in RPMI 1640 growth medium containing L-glutamine (Gibco, Carlsbad, CA; 1% streptomycin/penicillin added) and plated in Falcon 24-well primary plates. Cell viability was determined to be >95% by Trypan Blue exclusion dye test. Each well was plated with 1.5×10^5 cells in 1 mL growth medium and

allowed to settle overnight at 18° C in an air atmosphere. Formation of a cell monolayer was visually confirmed by microscopy after a 24-hour recovery period. After recovery, media was removed and replaced by experimental media. At termination of the incubations, media was replaced with TRI Reagent, and the cell suspension removed by pipette for nucleic acid isolation.

The lyophilized leptin used in the cell cultures was first dissolved in 500 µL of 0.1 mM PBS (pH 8.3) and this stock was then diluted into RPMI growth media to desired concentrations. We have shown in previous publications that human recombinant leptin is bioactive in reducing feeding in HSB (Won et al., 2012), and is effective in inducing prolactin secretion in tilapia (Tipsmark et al., 2008). **The effectiveness of recombinant human leptin has been shown to be overall comparable to homologous peptide in regulating glucose mobilization, tissue glycogen levels and the expression of many metabolic genes in tilapia (Baltzegar et al., 2014).** Mammalian GH, including bovine, was shown to be effective in stimulating IGFs in a variety of teleosts (Duguay et al., 1996; Shablott et al., 1995; Vong et al., 2003). The concentrations of treatment peptides used in these experiments were based on similar *in vitro* studies using fish (Tipsmark et al., 2008, Pierce et al., 2010).

2.3 *In vivo* effects of leptin on GHR1 and IGF-1 gene expression

The juvenile, phase I HSB used in the *in vivo* leptin injection study ($45 \text{ g} \pm 0.80 \text{ g}$; mean body weight $\pm$ SEM) were housed in six 75-liter freshwater tanks, which were randomly assigned as control or treatment groups ($N = 8$ fish/group). At the onset of the experiment, fish were anesthetized with MS 222 (Western Chemical, Ferndale, WA) and injected intraperitoneally with 50 µL saline (control) or leptin at doses of 100 ng/g or 1 µg/g body weight (BW) (Won et al., 2012). Leptin was dissolved in 0.1 mM PBS (pH 8.3) and then diluted with biological saline

immediately prior to injection. As a positive control to assess hormone delivery methods and to verify classic endocrine growth axis regulation by GH, a separate group was injected with GH (5 µg/g BW) to confirm IGF-1 responsiveness (data not shown). At 6 or 24 hrs post-injection, fish were again anesthetized using MS 222, decapitated, and liver samples taken. Fish were not fed during the day of the experiment. Liver was stored in RNAlater (Qiagen, Valencia, CA) until processing for measurement of hepatic GHR1 and IGF-1 mRNA. Nucleic acid samples from this experiment were lost due to a hardware malfunction prior to measuring GHR2 and IGF-2 mRNA levels.

2.4 Nucleic acid isolation and cDNA synthesis

Total RNA was isolated from cells with TRI Reagent (Molecular Research Center; Cincinnati, OH) using the manufacturer's procedure. RNA concentration and purity were determined by Nanodrop ND-1000 spectrophotometry (Nanodrop Technologies; Wilmington DE) and gel electrophoresis (1% agarose, 0.6 µg/ml ethidium bromide) before and after DNase treatment (DNA-free; Ambion; Austin, TX). Absorbance ratios of samples at 260:280 nm ($A_{260/280}$) ranged from 1.8–2.0. Complementary DNA (cDNA) was synthesized from 1 µg of DNase-treated total RNA using a High Capacity cDNA Reverse Transcription kit and the manufacturer's protocol (Applied Biosystems, Foster City, CA).

2.5 Quantitative RT-PCR gene expression measurement

Relative GHR1, GHR2, IGF-1 and IGF-2 mRNA expression was determined by quantitative RT-PCR (qRT-PCR) according to our previously established protocols (Picha et al., 2008). Gene-specific primers (Table 1) were designed using Vector NTI software (Invitrogen).

Analysis was performed on an ABI 7300 HT Sequence Detection System, using Brilliant SYBR Green II master mix (Stratagene; La Jolla, CA), 1.5 μ M primers, and 6 ng of cDNA in a total reaction volume of 25 μ L. The qRT-PCR cycling parameters were 95° C for 10 minutes followed by 40 cycles of 95° C for 15 seconds and 60° C for 1 minute. The absence of genomic DNA contamination was confirmed using water (no template control) and DNase-treated RNA (no amplification control) as negative control templates. Primer specificity was verified by gel visualization (2% agarose gel, 0.6 μ g/ml ethidium bromide) of a single PCR amplicon and by direct sequencing of the amplicon for each gene (University of Chicago Cancer Research Center), as well as by dissociation curve analysis following qRT-PCR cycles. For qRT-PCR analysis, cycle threshold (Ct) values for experimental samples were transformed using a standard curve of serially diluted cDNA versus Ct values ($R^2 > 0.97$) and then normalized to reflect the amount of template cDNA / ng total RNA loaded into each reaction (Metzger et al., 2012; Picha et al., 2008). Gene expression data is presented as a percentage of the control mean (% control $\pm$ SEM). The results from these experiments were substantiated by data normalized to 18S RNA and the housekeeping gene, β -actin (not shown). PCR efficiency was calculated using the slope of the standard curve in the formula: $\text{Efficiency} = -1 + 10^{(-1/\text{slope})}$. Efficiencies for primers ranged from 97-102%.

2.6 Statistics

Quantitative real-time PCR data from hepatocyte culture experiments were analyzed by one-way ANOVA (treatment) followed by Fisher's LSD test for significant differences using Statistica 7.0 software (Stat Soft; Tulsa, OK). Data from the *in vivo* trial were analyzed by two-way ANOVA (treatment $\times$ time) followed by Bonferroni's post hoc test using GraphPad Prism 6

software (La Jolla, CA). Gene expression data for the *in vivo* study was log transformed prior to ANOVA to satisfy Bartlett's test for equal variances. The threshold for statistical significance was set at $P < 0.05$.

3. Results

3.1 Hepatocyte culture validation: *in vitro* regulation of IGF gene expression by GH

In the first study, primary hepatocyte cultures were incubated with two concentrations of GH (5 or 50 nM) for 18 hrs (Figure 1). The 50 nM treatment increased IGF-1 by 16-fold ($P < 0.001$) and IGF-2 mRNA by 4.3-fold ($P < 0.001$). The 5 nM treatment did not have significant effects on IGF gene expression. In as much as GH is a classic stimulator of IGF-1 synthesis and secretion, this experiment serves to validate that the hepatocytes were functional following isolation and viable in the reagents and incubation conditions utilized in these investigations.

3.2 Regulation of IGF and GH gene expression by leptin in hepatocytes

In the second experiment, HSB primary hepatocyte cultures were incubated with low (5 nM) and high (50 nM) concentrations of human recombinant leptin for 8 or 24 hours to test if leptin modulates hepatic mRNA expression of GHRs and/or IGFs (Figure 2). Leptin treatment upregulated mRNA levels of both forms of GHRs and IGFs in hepatocytes. GHR2 gene expression was sensitive to both the low and high leptin concentrations by 8 hours, respectively rising 3.7 and 4.2-fold above control levels ($P < 0.001$). After 24 hours, the GHR2 response to 50 nM leptin was maintained at a 3.4-fold higher level than controls ($P < 0.001$). The higher leptin treatment increased GHR1 mRNA levels by 5.4-fold during 24 hour incubations ($P < 0.001$). The higher leptin concentration also directly upregulated IGF-1 mRNA by 3.3-fold ($P <$

0.001) and IGF-2 by 2.2-fold ($P < 0.001$) after a 24 hour incubation relative to untreated control cells.

3.3 *In vitro* regulation of IGF and GHR in hepatocytes co-incubated with GH and leptin

In the third experiment, hepatocytes were incubated with an intermediate concentration (25 nM) of GH, leptin, or both hormones for 24 hours (Figure 3) to determine if the two peptides might work synergistically in stimulating IGF mRNA expression; namely through upregulated GHR expression by leptin, which would theoretically enhance responsiveness of IGF to GH stimulation. There was no effect by GH alone on regulating mRNA levels of either GHR compared to controls. Leptin alone increased GHR1 (2-fold; $P < 0.05$), but not GHR2 mRNA levels after 24 h incubations. Levels of GHR1 and GHR2 mRNA were, however, both elevated by about 2-fold (up 84% and 115% respectively; $P < 0.05$) in the group co-incubated with leptin and GH. The level of IGF-1 mRNA was moderately increased above controls by leptin alone (2-fold; $P = 0.18$) and significantly by GH alone (3-fold; $P < 0.05$), as well as by the combination of leptin and GH treatments (4.8-fold; $P < 0.001$). The fold-increase in IGF-1 gene expression elicited by leptin and GH together was approximately the sum of the fold changes elicited by the treatment peptides individually. At the 25 nM dosage, no effect on IGF-2 mRNA levels ($P > 0.05$) by individual or combined hormone treatments was detected.

3.4 *In vivo* effect of leptin on hepatic GHR1 and IGF-1 gene expression

Hepatic GHR1 and IGF-1 mRNA levels were measured at 6 and 24 hours following 0 ng, 100 ng or 1 ug/g BW leptin injection (Figure 5). A 5 µg/g BW injection of GH significantly

increased hepatic IGF-1 mRNA by 50-fold after 24 hours (data not shown), validating hormone activity and delivery method. IGF-1 gene expression dropped in controls by about 6-fold between the 6 to 24 hour sample points, although the difference was not statistically significant ($P > 0.05$). After 24 hours, hepatic IGF-1 mRNA levels were significantly elevated by the 100 ng/g BW leptin injection (up 10-fold; $P < 0.01$) relative to controls, but not by the higher dose (up 2.5-fold; $P > 0.05$). Although the 1000 ng/g BW leptin dosage raised hepatic GHR1 mRNA levels by 2.3-fold at 6 hrs, the effect was marginal ($P = 0.12$). A 5 µg/g BW injection of GH significantly increased hepatic IGF-1 mRNA by 50-fold after 24 hours (data not shown), validating hormone activity and delivery method. No differences in GHR1 expression were detected between groups at 24 hours, or between control levels at 6 and 24 hours.

4. Discussion

The present studies explored the possibility that leptin might modulate endocrine growth axis signaling in the liver by regulating hepatic GHR and IGF gene expression. We show that GH increased IGF-1 and IGF-2 mRNA in cultured HSB hepatocytes, but was ineffective at regulating its own receptors, GHR1 and GHR2. Leptin treatment also enhanced *in vitro* hepatic gene expression of both forms of IGFs as well as GHRs, which may represent an autocrine or paracrine regulatory relationship considering that all of these components are expressed in the liver. Leptin injection maintained hepatic IGF-1 mRNA levels *in vivo* after 24 hrs of fasting compared to controls, which experienced a drop, suggesting leptin may have buffered the catabolic decline in mitogen expression that is typical during fasting. Together, these results suggest that leptin may serve as a stimulator of IGF-1 and IGF-2 production directly, as well as potentially contribute to enhanced GH sensitivity via its ability to upregulate GHR transcription.

In vivo and *in vitro* studies have clearly established that GH acts in its classic manner to stimulate growth through regulation of IGF-1 synthesis and secretion in teleosts through both endocrine and paracrine mechanisms (Leung et al., 2008; Pierce et al., 2005; Schmid et al., 2000; Shambloott et al., 1995). Research also suggests that IGF-2, which is synthesized in hepatic tissue throughout life in teleosts (Chen et al., 2000; Gabillard et al., 2006; Peterson et al., 2004; Picha et al., 2008; Terova et al., 2007), is similarly regulated by GH in a range of fishes (Carnevali et al., 2005; Gahr et al., 2008; Moriyama et al., 2008; Peterson and Small, 2005; Ponce et al., 2008; Tse et al., 2002). The degree of effectiveness that GH has in stimulating production of the two forms of IGF may vary, however. For example, native GH increased IGF-1 and IGF-2 mRNA levels by a similar magnitude (about 3-fold using 5-50 nM GH) in coho salmon hepatocytes (Pierce et al., 2010; Pierce et al., 2005). In contrast, native GH (0.5-50 nM) stimulated IGF-2 by around 8-fold in tilapia hepatocytes while it increased IGF-1 transcript by 2-fold (Pierce et al., 2011).

In accord with conventional growth axis signaling, we found that GH has a potent stimulatory effect on IGF-1 as well as IGF-2 gene expression in *Morone* hepatocytes, with IGF-1 exhibiting higher sensitivity than IGF-2 to GH. The mRNA levels of IGF-1 induced by 50 nM GH were about four times greater than those of IGF-2 (Figure 1), while a GH concentration as low as 25 nM was effective at stimulating IGF-1, but not IGF-2 gene expression (Figure 3). Whether the discrepancy between *Morone* and other species in *in vitro* responsiveness of the two IGFs to GH stimulation reflects fundamental interspecies differences, variable effectiveness of heterologous and native peptides, or different experimental conditions is uncertain. The response nevertheless demonstrates that hepatocytes used in these studies were functionally intact

following isolation, and that GH exerts direct effects at the level of the hepatocyte to stimulate production of not only IGF-1, but also IGF-2 in *Morone*.

Leptin's adipostatic role as an endocrine indicator of energy reserves and nutritional state is well documented in mammals (Arora and Arora, 2008; Copeland et al. 2011), in which leptin levels decline during states of fasting and rise with increased fat deposition (Ahima et al., 1996; Dubuc et al., 1998). In many fish, however, leptin is predominantly expressed in the liver where the majority of GH-mediated IGFs are also produced. Hepatic leptin (Won et al., 2012), GHR1, GHR2, IGF-1 and IGF-2 (Picha et al., 2008) show similar expression patterns under altered metabolic states in HSB suggesting a possible interaction. We found that leptin not only increased mRNA levels of both GHRs, but that transcription of both IGFs was also upregulated by leptin in the absence of exogenous GH (Figure 2). The response of GHR2 was more sensitive than GHR1 to leptin treatment, exhibiting upregulated mRNA levels at a lower leptin concentration and at an earlier time point, although the fold-increase in GHR1 mRNA was nearly 2-fold greater. To our knowledge, these results represent the first account of leptin regulating GHRs in any vertebrate, or it regulating IGFs in a teleost, suggesting that leptin may have a previously unrecognized influence on metabolism or growth through upstream regulation of the GH-IGF axis.

In order to test if the increase in GHR mRNA levels by leptin contributes to GH sensitivity, hepatocytes were incubated alone or in combination with intermediate leptin and GH concentrations (25 nM; Figure 3) based on preceding experiments. The elevated mRNA levels observed in both GHRs in this trial were likely attributable to the leptin treatment, as GH treatment alone did not affect mRNA levels of either form of its own receptor. While the reason for the lack of effect by leptin alone on GHR2 in this experiment is uncertain, the respective

responses to lower and higher leptin concentrations used in the previous trial suggests that the 25 nM leptin treatment may not have been sufficient to elevate or sustain GHR2 transcript over 24 hrs, but that an interaction between leptin and GH maintained elevated gene expression levels of this receptor; a possibility which may warrant further investigation.

Individual 25 nM leptin and GH treatments upregulated IGF-1 transcript to lesser extents (Figure 3) than the 50 nM concentrations tested in earlier experiments (Figures 1 and 2), while co-incubation with both treatment peptides together raised IGF-1 mRNA levels by approximately the sum of the marginal increases elicited by the hormones individually. This additive effect of the combined treatments on IGF-1 gene expression suggests that the stimulatory effect of leptin on GHRs may be limited to the transcriptional level, at least under the culture conditions and duration utilized here. Had translation of new functional GHRs taken place, in other words, the presence of exogenous GH and leptin should theoretically have had a synergistic effect on IGF-1 expression (*e.g.* Li et al., 2011) through enhanced growth axis signaling.

In contrast to IGF-1, IGF-2 transcript levels were not altered by the leptin or GH treatments, including co-incubation, likely due to the lower concentration of the treatment hormones used in this experiment. It would appear that a minimal threshold concentration between 25 and 50 nM of both treatment peptides is necessary to directly upregulate IGF-2 mRNA, as 5 nM had already been shown to have no significant effect (Figure 2). The fact that IGF-2 gene expression was not upregulated in the presence of GH despite elevated GHR mRNA levels nonetheless substantiates that leptin does not promote sensitivity of the cells to GH through enhanced production and placement of GHR proteins, but may rather be working instead to increase GHR transcription or mRNA stability as part of a larger mechanism responsible for

modulating growth axis signaling. Additional anabolic factors, such as metabolic substrates or insulin (Butler et al., 2003; Houston and O'Neill, 1991), may ultimately be needed in conjunction with leptin to induce the full translation of functional GHRs in liver. Furthermore, leptin is a demonstrated stimulator of suppressors of cytokine signaling (SOCS) (Jorgensen et al., 2013), raising the possibility that leptin signaling may disrupt the predicted synergistic increases in IGF-1 and IGF-2 mRNA during cotreatment with GH.

In support of the *in vitro* results, a 100 ng/g BW leptin injection upregulated hepatic IGF-1 mRNA levels *in vivo* at 24 hours relative to controls (Figure 4). Control fish experienced a marginal drop in IGF-1 gene expression at 24 hours compared to the earlier time point, likely due to feed restriction (Reinecke, 2006). The leptin treatment may therefore have buffered the response to fasting and contributed to the maintenance of hepatic IGF-1 mRNA levels. Chronic leptin treatment also raised hepatic IGF-1 mRNA levels in pigs (Ajuwon et al., 2003), as well as circulating IGF-1 levels in leptin deficient mice (Bartell et al., 2011b) and humans (Chan et al., 2008a). It is not clear, however, why only the lower leptin dose stimulated IGF-1 gene expression. While similar dosages of leptin significantly reduced feeding in HSB to about the same degree in a previous study (Won et al., 2012), the higher dosage resulted in a marginally weaker anorexigenic response. In tilapia, 0.5 µg/g BW injections of either human or native leptin had a strong stimulatory effect on prolactin gene expression, whereas a higher 5 µg/g BW dose had a lesser or no effect (Douroso et al., 2014). Leptin had a biphasic effect in regulating its own receptor in rat hypothalamic explants, with attenuated stimulation occurring at high treatment concentrations until the effect was ultimately reversed (Di Yorio et al., 2008). It is therefore possible that the range of dosages used in this trial approached an upper limit and became ineffective at the high dose.

Although the mechanism by which leptin stimulates IGF-1 gene expression *in vivo* is currently unknown, the hormone is likely to exert its effects, at least in part, through direct regulation of hepatocytes, as demonstrated here, as well as indirectly, possibly through enhanced GH signaling (Barb et al., 1998; Mizuno et al., 1999; Tannenbaum et al., 1998) or via other regulatory pathways. Exogenous leptin injection did not, however, significantly alter hepatic GHR1 mRNA levels (data for GHR2 was not available). Catabolic factors, such as somatostatins (Very and Sheridan, 2007) or cortisol (Small et al., 2006), which are known to inhibit transcription and translation of GHRs in fish, could have dampened the response *in vivo*, but would not likely have been convoluting factors in the *in vitro* experiment using isolated cells.

The stimulation of hepatic GHR and IGF gene expression by leptin observed in the current study raises the possibility that leptin might regulate the endocrine growth axis within the liver to help coordinate somatic growth and metabolism with nutritional status. In accord with this idea, our previous work shows that hepatic mRNA levels of **leptin** in HSB decrease during fasting and rise during refeeding (Won et al., 2012), paralleling the responses of colocalized GHRs and IGFs in the liver (Picha et al., 2008). For cases like HSB, in which leptin positively reflects endogenous energy availability, the present study suggests that leptin may serve to promote hepatic GHR and IGF gene expression when conditions are favorable for growth. Because leptin responds differently to nutritional state among the fish that have been studied to date, however, it is difficult to speculate if or how it might functionally interact with the growth axis in various species with different life histories. Depending on the tissue and leptin type, leptin appears to reflect energy status in some fish (Gorissen et al., 2009; Johnson et al., 2000; Kling et al., 2012; Nieminen et al., 2003), **although most fish show the opposite trend** (Frøiland et al., 2012; Gambardella et al., 2012; Kling et al., 2009; Trombley et al., 2012) or no long-term

relationship (Huising et al., 2006) between leptin and feeding history. Leptin might rather function to suppress lipolytic pathways in some fish during fasting. With these studies in mind, it would be counterintuitive for leptin to stimulate growth in cases where it is not reflective of energy deposition. In Arctic charr, for example, hepatic leptin mRNA levels were either maintained or rebounded during seasonal fasting as IGF-1 levels declined while, in liver slices of fasted fish, leptin was ineffective at regulating IGF-I gene expression (Jorgensen et al., 2013). Our group has observed that leptin gene expression increases with fasting in tilapia, while recombinant homologous leptin elicits a significant stimulatory effect on IGFs and GHRs in tilapia hepatocyte cultures (unpublished results). Further research is clearly needed to determine if the regulatory properties of leptin on hepatic growth axis genes are apparent in other fish despite interspecific differences in leptin expression patterns and, if so, if local or systemic leptin, or a particular leptin type, is responsible for carrying out these actions. Such insights may eventually help provide context(s) for the regulatory effects of leptin on growth axis transcripts relative to variable nutritional states among fishes. Expanded investigations could also determine if our findings have clinical relevance in describing an adipostatic regulator of growth in humans or other mammalian models that use leptin as a gauge of endogenous energy.

In summary, these experiments show that leptin and GH exerted direct effects at the level of the hepatocyte to stimulate IGF-1 and IGF-2 gene expression in HSB. In addition to upregulating IGF transcripts *in vitro*, leptin also stimulated the accumulation of hepatic IGF-1 mRNA *in vivo*, together indicating for the first time in a teleost that leptin may influence expression of these key growth regulatory hormones in the liver. These studies also establish, for the first time in vertebrates, a novel function for leptin in increasing GHR1 and GHR2 gene expression and the potential to modulate hepatic GH sensitivity. Considering that leptin is

predominantly produced in the liver of these and other fish, we postulate that the hormone may serve as a paracrine or autocrine regulator of hepatic GHR and IGF expression, ultimately modulating metabolism and somatic growth.

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FIGURE LEGENDS

Figure 1. Effect of 5 and 50 nM GH treatment on IGF-1 (top) and IGF-2 (bottom) mRNA levels in HSB hepatocytes incubated for 18 hrs. Gene expression levels are expressed as a percentage of control levels (mean $\pm$ SEM; N = 5-6 wells/group). Asterisks denote significant differences between treatment groups and control group ($***P < 0.001$).

Figure 2. Effect of 5 and 50 nM leptin treatment on GHR and IGF mRNA levels in HSB hepatocytes incubated for 8 and 24 hrs. Gene expression levels are expressed as a percentage of control levels at each time point (mean $\pm$ SEM; N = 4-6 wells/group). Asterisks denote significant differences between treatment groups and the control group at each time point ($***P < 0.001$).

Figure 3. Effects of leptin and GH (25 nM each) treatment on GHR and IGF mRNA levels in hepatocytes incubated for 24 hrs. Gene expression levels are expressed as percent control (mean $\pm$ SEM; N = 4-5 per group). Different letters denote significant differences between groups ($P < 0.05$).

Figure 4. Effect of interperitoneal leptin injection (0, 100 and 1000 ng/g BW) on *in vivo* hepatic IGF-1 (top) and GHR1 (bottom) mRNA levels. Samples were taken 6 (gray) and 24 (hatched) hrs post-injection. Gene expression data are expressed as cDNA/ng total RNA (mean $\pm$ SEM; N=6-8 per group). Asterisks denote significant differences between treatment groups and control group ($**P < 0.01$).

Table 1. SYBR Green primers used for relative quantification of IGF and GHR transcript levels using qRT-PCR.

	5'-Forward-3'	5'-Reverse-3'
IGF-1	AGCCACACCCTCTCACTACTGCTG	CAGGTGCACAGTACATCTCCAGGC
IGF-2	AAACAACAGACGGACCCAGAA	AGCAAGTTGAGGTCACAGCTA
GHR-1	TTAAAGGCATCGATCCAGAGCTGT	CATGTGGTTGCTGACGGGTTGTAG
GHR-2	TTGAGGAGCACAACGACAGACTGG	TGTTCCCTCAGGTGTCAGCAGCA

Figure 1

Figure 2

Figure 3

Figure 4