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Control of leptin by metabolic state and its regulatory interactions with pituitary growth hormone and hepatic growth hormone receptors and insulin like growth factors in the tilapia (*Oreochromis mossambicus*)

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Abstract

Leptin is an important cytokine for regulating energy homeostasis, however, relatively little is known about its function and control in teleost fishes or other ectotherms, particularly with regard to interactions with the growth hormone (GH)/insulin-like growth factors (IGFs) growth regulatory axis. Here we assessed the regulation of LepA, the dominant paralog in tilapia (*Oreochromis mossambicus*) and other teleosts under altered nutritional state, and evaluated how LepA might alter pituitary growth hormone (GH) and hepatic insulin-like growth factors (IGFs) that are known to be disparately regulated by metabolic state. Circulating LepA, and *lepa* and *lepr* gene expression increased after 3-weeks fasting and declined to control levels 10 days following refeeding. This pattern of leptin regulation by metabolic state is similar to that previously observed for pituitary GH and opposite that of hepatic GHR and/or IGF dynamics in tilapia and other fishes. We therefore evaluated if LepA might differentially regulate pituitary GH, and hepatic GH receptors (GHRs) and IGFs. Recombinant tilapia LepA (rtLepA) increased hepatic gene expression of *igf-1*, *igf-2*, *ghr-1*, and *ghr-2* from isolated hepatocytes following 24 h incubation. Intraperitoneal rtLepA injection, on the other hand, stimulated hepatic *igf-1*, but had little effect on hepatic *igf-2*, *ghr1*, or *ghr2* mRNA abundance. LepA suppressed GH accumulation and *gh* mRNA in pituitaries *in vitro*, but had no effect on GH release. We next sought to test if abolition of pituitary GH via hypophysectomy (Hx) affects the expression of hepatic *lepa* and *lepr*. Hypophysectomy significantly increases hepatic *lepa* mRNA abundance, while GH replacement in Hx fish restores *lepa* mRNA levels to that of sham controls. Leptin receptor (*lepr*) mRNA was unchanged by Hx. In *in vitro* hepatocyte incubations, GH inhibits *lepa* and *lepr* mRNA expression

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at low concentrations, while higher concentration stimulates *lepa* expression. Taken together, these findings indicate LepA gene expression and secretion increases with fasting, consistent with the hormones function in promoting energy expenditure during catabolic stress. It would also appear that LepA might play an important role in stimulating GHR and IGFs to potentially spare declines in these factors during catabolism. Evidence also suggests for the first time in teleosts that GH may exert important regulatory effects on hepatic LepA production, insofar as physiological levels (0.05–1 nM) suppress *lepa* mRNA accumulation. Leptin A, may in turn exert negative feedback effects on basal GH mRNA abundance, but not secretion.

Keywords

Leptin; Growth hormone; Insulin-like growth factors; Growth hormone receptor; Teleost fishes; Metabolic stress

1. Introduction

Leptin is a 16 kDa cytokine hormone known to regulate metabolism and energy expenditure in vertebrates (Zhang et al., 2013). In mammals, leptin increases during feeding as lipids accumulate, and acts to simultaneously promote satiety and induce lipolysis; thus the hormone has been canonically described as an adipostat that prevents excessive fat accumulation (Ahima and Flier, 2000). Consistent with this role, depletion of adipose stores during fasting acts to greatly reduce leptin abundance and expression (Harris et al., 1996; Sandoval and Davis, 2003). Evidence suggests, however, that the adipostatic model for leptin may not apply to teleost fish and perhaps other ectotherms, as the hormone tends to increase during fasting in most, but not all teleosts examined to date (see reviews by Copeland et al., 2011; Gorissen and Flik, 2014).

Through gene or genome-wide duplication events, multiple forms of leptin exist in fishes (Huising et al., 2006b) with the major form, leptin A (LepA) produced predominantly in the liver in most species including the tilapia (Angotzi et al., 2013; Douros et al., 2014; Trombley et al., 2012b). Evidence suggests that leptin increases in response to various stressors (e.g. fasting, hypoxia and acute salinity challenge; Baltzegar et al., 2014; Bernier et al., 2012; Kling et al., 2009b) and acts as a potent hyperglycemic factor, in part, through stimulation of glycogenolysis (Baltzegar et al., 2014). Consistent with this action, the preponderance of evidence in fishes indicates that leptin gene expression or circulating leptin rises with fasting when energy stores decline and falls during feeding when fat or carbohydrate stores accumulate as observed in fine flounder, Arctic charr, and rainbow trout (Fuentes et al., 2012; Jorgensen et al., 2013; Kling et al., 2009a). In other studies, fasting was shown to either stimulate (Volkoff et al., 2003) or exert little effect on leptin gene expression (Tinoco et al., 2012) in goldfish. In carp, fasting had little effect on leptin gene expression (Huising et al., 2006a), or in the case of hybrid striped bass, it may stimulate leptin gene expression in a manner similar to mammals (Won et al., 2012; Won and Borski, 2013). Research utilizing heterologous assays suggests that a leptin-like peptide may decrease with fasting in green sunfish (Johnson et al., 2000). Despite its wide commercial appeal as a foodfish, there is no information on leptin regulation by metabolic state in tilapia

and relatively few studies (outside of salmonids) have addressed the dynamics in both leptin gene expression and secretion in teleost fishes.

Somatic growth in fish is dependent on nutritional status and, as in most other vertebrates, is primarily regulated by the endocrine growth hormone (GH)/insulin-like growth factor (IGF) axis (Picha et al., 2008; Reinecke et al., 2005; Won and Borski, 2013). How leptin interacts with GH-IGF axis to regulate metabolism and growth in fishes is poorly understood. Various endogenous (nutrients, hormones) and exogenous factors (salinity, temperature) act either directly at the pituitary or through the hypothalamus to regulate GH synthesis and secretion (Canosa et al., 2007). Upon entering the circulation GH binds to one of the two major paralogs of its receptor (GHR1, GHR2) (Jiao et al., 2006) on target tissues of fish, including the liver and muscle, to induce the expression and secretion of IGFs (IGF-1 and IGF-2) which in turn stimulate cell differentiation and proliferation leading to increased somatic growth (Duan, 1997). Plasma IGF-1 subsequently acts in a negative feedback fashion to inhibit pituitary GH secretion (Fruchtman et al., 2002). Additionally, both IGF-1 and IGF-2 act in a classic insulin fashion to promote glucose uptake and glycogen synthesis in peripheral target tissues (Froesch et al., 1985). It is well established that feed restriction induces a hepatic GH-resistant state; reducing GHR and/or IGF expression and abundance as well as growth in the face of elevated GH in various vertebrates including numerous teleost fishes (Fox et al., 2006; Reinecke et al., 2005; Won and Borski, 2013). These results along with those described above for leptin indicate that the hepatic expression of GHRs and IGFs and circulating IGF-1 are regulated discordantly with leptin, whose dynamics more closely resemble that of pituitary GH than hepatic GHR/IGFs in fasted animals. However, no comprehensive assessment of leptin interactions with the pituitary GH- hepatic GHR/IGF growth axis has been undertaken in fish or other ectotherms.

In mammalian models, leptin appears to stimulate IGF-1 both *in vivo* and *in vitro* (Chan et al., 2008; LaPaglia et al., 1998), but information on its potential regulation of GH receptors is altogether lacking. Leptin had little effect on *in vitro* IGF-1 expression in arctic charr while it enhanced suppressors of cytokine signaling (SOCS), key proteins that feedback to reduce cytokine signals (Jorgensen et al., 2013). Our recent work shows human leptin stimulates hepatic IGFs and GHR expression in the hybrid striped bass (Won et al., 2016), but it is uncertain whether this response might occur with homologous hormone or is conserved among other teleosts, particularly in those species where leptin shows a discordant regulation to GHRs and IGFs with altered nutritional state, *i.e.* leptin increases and GHRs/IGFs decline with fasting-induced catabolism and vice versa with feeding.

In addition to its regulatory effects on IGFs, evidence suggests leptin stimulates pituitary GH gene expression and secretion in rodents and ungulates (Barb et al., 1998; Morrison et al., 2002). Growth hormone itself suppresses leptin secretion from adipocytes in humans and cow (Engstrom et al., 2003; Houseknecht et al., 2000), and recent evidence with chronic implants shows the hormone may exert a mildly suppressive effect on leptin secretion in Atlantic salmon (Kling et al., 2012). Despite the well-established effect of leptin in promoting pituitary GH secretion in mammals, nothing is known about its actions on GH cell function in teleosts.

Ultimately these studies were designed to test the hypothesis that leptin might serve as an intermediary signal for nutrient status to regulate metabolism and somatic growth. The aims of this study were to (1) assess the regulation of leptin A (LepA) under different metabolic states in tilapia (*Oreochromis mossambicus*), (2) examine how changes in LepA, the dominant leptin paralog in diploid fishes, might alter pituitary GH synthesis and secretion and hepatic gene expression of GHRs and IGFs, and (3) assess if GH itself might regulate gene expression of leptin and its receptor in the liver.

2. Materials and methods

All studies except for the hypophysectomy experiment were conducted at North Carolina State University and were approved by its Institutional Animal Care and Use Committee (IACUC). The University of Hawaii IACUC approved the hypophysectomy protocol.

2.1. Regulation of LepA by metabolic state induced through fasting and refeeding

Adult male tilapia (154 ± 35 g body weight) used in feed restriction studies were acclimated to freshwater (FW) recirculating tank systems (salinity 0–0.5 ppt, hardness 74–84 mg/L, alkalinity 126–178 mg/L, pH 8.0) kept at 24–26 °C with a photoperiod of 12:12 h of light and dark and fed daily for 2 weeks prior to the beginning of the study. Treatment fish were fasted for the initial 3 weeks (days 0–21) of the experiment, after which time they were placed on the control diet for 10 days (days 22–31). Fish ($n = 8$ /group) were sampled at 0, 7, 21, 24, and 31 days. All control fish were fed once daily to apparent satiation throughout the 31-day experiment. Body weight (Li et al.) and liver weights were recorded for each sampling point; hepatosomatic index (HSI) was calculated for each fish ([liver weight/BW] $\times$ (100)). Additionally, blood was collected via the caudal vein for an assessment of circulating LepA levels by homologous ELISA (Douros et al., 2014) while liver samples (~ 100 g) were taken and stored in RNAlater for subsequent processing of samples for gene expression analysis of hepatic *lepa* and *lepr* (Table 1).

2.2. In vitro effects of rtLepA and GH on expression of genes in isolated hepatocytes

We assessed the *in vitro* effects of recombinant tilapia LepA (rtLepA) and bovine GH (bGH) on the hepatic expression of genes for *igf-1*, *igf-2*, *ghr1*, *ghr2*, *lepa* and/or *lepr* using hepatocyte incubations according to our previously described methods (Douros et al., 2014). Hepatocytes harvested from male freshwater fish (95 ± 19 g) fed at 2–3% BW were used for the studies. Liver was removed and finely diced with a razor blade in a Ca^{2+} -free Hank's buffered salt solution (HBSS) containing 0.3 mg/mL type IV collagenase (Sigma, St. Louis, MO). Tissues were incubated in the HBSS-collagenase solution for 30–45 min at room temperature with gentle agitation. The digested tissue was pushed through a 260 μm mesh filter to remove structural tissue, and then allowed to drip through a second 60 μm mesh. Filtered hepatocyte suspension was collected in a beaker kept on ice. The cells were then washed 5x in HBSS containing 3 mM CaCl_2 and 1x MEM solution, with essential and nonessential amino acids (Gibco, Carlsbad, CA), then allowed to recover on ice for 1 h. After a 1 min centrifugation at 70g, cells were resuspended in RPMI 1640 growth media containing L-glutamine (Gibco, Carlsbad, CA; 1% streptomycin/penicillin mixture added). The hepatocytes were then plated on 24-well Falcon Primaria dishes at a density of $\sim 3.5 \times$

10⁵ cells/mL. Cell viability was determined to be >90% using the Trypan Blue exclusion test. After plating, cells were allowed to acclimate for 4 h at 24 °C in an air atmosphere. After acclimation, the media were removed, replaced by fresh experimental media containing either rtLepA, bGH or no hormone and then incubated for 18 h at 24 °C under air. At termination media was removed TRI Reagent added to lyse cells. The lysed cell suspension was aspirated with a pipette and held at 4 °C overnight prior to RNA isolation. The functionality of the hepatocytes was confirmed by assessing the stimulation of *igf-1* gene expression by bovine growth hormone.

Hormones were applied to cell incubations as follows. Lyophilized leptin was first dissolved in 1 mL PBS (pH 8.3) and this stock was then diluted into RPMI growth media to 0.1, 1, 10, 100 or 500 nM rtLepA. For treatment with GH, cells (n = 5 wells/group) were incubated in growth medium containing 0, 0.1, 1, 10 or 50 nM bGH (National Hormone and Peptide Program, Torrance, CA), previously shown to be bioactive in stimulating IGFs in *O. mossambicus*. (Pierce et al., 2011; Schmid et al., 2000).

2.3. In vivo effect of rtLepA on hepatic ghr and igf expression

For injection experiments, rtLepA was used and was previously validated for bioactivity in tilapia (Baltzegar et al., 2014; Douros et al., 2014). Adult male tilapia (95 ± 19 g mean) were acclimated for 3 weeks in brackish water recirculating tank systems (11–13 ppt, hardness 209–263 mg/L, alkalinity 127–152, pH 8.0) held at 24–26 °C with a photoperiod of 12:12 h of light and dark. Fish were anesthetized in MS-222 (Aquatic EcoSystems, Apopka, FL), weighed, and then administered with 0.5 or 5.0 µg/g BW of rtLepA in PBS or a PBS vehicle (n = 8 fish/group). Twenty-four hours post-injection, the fish were anesthetized, decapitated and a section of liver collected and stored in RNAlater prior to assessment of *igf-1*, *igf-2*, *ghr1*, or *ghr2* mRNA levels. The rtLepA doses were chosen based on their effectiveness in inducing hyperglycemia and regulating prolactin gene expression in tilapia (Baltzegar et al., 2014).

2.4. Effect of hypophysectomy and GH replacement on lepa and lepr expression

Adult male tilapia (*O. mossambicus*; 57–95 g) were reared in outdoor tanks (700 L) with a continuous flow of FW under natural photoperiod. Water temperature was maintained at 24–26 °C, and the animals were fed at 2–3% BW/day with a commercial feed (Skretting, Tooele, UT). Hypophysectomy (Hx) was performed by the transorbital technique (Breves et al., 2010). Briefly, fish were anesthetized in a combination of buffered MS-222 (100 mg/L, Argent Chemical, Redmond, WA) and 2-phenoxyethanol (0.3 ml/L, Sigma, St. Louis, MO), and a small hole was made through the neurocranium following removal of the right eye and underlying tissue. The pituitary was aspirated with a modified Pasteur pipette, and its complete removal was later confirmed by post-mortem inspection at sampling. The right orbit was packed with microfibrillar collagen hemostat (Ethicon, Somerville, NJ). Sham operations were carried out in the same manner, but without aspiration of the pituitary. The fish were allowed to recover for 4 days in 1/3 seawater (SW; 12 ppt) before receiving intraperitoneal (IP) injections of ovine GH (5.0 µg/g BW in 0.9% NaCl, Sigma) or vehicle (0.9% NaCl). Two consecutive injections were administered 24 h apart, with sham fish receiving only the saline vehicle. At 48 h after the second injection, fish were anesthetized in

buffered MS-222 and subsequently decapitated. Liver samples (n = 6–7/group) were placed in RNAlater overnight at 4 °C and then frozen at –80 °C prior to processing for RNA isolation as outlined below, and assessment of hepatic *lepa* and *lepr* gene expression. Due to requirements of other parallel studies adequate amounts of plasma were not available for assessment of circulating LepA levels.

2.5. Effect of rtLepA on pituitary GH gene expression and GH secretion in vitro

The *in vitro* pituitary proximal pars distalis (PPD) incubation procedure has been described previously (Helms et al., 1991). Male freshwater fish averaging 95 g body weight were used for the studies. The pituitary was removed and the PPD was dissected then pre-incubated for 2 h in Modified Kreb's bicarbonate Ringer (300 mOsmolal) containing glucose, L-glutamine, and 1x Eagles MEM (GIBCO, Grand Island, NY). Media was then replaced with the same media with and without rtLepA. Tissues were incubated on a gyratory platform (70 rpm) for the designated times at 24 °C under a humidified atmosphere of 95% O₂/5% CO₂.

Two parallel sets of experiments were performed to assess the effects of LepA on pituitary GH mRNA accumulation and GH secretion. PPDs (n = 3–4/group) were exposed for 24 h to various concentrations of rtLepA (0, 1, 10, and 100nM) previously shown to be effective in stimulating prolactin release from the tilapia pituitary rostral pars distalis (Tipsmark et al., 2008). Based on the results of this study, we then assessed the time course effects (0, 1, 4, and 18 h) of 10 nM rtLepA on *gh* expression and GH secretion. After incubations, tissues were removed and placed RNAlater until extraction for mRNA analyses. For experiments measuring GH release, tissues and media were removed and placed in radioimmunoassay (RIA) buffer. Media and tissue GH concentrations were assessed by radioimmunoassay as described previously (Ayson et al., 1993). GH secretion was calculated as a percentage of the amount of hormone released in the media divided by the total amount of hormone in the incubations (media + tissue).

2.6. RNA isolation and quantitative RT-PCR gene expression measurement

Total RNA was isolated from samples using TRI reagent (Molecular Research Center, Cincinnati, OH), coupled with on-column affinity purification and DNase treatment (Direct-zol minipreps, Zymo Research Corporation, Irvine, CA). RNA concentration and quality were calculated by OD₂₆₀:280 ratios (range 1.9–2.0) using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE) after initial RNA extraction from tissues or cells, and again after diluting RNA to 100 ng/uL for reverse transcription. Total RNA (0.8–1.0 µg) was reversed transcribed to cDNA using random priming hexamers (High Capacity cDNA Synthesis Kit, Life Technologies). Relative *lepa*, *lepr*, *gh*, *ghr1*, *ghr2*, *igf-1*, and *igf-2* mRNA expression was determined by quantitative RT-PCR (qRT-PCR) according to our previously established protocols (Douros et al., 2014). 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 µM primers, and 6 ng of cDNA in a total reaction volume of 10 µL. The qRT-PCR cycling parameters were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The absence of genomic DNA contamination was confirmed using water (No Template Control; NTC) and DNase-treated RNA (No-

Amplification Control; NAC) as negative control templates. Primer specificity was verified by dissociation curve analysis following qRT-PCR cycles. For qRT-PCR analysis, cycle threshold 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 per ng total RNA loaded into each reaction (cDNA/ng total RNA). We quantified expression levels of 18S ribosomal RNA as a reference gene and found its expression to be unchanged among treatments. Additionally, the results from the experiments presented here were substantiated by data normalized to 18S RNA. Gene expression data for each group is presented as a percentage of the control mean (% control $\pm$ SEM).

2.7. Statistics

Quantitative real-time PCR data from hypophysectomy, leptin injection, hepatocyte culture, and pituitary culture experiments with a single time point was analyzed by one-way ANOVA (treatment) followed by Dunnett's test for significant differences between treatment and control groups. Feed restriction, and pituitary culture experiments using multiple time points were analyzed by two-way ANOVA (treatment $\times$ time) followed by Tukey's HSD post hoc test for significant differences. All analyses were performed with Prism 6 (GraphPad; La Jolla, CA). The threshold for statistical significance in all experiments was set at $P < 0.05$.

3. Results

3.1. Leptin response to fasting and refeeding

Fish fasted for 21 days had significantly lower body weight (30%; $p < 0.05$). This was accompanied by a 35–44% reduction in HSI by 7 days ($p < 0.01$). Realimentation for 3 days (day 24) was sufficient to restore body weight to that of controls, with HSI achieving control levels after 10 days of refeeding (day 31; Fig. 1A–B). Both hepatic *lepa* and *lepr* mRNA levels increased within 1 day of fasting and remained elevated by as much as 2–8-fold over control fish throughout the feed restriction period (Fig. 1D–E; $p < 0.001$). The levels of *lepa* expression returned to that of fed controls within 3 days (day 24) and *lepr* by 10 days of realimentation (day 31). Circulating LepA titers were higher in fish fasted for 21 days (Fig. 1C; $p < 0.01$) and subsequently declined to levels insignificantly different from controls following realimentation.

3.2. In vitro regulation hepatic *igf* and *ghr* gene expression by rtLepA

Hepatic mRNA levels of *igf-1* and *igf-2* increased by 10- and 15-fold, respectively with 1 nM rtLepA and gradually declined thereafter with exposure to higher leptin concentrations (Fig. 2A and B, $p < 0.001$). The lowest (0.1 nM) and highest (100 and 500 nM) dosages were ineffective in regulating the *igf* genes. Leptin A increased mRNA levels of both *ghr* forms in a dose-dependent fashion with the lowest effective concentration of 10 nM (Fig. 2C and D; $p < 0.001$). A maximal, 7.5–10-fold elevation was seen with 10 and 100 nM rtLepA ($p < 0.001$).

3.3. In vivo regulation of ghr and igf by rtLepA

Since rtLepA was effective in stimulating expression of hepatic ghrs and igfs *in vitro*, we then assessed if the hormone might exert similar actions *in vivo*. Interperitoneal injection of either 0.5 or 5.0 µg/g rtLepA was ineffective in regulating hepatic gene expression of *ghr1*, *ghr2*, and *igf-2* (Fig. 3B–D). Leptin A at 5.0 µg/g BW, on the other hand, increased *igf-1* mRNA by over 2-fold ($p < 0.05$; Fig. 3A).

3.4. In vivo and in vitro GH regulation of hepatic lepa and lepr expression

Hepatic *lepa* mRNA expression increased in Hx fish ($p < 0.05$; Fig. 4A) compared to sham-operated, control animals. Replacement of GH to Hx animals restored hepatic *lepa* mRNA levels to that of control fish. Hepatic mRNA expression of *lepr* was not significantly altered in Hx or Hx animals treated with GH (Fig. 4B).

3.5. In vitro GH regulation of hepatic lepa and lepr expression

Growth hormone at the lowest dosage tested (0.1 nM) reduced *lepa* and *lepr* mRNA abundance (Fig. 5; $p < 0.05$). At higher concentrations (10 and 50 nM) GH stimulated both *lepa* and *lepr* ($p < 0.001$). Regulation of *igf-1* was also tested to validate that the cells responded appropriately to a classic GH stimulus. As expected, GH was effective at increasing *igf-1* gene expression at all dosages tested ($p < 0.01$; Fig. 5).

3.6. Leptin regulation of pituitary gh mRNA, GH abundance, and GH release

The dose-response effect of rtLepA on pituitary *gh* mRNA abundance and GH release was assessed during 24 h incubations of PPD. Pituitary *gh* mRNA abundance declined by >60% with 1 and 10 nM rtLepA (Fig. 6A; $p < 0.05$). The highest dose (100 nM) was ineffective in regulating *gh* gene expression. Recombinant tilapia LepA at concentrations ranging from 1 to 100 nM was ineffective in altering GH release from the PPD after 24 h (Fig. 6C). The time course over which rtLepA might regulate pituitary GH gene expression and GH release was then assessed using 10 nM rtLepA. The levels of GH mRNA increased gradually in control incubations peaking between 4 and 24 h (Fig. 6B; $p < 0.01$). Leptin A reduced *gh* mRNA levels by 50% after 4 h and 24 h incubations (Fig. 6B; $p < 0.01$). Pituitary GH release was not altered by rtLepA secretion at any time point (Fig. 6D).

4. Discussion

These studies examine the regulation of LepA, the major leptin paralog in the tilapia (Douros et al., 2014), by metabolic state and its regulatory interactions with key elements of the GH/IGF endocrine growth axis. Circulating LepA and hepatic *lepa* mRNA abundance were elevated in fasted tilapia and restored during refeeding (Fig. 1). The findings mirror the prevailing model of leptin regulation observed in most fish where hormone secretion and/or gene expression was shown to increase with fasting in a number of species, including the arctic charr (Froiland et al., 2012), fine flounder (Fuentes et al., 2012), rainbow trout (Kling et al., 2009a) Atlantic salmon (Trombley et al., 2012a) and goldfish (Volkoff et al., 2003). It appears that leptin sensitivity may also be enhanced with altered metabolic state in that hepatic *lepr* expression was increased along with LepA during fasting (Fig. 1). Leptin receptor gene expression also increases with *lepa* expression during acute seawater challenge

in tilapia (Baltzegar et al., 2014) and with hypoxia in common carp (Baltzegar et al., 2014; Wong et al., 2007), suggesting up regulation of the receptor may promote leptin function in metabolic adaptations to stress.

The dynamics of leptin observed here and with most fishes, but not all, contrasts with that seen in mammals, including humans and rodents, where leptin levels decline with fasting and increase with realimentation in concert with changes in lipid stores (Ahima and Flier, 2000), although the anorexigenic function of leptin appears to be conserved among most vertebrate taxa (Elias et al., 1999; Won and Borski, 2013). Similar to reports in other fishes (Froiland et al., 2012; Michel et al., 2016), leptin does not appear to serve as an adipostat in tilapia, since neither hepatic gene expression nor circulating hormone correlate with hepatic energy stores as measured by HSI (Fig. 1) or with peritoneal fat, which changed little over 3-weeks of food deprivation in the present investigation (data not shown).

The disparate regulation of leptin during fasting between mammals and most fishes may reflect a divergence in metabolic strategies between endotherms and ectotherms, generally. In mammals, leptin is produced predominantly in adipose tissue, circulates in proportion to lipid deposition, and promotes lipolysis (Ahima, 2008). Hence during fasting, leptin levels decline as adipose stores are depleted; however, during feeding leptin levels rise and act to promote satiety thereby keeping lipid levels in stasis. In tilapia and other teleosts, leptin is produced predominantly by the liver (Angotzi et al., 2013; Douros et al., 2014; Won et al., 2012), and to a lesser degree fat, depending on leptin type and fish species. The increase in LepA with fasting observed here (Fig. 1) and in other fishes (described above) suggests the hormone may play an important role in mobilizing energy under catabolic states. Evidence indicates the hormone induces hyperglycemia in fish and lizards and liberates hepatic glucose stores through glycogenolysis in tilapia (Baltzegar et al., 2014; Paolucci et al., 2006) suggesting it may work to promote carbohydrate catabolism. However, its action in regulating lipid metabolism remains poorly understood (for review see Copeland et al., 2011; Londraville et al., 2014). Interestingly, in this study LepA suppresses gene expression of GH (Fig. 6), a known lipolytic factor (Sheridan, 1986), while our previous work in tilapia shows LepA suppresses gene expression of hepatic hormone-sensitive and lipoprotein lipases (Baltzegar et al., 2014) suggesting a potentially lipid sparing effect of the hormone in tilapia and perhaps other fishes. As a whole, the data suggest that leptin is a key catabolic factor in tilapia, and perhaps other teleosts, as its production and/or secretion increase in response to various stressors including fasting, acute hyperosmotic challenge, and hypoxia (Baltzegar et al., 2014; Bernier et al., 2012; Fuentes et al., 2012; Kling et al., 2009b) and the hormone acts to mobilize carbohydrates and perhaps other energy substrates.

Fasting or certain other conditions that impair growth (e.g. Laron dwarfism in humans) induce a hepatic GH-resistant state that is accompanied by reduced GHR and/or IGF expression and abundance as well as growth in the face of elevated GH (Argetsinger and Carter-Su, 1996; Fox et al., 2006; Won and Borski, 2013). Because hepatic or muscle GHRs/IGFs and LepA are disparately regulated during fasting in tilapia and other fishes (Fox et al., 2006; Picha et al., 2008) we sought to test if LepA might modulate hepatic GHRs and IGFs. Recombinant tilapia LepA up regulates *igf-1* and *igf-2* expression at low doses, while higher concentrations (>10 nM) were sufficient to increase *ghr1* and *ghr2* mRNA abundance *in*

vitro (Fig. 2). The effects observed are physiological as the lower rtLepA concentrations reflect those within the range found in tilapia circulation (Douros et al., 2014) while higher concentrations may also be relevant given the hormone is produced and secreted into the dense hepatic capillary network and act in a localized autocrine or paracrine fashion.

The increased response of hepatic *igf-1* seen *in vitro* was also observed *in vivo* following a single injection of rtLepA. It is likely therefore, that rtLepA increases hepatic *igf-1* expression, in part, through direct actions at the level of the hepatocytes. Leptin treatment elevates *igf-1* mRNA levels in pig (Ajuwon et al., 2003), as well as circulating IGF-1 levels in mice (Bartell et al., 2011) and humans (Chan et al., 2008), suggesting this action of leptin may be well conserved among vertebrates. In contrast to effects observed *in vitro*, exogenous leptin did not significantly alter hepatic *ghr1*, *ghr2* or *igf-2* expression in tilapia liver, suggesting that endogenous factors, such as somatostatins (Very and Sheridan, 2006) 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*.

Like that observed for tilapia here, work also shows heterologous leptin stimulated both hepatic *igfs* and *ghrs* *in vitro* and *igf-1* *in vivo* in hybrid striped bass (Won et al., 2016). In these animals leptin is elevated during feeding as a possible means to stimulate the growth axis when nutrient availability is high (Jorgensen et al., 2013; Won et al., 2012), similar to that which may occur for mammals. We postulate that stimulation of hepatic GHR/IGF by leptin in the tilapia and perhaps other fishes where leptin increases during fasting may serve as a mechanism to limit abrupt catabolic declines in the expression of GHRs or IGFs that have been reported previously (Fox et al., 2006; Picha et al., 2014). Furthermore, LepA stimulation of IGFs may serve to promote peripheral glucose uptake in accordance with IGFs established insulin-like actions and LepA glucose mobilizing effects (Baltzegar et al., 2014; Froesch et al., 1985). Considering the actions of GH and IGF-1 in seawater adaptation (Sakamoto and Hirano, 1993; Borski et al., 1994; Mancera and McCormick, 1998; Riley et al., 2003) and studies that demonstrate hepatic LepA gene expression rises with acute salinity challenge in tilapia (Baltzegar et al., 2014) it is also possible that LepA may serve a role in enhancing GH sensitivity and IGF production for promoting seawater adaptation in fishes, in addition its actions in promoting energy expenditure during the process. Regardless, the results in tilapia and hybrid striped bass suggest that leptin may be an important stimulator of hepatic *igfs* and *ghrs* in teleost fishes

These studies also assess the regulation of leptin and its receptor by GH. As previously reported, *lepa* expression is under constitutive suppression by pituitary factors (Douros et al., 2014). As such, hypophysectomy (pituitary removal) significantly increases *lepa* gene expression *in vivo*. Here we see a similar response and show that exogenous GH reduces *lepa* gene expression in hypophysectomized tilapia to that of levels in intact animals (Fig. 3A). We confirm this effect *in vitro* by demonstrating that GH provided at basal physiological concentrations (0.1 nM) (Fox et al., 2006) suppresses *lepa* and *lepr* gene expression in cultured hepatocytes (Fig. 6). However, higher physiological levels of circulating GH as seen with seawater adaptation (1.0 nM; Auperin et al., 1997) and supraphysiological doses the peptide (10 and 50 nM), either exerted little effect or increased *lepa* expression *in vitro* (Fig. 6), respectively. The differential dose effects of GH on *lepa*

seen with tilapia here are clearly specific as we found GH at all concentrations tested enhanced *igf-1*. While we are uncertain of the nature of this biphasic response, it would appear that fluctuations of GH levels might exert disparate responses on the synthesis of leptin in hepatic tissue. Considering basal serum GH levels are around 0.1 nM (Auperin et al., 1997; Melamed et al., 1994) it would appear the hormone might function to suppress leptin, a carbohydrate catabolic hormone, while promoting growth anabolic processes and lipolysis during normal nutritional plane. Studies in Atlantic salmon show sustained GH treatment (up to 6 days) inhibits hepatic *lep1a* (Kling et al., 2012). Thus it would appear that acute and chronic exposure to GH inhibits hepatic leptin mRNA abundance and potentially secretion.

The elevations in GH seen with fasting in tilapia and other fish (Fox et al., 2006; Picha et al., 2014), combined with potential increases in LepA resulting from higher levels of GH and LepA actions in promoting *ghr* and *igf* expression itself, might provide a mechanism for sparing declines in GHR and IGFs during states of GH resistance (Fig. 7).

The regulatory effect of leptin on pituitary GH in teleosts is unknown. While pituitary GH gene expression and secretion is stimulated by leptin in pigs and sheep (Barb et al., 1998; Morrison et al., 2002), the hormone has little effect on spontaneous GH secretion in rats (Carro et al., 1997). Our data suggest that rtLepA reduces pituitary *gh* expression in as early as 4 h *in vitro* in tilapia. However, basal GH secretion was not effected by LepA. It appears therefore that LepA may suppress accumulation of GH mRNA and perhaps the synthesis of the hormone while exerting little action on secretion. Further studies are required to determine if LepA alters the rate of GH synthesis or might suppress GH secretion under stimulatory conditions.

In summary, the studies indicate the synthesis and secretion of leptin and hepatic expression of its receptor is sensitive to metabolic state, increasing with fasting and rising with feeding. We postulate the enhanced production and responsiveness of LepA, a hormone whose central function is postulated to promote catabolism, is fundamental to maintaining energy homeostasis in animals during the adaptive endocrine stress response linked to low nutrient availability. The findings also suggest the hormone may act locally on the liver to stimulate gene expression of hepatic GHRs and IGFs possibly to limit abrupt catabolic declines in these hepatic factors. This, along with findings that show GH suppresses hepatic *lep* and that LepA attenuates GH transcript accumulation, suggests GH and LepA may interact to regulate energy homeostasis and metabolism in fish.

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Fig. 1. (A) Body weight, (B) hepatosomatic index, (C) circulating LepA, (D) hepatic *lepa* mRNA abundance and (E) hepatic *lepr* mRNA abundance of tilapia fed daily to satiation (solid line) and fish subjected to 3 weeks fasting followed by 10 days of refeeding to satiation. Asterisks represent significant differences between groups at each time point (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; $n = 8$ fish/group; mean $\pm$ SEM).

Fig. 2.

In vitro effect of rtLepA on (A) *ghr1* (B) *ghr2* (C) *igf-1* and (D) *igf-2* mRNA abundance in hepatocytes over 18 h incubation. Data were normalized to 18 s ribosomal RNA levels and then expressed as fold change relative to control group. Asterisks denote significant differences from control group (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; $n = 3-4$ wells/group; mean $\pm$ SEM).

Fig. 3.

In vivo effect of recombinant tilapia leptin A (LepA) on hepatic (A) *igf-1*, (B) *igf-2*, (C) *ghr1* and (D) *ghr2* mRNA abundance. Fish were injected intraperitoneally with 0.5 or 5.0 ug/g BW LepA and sampled 24 h later. Data were normalized to 18 s ribosomal RNA levels and then expressed as fold change relative to control group. Asterisks denote significant differences from control (* $p < 0.05$; $N = 8$ fish/group; mean $\pm$ SEM).

Fig. 4.

Effect of hypophysectomy and oGH replacement on hepatic mRNA levels of (A) *lepa* and (B) *lepr*. Data were normalized to 18 s ribosomal RNA levels and then expressed as fold change relative to sham/saline group. Asterisks denote significant differences from sham/saline group (* $p < 0.05$; $n = 6-7$ fish/group; mean $\pm$ SEM).

Fig. 5.

In vitro effect of GH on *lepa*, *lepr* and *igf-1* mRNA abundance in 18-h primary hepatocyte incubation. Data were normalized to 18 s ribosomal RNA levels and then expressed as fold change relative to control group. Asterisks denote significant differences from control group (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; $n = 5$ wells/group; mean $\pm$ SEM).

Fig. 6.

Dosage (1–100 nM, 24 h incubation) and time-course (10 nM, 1–24 h incubation) effect of rtLepA on pituitary GH mRNA abundance (A,B), and GH release (C,D). GH mRNA was normalized to 18 s ribosomal RNA levels and then expressed as fold change relative to control group. GH release reflects the percentage of hormone released in the media to that of the total amount in the incubation (tissue + media levels). Black bars represent control and white bars represent leptin treated groups. Asterisks denote significant differences from control group (* $p < 0.05$, $n = 4/\text{group}$; mean $\pm$ SEM).

Fig. 7.

Illustration of the proposed regulatory relationship between leptin A and the hepatosomatic growth axis in the tilapia (*Oreochromis mossambicus*). Growth hormone at physiological levels acts to reduce hepatic *lepa* mRNA expression and LepA secretion. Conversely, elevated LepA in circulation during, for instance, fasting may act in a counter regulatory manner to reduce hypophyseal GH expression during time periods when it is normally stimulated. Similarly, elevated LepA during fasting may stimulate *igf-1* and *igf-2*, as well as *ghr-1* and *ghr-2* expression in the liver in order to keep these transcripts at a threshold level as a means for priming the hepatosomatic growth axis for growth when feeding resumes.

Table 1

List of PCR primer pairs used for qPCR analysis of various genes in tilapia.

Gene	Accession Number	Forward (5'-3')	Reverse (5'-3')	Amplicon Size	Primer Efficiency (%)
Leptin a (lepa)	KC354702	GGGTCTCCACAGATCAAGTACGA	TGCCGCCACAGATGAATG	61	99.9
Leptin receptor (lepr)	KC354703	AAATTCAACCGGAAGCAAAACCT	TGCAGCCGGGACTGTGT	56	102.6
Growth hormone (GH)	M26916	GCAACGTCAGCTCAACAAAA	CAGCCTTGGTGAAATCTGGT	202	100.3
Insulin-like growth factor-1 (IGF-1)	EU272149	CCAAGAGCACCCAAAGGTTAG	TTTGTCATTGTGCTCCTTCC	206	105.3
Insulin-like growth factor-1 (IGF-2)	ABY88873	GAACGGAGAACACAGCAAGATG	GTTGTTACCCCTGCTGGTTG	200	105.4
GH receptor 1 (GHR1)	AY973232	TAAGAAAAGAGCCCTCCTACCA	ACTGTCGCTGAATGTCCAAT	377	101.3
GH receptor 2 (GHR2)	AY973233	AGCACTGAGACGCCAAACGA	CCAAAGATGAGCAAAAGCCAC	494	102.7
Ribosomal 18 s	U67340	ATTACTGGCTACACCGAGCG	AGTCCTGACTCTCCTCCGTC	252	92