PRODUCTION OF RECOMBINANT CARP LEPTIN AND ITS EFFECTS ON LIPID METABOLISM IN THE COMMON CARP (CYPRINUS CARPIO)

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PRODUCTION OF RECOMBINANT CARP LEPTIN AND ITS EFFECTS ON LIPID METABOLISM IN THE COMMON CARP (CYPRINUS CARPIO)

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ABSTRACT

The protein hormone leptin has emerged as a major contributor in the regulation of energy balance. However, most of our knowledge of leptin stems from mammalian studies that have focused primarily leptin’s role in human pathologies (e.g. obesity). Little is known about how this hormone evolved and how it functions in more basal vertebrates. Recent efforts have identified the gene for leptin in many non-mammalian taxa including several fish species. Common carp (Cyprinus carpio) represents one such species and is an excellent system to study leptin because their otherwise well-studied physiology provides a plethora of context to interpret the effects of leptin. In particular, leptin has potent effects on lipolysis (i.e. triglyceride hydrolysis) and lipid oxidation (β-oxidation of fatty acids) in mammals; a function that may be conserved in fishes.

In order to evaluate the effects of leptin on lipid metabolism in carp, I first produced recombinant carp leptin-I using a bacterial expression system. Carp leptin was injected into the intraperitoneal cavity of koi (domesticated common carp) then, after a three hour recovery period, sampled for plasma and liver tissue. To evaluate the effects of leptin on lipolysis, plasma non-esterified fatty acids (NEFA) concentrations were measured. Additionally, hepatic enzyme activity of carnitine palmitoyltransferase-I (CPT) was measured to assess changes in fatty acid oxidation. Leptin injections increased plasma NEFA concentrations significantly but had no effect on hepatic CPT activity. These results suggest that leptin stimulates lipolysis but may not affect fatty
acid oxidation as it does in mammals. I suggest that additional studies are needed to look at more specific indicators of lipid catabolism and oxidation, and higher more chronic doses of leptin should be used to thoroughly evaluate leptin’s role in the lipid metabolism of fishes.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>List</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
</tbody>
</table>

## CHAPTER

1. PRODUCTION OF RECOMBINANT CARP LEPTIN AND ITS EFFECTS ON LIPID METABOLISM IN THE COMMON CARP (*CYPRinus CARPIO*) .............1

   Introduction...............................................................................................................1

   Materials and Methods........................................................................................................6

   Results...............................................................................................................................13

   Discussion.........................................................................................................................21

LITERATURE CITED...........................................................................................................27
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Summary of fish leptin studies</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Condition matrices for leptin expression</td>
<td>16</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SEC chromatogram</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>SDS-PAGE solubility analysis</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>SDS-PAGE analysis with chaperone proteins</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>SDS-PAGE analysis after immobilized metal affinity chromatography</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>SDS-PAGE analysis after size exclusion chromatography</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>Western blot analysis of recombinant carp leptin</td>
<td>18</td>
</tr>
<tr>
<td>7</td>
<td>Mass spectrometry analysis of leptin peptides</td>
<td>19</td>
</tr>
<tr>
<td>8</td>
<td>Effects of leptin injection on plasma non-esterified fatty acids</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>Effects of leptin injection on liver carnitine palmitoyltransferase activity</td>
<td>21</td>
</tr>
</tbody>
</table>
CHAPTER I

PRODUCTION OF RECOMBINANT CARP LEPTIN AND ITS EFFECTS ON LIPID METABOLISM IN THE COMMON CARP (CYPRINUS CARPIO)

Introduction

Arguably, the greatest challenge that organisms face is the allocation of energy towards generally adaptive processes. The complexity of this challenge is often compounded in metazoan taxa that possess many “choices” in terms of where this energy can be directed (e.g. growth, reproduction, immune function, and social interactions). Thus, it is not surprising that animals possess signaling molecules (hormones and neurotransmitters) that respond to the environment and coordinate with other organs to meet the metabolic needs of the animal. Although many hormones are involved in this process, one has emerged as being a key player in the energy balance equation. Leptin, the product of the obese (ob) gene, is a protein hormone that was first characterized in mice (Zhang et al. 1994). A mutation of this gene was discovered to be the sole cause of morbid obesity in a strain of mice created over half a century ago (Ingalls et al. 1950).

Once the gene was characterized, an explosion of research ensued (>99000 published manuscripts; ISI search June 2012) aimed primarily at understanding leptin’s role in the emerging obesity epidemic. Consistently, over 99% of these studies are in
mammals (ISI search, May 2012) and a large portion of these are focused specifically on leptin’s role as an anorexigen (appetite inhibiting molecule). However, in addition to influencing food intake, leptin is also involved in fat metabolism, reproduction, body temperature regulation, growth, immune function, stress, inflammatory responsiveness, hematopoiesis, and angiogenesis (Pelleymounter et al. 1995; Barash et al. 1996; Bennett et al. 1996; Grunfeld et al. 1996, Fruhbeck et al. 1997, Sierra-Honigmann et al. 1998, Steppan et al. 2000, Dagogo-Jack et al. 2005, Lafrance et al. 2010, and reviewed in Swoap 2008, Freidman 2009). Given such pleiotropy, it can be argued that leptin tends to be involved in any activity that is metabolically expensive. Additionally, serum leptin concentrations correlate strongly with body fat and/or BMI in mice and humans (Maffei et al. 1995) suggesting that the metabolic currency is primarily lipid-based.

Once leptin’s paramount role in energy homeostasis was established in mammals, efforts began to characterize the gene in non-mammalian vertebrates. The first evidence of leptin in fishes (based on immunoreactivity with mammalian leptin) was published in 2000 (Johnson et al. 2000). They found that blood leptin was higher in fed versus food restricted green sunfish (*Lepomis cyanellus*), although they did not have higher body fat concentrations. Several years later, Kurokawa and colleagues (2005) identified the coding sequence for pufferfish (*Takifugu rubripes*) leptin using gene synteny, noting tremendous divergence in the amino acid sequence (13.2% identity to human leptin). Furthermore, they found that the primary site of leptin expression was liver as opposed to adipose tissue as in mammals. Over the course of the next seven years, studies on leptin’s effects in fishes and amphibians revealed interesting yet inconsistent results with
respect to the mammalian paradigm of leptin’s function. Leptin exhibits a range of responses in fishes making it difficult to assign a definitive function (Table 1).

### Table 1. Summary of fish leptin studies

<table>
<thead>
<tr>
<th>Species</th>
<th>Nature of study</th>
<th>Main Findings</th>
<th>Reference Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atlantic salmon (Salmo salar)</td>
<td>Measured leptin mRNA expression and plasma protein from fish either fed to satiation or feed restricted</td>
<td>Feed restricted salmon had higher leptin mRNA expression and plasma protein concentrations than fully fed controls</td>
<td>Trombley et al. 2012</td>
</tr>
<tr>
<td>rainbow trout (Oncorhyncus mykiss)</td>
<td>Measured response to Intraperitoneal injections of recombinant trout leptin</td>
<td>Acute decrease in food intake and NPY expression, increase in POMC expression</td>
<td>Murashita et al. 2008</td>
</tr>
<tr>
<td>salmonid radioimmunoassay validation and application to feeding regime effects</td>
<td>Trout showed higher plasma leptin and growth hormone concentrations during 3-week fasting</td>
<td></td>
<td>King et al. 2009</td>
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<td>goldfish (Carassius auratus)</td>
<td>Measured gene expression of 2 leptin isoforms under different feeding regimes</td>
<td>Prolonged overfeeding and fasting did not affect leptin expression. Leptin expression acutely increased after feeding</td>
<td>Tinoco et al. 2012</td>
</tr>
<tr>
<td>common carp (Cyprinus carpio)</td>
<td>Measured gene expression of 2 leptin isoforms under different feeding regimes</td>
<td>Increased hepatic expression immediately following feeding but no long term differences between fed and fasted groups</td>
<td>Haising et al. 2006</td>
</tr>
<tr>
<td>Arctic char (Salvelinus alpinus)</td>
<td>Studied the effects of hypoxia on food intake and leptin-I -II and leptin receptor expression</td>
<td>Chronic hypoxia caused an increase in leptin and leptin receptor expression</td>
<td>Bernier et al. 2012</td>
</tr>
<tr>
<td>Crucian carp (Carassius carassius)</td>
<td>Measured leptin receptor expression in gill tissue in response to and hypoxia</td>
<td>Hyoxia caused an increase in expression of two isoforms of leptin receptor</td>
<td>Cao et al. 2011</td>
</tr>
<tr>
<td>zebrafish (Danio rerio)</td>
<td>Exposed adults to hypoxia and embryos to CoCl$_2$ to stimulate hypoxia inducible factor</td>
<td>Hyoxia and hypoxia-inducible factor 1 increased leptin expression</td>
<td>Chu et al. 2010</td>
</tr>
<tr>
<td>channel catfish (Ictalurus punctatus)</td>
<td>Measured the response of a putative leptin to exposure of an infectious bacterium</td>
<td>Exposure to Edwardsiella ictaluri increased putative leptin expression in liver</td>
<td>Kobayashi et al. 2011</td>
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I assert that these studies reveal an adaptive role for leptin in facilitating critical processes. More specifically, I hypothesize that leptin evolved to stimulate lipolysis and promote fatty acid oxidation. Furthermore, through its stimulatory actions on lipid metabolism, leptin aids in the regulation of developmental, homeostatic, and stress responsive processes. In mammals, chronic leptin injections can stimulate non-esterified fatty acid (NEFA) release from adipose triglyceride (TAG) stores into the bloodstream (Ajuwon et al. 2003). However, others have seen only glycerol release into circulation in
response to leptin injections (Wang et al. 1999, Reidy & Weber 2002). Whether or not changes in TAG metabolites are observed is likely to depend on the response of other physiological systems. Reidy & Weber (2002) demonstrated that leptin stimulates TAG catabolism resulting in glycerol and NEFA production. However, most of the NEFAs were reesterified back into TAGs before being released into circulation. Therefore, it is clear that leptin stimulates TAG catabolism but the net effect of this stimulation will depend on whether other tissues utilize these substrates. For example, a serum NEFA response may be obscured by leptin’s stimulatory effects on lipid oxidation (i.e. NEFA release is matched by NEFA oxidation). Leptin stimulates fatty acid oxidation by upregulating expression and/or activity of enzymes such as adipose triglyceride lipase and carnitine palmitoyltransferase-I (Gallardo et al. 2007, Wein et al. 2007), and inhibiting lipogenic enzymes such as acetyl CoA carboxylase (Minokoshi et al. 2002).

Taken together, these studies confirm both the presence and complexity of leptin’s role in fat metabolism. Moreover, the physiological response of the animal is likely to be tailored toward its life history strategy, and must be considered when inferring general roles for leptin. Although no study has demonstrated direct lipolytic actions of leptin in a fish species, there is indirect evidence that leptin may influence this process. Starved rainbow trout exhibit both elevated plasma leptin and NEFA concentrations (Kling et al. 2009, Farbridge & Leatherland 1992). Additionally, hypoxia exposure, which increases leptin and leptin receptor expression in fishes (Chu et al. 2010, Cao et al. 2011, Bernier et al. 2012), results in elevated lipolysis in a hypoxia-tolerant goby (Gracey et al. 2011). Gracey et al. did not measure leptin directly but they found increased expression of adipose triglyceride lipase, a major lipolytic enzyme that has
been previously shown to be stimulated by leptin (Gallardo et al. 2007). Although these data are only correlational, they identify leptin as a reasonable candidate for an effector, considering its effects in mammalian systems.

Among fish species for which leptin genes have been characterized, common carp (Cyprinus carpio) represent a particularly unique animal for leptin studies. Carp are eurythermal and hypoxia tolerant and a recent study showed that hypoxia led to an increase in leptin expression in these animals (Bernier et al. 2012). Interestingly, Bernier et al. did not find associated changes in plasma NEFA concentrations; however this does not preclude the possibility of changes in lipolysis. As mentioned above, changes in levels of metabolic products of lipolysis may not be observed if the rate of their production is matched to the rate of utilization. Like other fish species, carp express leptin primarily in the liver (Huising et al. 2006); this organ also represents a major site of lipid storage and metabolism. Additionally, carp physiology has been widely studied due to its popularity in aquaculture, allowing functional studies to be interpreted with ample physiological context. Finally, leptin represents a candidate signaling system that can extend our knowledge of lipid metabolism in fishes. As noted by van den Thillart et al. (2002), little is known regarding the regulation of lipid metabolism in teleost fishes and it is my aim to narrow this knowledge gap. I contend that leptin may be a key player in mediating adaptive energy responses as evidenced by its responsiveness to environmental challenges such as starvation and hypoxia. Both of these challenges lead to changes in lipid metabolism in fishes and so I sought to study leptin’s role in lipolysis as a basis for understanding how leptin might aid in the stress response of fishes.
Materials and Methods

**Recombinant carp leptin expression**

Primers encoding *Cyprinus carpio* leptin-I (Accession # AJ830745.3) were used to amplify the coding sequence (Forward: 5'ATTCCCATTCATTCA 3' and Reverse: 5'GCAGCTTTTCACTG 3'); this excluded the N-terminus 20 amino acid signal peptide (predicted by SignalP 4.0). PCR was performed with GoTaq® Green master mix (Promega, Madison, WI), 100ng of carp liver cDNA, and 500nM final concentration of forward and reverse primers (cycling regime: 2 minutes at 95°C followed by 40 cycles of 95°C, 55°C, and 72°C, with a final step of 72°C for 5 minutes). The PCR reaction was separated on a 1% (w/v) low melting agarose gel with a 100bp ladder. The 453bp amplicon was cut from the gel and purified using MinElute PCR purification kit (Qiagen, Valencia, CA). The amplicon was cloned in-frame into an expression vector (Talon; Clontech, Mountain View, CA) containing an N- or C terminus sequence of alternating histidine and arginine residues (His-tag) and transformed into chemically competent BL21 cells (Invitrogen, Grand Island, NY). Finally, the product was sequenced on 3130xl genetic analyzer (ABI, Carlsbad, CA) to confirm sequence and coding frame.

**Solubility analysis of recombinant carp leptin**

Two leptin clones were used primarily to express leptin (#12 and #15). First, two 1L flasks of LB broth containing 100ug/ml ampicillin were inoculated with a single colony of clone #15 and grown until the optical density at 600nm reached 0.6. At this point Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1mM to one of the flasks and the induction was grown for 4 hours at 37°C on an
orbital shaker (150RPM). The induction was centrifuged at 4000*g for 10 minutes at 4°C and cells were lysed by sonication (2X for 30 seconds on ice) in 9 volumes of wash/equilibration buffer (40mM HEPES, 160mM NaCl, pH 7.5). An analysis of solubility was performed by centrifuging the lysate at 25000*g for 30 minutes at 4°C. The (soluble) supernatant was removed and the pellet resuspended in wash/equilibration buffer containing 6M urea. The pellet was solubilized by incubating for 2 hours on a rotary shaker followed by centrifugation at 25000*g for 30 minutes at 4°C and collecting the supernatant. All fractions were analyzed using SDS-PAGE. For both the soluble and insoluble fractions (induced and uninduced), 20µg of total protein was loaded (as determined using Bradford protein assay with bovine serum albumin, BSA, as a standard) along with 4X Laemmli buffer and 50mM TCEP. Samples were separated on 4-20% acrylamide gels. Results from the analysis revealed that clone #15 produces leptin primarily as insoluble inclusion bodies. Consequently, efforts were directed at soluble production of leptin.

*Optimizing soluble leptin expression*

In an effort to increase soluble production, a Takara Chaperone Plasmid Set encoding a team of chaperone proteins (dnaK, dnaJ, grpE; Clontech, Mountain View, CA) was transformed into clone #12 following manufacturers protocol. These clones were subsequently grown in 1L of LB broth; chaperones were induced first with 3mg/ml L-arabinose at an optical density of 0.4, followed by 1mM IPTG to induce leptin at an optical density of 0.6. The induction was grown for 4 hours on an orbital shaker (150RPM) and subsequently harvested and analyzed as described earlier. This effort was unsuccessful (see Results). Consequently, the insoluble fraction was solubilized with
wash/equilibration buffer containing 6M urea. Yields were substantially greater (>10mg/L) and after being partially purified, and leptin was dialyzed at 4°C against a series of urea solutions: 4M urea for 24 hours, 2M urea for 24 hours, 1M urea for 24 hours, 500mM urea for 24 hours (at this point 0.1mM oxidized glutathione and 1mM reduced glutathione were added to the dialysate), 200mM urea, 100mM urea. Before urea could be completely removed, leptin precipitated irreversibly. A second refolding attempt employed a commercially available refolding kit (ProFoldin, Hudson, MA) that uses prepacked columns with proprietary buffers to renature proteins. Leptin was refolded according to manufacturer’s instruction, however leptin yields proved to be low (<10% recovery) and prone to precipitation when attempting buffer exchanges so efforts were redirected at soluble expression. Soluble production of leptin was successfully scaled up using the following conditions: 3L of LB broth was inoculated and grown at 37°C until the optical density at 600nm reached 0.4. The broth was cooled to 20°C, induced with IPTG to a final concentration of 0.2mM, and was grown for 16 hours on an orbital shaker (120RPM). Cells were harvested as described above.

*Recombinant carp leptin purification*

Crude bacterial lysates underwent three steps of purification. Beginning with immobilized metal affinity chromatography (IMAC), the soluble protein from the lysate was incubated at room temperature with cobalt-based IMAC resin (Clontech, Mountain View, CA) for 20 minutes. Next, samples were centrifuged for 5 minutes at 800g and ~90% of the supernatant was removed without disturbing the resin pellet. The remaining supernatant was used to resuspend the pellet and the entire slurry was pipetted onto a chromatography column. Once the column began to settle, the lysate was allowed to
The column was washed with wash/equilibration buffer until the eluate had a protein concentration below 0.05mg/ml. Wash/equilibration buffer containing 10mM imidazole was then added (serving to remove additional contaminating proteins) until the protein concentration once again reached below 0.05mg/ml. Finally, bound proteins were eluted by adding wash/equilibration buffer containing 150mM imidazole (pH 7.5). After this step, leptin was ~50% pure. Size exclusion chromatography (SEC) using a HiLoad™ 16/60 Superdex 75 PG column (GE, PA) was performed; 2ml samples of leptin (3mg/ml) were loaded onto the column and ran at 4°C at a flow rate of 0.4ml/min. A sharp peak eluted beginning at 48ml and ending at 58ml (Figure 1). These fractions were analyzed using SDS-PAGE and showed a single dominant band corresponding to leptin’s molecular mass. Samples were concentrated to the threshold of solubility (~3mg/ml total protein) using an Amicon® stirred cell (Millipore, Billerica, MA), using a 10kDa filter membrane and 30psi of nitrogen gas pressure. After this step, leptin was >90% pure as determined by SDS-PAGE. Finally, since bacterial endotoxins can stimulate an increase in leptin expression (Grunfeld et al. 1996), endotoxins were removed using ToxinEraser™ endotoxin removal kit (Genscript, Piscataway, NJ) following manufacturer’s instruction. Leptin was maintained at 4°C for less than one week in wash/equilibration buffer at a concentration of 0.12mg/ml.
Proteomic analysis of recombinant carp leptin

To confirm the presence of leptin, western blotting was performed using an antibody raised against recombinant zebrafish leptin A. First, 3µg of the purified sample was separated using SDS-PAGE as described above. The proteins were transferred to PVDF membrane using a semi-dry blotting system using buffer pads saturated with transfer buffer (48mM Tris, 39mM glycine, 20% v/v methanol, 0.0375% w/v SDS). A 50cm² gel was transferred with 70mA of current for 2 hours. The blot was incubated in blocker solution (4% w/v non-fat dried milk in 1X PBS, phosphate buffered saline: 3.2mM Na₂HPO₄, 0.5mM KH₂PO₄, 1.3mM KCl, 135mM NaCl, pH 7.4) for 2 hours, rinsed in 1X PBS, then placed into a primary antibody solution containing 1X PBS, 0.1% w/v BSA, and 1:100 v/v rabbit anti-zebrafish leptin antibody. The blot was incubated for 4 hours at room temperature, rinsed with 1X PBS, then placed into secondary antibody
solution containing 1X PBS, 0.1% w/v BSA, and 1:1000 v/v goat anti-rabbit HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The blot was incubated for 3 hours at room temperature, rinsed, and developed using Immobilon™ western chemiluminescent HRP substrate (Millipore, Billerica, MA). After adding the substrate, the blot was exposed for 15 seconds on a G-Box (Syngene, Frederick, MD).

To supplement the western blot analysis, a MALDI mass spectrometry analysis was performed. Putative leptin bands from SDS-PAGE were excised from the gel, minced, and placed into a siliconized microcentrifuge tube. The sample was washed and destained with wash reagent (50% ethanol, 5% acetic acid), and reduced and alkylated with DTT and acetonitrile, respectively. The sample was rehydrated with 100mM ammonium bicarbonate and dehydrated with acetonitrile, then dried using a SpeedVac® centrifuge. Protein was digested overnight using sequencing grade trypsin (Promega, Madison, WI) and peptides were extracted using extraction reagent (50% acetonitrile, 5% acetic acid) and dried to less than 10µl in SpeedVac® centrifuge. The digested samples were analyzed by The Lerner Research Institute’s Mass Spectrometry Lab for Protein Sequencing (Cleveland Clinic, Cleveland, OH) on a Finnigan LTQ linear ion trap mass spectrometer system.

Fish husbandry, leptin injections, and tissue collection

Koi (domesticated carp) were purchased from Amore’s Koi Farm (Coshocton, OH) and maintained in a 300 L recirculating tank (Living Systems, Toledo, OH) for 12 months. Koi were maintained at 21°C on a 14:10 light:dark cycle and fed twice daily ad
on commercial koi pellets (38% protein, 8% fat, 4% fiber; Blackwater Creek, Eustis, FL). Seven days before the experiment, 14 koi (average body mass 15.5g ± 6.8) were taken from group housing and placed into individual, opaque 10L chambers within the 300L recirculating tank. On the day of the experiment, koi were anesthetized with 0.01% MS-222 (tricaine methane sulfonate buffered to 7.4 with sodium bicarbonate), weighed, photographed on a 1mm grid, and injected in the intraperitoneal cavity with either recombinant carp leptin (0.3µg/g body mass) or vehicle only (wash/equilibration buffer), and allowed to recover for three hours. Fish were then euthanized with 0.02% MS-222 and immediately sampled for blood using caudal venipuncture. Plasma was collected after centrifuging blood samples in tubes containing K$_2$EDTA. Additionally, liver and brain tissue was collected and flash frozen in liquid nitrogen. All tissues were collected within four minutes of euthanizing. Body metrics (standard/total length, body depth) were later determined by analyzing photographs.

\textit{Lipid metabolism assays}

Plasma non-esterified fatty acids (NEFA) were analyzed using a free fatty acid quantification kit (Biovision, Milpitas, CA) per manufacturer’s protocol. Briefly, the assay employs a 96-well plate and a proprietary reaction mixture that is mixed with plasma samples and incubated for 30 minutes at 37°C. The plate is read at 570nm and NEFA concentrations are determined by comparing samples to a palmitic acid standard curve.

Carnitine palmitoyltransferase-I activity was measured in liver tissue using a spectrophotometric assay adapted from Londraville and Duvall (2002). Liver was
homogenized by sonication on ice in 40mM HEPES buffer containing 1.5mM EDTA (pH 7.8). Liver homogenates were combined with assay reagent containing 40mM HEPES, 1.65mM EDTA, 0.27mM DTNB, and 0.038mM palmitoyl CoA, and 0.1% BSA (w/v). Finally, the reaction was initiated by adding 40µl of 1.37mM carnitine HCl and was monitored at 412nm for three minutes at 25°C. Slopes (Δabs/min) were determined by subtracting changes in absorbance for background (uninitiated) samples from their respective carnitine-initiated samples. Enzyme activity was determined using the following formula:

\[ \Delta \text{abs/min} \times 13.7 \times 1/1 \times 0.000272 \times 1000 = \text{micromoles product/min} \]

where 13.7 is the molar extinction coefficient, 1 is pathlength in centimeters, 0.000272 is the reaction volume in liters, and 1000 is the number of micromoles in 1 millimole (to provide answer in Units). Final activity was expressed as U per mg protein, U per g wet tissue, and enzyme capacity (U/gm liver * liver mass).

Statistical analysis

Data were analyzed for normality of the distribution using Shapiro-Wilk test and equality of variances between groups using Levene’s test. CPT data met the assumptions for a t-test and \( \alpha = 0.05 \) was set as the criterion for significance. Plasma NEFA data were normally distributed, but with unequal variances. A Mann-Whitney rank sum test was performed using \( \alpha = 0.05 \) as the criterion for significance.

Results

SDS-PAGE analysis of clone #15 expression clearly showed that carp leptin is preferentially expressed as insoluble inclusion bodies (Figure 2). Consequently, efforts
were directed at improving soluble production. Clone #12 proved to be the most effective at producing soluble leptin but still produced very low yields (<1mg per liter). The coexpression of chaperone proteins marginally increased soluble leptin expression (1.5mg/L) but further processing revealed that the constituent proteins also contributed greatly to contaminant protein load during purification (Figure 3). As a result, the insoluble fraction was used, but it required solubilizing and refolding. Refolding involved stepwise dialysis to remove excess urea, and the addition of glutathione to create the proper redox state (to facilitate disulfide bond formation). However, refolding by this method was not successful as the protein sample would precipitate irreversibly once urea concentrations reached below 1M. A second refolding method was attempted that used commercially produced pre-packed columns, comprised of a proprietary buffer and resin system that refolds proteins as they pass through the column. Although the refolding kit was able to refold leptin, as evidenced by the sample’s solubility in a non-chaotrophic buffer, the yields were too low to be cost effective (~$600/mg).

Figure 2. SDS-PAGE analysis of leptin expression. Lane 1 is the insoluble fraction (induced with IPTG), lane 2 is the insoluble fraction (uninduced), lane 3 is a protein ladder, lane 4 is the induced soluble fraction, and lane 5 is the uninduced soluble fraction. Ladder key is shown on right.
A final effort to produce soluble leptin was approached using a matrix of different conditions that are suspected to influence the quality of protein expression. Four variables were manipulated: length of induction, concentration of IPTG, temperature, and optical density (i.e. cell density) at the time of induction with IPTG. This effort was divided into two sequential matrices, with IPTG concentration and temperature being manipulated and optimized first, followed by optimization of induction time and OD at the time of induction with IPTG (Table 2). It was determined that the preferred conditions for producing soluble leptin are inducing relatively early (0.3 OD), with a low concentration of inductant (0.2mM IPTG), and incubating at cooler temperatures (20°C) for 16 hours. This result was based on measuring the protein concentration after the first round of purification (IMAC), since no differences in band intensity were visible after
SDS-PAGE. After scaling up the production of soluble leptin, IMAC was performed to remove contaminant proteins.

Table 2. Condition matrices used to optimize soluble leptin expression

<table>
<thead>
<tr>
<th>IPTG conc. (mM)</th>
<th>Temperature °C</th>
<th>Length of Induction (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>20</td>
<td>30</td>
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Initial efforts yielded relatively low purity (~30%). Attempts were made to improve purity, including addition of 10% glycerol to wash/equilibration buffer (to reduce hydrophobic interactions) and addition of a low strength imidazole wash (10mM) before elution. After these modifications, purity was increased to ~50% (Figure 4), however additional purification was still needed. Size exclusion chromatography (SEC) further removed contaminants and was successful at removing ~90% of unwanted proteins. SDS-PAGE revealed one prominent band at ~23kDa (Figure 5). Although carp leptin is only 18kDa with the His-tag, western blot has confirmed that leptin migrates slightly higher than its molecular mass (~23kDa) as a monomer, and ~46 kDa as a dimer (Figure 6).
Figure 4. IMAC fraction showing partially pure leptin (~50%). Lane 1 is a protein ladder (MW in kDa) and lane 2 is eluate containing leptin.

Figure 5. SEC fractions showing nearly pure (>90%) leptin. Lane 1 is a protein ladder, lanes 2 and 3 are leptin containing fractions.
Mass spectrometry was used to confirm the presence of leptin and revealed a bacterial contaminant protein (Figure 7). cAMP regulatory protein (CRP), a bacterial protein, was present in the leptin sample even though SDS-PAGE showed only a single band. For the soluble (injected) leptin sample, CRP represented the majority of the sample composition, although it was not detected in the insoluble leptin fraction. This contaminant required that the dose received by the koi be recalculated to reflect this impurity. Insoluble leptin (without CRP) was separated on SDS-PAGE along with the soluble leptin (contaminated with CRP) and analyzed via immunoblot against leptin. Total leptin signal on the blot vs. total protein loaded was compared between the pure leptin (insoluble) and soluble fractions to determine the percent leptin in the soluble fraction. The result of this calculation revealed that only 18% of the protein sample that was injected was actually leptin. Therefore, although 1.6µg of protein per gram body mass was injected, only 0.3µg of the 1.6µg was actually leptin. Because mass
spectrometry results were received after the injection experiment, the dose was not able to be adjusted prior to injections.

Leptin-injected koi had significantly higher plasma NEFA concentrations compared to controls (p = 0.014; Figure 8). No significant differences were observed for any measures of liver CPT activity (CPT U/g wet tissue, p = 0.895; CPT U/mg protein, p = 0.614; CPT liver capacity, p = 0.456; Figure 9). These activities are highly similar to those reported for grass carp fed a high fish oil diet (Du et al. 2008). Statistical analyses for both plasma NEFA and liver CPT did not correct for body size by using residuals. A
regression analysis showed that body mass, body length, and body height have negligible effects on these parameters ($r^2 < 0.05$; $p > 0.05$).

Figure 8. The effect of leptin injection on plasma NEFA concentrations. Asterisk indicates significance at $p \leq 0.05$. Errors bars represent ±1 SEM.
The process of producing recombinant proteins can be very difficult and inefficient, especially when expressing eukaryotic proteins in non-eukaryotic systems. The protein is being synthesized in an environment that has vastly different characteristics than its native cell. It is a milieu consisting of different proteins with different functions. Also, with prokaryotic systems such as *E. coli*, secretion is not possible and combined with the effect of overexpression (high rates of protein synthesis), the protein will accumulate in this relatively small cell to the point where solubility reaches unfavorable concentrations. Leptin was no exception to these pitfalls. After countless efforts using many technologies, it was still not possible to produce more than...
microgram quantities of recombinant carp leptin. To begin with, leptin was preferentially expressed as insoluble inclusion bodies. In fact, when solving human leptin’s structure, a soluble crystal could not be grown until the sequence was modified (Zhang et al. 1997). Such challenges are not uncommon in bacterial expression systems and are known to occur for recombinant leptin from other species (Jeong & Lee 2000, Yacobovitz et al. 2008).

The process of refolding was not possible to achieve in a cost effective manner. The traditional method of dialysis and glutathione was unsuccessful and the use of commercially produced refolding columns was too expensive to be scaled up. Moreover, even if the protein is successfully solubilized it may not be folded in its native state (Thomas Leeper, personal communication). Once efforts were directed towards soluble leptin expression, it was clear that while possible, the process is remarkably inefficient. The combination of low initial yields and multiple purification steps resulted in tremendous loss of leptin by the time injections were performed. In fact, it required 800 grams (wet cell mass) of leptin-expressing E. coli (>30 liters of culture) to produce a final yield of 80 micrograms of carp leptin.

Mass spectrometry analysis revealed that the SDS-PAGE band corresponding to leptin was impure. cAMP regulatory protein (CRP) was found to be the dominant protein in the injected sample. CRP is specific to bacteria and has no known effect in carp. It is therefore unlikely that CRP contributed to the observed responses in the leptin injected koi. However, it is possible that CRP elicited an immune response which can in turn stimulate leptin, thereby enhancing the observed responses. IMAC purifies recombinant proteins by interacting with a series of alternating histidine and arginine residues that are
encoded by the vector to be present on the N or C terminus of the protein. Specifically, the beads IMAC resin has immobilized (chelated) cobalt ions (Co$^{2+}$), which in turn interacts covalently with the imidazole side chains of histidine residues. Consequently, contaminant proteins that possess similar histidine sequences are likely to interact with IMAC resin. *E. coli* CRP (Accession # AEH68826.1) contains the sequence, H-C-H-I-H that is very similar to recombinant carp leptin’s His-tag sequence: H-R-H-R-H. Furthermore, once CRP evaded purification by IMAC, SEC was unlikely to remove CRP based on its inherent principle. SEC separates molecules on the basis of molecular mass and since leptin and CRP are nearly identical in size, they cannot be separated by SEC. The presence of this contaminant protein required that the injected dose be recalculated to reflect the effective dose. This value was calculated to be 0.3µg of leptin per g body mass, and while this was much less than intended, it was sufficient to raise plasma NEFA concentrations significantly. Moreover, this response was elicited at physiological concentrations whereas all fish leptin injection studies to date have used pharmacological doses with concentrations of at least 1µg/g body mass.

Plasma NEFA concentrations increased significantly in leptin injected koi. This is in agreement with Ajuwon et al. (2003) where a similar response was seen in pigs. However, this is the first report of plasma NEFA concentrations responding to leptin in any fish. The increase in plasma NEFA concentrations is most likely the result of leptin stimulating lipolytic pathways. In mammals, leptin upregulates lipolytic enzymes including ATGL and hormone sensitive lipase, and stimulates enzymes involved in β-oxidation such as CPT and malonyl-coenzyme A decarboxylase (Gallardo et al. 2007). This can result in a decrease in adipose and hepatic lipid stores (Minokoshi et al. 2002;
Wein et al. 2007) and an increase in plasma NEFA and glycerol concentrations (Ajuwon et al. 2003, Wang et al. 1999). If the same mechanism is responsible for the response seen in the current study, it represents a conserved function of leptin that has been maintained between fishes and mammals. Such a finding would be significant because few, if any, of leptin’s effects have been definitively shown to be conserved between fishes and mammals.

An increase in plasma NEFA concentrations may also be explained by a decrease in fatty acid utilization. Due to the simplicity of the free fatty acid assay, it is not possible to know whether fatty acids were mobilized into circulation or merely accumulated as a result of decreased lipid oxidation, or both. A follow up study could address this point of ambiguity by measuring lipolytic enzyme expression and activity, or by measuring enzymes involved in lipogenesis and lipid oxidation. Additionally, measuring the animal’s respiratory quotient can provide some indication of substrate utilization and can at least provide directionality in terms of whether or not lipid metabolism has been upregulated. It is worth noting however that no study has shown leptin to inhibit lipolysis or fatty acid oxidation.

Liver CPT activity was not affected by leptin administration. There are three possible explanations for the lack of response from CPT. First and foremost, leptin simply may not stimulate CPT in carp as it does in mammals. However, given the conserved role of leptin in stimulating lipid metabolism across many different species, I suspect it is much more likely that the dose given was simply insufficient to stimulate a significant response. Studies showing significant increases in CPT expression or activity in response to leptin administration either used higher (i.e. pharmacological) doses and/or
chronic administration (Zhou et al. 1999, Wein et al. 2007). Finally, it is possible that a low sample size did not confer enough statistical power to detect differences between the two groups. Based on the effect size, the power of the test was 0.05 (β = 0.95), far below the desired power of 0.8 (β = 0.2). The low power of the analysis greatly increases the probability of accepting a false negative (type II error). Therefore, the lack of response should be interpreted cautiously. A follow up study should possess a greater sample size and employ either higher or chronic doses to more definitively evaluate leptin’s potential effect on CPT. Additionally, measuring CPT in other tissues may be informative. Johnston et al. (1985) found that cold acclimation increased CPT activity in oxidative muscle. Although the link between temperature and leptin has not been well studied in fishes, leptin responds to cold exposure in mammals (Zeyl et al. 2004, Zhang et al. 2012) and may be under similar influences in fishes.

This study highlights the challenges associated with recombinant protein production. However, in attempting to characterize the function of leptin in common carp and other more basal vertebrates, it is critical to have access to the native hormone. Previous studies in other non-mammalian vertebrates have demonstrated conserved responses to mammalian leptin injections such and reduced food intake and increased body temperature (de Pedro et al. 2006, Niewiarowski et al. 2000), however it cannot be assumed that the species own circulating leptin signals in the same manner. Results from this study indicate that leptin influences lipid metabolism in carp and furthermore suggests that its effects on lipolysis are similar to mammals. This is significant because leptin has scarcely been shown to affect fish physiology in a similar manner to mammals. In fact, many studies show marked departures from the mammalian paradigm of leptin.
signaling (e.g. no correlation between leptin and body fat, and increased plasma leptin during starvation).

I argue that leptin’s most basal and critical function is to stimulate lipolysis and that this function has been maintained throughout millions of years of evolution. Utilization of stored lipids is the one process that can connect all the observed effects and responses of leptin including those observed in this study. Whether leptin upregulates or downregulates some process is likely to be explained by the animal’s life history rather than assuming a complete co-optation of leptin’s function in mammals. Future studies should test the hypothesis that leptin's most conserved function is to stimulate lipolysis and through this action modulate lipid-responsive processes (e.g. reproduction, development, and stress responses). Far too much attention has been devoted solely to measuring the myriad gene responses that occur when leptin is manipulated. Few have followed up these findings by studying organism-level physiology and ecology. Undoubtedly, this knowledge will be critical if we are to gain a comprehensive understanding of leptin's evolution.
LITERATURE CITED


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