ABSTRACT

DOUROS II, JONATHAN DAVID. The Function and Regulation of Leptin in Teleost Fish (Under the direction of Dr. Russell J. Borski).

Leptin is classically described as an adipostat in mammals, where the hormone circulates in proportion to adiposity to prevent excess fat accumulation by inhibiting appetite and stimulating lipolysis, metabolic rate, and overall energy expenditure. However, the a si function of leptin in controlling energy homeostasis is poorly understood in ectotherms whose metabolism may differ substantially from homeotherms. This research focuses on characterizing the function and regulation of leptin in a euryhaline teleost fish, the Mozambique tilapia (Oreochromis mossambicus), a representative erciforme fish, the largest extant vertebrate order.

Studies focused on production of homologous leptin and the development of a specific ELISA needed to reliably study the hormone. Tilapia leptin A (LepA) is the major form of leptin produced primarily in the liver where it is expressed at 280-fold higher levels than the minor paralog, lepb. Recombinant tilapia LepA was produced in bacterium, purified, and verified for bioactivity. It stimulated hyperglycemia, glycogenolysis and growth hormone receptor (ghr) expression with equal efficacy as human leptin, while suppressing hepatic lipase expression in a manner distinct from the human form, confirming the value of homologous hormone. An ELISA assay was developed for detection of LepA. The assay displays a detection range of 0.25-1000 nM with intra- and interassay variations of 9 and 14% respectively.

Pituitary prolactin (PRL) is a pleiotropic hormone whose regulatory interactions with LepA are unknown. Hypophysectomy (Hx i.e. pituitary removal) increases LepA secretion and synthesis, while PRL replacement restored levels to sham controls. Prolactin also
reduced hepatic lepa mRNA in vitro. Leptin A increased pituitary PRL mRNA. These results demonstrate LepA enhances prolactin synthesis, while PRL in turn inhibits hepatic leptin secretion and synthesis.

Further studies evaluated LepA regulation under different metabolic states, and examined how LepA might interact with the pituitary growth hormone (GH)/hepatic insulin-like growth factor (IGF) axis central to growth and metabolic regulation. Circulating LepA, and hepatic lepa and lepr expression increased with fasting and was restored following refeeding, consistent with the hormones function in promoting energy expenditure during catabolic stress and confirming the hormone is not an adipostat as seen for mammals. Leptin A treatment stimulated hepatic igf-1, igf-2, ghr1 and ghr2 expression in vitro, and hepatic igf-1 in vivo. In vivo experiments using an Hx tilapia model show that GH inhibits lepa synthesis, while in vitro GH treatment exerted a biphasic effect on hepatic lepa and lepr mRNA. The responses were accompanied by increases in igf-1 expression. These data indicate GH is an important negative regulator of LepA synthesis and sensitivity and that LepA stimulates the GH/IGF axis to potentially spare catabolic declines in these factors during long-term stress.

Glycolysis is a critical process for energy production in all organisms. Studies assessed cellular gene networks modulated by leptin and its effects on glycolysis. Pituitary transcriptome analyses show rtLepA stimulates the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in a covariable manner to the hypoxic stress gene network. Leptin also increased phosphofructokinase (PFK) mRNA, the rate limiting glycolytic enzyme. In orthogonal tests rtLepA dose-dependently increased GAPDH and PFK mRNA expression, PFK activity and total glycolytic output. A STAT3, but not an ERK
blocker, suppressed leptin-induced elevations in PFK activity and glycolysis, indicating leptin stimulates glycolysis through a STAT3-dependent mechanism. Leptin A also stimulated PFK gene expression and glycolytic activity in cultured hepatocytes. Results identify a novel action of leptin on glycolysis, indicating the hormone may induce glucose catabolism to provide energy needed for the adaptive stress response, including those linked to hypoxia, cancer, and osmotic challenge.
The Function and Regulation of Leptin in Teleost Fish

by
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DEDICATION

This work is dedicated to the love of One who was, is, and will be absolutely faithful, even as I am utterly faithless.
BIOGRAPHY

Jonathan David Douros II was born in Atlanta, GA in 1987 to Jonathan Sr. and Kendra Douros. He grew up in Fuquay-Varina, N.C., graduating from the Wake Christian Academy despite attending to everything but his studies. He attended Shaw University in Raleigh, N.C., where he learned, to his eternal gratitude, that his future lay in academia and not athletics. He graduated with a Bachelor of Science in Biology in 2010. In 2011, motivated by equal parts pride and naiveté, he joined Dr. Russell Borski’s laboratory at N.C. State University to begin work on his Ph.D. He completed his degree in 2015, thankful to those who broke his pride and sharpened his thinking. Jonathan and will continue his research endeavors as a postdoctoral fellow studying metabolic endocrinology.
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Finally, to my wife Katie, my family Jon, Kendra, Kelsie, and my community, thank you for your sympathy in my failure, celebration in my success, and endless love.
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Chapter I

Production of bioactive recombinant tilapia (*Oreochromis mossambicus*) leptin A and development of homologous enzyme-linked immunosorbent assay for its measurement\(^1\)\(^2\)

Abstract

High sequence divergence amongst leptin orthologs in vertebrates necessitates the development of species-specific molecular tools, including homologous recombinant peptides and immunoassays, to investigate the hormone’s biology. This work presents the development of both a recombinant tilapia leptin A (rtLepA) and homologous tLepA enzyme linked immunosorbent assay (ELISA). To produce rtLepA, the tilapia leptin a gene (*lepa*) was cloned into a plasmid vector, inserted into competent *Escherichia coli*, and expressed in high quantity. The recombinant protein was then isolated and purified using a system of nickel-affinity and size filtration chromatography. The purity of the rtLepA was determined to be 96% relative to a commercial recombinant human leptin (rhLep), while the peptide’s bioactivity was confirmed by assessment of its effect on hepatic *ghrl* gene expression *in vitro*. The ELISA was developed using a polyclonal antibody specific to a 16 amino acid segment of the tLepA peptide. The antibody shows strong reactivity with rtLepA, and a corresponding 16 kDa circulating protein in both tilapia and hybrid striped bass, another Perciforme. The primary antibody does not cross react with rhLep. The ELISA displays a

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Research in this chapter has been published in the following journals:


linear detection range of 0.25–1000 nM, with intra- and interassay variability of 9% and 16%, respectively. Additionally, this work presents a quantitative tissue expression profile of teleost leptin paralog, lepa and lepb. The liver is shown to be the primary site of lepa and lepb expression in teleosts, with lepa proving to be the dominant paralog, showing >280-fold higher expression relative to lepb.
Introduction

While there is conclusive evidence of leptin expression in teleost fish like the tilapia (Oreochromis mossambicus), the action and regulation of the hormone in these animals remains largely uncharacterized (Won et al., 2012; Zhang et al., 2013). The comparative study of leptin biology is frequently hindered by the unavailability of necessary tools for its measurement. For example, multiple forms of the hormone exist in both diploid (lepa and lepb) and tetraploid (lepa1, lepa2) fish species due to gene or genome-wide duplication events, (Figueroa et al., 2005; Huising et al., 2006). It is currently unknown which leptin paralog (lepa, lepb) is dominantly expressed in the tilapia, or which tissue is primarily responsible for producing the hormone. Additionally, the function of each leptin paralog is unknown as they may have subfunctionalized or neofunctionalized throughout their evolutionary history. This makes the use of commercially available mammalian leptin orthologs inappropriate as they may exert altogether different actions than native leptins. Therefore, characterizing tissue distribution of leptin gene expression and developing a bioactive, homologous recombinant tilapia leptin are necessary hurdles to clear in any investigation of leptin(s) function in the tilapia.

Investigations assessing teleost leptin biology are further hindered by the lack of species-specific antisera and validated assays for measuring circulating leptin. Teleost leptin homologs exhibit a considerable degree of sequence divergence, both within fish taxa (zebrafish to Fugu, 20% identity), as well as to other vertebrates (tilapia to mouse or human, 14–17%). This high level of sequence divergence makes the use of heterologous assays undesirable for functional studies, however, only a single homologous leptin radioimmunoassay has been developed in fish. This assay is specific to salmonid leptin,
which is a considerably more ancient group among extant teleosts (*Protacanthopterygii*) (Kling et al., 2009). The high degree of evolutionary distance of leptin(s) among teleosts, and other vertebrate groups, requires the development of specific assays for the reliable detection of protein abundance necessary for studying leptin biology. Thus, this work also aims to develop and validate an enzyme linked immunosorbant assay (ELISA) specific to LepA in the tilapia.

This work will provide the requisite biotechnology tools to properly assess the biology of leptin in the tilapia, a Perciforme fish, which constitute the largest extant order of vertebrates (Helfman et al., 2009).

**Materials and Methods**

**Animals**

The NCSU Institutional Animal Care and Use Committee (IACUC) approved all protocols. Fish used for the development of the recombinant tilapia leptin A (rtLepA), the tilapia leptin A enzyme linked immunosorbant assay (tLepA ELISA), and *lepa* tissue distribution were housed in 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.

**Recombinant Tilapia Leptin Production, Purification and Bioactivity**

*Cloning lepa:* Total RNA from Mozambique tilapia (*Oreochromis mossambicus*) hepatic tissue was isolated using standard TRIReagent ® protocol (Baltzegar, 2013; Douros et al., 2014). The *lepa* cDNA sequence (GenBank: KC354702) was PCR amplified using *lepa* specific primers (Table 1), and inserted into the Champion pET151 TOPO plasmid using a
topoisomerase cloning method (manufacturers protocol; Life Technologies).

**Bacterial Transformation and Protein Induction:** The pET151 TOPO plasmid containing the tilapia *lepa* sequence was transformed into competent *Escherichia coli* (BL21; Life Technologies), which were grown on LB/Ampicillin plates and PCR screened for positive clones. One positive colony was grown in culture (LB/Ampicillin/20% glucose broth) to an OD$_{600}$ ~ 0.2 at 37°C (24-26 hours). The production of rtLepA protein was induced by IPTG (0.5 mM) and incubated overnight at 37°C. Bacteria were harvested by centrifugation and total cell protein extracted via sonication for 30 seconds and lysozyme digestion for 2 h. (Thermo Pierce; Walthman, MA).

**Protein Purification:** The propagated cells were induced and lysed with a native isolation buffer (Life Technologies, Grand Island, NY) for selective purification of rtLepA from free cytosolic proteins. The rtLepA was then purified by two rounds of double nickel-affinity chromatography (Life Technologies). The preparation was further purified by filter centrifugation (5000 g for 15 min) through Amicon Ultra protein purification columns with 30 and 10 kDa molecular weight cutoffs respectively (EMD Millipore, Billerica, MA, USA) in order to eliminate any potential rtLepA dimers. The retained protein fraction was then dialyzed in 2 changes of NaCO$_3$ at 4°C for 2h each and lyophilized overnight.

**Purity determination:** The relative purity of rtLepA was determined with quantitative SDS–PAGE blot imaging (Odyssey Infrared Imager, LI-COR Biosciences, Lincoln, NE, USA) using Coomassie Fluor Orange Reagent (Invitrogen) and found to be 96.1% relative to that of the commercially prepared rhLep (> 90% absolute purity) (Baltzegar et al., 2014).

**Validation of bioactivity:** The bioactivity of the leptin(s) was evaluated *in vitro* using cultured hepatocytes from hybrid striped bass (*Morone chrysops x M. saxatilis*). In
previous studies, cells incubated with 50nM human leptin showed significant increases in growth hormone receptor 1 (ghr1) mRNA expression after 24 h (Won, 2013).

**Tilapia LepA ELISA development and validation**

*Development of Anti-tLepA polyclonal antibody:* Using the mRNA coding sequence of tilapia lepa, an 11-amino acid polypeptide (GS-177060) was synthesized by Gen-Script Inc. (Piscataway, NJ, USA), after initial epitope site-selection to confer high antigenicity (Figure 5). Additional cysteine residue addition to the N-terminus of the peptide and KLH conjugation was performed to enhance immunization efficiency. The antibody was raised in rabbit against the GS-177060 antigen, and subsequently purified by affinity chromatography.

*Western blotting:* The specificity of the antibody was assessed by Western blot using rtLepA, rhLep, tilapia plasma, and plasma from another perciform teleost, the hybrid striped bass (HSB, *M. chrysops x M. saxatilis*). The plasma samples were diluted 1:20 (v/v) in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4). Ten microliters of either molecular weight marker or experimental samples were mixed in NuPAGE LDS sample buffer (4:1 v/v dilution; Life Technologies, Grand Island, NY), and heated to 70 °C for 10 min prior to loading on a Nu-PAGE 4–12% Bis-Tris gradient gel. Samples were electrophoresed for 50 min at 200 V (XCell electrophoresis system and blot module, Life Technologies, Grand Island, NY). Proteins were transferred to a nitrocellulose membrane by soaking the gel for 5 min in transfer buffer (2.4 g Tris HCl, 8.06 g NaCl, 1 L dH2O, pH 7.6), followed by 1 h electroblotting at 30 V. For immunodetection of leptin, the membrane was first blocked overnight at 4°C using Odyssey Blocking buffer (LI-COR, Lincoln, NE), and then incubated for 1 h with anti-tLepA (1:30,000 in PBS). Membranes were subsequently washed 4x in PBS/0.1% Tween-20 for 5 min with gentle agitation. Membranes were then
incubated for 1 h with a secondary antibody (goat anti-rabbit IgG) conjugated to Alexa IR-Dye 680 (LI-COR; 1:15,000 in PBS) at room temperature. After a final wash (PBS/0.1% Tween-20), the membrane was imaged using an Odyssey CLx infrared imager (LI-COR).

**ELISA procedure and validation:** The following protocol outlines an indirect ELISA procedure for measuring plasma leptin in tilapia. All steps were performed at room temperature (Hornbeck et al., 2001). Costar 3590 EIA 96-well polystyrene plates (Corning Life Sciences, Tewksbury, MA) were coated with 200 µL of tilapia LepA standard (GS-177060) or diluted plasma (1:1 v/v) for 4 h. Coated wells were washed (4x in 400 µL PBS), blocked with 400 µL of 5% non-fat milk in PBS for 2 h, washed 1 time in 400 µL PBS, and then incubated for 2 h with 200 µL of the anti-tLepA primary antibody diluted 1:3000 in PBS. The wells were then washed (PBS) and incubated for 1 h with 200 µL of HRP-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology Inc., Dallas, TX) diluted 1:5000 in PBS. Following incubation, wells were washed 4x in PBS before addition of 200 µL of o-Phenylenediamine (OPD; Thermo Scientific; Rockford, IL) solution (2 µL H2O2, 4 mg OPD per mL PBS). The color development was measured at 490nM using an ELx800 microplate reader (BioTek Instruments, Winooski, VT).

A series of initial assays were performed to optimize the ELISA procedure for primary antibody dilution, effective assay range, and specificity. Primary antibody dilutions, ranging 1:100 to 1:100,000, were tested for maximal binding with a 1:3000 dilution found to elicit optimal signal with non-specific binding of <10%. The effective (linear) range of the assay was calculated from a 10-fold serial dilution of GS-177060 antigen ranging from 0.01 to 1000 nM. Specificity for native LepA was determined by comparing the standard curve to that of pooled, serially diluted fish plasma from tilapia and HSB. Plasma samples were
diluted from 1:1 to 1:4 (v/v in PBS) to a final volume of 200 µL. Cross reactivity of mammalian leptin was also evaluated in the tilapia LepA ELISA using serially diluted preparations of rhLep (1–1000 nM). The ELISA detection limits were calculated from the mean ± 2 SD of the lowest point on the linear range for 12 replicated standard curves, using segmental linear regression analysis in GraphPad Prism (v6; San Diego, CA). The intra-assay variation was calculated as the mean percentage variation ($V_{\text{intra}} = \frac{\text{Variance}}{\text{Mean}} \times 100$) for 8 replicate-pooled samples on a single ELISA plate; the inter-assay variation ($V_{\text{inter}}$) was calculated as the mean percentage variation across 8 plates ($V_{\text{inter}} = \frac{\text{Variance}}{\text{Mean}} \times 100$). Specificity was calculated by the degree of parallelism for serially diluted plasma or recombinant hormone relative to the standard curve, which was calculated as ($m_{\text{plasma}}/m_{\text{standard}}) \times 100$. Non-specific binding was calculated as the fractional percentage of background absorbance to that of the lowest detection limit in the assay.

**Tissue distribution of lepa and lepb**

Tilapia *lepa* and *lepb* mRNA expression profiles were evaluated by SYBR Green quantitative real-time PCR (qPCR) in the pituitary, whole brain, hypothalamus, heart, gill, kidney, pancreas, liver, muscle, fat, intestine, and testes. Fish were anesthetized in buffered tricaine methanesulfonate (MS-222; 100 mg/L) and then decapitated prior to tissue collection. Tissues were rapidly dissected and placed in RNAlater (Life Technologies) overnight at 4°C and then frozen at -80°C prior to processing for RNA isolation.

Total RNA was isolated from samples using TRI reagent (Molecular Research Center, Cincinnati, OH), coupled with on-column affinity purification and DNAse treatment (Directzol 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). RNA integrity was confirmed by agarose gel electrophoresis. Total RNA (0.8–1µg) was reversed transcribed to cDNA using random priming hexamers (High Capacity cDNA Synthesis Kit, Life Technologies) and the cDNA tested for viability using 18S rRNA amplification as a positive control. Following qPCR, cycle threshold (Ct) values were compared to copy number standard curves for tilapia lepa and lepb (serial dilutions of plasmid DNA containing the gene coding sequence). Copy number values were then normalized to total RNA used in the cDNA synthesis reactions. The qPCR primers are provided in Table 1.

Results

Production and Purification of rtLepA

Cloning and Bacterial Transformation: The lepa gene sequence was cloned from tilapia hepatic cDNA resulting in a single band at ~420 bp. The clone was ligated into pET151 plasmid (Figure 1A), and transformation of competent E. coli was verified by a subsequent PCR screen (Figure 2).

Protein Purification: One positive colony (#5; Figure 2) was be grown in 10 mL LB/AMP broth at 37ºC overnight and subsequently transferred to a nutrient rich media (5L) for another period of overnight growth at 37ºC. IPTG (5mM) was be added to induce rtLepA expression, and culture was incubated at 37ºC for 8 hours.

After protein expression induction, cell lysates were purified via nickel resin affinity chromatography (Invitrogen protocol) and double filtration (Figure 2B). The retained protein fraction was dialyzed in 10mM NaCO3, then lyophilized overnight resulting in ~43 mg of powdered protein. This protein is ~16 kDa (Figure 3A, lanes 6-8) and reactive with tLepA specific antibody (Figure 6A).
Purity determination of rtLepA: SDS-PAGE blots (Figure 3A) of recombinant tilapia leptin A (rtLepA) and rhLep (97±2 % purity; National Hormone and Peptide Program) both display a single, 16 kDa band on when stained with Coomassie Fluor Orange reagent (Invitrogen). The blot contains a molecular marker (62 to 3 kDa range; Lane 1), serial dilution of rhLep (17.3-2.5 µg loaded; Lanes 2-5); and triplicate preparations of rtLepA (4.1- 6.6 µg loaded; Lanes 6-8). The relative purity estimate for rtLepA (96.1%) was determined as a percentage of the observed and expected (linear regression of recombinant hLep curve; Figure 3B) fluorescence values of the 16 kDa band.

Validation of rtLepA bioactivity: Growth hormone receptor 1 (ghr1) mRNA expression in cultured hybrid striped bass hepatocytes was upregulated ~ 10 fold following 24 h incubation with 50 nM rtLepA (p < 0.01; Figure 4). A lower dose of rtLepA (5 nM) was ineffective in regulating ghr1 mRNA expression.

Tilapia LepA ELISA development and validation

Western blotting against anti-tLepA polyclonal antibody: Western blotting using the anti-tLepA antibody identified a single band of ~16 kDa in plasma samples from both tilapia and HSB, and in recombinant tilapia leptin A (rtLepa) preparations (Figure 6A). The tLepA antibody was ineffective in detecting recombinant human Lep (Figure 6A).

ELISA validation: For the ELISA, the detectable range of the tilapia LepA peptide antigen (GS-177060) standard curve was 0.25–1000 nM (Figure 6B), with inter- and intra assay variation calculated as 16% and 9%, respectively. Non-specific binding was <9%. Serially diluted tilapia and HSB plasma pooled from 8 adult fish for each species, displayed parallelism of >98% relative to the antigen standard (Figure 1C). Recombinant human Lep, however, displayed little parallelism (5%) to the standard curve at concentrations ranging
from 0.1 to 1000 nM. Physiological levels of human leptin (0.2–20 nM) were undetectable and fell below background levels in the ELISA (OD490 ± 2SD < 0.27; Figure 6B and C).

**Tissue distribution of lepa and lepb mRNA**

Tissue copy number expression of *lepa* and *lepb* was assessed by qPCR. Tilapia *lepa* mRNA expression was nearly 10-fold higher in the liver than in any other tissue source examined, however, nominal levels of *lepa* were detected in the pituitary, hypothalamus, kidney, muscle, fat, pancreas and the posterior intestine (Figure 7). Relative to tilapia *lepa* expression, the *lepb* paralog showed lower levels in tissues where they are coexpressed, but was also most abundant in the liver (Figure 7). The liver abundance of tilapia *lepa* was 280-fold greater than *lepb*. Low levels of *lepb* were detected in the pancreas, gill, muscle and testes, while little detection was found in the pituitary, brain, hypothalamus, kidney, fat, or intestine.

**Discussion**

The aims of the present studies were to produce a bioactive rtLepA, develop a homologous LepA ELISA needed for characterizing the function and regulation of the hormone, and to characterize tissue specific distribution and expression levels of *lepa* and *lepb*.

The rtLepA produced was highly pure peptide (96.1% relative to rhLep), free of dimerization, and effective in stimulating the expression of *ghr1* mRNA in cultured hepatocytes. The effects of rtLepA to stimulate glycogenolysis and suppress *prl1* and *prl2* gene expression *in vivo* have been reported elsewhere (Baltzegar et al., 2014; Douros et al., 2014). Interestingly, while rhLep and rtLepA exert similar effects in stimulating glycogenolysis, however the two hormones exert disparate effects on hepatic lipase
(hormone sensitive lipase and lipoprotein lipase) gene expression (Baltzegar et al., 2014). These data suggest that the use of heterologous (non-native) peptides may be undesirable. Thus, this highly purified, bioactive compound represents an important tool in furthering comparative studies regarding the function of leptin in teleost fish like the tilapia.

The rtLepA ELISA specifically and reliably measures plasma LepA, which we found to be the dominant paralog produced in tissues of the tilapia. The existence of multiple leptin paralogs, coupled with high sequence divergence, underscores the need for developing specific proteins and assays to assess hormone action and measure protein levels of this important hormone in fishes. Western blotting with the homologous tilapia LepA antibody identified a single, 16 kDa protein in plasma taken from both tilapia and the hybrid striped bass, a band of similar size to that identified in preparations of recombinant tilapia LepA (rtLepA; Figure 6A). No protein was detected by Western blot with preparations of rhLep, suggesting little or no cross reactivity of the tilapia LepA polyclonal antibody to mammalian leptins (Figure 6A). This specificity was consistent with results from the tilapia LepA ELISA, as serial dilutions of tilapia and HSB plasma showed high parallelism with the standard curve (Figure 6B–C). In contrast, detection of serially diluted rhLep within a physiologically relevant range was negligible. Detection of rhLep above background was achieved only at micromolar concentrations of hormone. Currently, recombinant tilapia leptin B (LepB) is unavailable for cross-reactivity testing with the LepA antibody. However, the LepA peptide sequence from which the antibody was generated (GS-177060) shares only 28.5% identity to both human leptin and tilapia LepB. As human leptin shows no significant cross reactivity by Western blot or in the tilapia LepA ELISA, it is unlikely that tLepB will be appreciably detected by the tilapia LepA antibody. Tilapia plasma LepA concentrations
ranged between 0.8 and 3.9 nM, while hybrid striped bass plasma had slightly higher mean LepA levels (5.18 nM). Kling and colleagues (2009), using a radioimmunoassay developed for salmonid leptin, reported circulating levels of 0.3–5.0 nM in rainbow trout and Atlantic salmon. Similarly in humans, circulating leptin levels typically range from 0.5 to 5 nM (Shek, 1998). Collectively, these results indicate that the ELISA developed here reliably detects tilapia LepA within a concentration range found in other vertebrates.

Tissue mRNA expression profiles identify LepA as the dominant leptin produced in the tilapia. Tilapia lepa mRNA was 10- to 50-fold higher than lepb in all tissues examined (Figure 7), which is similar to patterns observed in grouper (Epinephelus coioides) (Zhang et al., 2013). The liver was the primary expression site for both paralogs, with lepa showing >280-fold higher expression relative to lepb (Figure 7). These results confirm the liver is the major source of leptin production in teleosts (Gorissen et al., 2009; Kurokawa et al., 2005; Won et al., 2012), in contrast with adipose tissue in mammals. The dramatically higher expression of lepa in the liver suggests this paralog is the predominant form found in circulation, while extensive tissue expression profiles of both lepa and lepb suggest the hormones may also act in an autocrine and paracrine fashion to elicit pleiotropic effects.

In summary, the present investigation establishes the production and purification of rtLepA, presents the validation of a homologous ELISA for reliable measures of LepA in this species, as well as other perciforms, and demonstrates that LepA is the dominant leptin paralog produced in tilapia, mainly from the liver.
References


Won, E.T., 2013. Cloning and characterization of leptin in a teleost fish and its role in
mediating appetite and growth.


Table 1. List of PCR primer pairs used for qPCR analysis in Mozambique tilapia.

<table>
<thead>
<tr>
<th>Gene</th>
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Figure 1: Flow chart outlining for rtLepA production and purification. (A) the pET151 TOPO plasmid sequence and attributes including T7 promoter, IPTG inducible lacO operon, ribosomal binding site, 6-histidine tag, and topoisomerase insertion point for the gene of interest (B) size exclusion chromatography purification process for rtLepA.
Figure 2: PCR screen for positive (pET151-lepa transformed) *E. coli* clones using *lepa* specific primers. The presence of a 420bp amplicon indicates the presence of the full coding *tilpia lepa* sequence.
Figure 3: Purity comparisons of recombinant tilapia leptin A (rtLepA) relative to commercial recombinant human leptin. (A) Leptin protein blot (SDS-PAGE) stained with Coomassie Fluor Orange reagent (Invitrogen): Lane 1, molecular marker (62-3 kDa); Lanes 2-5, serial dilution of rhLep (17.3-2.5 µg loaded); Lanes 6-8, triplicate preparations of rtLepA (4.1-6.6 µg loaded). (B) Purity analysis of rtLepA relative to rhLep, using quantitative fluorescence imaging (LI-COR Odyssey; 700 nm). The relative purity estimate for rtLepA (96.1%) was determined by the observed and expected fluorescence values of the 16 kDa band calibrated to a BSA standard curve.
Figure 4: Growth hormone receptor 1 (ghrl) mRNA expression in cultured hybrid striped bass hepatocytes following 24h incubation with recombinant tilapia leptin A (rtLepA). Asterisks represent dose-dependent effects relative to control fish (** p < 0.01); letters identify significant effects across treatment (dosage) groups (P < 0.05). The GHR1 mRNA expression data was normalized to beta actin RNA expression and expressed as relative fold change to the control treatment (0 nM rtLepA). Values represent means ± SEM (n = 4).
Figure 5: Tertiary structures of (A) tilapia leptin and (B) tilapia leptin gene and peptide sequence. The signal peptide (gray), Helices A-D (underlined), and peptide sequence against which the tLepA antibody was raised (yellow) are highlighted.
Figure 6: Validation of a homologous leptin A ELISA in the tilapia. (A) Western blot testing cross-reactivity of the tilapia leptin A antibody (diluted 1:3000) to recombinant tilapia leptin A (rtLepA), recombinant human leptin (rhLep), tilapia plasma, and hybrid striped bass (HSB) plasma. (B) Enzyme-linked immunosorbent assay for tilapia leptin A, measuring the antigen peptide (standard curve, 0.01 – 1000 nM), serially diluted tilapia plasma, HSB plasma, and rhLep (in PBS). (C) Linear range (of B) of the leptin A ELISA (0.25 – 1000 nM). Lines depict sigmoidal (B) and linear (C) regressions for the antigen standard curve.
Figure 7: Tissue mRNA expression profile of leptin A (lepa) and leptin B (lepb) in the tilapia. Expression was determined by qPCR amplification of lepa and lepb in the pituitary, brain, hypothalamus, heart, gill, kidney (whole), pancreas, liver, white muscle, fat, posterior intestine, and testes. Cycle threshold values were compared to a copy number standard curve for each leptin ($R^2 = 0.98$), and normalized to nanograms of total RNA used in the cDNA reaction. Values depict mean $\pm$ SEM (n = 8).
Chapter II.

Prolactin is a major inhibitor of hepatic Leptin A synthesis and secretion.

Abstract

Prolactin (PRL) is the most complex among all pituitary hormones both in its number of actions and in the diversity of factors that control its synthesis and secretion. Leptin is also a pleiotropic hormone best known for its functions in regulating metabolism and reproduction, among other physiological processes. The present study evaluates the regulatory interactions between PRL and leptin for which little is known in vertebrates. In the tilapia (*Oreochromis mossambicus*) removal of the pituitary (hypophysectomy; Hx) increased circulating LepA and *lepa* mRNA levels in the liver, the dominant source of hormone production. Administration of ovine PRL (oPRL, 5 µg /g BW) to Hx fish restored circulating LepA and hepatic *lepa* mRNA levels to those of control fish. Additionally, oPRL reduced *lepa* mRNA levels in a dose-dependent fashion in cultured hepatocytes following 18 h incubation. Previous work in our lab demonstrates that recombinant human leptin stimulates PRL release *in vitro* from tilapia pituitaries. Here, both recombinant tilapia LepA (rtLepA) and recombinant human leptin (rhlep; 0.5 µg g/g BW) increased mRNA expression of both tilapia prolactins (*prl1*, *prl2*) *in vivo*. These results along with our previous work demonstrate that LepA enhances pituitary prolactin synthesis and release, while PRL may, in turn, inhibit hepatic leptin secretion and synthesis in teleosts. We postulate this regulatory interaction may

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be necessary for mobilizing energy reserves during acute hyperosmotic adaptation.
Introduction

Leptin is a 16 kDa cytokine hormone regulating metabolism and energy expenditure in vertebrates (Zhang et al., 1994). Primarily secreted from adipocytes in mammals, leptin acts as a potent lipolytic and anorexigenic factor that is secreted in proportion to fat reserves (i.e., an adipostat) (Ahima, 2008). In fishes and other poikilotherms, the function and regulation of leptin is largely unknown. Despite conservation in suppressing appetite the action(s) of leptin in fishes appear to differ considerably from those demonstrated in mammals (Trombley et al., 2012; Won et al., 2012). This includes the liver, rather than adipose, as a primary secretory tissue for the hormone (Gorissen et al., 2009; Huising et al., 2006; Kurokawa and Murashita, 2009; Kurokawa et al., 2005), potent hyperglycemic actions during acute salinity stress in the tilapia (Baltzegar et al., 2014), elevated synthesis and secretion of leptin during periods of fasting, and declines with feeding (Trombley et al., 2012). Currently, very little is known of the endocrine control or actions of leptin in basal vertebrates.

Both leptin and prolactin (PRL) govern numerous physiological processes in vertebrates, including metabolism and growth, reproduction, and hydromineral balance (Ahima and Flier, 2000; Bernichtein et al., 2010; Copeland et al., 2011; Manzon, 2002). However, the regulatory interactions between these two hormones remain unclear, as studies have produced equivocal findings. Leptin stimulates both central (pituitary) and peripheral (endometrial cells) PRL secretion in rat (Gonzalez, 1998; Tennekoon et al., 2007; Yu, 199u), and cow, but not from porcine pituitary cells (Accorsi et al., 2007; Nonaka et al., 2006). In the tilapia (Oreochromis mossambicus), recombinant human leptin potently increases the release of both tilapia PRLs (PRL1 and PRL2 or PRL177 and PRL188) in vitro through an
ERK 1/2-dependent pathway (Tipsmark et al., 2008), but it is unclear whether the hormone similarly alters PRL synthesis. In contrast to leptin regulation of PRL release, studies to date suggest PRL may exert sex-specific regulatory actions on leptin. Prolactin stimulates adipose leptin secretion in female rats, yet inhibits leptin secretion, lipolysis, and anorexigenic actions in male rats (Brandebourg, 2007; Naef and Woodside, 2007). In prolactin receptor-deficient male mice, no changes were observed in circulating leptin titer, relative to that of normal genotypes, yet female mice exhibit significant decreases in leptin abundance (Freemark, 2001). It is currently unclear if PRL may modulate leptin synthesis and secretion in teleost fishes or other non-mammalian vertebrates.

The aims of the present studies were to assess the role of pituitary PRL in modulating
in vivo leptin synthesis and secretion, as well as in vitro lepa gene expression. To further assess the operation of a PRL-LepA regulatory interaction, we also assessed the in vivo effects of recombinant tilapia LepA on pituitary gene expression of the two tilapia prolactins (prl1, prl2).

Materials and Methods

Animals

All studies except the hypophysectomy experiments were conducted at North Carolina State University (NCSU). The NCSU Institutional Animal Care and Use Committee (IACUC) approved all protocols. For injection studies utilizing recombinant leptin(s), adult male tilapia (95 ± 19 g mean Body Weight) 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. All fish were fed daily
(1–2% BW/day).

**Hypophysectomy and hormone replacement**

Hypophysectomy (Hx) studies were performed at the Hawaii Institute of Marine Biology (Kaneohe, HI) with surgical and experimental procedures approved by the University of Hawaii IACUC. 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 was performed by the transorbital technique (Borski et al., 1994; Breves, 2010). Briefly, fish were anesthetized in a combination of buffered MS-222 (100 mg/L, Argent Chemical, Redmond, WA) and 2-phenoxy-ethanol (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 PRL (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 blood was collected from the caudal vein with heparinized syringes. Plasma was separated by centrifugation at 4°C and stored at -20°C. Animals were subsequently decapitated and liver samples were placed in RNAlater overnight at 4 °C and then frozen at -80 °C prior to processing for RNA
isolation as outlined above. The sample sizes for all groups were 3–4.

Intraperitoneal injection of recombinant leptins

Fish held in brackish water (11–13 ppt) at North Carolina State University received a single IP injection of either recombinant human leptin (rhLep) or recombinant tilapia leptin A (rtLepA). Fish were anesthetized in buffered MS-222 (Aquatic Eco-Systems, Apopka, FL), weighed, and then administered one of the following injections: 0.5 or 5.0 µg /g BW of rhLep dissolved in PBS (45 mM NaCl, 3 mM Na₂HPO₄, 0.6 mM NaH₂PO₄, pH 8.0, 325 mOsmolal); 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 their pituitaries removed and stored in RNAlater prior to assessment of prolactin gene expression. The leptin doses were chosen following preliminary range-finding experiments for the effects of leptin on plasma glucose (Baltzegar et al., 2014).

Commercially prepared rhLep was obtained from Dr. A.F. Parlow (National Hormone & Peptide Program). The production, purity, and bioactivity of rtLepA was previously described by our laboratory (Baltzegar et al., 2014; Douros et al., 2014).

In vitro examination of lepa response to oPRL treatment

In vitro experiments on tilapia hepatocytes were conducted to test effects of oPRL treatment on lepa mRNA expression. Hepatocytes were harvested from the livers of six tilapia as previously described (Pierce et al., 2004). This entailed lethal anesthesia (MS-222) followed by removal of the liver, which was finely excised with a razor blade in a calcium-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 liver tissue was gently pushed
through a 260 $\mu$m mesh filter to remove structural tissue, and then allowed to drip through a second 60 $\mu$m mesh. Filtered hepatocytes were collected in an ice-cold beaker. The cells were washed in HBSS containing 3 mM CaCl$_2$ and 1x MEM solution, with essential and nonessential amino acids (Gibco, Carlsbad, CA), and allowed to recover on ice for 1 h. After centrifugation 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 plates at a density of 3.5 x 10$^5$ cells/mL. Cell viability was determined to be >90% using a Trypan Blue exclusion test. After plating, cells were allowed to acclimate for 4 h at 24 °C in an air atmosphere. After acclimation, the growth media were removed and replaced by experimental media (oPRL or control). The PRL incubations were terminated after 18 h when media was removed and replaced with TRI Reagent. The cell suspension was aspirated with a pipette and held at 4 °C overnight prior to RNA isolation. The functionality and viability of the hepatocytes was confirmed by assessing the stimulation of insulin-like growth factor-1 (igf-1) gene expression by bovine growth hormone.

**Quantitative polymerase chain reaction (qPCR)**

Total RNA extraction and cDNA synthesis were carried out as described above. Leptin A (lepa), leptin receptor (lepr), prolactin 1 and 2 (prl1, prl2) and 18s ribosomal RNA were measured using primers designed by ABI Primer Express software (v3.0; Table 2). Gene expression was measured using Brilliant II SYBR Green Quantitative Real-Time PCR reagents (Agilent, Santa Clara, CA) with 12–25 ng of cDNA template and 75 nM primer concentrations. Duplicate runs for all samples, standards, and negative controls were measured on an ABI 7900HT sequence detection platform using the following protocol: 1
cycle-95 °C for 10 min; 40 cycles-95°C for 30s, 60°C for 60s, and 72°C for a 60s extension. Pooled cDNA samples were used for across-plate normalizations with negative controls run on each plate. Cycle threshold (Ct) values were analyzed by absolute quantification using standard curves derived from serially diluted cDNA for each tissue ($R^2 = 0.98$). Data were then normalized to the expression of 18S ribosomal RNA, whose levels were found to be similar across treatment groups, and expressed as relative-fold change to the mean (calibrator) of the control groups in the hypophysectomy, leptin injection, and in vitro hepatocyte culture studies.

**Statistics**

Statistical analysis was performed using JMP software (v9, SAS Institute, Cary, NC). A one-way ANOVA procedure was employed to test for model significance, with significant differences among groups evaluated using Tukey’s HSD post hoc test. Where necessary, data were log-transformed to pass homogeneity of variance tests. The nominal level of significance for all tests was $P < 0.05$.

**Results**

**Effect of hypophysectomy and oPRL treatment on leptin gene expression and secretion**

Plasma levels of leptin A (LepA) were significantly elevated following Hx relative to sham control fish ($P < 0.01$; Figure 8A). Following treatment with oPRL, Hx tilapia showed a decline in circulating LepA, to levels similar to those of sham-control fish (Figure 8A). Similar to that observed with circulating hormone, hepatic lepa mRNA levels increased in Hx fish ($P < 0.01$; Figure 8B). Replacement with oPRL caused a significant decline in hepatic lepa mRNA relative to that in Hx fish given vehicle injection ($P < 0.05$; Figure
these levels were slightly higher than sham-surgery fish (P < 0.05). Hepatic mRNA expression of lepr was not significantly altered by hypophysectomy or oPRL injection (Figure 8C).

**In vitro effect of PRL on hepatocyte lepa expression**

During 18 h hepatocyte incubations, oPRL reduced lepa expression in a dose-dependent fashion (Figure 9; P < 0.01). The lowest effective dose of oPRL that inhibited lepa was 1 nM (P < 0.05) with maximum inhibition occurring with 10 nM and higher concentrations (P < 0.01).

**Effects of leptin injection on pituitary prolactin expression**

Our previous research showed that rhLep stimulates in vitro release of PRL1 (177 amino acids) and PRL2 (188 amino acids) from the tilapia pituitary in a dose-dependent fashion (Tipsmark et al., 2008). Here we assessed if rhLep and homologous tilapia LepA might be effective in regulating expression of the PRL genes (prl1, prl2) in vivo. The low dose (0.5 µg/g) of both rtLepA and rhLep significantly increased pituitary prl1 and prl2 mRNA expression 24 h following i.p. injection (P < 0.001; Figure 10). At the high dosage of rtLepA (5 µg/g), mRNA expression of prl1 and prl2 was significantly reduced relative to that in control fish (P < 0.01; Figure 10). The high dosage of rhLep elicited a similar, yet less robust response relative to rtLepA (P < 0.05; Figure 10) in reducing prl1 and prl2 expression.

**Discussion**

The aim of the present studies was to identify regulatory interactions between LepA and PRL. We identify an important regulatory interaction between LepA and PRL, whereby PRL reduces hepatic LepA synthesis and secretion, while LepA induces a biphasic effect
on prl1 and prl2 *in vivo*, with lower dosages increasing mRNA levels of these genes. We found that hypophysectomy increased both hepatic lepa mRNA expression and circulating hormone levels. Subsequent replacement of PRL was sufficient to restore plasma LepA levels and reduce lepa mRNA by 5-fold in the liver. Similarly, we found physiological concentrations (1 nM) (Auperin, 1997; Seale et al., 2012) of PRL were effective in inhibiting lepa mRNA levels in tilapia hepatocytes in vitro. Collectively, these data suggest PRL is a negative regulator of LepA synthesis and secretion (Fig. 6) and that its actions are likely to occur, in part, through direct regulation at the level of the hepatocyte. In mammals, the limited studies to date suggest PRL regulates leptin secretion in a sexually dimorphic manner, inhibiting leptin secretion from white adipocytes of male rats, but stimulating synthesis and/or secretion from female adipocytes and endometrial cells (Brandebourg, 2007; Gualillo, 1999; Tennekoon et al., 2007). Whether a similar differential response with sex occurs in tilapia remains unclear, as only males were evaluated in the present study. Nonetheless, the actions of PRL reported here appear specific, insofar as the hormone was ineffective in altering he expression of other genes in the liver, including lepr mRNA (Figure 8C) or 18S ribosomal RNA (data not shown).

In contrast with PRL’s inhibitory actions on LepA synthesis and secretion, leptin also appears to modulate PRL cell function. Previous studies have identified a stimulatory role for leptin on pituitary PRL secretion in both fish and mammals. In rat, treatment with leptin (1–1000 ng/mL) significantly enhanced PRL secretion from the pituitary in vitro and *in vivo* and endometrial stromal cells *in vitro* (Gonzalez, 1998; Tennekoon et al., 2007; Yu, 1997). In tilapia, Tipsmark and colleagues (2008) found that rhLep concentrations ranging from 1 to 10 nM increased the in vitro release of both tilapia PRLs (PRL1 and PRL2) from the pituitary
by as much as 350–600% after 1 h incubation. In the present study, pituitary mRNA expression of both prolactin paralogs \((prl1, prl2)\) was stimulated 4-fold, 24 h following a single, low-dose injection \((0.5 \mu g \text{ g/g})\) of either rhLep or rtLepA (Figure 10). Interestingly, the high \((5.0 \mu g \text{ g/g})\) dose of both leptins administered in our study seemed to exert little effect or inhibited both \(prl1\) and \(prl2\) (Figure 10). While we are uncertain of the nature of this biphasic response, it would appear that fluctuations in leptin levels might exert disparate responses on accumulation of prl mRNA in the tilapia pituitary. Regardless, this and our previous work (Tipsmark et al., 2008) clearly demonstrate that low concentrations of leptin enhance \(prl\) gene expression and secretion in the tilapia. The temporal differences observed between rapid PRL release observed in vitro \((1–4 \text{ h})\) (Tipsmark et al., 2008), and the increases in pituitary \(prl1\) and \(prl2\) mRNAs reported here suggests that the pituitary responds to peripheral leptin signals with acute PRL secretion followed by enhanced transcription and presumably the synthesis of protein needed to replenish levels of the stored hormone. Future studies are required to address the time course over which tilapia LepA might alter \(prl\) gene expression, synthesis and secretion. Overall, it would appear leptin not only stimulates PRL secretion, but also enhances transcription of the \(prl\) genes in tilapia, and perhaps other vertebrates.

The functional significance of the regulatory interactions identified in the present study between teleost LepA and PRL is unclear, but we postulate that it may underlie important mechanisms for regulating hydromineral balance and energy homeostasis in fishes and perhaps other vertebrates. It is well established that PRL is an important freshwater (FW)-adapting hormone in teleosts and that the PRL cell responds accordingly, with PRL synthesis and secretion rising during FW adaptation and declining with seawater (SW)
exposure in tilapia (Seale et al., 2012). We show during acute SW adaptation (24 h), when energy requirements are high, that hepatic lepa mRNA expression rapidly increases (up to 25-fold) and that the hormone exerts hyperglycemic effects through induction of hepatic glycogenolysis in tilapia (Baltzegar et al., 2014). Considering circulating PRL titer (PRL1, PRL2) and pituitary mRNA expression (prl1, prl2) rapidly decline (Seale et al., 2012) during SW adaptation, we postulate that the alleviation of a tonic inhibition of LepA by PRL may contribute to the rise in LepA during acute SW challenge. It is also possible that LepA and PRL may antagonize each other in regulating energy homeostasis and appetite independent of salinity. In mammals, PRL is known to abolish the anorexigenic effects of leptin (Naef & Woodside, 2007) and directly inhibit leptin secretion in white adipocytes (Brandebourg, 2007). The anorexigenic actions of leptin in mammals appear to be conserved in teleosts (Volkoff, Joy Eykelbosh, & Ector Peter, 2003; Won et al., 2012), and future studies should evaluate whether PRL antagonizes leptin’s effects on appetite as well as other metabolic processes, much of which remain to be identified.

In summary, the present investigation identifies key regulatory interactions between LepA and PRL, whereby LepA upregulates mRNA abundance of prl1 and prl2 in vivo in conjunction with its known stimulatory action on PRL secretion (Tipsmark et al., 2008). Evidence also indicates that PRL may serve as a key pituitary hormone involved in suppressing both the synthesis and secretion of LepA in teleosts.
References


Tipsmark, C.K., Strom, C.N., Bailey, S.T., Borski, R.J., 2008. Leptin stimulates pituitary


Table 2. List of PCR primer pairs used for qPCR analysis in Mozambique tilapia.

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Figure 8: The effects of hypophysectomy (Hx) and ovine prolactin (oPRL; 5 µg g/g) replacement on leptin. (A) circulating leptin A levels, (B) hepatic leptin A (lepa) mRNA expression, and (C) hepatic leptin receptor (lepr) mRNA expression in the tilapia. Asterisks represent treatment-dependent effects relative to sham fish (***P < 0.001, **P < 0.01, and (*)P < 0.05); letters identify significant effects across all treatments (P < 0.05). Gene expression data were normalized to 18S rRNA expression, and are expressed as relative fold change relative to the sham fish group. Values represent means ± SEM (n = 3–4).
Figure 9: Dose-response curve of leptin A (lepa) mRNA expression in cultured tilapia hepatocytes following 18h incubation with ovine prolactin (oPRL). Asterisks represent dose-dependent effects relative to control fish (/P<0.01, /P<0.05); letters identify significant effects across treatment (dosage) groups (P < 0.05). The lepa mRNA expression data was normalized to 18S rRNA expression and expressed as relative fold change to the control treatment (0 nM PRL). Values represent means ± SEM (n = 4).
Figure 10: Effects of recombinant tilapia leptin A (rtLepA) and human leptin (rhLep) on pituitary prolactin mRNA expression. The expression of (A) prolactin 1 (prl1) and (B) prolactin 2 (prl2) were measured 24 h following *in vivo* leptin injection (0.5 and 5.0 µg g/g). Asterisks represent treatment-dependent effects relative to control fish (**P < 0.01, *P < 0.05); letters identify significant effects across treatment groups (P < 0.05). Gene expression data were normalized to 18S rRNA and expressed as relative fold change to control fish. Values represent means ± SEM (n = 6–8).
Figure 11: Proposed negative feedback regulatory model of leptin A (LepA) and prolactin (PRL) hormone interactions in the tilapia. LepA stimulates pituitary prl mRNA expression (prl1 and prl2; Figure 10) and hormone secretion (PRL1, PRL2; Tipsmark et al., 2008) in the pituitary. Circulating PRL inhibits mRNA expression of hepatic leptin A (lepa) and circulating LepA titer.
Chapter III.

Metabolic stress stimulates leptin A (LepA) in the tilapia (*Oreochromis mossambicus*): Implications for the regulatory interactions between LepA and the growth hormone (GH)/insulin-like growth factor (IGF) axis.

Abstract

Leptin is a cytokine important for regulating energy homeostasis. Relatively little is known on the function and control of this hormone in teleost fishes. These studies evaluate the regulation of leptin A (LepA), a putative catabolic stress hormone, under different metabolic states in tilapia (*Oreochromis mossambicus*), and examine how subsequent changes in LepA, the dominant leptin paralog in diploid fishes, might alter pituitary growth hormone (GH) and hepatic gene expression of GH receptors (GHRs) and insulin-like growth factors (IGFs). Additionally, the studies examine regulation of hepatic leptins and leptin receptor (LepR) by GH. This work reveals that circulating LepA, and lepa and lepr gene expression are elevated after 3-week feed restriction and decline to control levels 10 days following refeeding. We then evaluated if LepA might alter GH and IGFs, which show discordant regulation with fasting and refeeding, respectively, where GH tends to rise and GH receptor, IGF-1 and IGF-2, and somatic growth decline during fasting. *In vivo* treatment with recombinant tilapia LepA (rtLepA) via *i.p.* injection stimulated hepatic *igf-1*, but had little effect on hepatic *igf-2, ghr1*, or *ghr2* mRNA abundance. Corresponding *in vitro* experiments demonstrate that rtLepA increases the mRNA levels of all these factors in a dose-dependent fashion from hepatocytes after 24 h incubation. By contrast, LepA suppressed *gh* expression from pituitaries *in vitro*. To test the regulatory action of GH on hepatic *lepa* and *lepr*, animals were

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4 Research in this chapter is planned for submission to Journal of Endocrinology.
hypophysectomized, to effectively abolish pituitary GH. Hypophysectomy (Hx) significantly increased hepatic lepa mRNA abundance, while GH replacement in Hx fish restores lepa gene expression to control levels. Hx did not alter lepr. Growth hormone inhibited lepa and lepr mRNA expression at low concentration while higher concentration stimulated the abundance of lepa, but not lepr transcripts. The responses were accompanied by increases in igf-1 expression. These findings indicate LepA synthesis and gene expression increases with fasting, consistent with the hormones function in promoting energy expenditure during catabolic stress. It would also appear that LepA may play an important role in stimulating GHR and/or IGFs to potentially spare declines in these factors during catabolism. Evidence also suggests for the first time that GH may exert important regulatory effects on hepatic LepA production in teleosts, suppressing its synthesis and/or secretion as well as sensitivity. Leptin A, may in turn exert negative feedback effects on GH synthesis. Whether it also suppresses GH secretion requires further study.
Introduction

Leptin is a 16 kDa cytokine hormone known to regulate metabolism and energy expenditure in vertebrates (Zhang et al., 2013). Adipose derived mammalian leptin increases during feeding as fat depots accumulate, and act 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, 2003). Evidence suggests, however, that the adipostatic model for leptin may not apply to teleost fish and perhaps other ectotherms.

Through gene or genome-wide duplication events, multiple forms of leptin exist in fishes (Figueroa et al., 2005; Huising et al., 2006b) with the major form, LepA produced predominantly in the liver in most species (Douroš et al., 2014). Leptin increases during stress (e.g. hypoxia and acute salinity stress) and acts as a potent hyperglycemic factor (Ahima and Flier, 2000; Baltzegar, 2013; Gorissen et al., 2009; Huising et al., 2006b; Kurokawa and Murashita, 2009; Kurokawa et al., 2005), in part, through stimulation of glycogenolysis. In contrast to mammals, the preponderance of evidence in fishes indicates that leptin gene expression or circulating leptin rises with fasting when energy stores decline and declines during feeding when fat or carbohydrate stores accumulate (Fuentes et al., 2012; Jorgensen et al., 2013; Kling et al., 2009a). Other studies show fasting is ineffective in regulating leptin (Huising et al., 2006a), or in the case of hybrid striped bass, it may stimulate leptin gene expression in a manner similar to mammals (Londraville et al.; Won et al., 2012; Won and Borski, 2013). Outside of acute salinity challenge, there is no information on leptin
regulation by metabolic state in tilapia and relatively few studies have addressed the
dynamics in both leptin gene expression and secretion.

Growth in fish, as in most other vertebrates, is primarily regulated by the highly
conserved endocrine growth hormone (GH)/insulin-like growth factor (IGF) axis (Oksbjerg
et al.; Picha et al., 2008; Reinecke et al.; Wood). 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 isoforms of its receptor (GHR1, GHR2) on
target tissues, including the liver and muscle, to induce the expression and secretion of IGFs
(IGF-1 and IGF-2) (Jiao et al., 2006; Kittilson et al., 2011), which in turn stimulate cell
differentiation and proliferation leading to increased somatic growth (Blaise et al., 1996;
Duan, 1997). Plasma IGF-1 subsequently acts in a negative feedback fashion to inhibit
pituitary GH secretion (Fruchtman et al.). 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 (Fox et al., 2006; Won and Borski, 2013). Leptin and tissue GHR and
IGF appear to be discordantly regulated by metabolic state in fish, however no
comprehensive assessment of leptin interactions with the pituitary-hepatosomatic growth axis
has been undertaken in fish or other ectotherms whose energy requirements and caloric
intake can differ dramatically from that of homeotherms.

In mammalian models, leptin appears to stimulate IGF-1 both in vivo and in vitro
(Chan et al., 2008b; 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 leptin stimulates hepatic IGF and GHR expression in the hybrid striped bass (Won, 2013), but it is uncertain whether this response might occur with homologous hormone or be conserved among other teleosts, particularly in those species where leptin shows a discordant regulation to GHRs and IGFs with altered nutritional state.

In addition to its regulatory effects on IGFs, substantial evidence indicates leptin stimulates pituitary GH gene expression and secretion in rodents and ungulates (Barb et al., 1998; LaPaglia et al., 1998; Morrison et al., 2002). Growth hormone itself suppresses leptin secretion from adipocytes in humans and cow, and recent evidence with chronic implants shows the hormone may exert a mildly suppressive effect on leptin secretion in Atlantic salmon (Engstrom et al., 2003; Houseknecht et al., 2000; 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.

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 and hepatic gene expression of GHRs and IGFs, and 3) assess if GH itself might regulate leptin and its receptor in the liver.

**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 study.

**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, 14, 21, 24, and 31 days. All control fish were fed once daily to apparent satiation throughout the 31-day experiment. Body weight (BW) and liver weights were recorded for each sampling point; hepatosomatic index (HSI) was calculated for each fish ([liver weight/BW]*100). Additionally, blood was collected via caudal vein puncture for an assessment of circulating LepA levels by homologous ELISA (Douros et al., 2014) while liver samples (~100g) were taken and stored in RNAlater for subsequent processing of samples for lepa and lepr gene expression analysis.

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

We assessed the in vitro effects of rtLepA and GH on the hepatic expression of genes using hepatocyte incubations according to our previously described methods (Douros et al, 2014). Hepatocytes harvested from 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) at 18 °C. 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 260µm mesh filter to remove structural tissue, and then allowed to drip through a second 60µm mesh. Filtered hepatocyte suspension was collected in a beaker kept on ice. The cells were then washed 5x in HBSS containing 3 mM CaCl₂ 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 70 x g, 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 ~3.5 x 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 growth media were removed, replaced by experimental media (control, rtLepA, or bovine GH) 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 500nM rtLepA. For treatment with GH, cells (n = 5 wells/group) were incubated in growth medium containing 0, 0.1, 1, 10 or 50 nM bovine GH (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).
In vivo effect of rtLepA on hepatic ghr and igf expression

For injection experiments, recombinant tilapia leptin A (rtLepA) was used. 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 gene expression. The rtLepA doses were chosen based on their effectiveness in inducing hyperglycemia and regulating prolactin gene expression in tilapia (Baltzegar, 2013; Baltzegar et al., 2014).

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, 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.
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 24h 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.

Effect of rtLepA on pituitary GH gene expression in vitro

The in vitro pituitary proximal pars distalis (PPD) incubation procedure has been
described previously (Douros et al., 2014; Helms et al., 1991; Rodgers et al., 1992). Male
freshwater fish were used (95 g BW) for the studies. The pituitary was removed and the PPD
was dissected then pre-incubated for 2h in Modified Kreb’s bicarbonate Ringer (300
mOsmolal) containing glucose, L-glutamine, and Eagles MEM (GIBCO, Grand Island, NY).
Media was then replaced with experimental medium and tissues were incubated for 18 h at
24°C under a humidified atmosphere of 95% O₂/5%CO₂. Tissues were removed and placed
in RNALater until extraction.

Two experiments were performed. First PPDs (n = 3-4/group) were exposed to
various concentrations of rtLepA (0, 1, 10, and 100nM) that we showed are 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 18h) of 10 nM rtLepA on gh expression.
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). RNA integrity was confirmed by agarose gel electrophoresis. 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, lepb, 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 3) 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 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; 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). The results were normalized to 18S RNA expression. Gene
expression data for each group is presented as a percentage of the control mean (% control ± SEM).

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 Dunnet’s test for significant differences. Feed restriction, and pituitary culture experiments using multiple time points were analyzed by two-way ANOVA (treatment x 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 $P < 0.05$.

Results

Leptin response to fasting and refeeding

Fish fasted for 21 days had significantly lower body weight (30%; $p < 0.05$). This was accompanied by lower HSI by 7 days (35-44%; $p < 0.01$). 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; Figure1A-B). Gene expression of both lepa and lepr increased after 1, 7, and 21 days of fasting (Figure 12D-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 titer was significantly elevated after 21 days of fasting (Figure 12C; $p < 0.001$) and levels were restored to controls by 3 days (day 24) of refeeding.

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 (Figure 13A, 2B, p < 0.001). The low (0.1 nM) and high (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 (Figure 13C, 13D; p < 0.001). A maximal, 7.5 – 10 fold elevation was seen with 100 nM rtLepA (p < 0.001).

**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*. Neither 0.5 nor 5.0 µg/g rtLepA *i.p.* injection were effective in regulating hepatic expression of *ghr1*, *ghr2*, and *igf-2* (Figure 14B-D). Leptin A at 5.0 µg/g BW, on the other hand, increased *igf-1* mRNA by over 2-fold (p < 0.05; Figure 14A).

**In vivo GH regulation of hepatic lepa and lepr expression**

Hepatic *lepa* mRNA expression increased in Hx fish (p < 0.05; Figure 15A) 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 (Figure 15B). Circulating LepA was not measured in these fish as blood was used for other studies on osmoregulatory physiology.

**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 (Figure 16; 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 \( (p < 0.01; \text{Figure 16}) \).

**Leptin regulation of pituitary gh mRNA abundance**

Pituitary gh mRNA abundance declined by > 60% with 1 and 10 nM rtLepA following 18 h incubation \( (\text{Figure 17A}; p < 0.05) \). The highest dose (100nM) was ineffective in regulating gh gene expression. The time course over which rtLepA suppressed gh mRNA was then assessed using 10 nM rtLepA. The levels of GH mRNA increased gradually in control incubations peaking between 4 and 24 h \( (\text{Figure 17B}; p < 0.01) \). Leptin A effectively reduced gh mRNA levels by 50% by as early as 4 h of incubation \( (\text{Figure 17B}; p < 0.01) \).

**Discussion**

These studies examine the regulation of LepA, the major leptin paralog in the tilapia \( (\text{Douros et al., 2014}) \), by metabolic state and its regulatory interactions with key elements of the GH/IGF endocrine growth axis. Using a homologous ELISA recently developed for tilapia LepA, circulating LepA as well as hepatic lepa mRNA abundance were found to increase with fasting and were restored upon refeeding in tilapia \( (\text{Figure 12}) \). 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 \( (\text{Froiland et al., 2012}) \), fine flounder \( (\text{Fuentes et al., 2012}) \), rainbow trout \( (\text{Kling et al., 2009a}) \) Atlantic salmon \( (\text{Trombley et al., 2012}) \) and goldfish \( (\text{Volkoff et al., 2003}) \). It appears that leptin sensitivity may also be enhanced with altered metabolic
state insofar as hepatic lepr expression was increased along with LepA during fasting (Figure 12). Leptin receptor gene expression also increases with lepa expression during acute seawater challenge in tilapia and with hypoxia in medaka (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 other fishes contrasts with that seen in mammals, including humans and rodents, where leptin levels decline with fasting and increases with realimentation in concert with changes in lipid stores (Ahima and Flier, 2000; Won et al., 2012). This despite the well conserved anorexigenic function of leptin among vertebrate taxa (Elias et al., 1999; Won et al., 2012). Similar to reports in other fishes, 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 (Figure 12) 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 starvation between mammals and fish may reflect a divergence in metabolic strategies between endotherms and ectotherms, generally. Specifically, teleost leptin may initiate carbohydrate catabolism as the hormone induces hyperglycemia in fish and lizards and liberates hepatic glucose stores through glycogenolysis in tilapia (Ahima and Flier, 2000; Baltzegar et al., 2014; Greene and Selivonchick, 1987; Paolucci et al., 2006; Reidy, 2000; Sheridan, 1988; Tseng and Hwang, 2008). Furthermore, in these studies LepA suppresses gene expression of GH (Figure 17), a known lipolytic factor (Sheridan, 1986), while others have demonstrated a potentially lipid sparing effect of leptin as the hormone suppresses lipase gene expression (Baltzegar et al., 2014). As a whole, these data suggest that leptin might preferentially mobilize
carbohydrate rather than lipids in tilapia and perhaps other teleosts, particularly in response to stressors like starvation, osmotic shock, and hypoxia that are known to stimulate leptin (Figure 12) (Bernier et al., 2012; Fuentes et al., 2012; Kling et al., 2009b). Conversely, leptin function may have evolved in mammals to preferentially promote lipid catabolism (Flier, 1998). Thus, it appears that leptin has a conserved function amongst vertebrates in that it stimulates catabolism of discrete energy stores, while sparing others as a means of adaptation to fasting, however there may be an evolutionary divergence in preferred energy substrate utilized depending on life history and metabolic strategy.

Fasting or certain other conditions that impair growth (e.g. Laron dwarfism) induce a hepatic GH-resistant state in tilapia and other fishes 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 we sought to test if LepA might modulate GHRs and IGFs during catabolism. Recombinant tilapia LepA up regulates \textit{igf-1} and \textit{igf-2} expression at low doses, while higher concentrations (>10 nM) were sufficient to increase \textit{ghr1} and \textit{ghr2} mRNA abundance \textit{in vitro} (Figure 13). 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 are also likely 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 \textit{igf} seen \textit{in vitro} was also observed \textit{in vivo} following a single injection of rtLepA. It is likely therefore, that rtLepA increases hepatic \textit{igf-1} expression, in part, through direct actions at the level of the hepatocytes. Leptin
treatment elevates \textit{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., 2008a), suggesting this action of leptin has been well conserved in vertebrates.

Our previous work also showed leptin stimulated both hepatic \textit{igfs} and \textit{ghrs in vitro} in hybrid striped bass. 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, 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 promote peripheral glucose uptake in accordance with IGFs established insulin-like actions and LepA glucose mobilizing action (Baltzegar et al., 2014; Froesch et al., 1985). In contrast to effects observed \textit{in vitro}, exogenous leptin did not significantly alter hepatic \textit{ghr1, ghr2} or \textit{igf-2} expression in tilapia (Figure 14). Catabolic 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 \textit{in vivo}, but would not likely have been convoluting factors in the \textit{in vitro} experiment using isolated cells.

These studies also assess the regulation of leptin and its receptor by GH. As previously reported, \textit{lepa} expression is under constitutive suppression by pituitary factors (Douros et al., 2014). As such, hypophysectomy (pituitary removal) significantly increases \textit{lepa} gene expression \textit{in vivo}, while oGH injections in hypophysectomized tilapia reduced \textit{lepa} gene expression to that of intact animals (Figure 14A). We confirm this effect \textit{in vitro}
by demonstrating that GH provided at basal physiological concentrations (0.1 nM) (Fox et al., 2010; Fox et al., 2006) suppresses *lepa* and *lepr* gene expression in cultured hepatocytes (Figure 17). However, high physiological for instance seen with seawater adaptation (1.0 nM; Auperin et al. 1997) and supraphysiological doses of GH (10 and 50 nM), either exerted little effect or increased *lepa* expression *in vitro* (Figure 17). The differential dose effects of GH on *lepa* 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, 1997) 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. The elevations in GH seen with fasting in tilapia and other fish (Fox et al. 2006), combined with potential increases in LepA resulting from higher levels of GH and LepA actions in promoting *ghr* and *igf* expression itself, would provide a mechanism for sparing declines in GHR and IGFs during states of GH resistance.

The potential regulatory effect of leptin on pituitary GH in teleosts is unknown. While pituitary GH gene expression and secretion is stimulated by leptin in mammals (Barb et al., 1998; LaPaglia et al., 1998; Morrison et al., 2002), our data suggest that rtLepA reduces *gh* expression as early as 4 h *in vitro*. While further studies are clearly needed to assess LepA role in modulating GH protein production and secretion in the pituitary, this data suggest the possibility that LepA may contribute to a reduction in GH transcription. The physiological meaning of this action is unclear, but may again be associated with a leptin stress response
whereby it promotes carbohydrate catabolism and counters any potential growth anabolic and lipid catabolic effects of GH.

In summary, the studies indicate the synthesis and/or secretion of leptin and 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 to promote carbohydrate 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 during long term fasting. This, along with our findings that definitively show for the first time in teleosts that GH suppresses hepatic lepa and also attenuates GH transcript accumulation, provide a potential mechanism by which GH and LepA may interact to balance energy expenditure between carbohydrate and lipid catabolism.
References


Naef, L., & Woodside, B. (2007). Prolactin/Leptin interactions in the control of food intake


Table 3. List of PCR primer pairs used for qPCR analysis in Mozambique tilapia.

<table>
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<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Forward (5’ – 3’)</th>
<th>Reverse (5’ – 3’)</th>
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<td>leptin a (lepa)</td>
<td>KC354702</td>
<td>GGGTCTCCCAGATCAAGTACGA</td>
<td>TGCCGACCAGATGAGAATG</td>
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<tr>
<td>leptin b (lepb)</td>
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<td>GTGCGCTCTTCGCCCTTAA</td>
<td>CTGTACTGCTGTTTGCCGTTTA</td>
</tr>
<tr>
<td>leptin receptor (lepr)</td>
<td>KC354703</td>
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<td>TGCAGCGGGACTGTTGT</td>
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<td>growth hormone (GH)</td>
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<tr>
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</table>
Figure 12: (A) Body weight (B) Hepatosomatic index (C) circulating leptin (D) hepatic lepa mRNA abundance and (E) hepatic lepr mRNA abundance of control tilapia fed to satiation daily and treatment fish subjected to 3 weeks fasting (red bar) followed by 1.5 weeks of refeeding daily 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).
Figure 13: Effect of hypophysectomy and oGH treatment on hepatic gene expression of (A) *lepa* (B) *lepr*. The data reported are expressed as fold change is normalized to 18s ribosomal RNA expression and reported as Mean ± SEM.
Figure 14: In vivo response of hepatic (A) *igf*-*1* and (B) *igf*-2 (C) *ghr*1 (D) *ghr*2 gene expression to recombinant leptin injections of 0.5 and 5 ug/g BW. The data reported are expressed as fold change is normalized to 18s ribosomal RNA expression and reported as Mean ± SEM.
Figure 15: (A) Dose effect and (B) Time-course effects of leptin (10 nM) on GH from mRNA abundance in somatotrophs in isolated PPDs. Values are mean fold change ± S.E.M normalized to 18s RNA abundance.
Figure 16: In vitro regulation of (A) *igf*-1 (B) *igf*-2 (C) *ghr1* and (D) *ghr2* gene expression in hepatocytes by rtLepA in doses of 0.1, 1, 10, 100 and 500 nM. The data reported are expressed as fold change is normalized to 18s ribosomal RNA expression and reported as Mean ± SEM.
Figure 17: In vitro regulation of *lepa*, *lepb*, *lepr* and *igf-1* gene expression in primary hepatocyte cultures to bGH treatment in 0.1, 1, 10, 50 nM doses. The data reported are expressed as fold change is normalized to 18s ribosomal RNA expression and reported as Mean ± SEM.
Chapter IV

Leptin directly stimulates glycolysis through a STAT3 dependent mechanism

Abstract

Glycolysis is one of the most ancient biochemical processes critical for energy production in all organisms. Here, we assessed if leptin, a cytokine known to enhance energy expenditure by promoting lipid or carbohydrate catabolism, might directly regulate cellular glycolysis. This possibility was first identified by transcriptomic analysis of the tilapia (*Oreochromis mossambicus*) pituitary rostral pars distalis (RPD) where clustering algorithms showed recombinant leptin (rtLepA) administration in vitro stimulated the gene expression of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in a covariable manner to the hypoxic stress gene network. Leptin also increased the gene expression of phosphofructokinase (PFK), the rate limiting glycolytic enzyme. In orthogonal tests rtLepA dose-dependently increased GAPDH and PFK mRNA expression in the RPD within 6 h. Leptin also stimulated PFK activity and total glycolytic output. A STAT3, but not an ERK blocker, suppressed leptin-induced elevations in PFK activity and glycolysis, indicating leptin stimulates glycolysis through a STAT3-dependent mechanism. Similarly, in cultured primary hepatocytes, rtLepA stimulated PFK gene expression and glycolytic activity. These results identify a novel action for leptin to directly stimulate glycolysis across tissue types. The findings suggest that leptin may promote energy expenditure, in part, by stimulating glycolysis and thereby facilitating the energetic needs associated with various stressors such as hypoxia, osmotic perturbations, and cancers that are accompanied by increased leptin.

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5 Research in this chapter is planned and organized for submission to Proceedings of the National Academy of Sciences, USA.
Introduction

Leptin is a pleiotropic cytokine implicated in multiple pathologies, including obesity, diabetes, and cancer (Ahima and Flier, 2000; Garofalo and Surmacz, 2006). Leptin is classically described as an adipostat, whereby the hormone circulates in proportion to total body lipid content, while acting to promote satiety and energy expenditure through stimulation of fatty acid oxidation and metabolic rate (Ahima and Osei, 2004; Ahima et al., 2000; Chinookoswong, 1999; Garofalo and Surmacz, 2006) preventing excessive lipid accumulation. Impairment of leptin production or signaling leads to obesity and reduced metabolic rate (Mistry et al., 1997). Additional evidence also suggests leptin may stimulate energy expenditure through carbohydrate catabolism as the hormone promotes hepatic glycogenolysis and peripheral glucose uptake (Benomar et al., 2006; Chinookoswong, 1999; Kamohara et al., 1997). Much of the catabolic actions of leptin appear associated with the general stress response, for instance with hyperosmotic perturbations that are accompanied by glucose mobilization (glycogenolysis), increased metabolic rate (O$_2$ consumption), and a 20-fold elevation in hepatic leptin gene expression (Baltzegar et al., 2014; Chu et al., 2010; Dalman et al., 2013; Druder, 2006; Mistry et al., 1997; Rodriguez et al., 2014).

Paradoxically, leptin is elevated during hypoxia in a wide range of vertebrates, when metabolic rate is suppressed (Bernier et al., 2012; Chaiban et al., 2008; Wang et al., 2008); thus leptin may act to elevate overall energy expenditure even when oxidative respiration is less viable, possibly by promoting anaerobic pathways (Gautier, 1996). The mechanism by which leptin promotes energy expenditure particularly during the adaptive stress response under normoxic and hypoxic conditions is unclear, however it is possible that the hormone
accomplishes this function by directly regulating one of the most critical aspects of basal metabolism, glycolysis.

Glycolysis, the enzymatic conversion of glucose to pyruvate, is the foundational biochemical pathway for ATP synthesis (Mason et al., 2014). This process is regulated in concert with metabolic rate often under conditions where leptin increases as a possible means to meet the energetic demands for maintaining homeostasis. Metabolic rate, glycolytic activity, and glucose levels increase with tumorigenesis and acute osmoregulatory stress (Beitner and Kalant, 1971; Fiol et al., 2006; Kobayashi and Neely, 1979; Macbeth and Bekesi, 1962; Morgan and Iwama, 1998; Tseng and Hwang, 2008; Wu et al., 2005). Metabolic suppression as seen with hypoxia and hibernation is also associated with enhanced glycolysis, as animals attempt to meet energy requirements through anaerobic pathways (Gautier, 1996; Papandreou et al., 2006; Storey and Storey, 1990). Cellular metabolic rate is regulated by various factors principally the substrate supply that may include pyruvate, intermediaries of the tricarboxylic acid cycle (TCA) cycle; overall metabolic demand for ATP (e.g., ADP availability); as well as hormonal signals that control metabolism (Rolfe and Brown, 1997, Krebs 1959). Sympathetic catecholamines (α,β-adrenergic) and osmoregulatory peptides (e.g. angiotensin, vasopressin) act via cAMP and Ca^{2+} mediated signals (Pilkis and El-Magharbi, 1988) to suppress glycolysis; while insulin, hypoxia-inducible factor 1α, and other growth factors work through cAMP phosphodiesterase activators and signal transducers and activators of transcription (STATs) to stimulate glucose catabolism (Beitner and Kalant, 1971; Coelho et al., 2007; Demaria et al., 2010; Hue and Rousseau, 1993; Konno et al., 2005). The full complement of endocrine factors that directly regulate glycolysis, particularly during stress (e.g. lipotoxicity, oncogenesis,
hyperosmolality, and hypoxia) is unknown. Considering that leptin increases under various stressors associated with enhanced glycolysis we postulate that the hormone may act directly to stimulate glycolysis, thereby increasing energy expenditure, and that this basal action may provide requisite energetic substrates to maintain cellular homeostasis (Baltzegar et al., 2014).

Given the evidence suggesting a functional link between leptin and glycolysis, we assess herein, leptin direct effects on glycolysis using a variety of approaches. We have characterized the leptin-induced changes in global gene expression using an advanced clustering analysis of the pituitary transcriptome, and further test these findings by measuring leptin effects on gene expression and activity of glycolytic enzymes, total glycolytic activity, and STAT3 and ERK1/2 activation in a series of classical in vitro experiments using both cultured pituitaries and hepatocytes. The data definitively illustrate leptin directly stimulates glycolysis by increasing the expression of glycolytic genes, activity of glycolytic enzymes, and overall glycolytic output through a STAT3 dependent mechanism.

Results

Effects of leptin on the pituitary transcriptome: The effect of homologous recombinant LepA (100nM), the dominant leptin paralog in teleost fish (Douros et al., 2014), on the global gene expression profiles of the tilapia (Oreochromis mossambicus) pituitary rostral pars distalis (RPD) transcriptome was examined using an RNA-seq approach. The tilapia RPD, like that of most fishes, is an ideal model for examining endocrine cell metabolism, as it consists of a nearly pure population of prolactin producing cells that can be studied in their natural in situ aggregated form. The lactotrophs in tilapia and other vertebrates are highly sensitive to
stimulation by leptin (Douros et al., 2014; Tipsmark et al., 2008). Illumina sequencing of the RPD returned ~17 million reads of 150bp (paired end reads) which align to the O. niloticus reference genome with >93% accuracy. The rtLepA treatment significantly regulates 1,994 genes (1,283 stimulated; 711 suppressed) compared with controls (p < 0.05; Differentially Expressed Genes, DEGs, Supplemental Table 4). Two glycolytic genes of particular interest for further studies, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphofructokinase (PFK), are stimulated 2.25 and 2.7 fold, respectively. A reduction of data dimensionality (RDD) demonstrates that at least 400 highly significant DEG inputs are required to build a predictive artificial neural network (ANN) model of leptin signaling (Figure 18A; Supplemental Table 2). Modulated modularity clustering (MMC) groups the 400 highly significant DEGs into 11 functionally interactive modules (Figure 18C-M). The 11 MMC modules display GO term enrichment (functionality) in metabolism, translational processes (protein processing, transport and metabolism), cell cycle, and hypoxia response among others (GO terms: Table 4; MMC modules: Supplemental Table 3).

Effect of leptin administration on glycolysis:

Based on the stimulatory effects of leptin on glycolytic enzyme gene expression observed from the transcriptomic analyses, a time-course study evaluating leptin regulation of glycolytic activity in RPD incubations was conducted. Lactate secretion, a proxy for glycolytic activity, was not effected at 0.5 and 1 h but significantly increased following 6 h of exposure to 100 nM rtLepA (p < 0.0001; Figure 19A). The effect is dose-dependent with 1 and 10nM rtLepA increasing lactate secretion by 18% and the 100 nM dosage by 30% following 6 h incubations (p < 0.005; Fig.2B).
The changes observed in glycolysis were accompanied by elevations in both GAPDH and PFK mRNA expression. Leptin dose-dependently increased PFK and GAPDH mRNA by 1.7 and >2.5 fold, respectively (p < 0.01; Figure 19C,D). Lactate secretion correlated significantly to PFK ($r^2 = 0.1332; p < 0.05$) but not GAPDH gene expression.

To evaluate the potential signaling mechanisms underlying the glycolytic actions of leptin studies assessed the effect of STAT and ERK inhibitors on glycolytic output and PFK activity. Leptin at 10 and 100nM concentration stimulates lactate secretion by 66 - 87% (p < 0.05; Figure 20A) and PFK enzyme activity by 2-fold (p < 0.001; Figure 20B) relative to controls. Coincubation of RPDs with rtLepA and the STAT3/5 inhibitor Stattic (100uM) significantly suppresses both lactate secretion and PFK activity by 77% and 1.5 fold, respectively compared to rtLepA treatment alone (p < 0.0001; Figure 20A-B). No differences in lactate secretion or PFK activity was observed between tissues coincubated with rtLepA and PD98059, an ERK1/2 blocker (30µM), versus those incubated with rtLepA alone (Figure 20A-B). Pituitary lactate secretion significantly correlates to PFK activity ($r^2 = 0.1438; p < 0.05$). STAT3 (~25-40%) and ERK1 (~15-20%) activity as measured by phosphorylation were significantly increased by rtLepA treatments at 6 h (Figure 20D,F). The total amount of STAT3 and ERK1 was not affected by rtLepA (Figure 20E,G).

Effect of leptin on hepatic glycolysis:

Leptin effects on glycolysis and PFK and GAPDH gene expression were characterized in primary hepatocytes to determine if the hormone’s actions might be broadly applicable in other cell types (Study 5). Leptin was effective in stimulating PFK gene expression by >1.8 fold (p < 0.05) and lactate secretion by >85% (p < 0.005; Figure 20A,B). GAPDH gene expression changed little in response to leptin (Figure 20C). Like that
observed with RPD, hepatic lactate secretion correlated significantly to PFK expression ($r^2 = 0.2540; p < 0.05$).

**Discussion**

We identify a new function for leptin as a stimulator of cellular glycolysis by utilizing, in part, an advanced neural network analyses of transcriptomic data that predict functional pathways regulated by leptin. *In vitro* leptin administration differentially regulates 1,994 genes in pituitary RPD, including the glycolytic genes PFK and GAPDH (Supplemental Table 4). Of these DEGs, 1,283 (64.3%) are up-regulated, while only 711 (35.6%) are down-regulated, suggesting leptin exerts largely stimulatory actions on pituitary gene expression consistent with that observed for transcriptome analyses of other stress signals (Landis et al., 2004; Lucau-Danila et al., 2005). We additionally assess the functional effects of leptin by the use machine-learning algorithms (Multilayer Perceptron; MLP), which identify 400 highly significant DEGs predicted to be central to leptin action (Supplemental Table 2; Figure 18A)(Holmes and Cunningham, 1993; Reading et al., 2013). These 400 highly significant DEGs cluster into 11 functionally interactive modules based on their shared covariability using Modulated Modularity Clustering (MMC; Figure 18B) (Gene Ontology, 2008) (Stone and Ayroles, 2009). These modules are involved in a variety of broad cellular functions, including: metabolism, protein processing, cell cycle, and hypoxia adaptation (Table 4). The hypoxic adaptation module (Module 7: Figure 18C) contains the glycolytic enzyme GAPDH along with many known hypoxic and hyperosmotic-sensitive genes including: chaperonin containing TCP1 (*CCT*), chromodomain-helicase-DNA-binding protein 4 (*chdb-4*), glycerophosphocholine phosphodiesterase (*gpcpd1*), increased sodium
tolerance-1 (*IST1*), heat shock protein beta 1 (*HSP90b*), inhibitor of NF Kappa B kinase (*ikbkg*), peptidylprolyl isomerase A (*ppiaa*), and ribosome assembly subunit (*UTP20*) (Supplemental Table 3). The genes *CCT*, *HSP90*, and *ppiaa* in particular are known to promote protein folding, acting to protect protein tertiary structure under extreme cellular conditions, suggesting a mechanism for leptin stimulated hypoxic and osmotic adaptation (Casazza et al., 2013; Chen and Meyrick, 2004; Mazurais et al., 2014). Additionally, a notable cluster-to-cluster interaction can be observed between module 7 (hypoxia adaptation) and 10 (cytokine signaling), reinforcing the idea that leptin, a cytokine known to rise during hypoxic events in many vertebrates (Chu et al., 2010; Papandreou et al., 2006) (Figure 18B; Table 4), regulates hypoxia adaptation. While greater characterization of all interactions within the transcriptome lies outside the scope of the present studies, these data serve to link the known role of leptin in hypoxic stress adaptation with increases in glycolytic gene expression, and most likely, increased glycolytic activity.

A series of orthogonal tests *in vitro* confirm the findings of the transcriptome analysis, and establish that leptin increases glycolysis directly at the cellular level. Several experiments conducted independently show that leptin, within a physiological range (Douros et al. 2014), stimulates glycolytic activity as measured by lactate secretion (Bujara et al., 2011; Cuendet et al., 1976; Hue and Rousseau, 1993; Kobayashi and Neely, 1979; Lopez et al., 1988) within 6 h (Figure 19A, 20A). These elevations in lactate occur concomitantly with increases in PFK and GAPDH mRNA abundance (Figure 19B-C) and PFK activity (Figure 20B). Both PFK activity and mRNA expression are significantly correlated with lactate secretion, consistent with PFK being a rate-limiting enzyme of glycolysis (Bujara et al., 2011; Hue and Rousseau, 1993; Mason et al., 2014). The simultaneous, correlated increase
in glycolysis, PFK activity, and PFK mRNA expression over a 6 h period, and not earlier
time points, suggest that glycolytic stimulation by leptin may be dependent on increased
transcription and production of PFK enzyme, rather than a modulation of PFK enzyme
kinetics. This is further supported by evidence that the effects of leptin are mediated by
STAT whose primary function is to regulate gene transcription (Rawlings et al., 2004).

Recombinant tilapia LepA significantly increased protein abundance of both
phosphorylated (activated) STAT3 and ERK1/2 proteins (Figure 20C,D,F). Although
coincubation with both STAT3 (Stattic) and ERK1/2 (PD98095) blockers were effective in
abolishing phosphorylated protein abundance (Figure 20C,D,F), only STAT3 inhibition
abolished leptin’s stimulatory effects on PFK activity and glycolysis (Figure 20A,B). This
suggests the effects of leptin on cellular glycolysis in the RPD are likely mediated through a
canonical JAK/STAT leptin signaling pathway, in keeping with previous reports of elevated
glycolysis in cell lines with constitutively activated STAT3 proteins (Demaria et al., 2010).
However, ERK1/2 signaling may be critical component of leptin regulation of pituitary
lactrophs in the RPD, as we previously show leptin potently stimulates PRL gene expression
and secretion, a response that was mediated by ERK1/2 activation.

The stimulatory effect of leptin on glycolysis shown for the pituitary RPD was
confirmed in another tissue type. Both lactate secretion and PFK mRNA increased in a dose
dependent fashion in response to rtLepA in isolated hepatocytes after 6h incubation (Figure
21A,B). However, leptin was ineffective in regulating GAPDH mRNA expression in
hepatocytes. Although GAPDH expression is not significantly correlated to glycolytic
activity in either cell type examined, the increase in GAPDH mRNA with leptin treatment in
RPD may, nevertheless, be an important component of glycolytic induction or hypoxic
adaptation in lactotrophs where a substantial amount of energy is required for cell proliferation and protein synthesis particularly during lactation in mammals and freshwater adaptation in euryhaline teleosts (Rodgers et al., 1992a; Seale et al., 2012). Regardless, it would appear that leptin acts on various on cell types to promote glycolysis primarily through a STAT-3 dependent stimulation of PFK mRNA expression and PFK activity.

These results identify a novel action for leptin to directly stimulate glycolysis across tissue types. The findings suggest that leptin may promote energy expenditure, in part, by stimulating glycolysis and thereby facilitating the energetic requirements associated with various stressors across vertebrate taxa, ranging from hypoxia to salinity adaptation. One primary function of leptin is to stimulate cell proliferation and its levels are known to increase with certain malignancies (Garofalo and Surmacz, 2006). This hormone may promote glycolysis, thereby providing the energy necessary for sustaining rapid cell division in tumors that are oxygen starved (Warburg effect) (Demaria et al., 2010; Macbeth and Bekesi, 1962) and whose increased glycolytic activity is STAT dependent. The research also argues that leptin may enhance metabolic rate and overall energy expenditure, in part, through stimulation of glycolysis and the production of pyruvate, a key precursor to the TCA cycle substrate Acetyl CoA.

Materials and Methods

Animals were housed in freshwater (FW) recirculating tank systems (salinity 0-0.5 ppt, hardness 74-84 mg/L, alkalinity 126-178 mg/L, pH 8.0) at 24-26 °C with a photoperiod of 12:12 h of light and dark and fed daily (1-2 % BW/day). Fish were anesthetized in buffered tricaine methanesulfonate (MS-222) and decapitated prior to sampling of pituitary and
liver. All animal protocols were approved by the NCSU Institutional Animal Care and Use Committee.

**Pituitary and hepatocyte incubations:** Procedures for pituitary RPD isolation and incubations have been described previously (Douros et al., 2014; Helms et al., 1991; Rodgers et al., 1992b). The pituitary was removed and the RPD was dissected and placed individually in wells of a 96-well plate containing Modified Kreb’s bicarbonate Ringer (320 mOsmolal) solution with addition of glucose, L-glutamine, and Eagles MEM (GIBCO, Grand Island, NY). Following a 2 hr preincubation the media was replaced with control or experimental media containing rtLepA and/or pharmacological agents at concentrations and time periods provided in the figures (Tipsmark et al., 2008). Media was removed and frozen at -20°C for subsequent lactate measurement. Tissue were removed and placed in either RNALater at 4°C for assessment of gene expression or in PFK assay buffer and stored at -20°C for later Western blot analyses and PFK activity determinations.

Hepatocytes were isolated, plated as a monolayer of 3x10⁵ cells per well (24-well plate), and incubated (n = 6/treatment, 4 groups) in RPMI containing 5 mM glucose for 4 hr as previously described (Douros et al., 2014). The preincubation media was then replaced with control or treatment media and hepatocytes were incubated for 6 h at 24°C under ambient air conditions. At termination, media lactate was measured and cells were collected in Tri-reagent and stored at 4°C until RNA isolation for gene expression analysis of PFK and GAPDH.

**Lactate, PFK activity, and Western blot assays:**
Media lactate secretion was assessed as a proxy for total glycolytic activity (Bujara et al., 2011; Lopez et al., 1988). Lactate released in media was quantified using a commercial colorimetric assay that measures NADH derived from lactate oxidation (Abcam, Cambridge, MA). PFK enzymatic activity was measured by a commercial colorimetric assay that directly quantifies ADP formation derived by conversion of PFK substrate, fructose-6-phosphate, to fructose-1,6-diphosphate (Sigma-Aldrich, St. Louis, MO). Lactate secretion and PFK activity were normalized to tissue protein concentration.

Cellular ERK1 and STAT3 were separated by Western blot and detected and quantified by an LI-COR Odyssey infrared imaging system as described previously (Douros et al., 2014; Tipsmark et al., 2008). Phosphorylated (active) ERK1 and STAT3 were detected by polyclonal phospho-p44 MAPK (Thr202)(#4372S, Cell Signaling, Beverly, MA) and polyclonal anti-STAT3 p-Y705 (AB76315, Abcam) using 1:2000 and 1:5000 dilution, respectively. Total were detected by anti-ERK1 (AB137766) and anti-STAT3 (AB5073) at 1:2000 and 1:5000 dilution. Following washing, membranes were incubated 1 hr with goat anti-rabbit secondary antibody conjugated to Alexa IRDye 680 (LI-COR). Active (phosphorylated) ERK1 and STAT3 were normalized to total ERK1 and STAT3 content.

**Single gene expression analysis by qPCR:** Hepatic and pituitary RNA (Studies 1, 3, 5) was isolated 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) as described previously (Baltzegar et al., 2014; Douros et al., 2014). For gene expression studies using qRT-PCR (Studies 3, 5), total RNA (0.8-1 µg) was reversed transcribed to cDNA using random priming hexamers (High Capacity cDNA
Synthesis kit, Life Technologies). Gene expression of PFK and GAPDH (primers in Table 2) was quantified via SYBR green qPCR (Applied Biosystems HT7300) then normalized to total RNA and 18s ribosomal RNA as described previously (Douros et al., 2014).

RNA-seq and bioinformatic analyses of the pituitary transcriptome:

For RNA-seq studies assessing leptin modulation of the pituitary RPD transcriptome, tissues were placed in wells of a Falcon 6-well plate in 3mL of either hormone-free (control) or leptin-containing medium and incubated for 6 h (n = 30/treatment; 10 RPD/well, 3 wells/treatment). Tissues from each well were pooled in 2mL of RNAlater at 4°C until RNA isolation and Illumina cDNA library preparation.

Illumina Sequencing—Pituitary mRNA (10µg; Study 2) was submitted to N. C. State University Genomic Sciences Laboratory (Raleigh, NC) for Illumina cDNA library construction. The cDNA was synthesized using the SuperScriptTM Double-Stranded cDNA Synthesis Kit (Invitrogen) and tagged with a 5’, four nucleotide barcode. Illumina cDNA libraries (n = 3) were prepared for each treatment (control or leptin). Sequencing was performed on the Illumina MySeq platform using 150bp, paired-end reads in triplicate (Reading et al., 2012).

Differential Gene Expression The FASTQ sequencing output files from each group were trimmed for barcode removal and standard quality control (Phred score > 36; FastQC) and, then aligned to the Oreochomis niloticus genome (Ensembl) using a local, short read aligner (Bowtie 2-2.1) (Langmead and Salzberg, 2012; Leggewie et al., 2013). The fragments per kilobase of exon per million fragments mapped (FPKM i.e. copy number) was calculated for all annotated genes using Cufflinks-2.2.1. Statistical comparisons between control and
leptin treatments were made using Cuffdiff-2.2.1 to determine significant differentially expressed genes (DEGs) (Flicek et al., 2014; Trapnell et al., 2013; Trapnell et al., 2012; Trapnell et al., 2010).

**Functional Analysis of Transcriptomic Modulation:** A reduction of data dimensionality (RDD) was conducted in order to empirically determine which DEGs are required to build a predictable model of leptin effects on the RPD transcriptome (i.e. “highly significant” DEG). This was accomplished by inputting FPKMs of each DEG into a multilayer perceptron (WEKA) (Holmes and Cunningham, 1993) and generating artificial neural networks (ANN; i.e. model for leptin signaling). Modulated Modularity Clustering (MMC) was used to group highly significant DEGs into small “modules” (i.e. subsets of covariable, functionally interactive genes) (Gene Ontology, 2008) (Stone and Ayroles, 2009). The FPKMs and residuals of each highly significant DEG are input into the MMC program available via Stone et al. (Stone and Ayroles, 2009). The Gene Ontology enrichment (GO term; i.e. function) of each covariable module was assessed using DAVID Bioinformatics suite (Huang da et al., 2009). Subsequent visualizations of each module were constructed using Cytoscape (Cline et al., 2007).

**Statistics:** RNA-seq was assessed statistically within Cuffdiff-2.2.1 followed by Benjamini-Hochberg correction (Trapnell et al., 2013; Trapnell et al., 2012). Time course experiment was analyzed using two-way ANOVA (treatment x time) followed by Tukey’s HSD post-hoc test (GraphPad Prism6). All other statistics were assessed using one-way ANOVA followed by Tukey’s HSD post-hoc test.
References


Molecular and cellular endocrinology 319, 143-146.


occur frequently prior to treatment in HIV-coinfected patients with acute hepatitis C. AIDS 27, 2485-2491.


Tseng, Y.C., Hwang, P.P., 2008. Some insights into energy metabolism for osmoregulation


Table 4. Gene ontology enrichment by leptin in the rostral pars distalis

<table>
<thead>
<tr>
<th>Process -- Module</th>
<th>Function (GO Term Number)</th>
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<td>pyruvate metabolism (0006090)</td>
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<td>Phosphorus/phosphate metabolism (0006793/0006796); neg. reg. of macromolecule metabolism (0010605);</td>
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<td>tRNA amino acylation for protein translation (0006418)</td>
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<td>7</td>
<td>response to oxygen levels/hypoxia (0070482/0001666)</td>
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Figure 18: (A) Reduction of data dimensionality reported as the accuracy (percentage) with which the multilayer perceptron (machine learning program) is able to learn and recognize leptin modulation of the transcriptome given as a function of the number of gene inputs (FPKM of DEGs given in triplicate). (B) MMC heat map of 400 correlated genes displaying 11 modules. (C-M) Cytoscape diagrams represent the genes within each module and their interactions. Module 7 (C) represents “Regulation of Hypoxia” GO term enrichment. The genes involved and the GO term enrichment of each module can be found in Table 4 and Supplemental Table 3 respectively.
Figure 19: (A) Effect of leptin (100nM ■) on lactate secretion in cultured pituitary RPDs compared to control group (0nM rtLepA □) at 0.5, 1, and 6h (n = 5/group). In vitro pituitary (B) lactate secretion, (C) PFK mRNA, and (D) GAPDH mRNA in response to rtLepA treatment (0, 1, 10, 100nM) at 6 h (n = 10/group). Asterisks denote significance differences relative to control (0 nM LepA)(mean ± SEM; * = p < 0.05, ** = p < 0.01, *** = p < .001).
Figure 20: (A) Lactate secretion and (B) PFK activity in response to rtLepA treatment (0, 1, 10, 100nM), rtLepA (100nM) + Stattic (S; 1mM), and rtLepA (100nM) + PD98095 (PD; 30µM) at 6 h (n = 7/treatment). (C-E) STAT3 and (C, F-G) ERK1 phosphorylation were determined by quantitative Western blotting (Schust, Sperl, Hollis, Mayer, & Berg, 2006; Tipsmark et al., 2008).
Figure 21: Hepatocytes were cultured and plated at a density of $3 \times 10^5$ cells per well (n = 6/treatment) (A) Hepatic lactate secretion in response to rtLepA treatment (0, 1, 10, 100nM) reported as mean concentration (nM) per mg protein ± SEM. (B) PFK and (C) GAPDH mRNA abundance normalized to 18s RNA as a housekeeping gene and reported as mean fold change ± SEM (n = 4).