Mammalian and clinical studies show that leptin circulates as an endocrine reflection of fat deposition that relays information about energy reserves to the brain and periphery through its ubiquitously expressed receptor (OBR). The leptin system thereby modulates appetite and energy expenditure according to endogenous energy levels in what is referred to as a lipostatic model of energy homeostasis. Due to difficulty in sequencing leptin and OBR genes in lower vertebrates, however, limited information has been gathered about this system in ectotherms generally, including teleost fishes. Upon cloning a putative leptin gene in striped bass (*Morone saxatilis*), representing the first leptin identified in Perciformes, the largest and most diverse order of fish, we profiled its tissue distribution, measured gene expression during fed, fasted and refed conditions, assessed its effect on appetite and tested its regulatory influence on key elements of the endocrine growth axis. The OBR gene was also cloned and its mRNA measured under similar metabolic states in brain regions associated with appetite regulation. This research portrays a leptin system in *Morone* that adjusts energy intake and expenditure according to nutritional state. Leptin may thereby influence the shift away from feeding and toward growth during positive energy states, and therefore serve as an anabolic switch for metabolically expensive processes.

While genome duplication events have resulted in leptin paralogs in some fish lineages, the single *Morone* leptin appears to be orthologous to the medaka A-type. HSB
leptin mRNA was exclusively expressed in the liver, which is an important energy storage organ in many fish, and is a prominent site of leptin gene expression in all fish studied to date. Hepatic leptin mRNA levels rose during feeding (anabolism) and decreased during fasting (catabolism) in hybrid striped bass (HSB; *M. chrysops x M. saxatilis*). Leptin injection suppressed appetite. Together, its function as an anorexigen and its regulation according to metabolic state in HSB suggests that leptin may play a role in maintaining energy homeostasis in these fish.

The *Morone* OBR peptide sequence possesses all major features common to vertebrate OBRs. Central OBR transcript temporarily rose in the telencephalon during fasting, when leptin levels are expected to be low, and again during refeeding. The modulation of OBR gene expression during different metabolic states may be a means of adapting sensitivity to fluctuating plasma leptin concentrations in order to regulate feeding or leptin’s other pleiotropic functions. Gene expression of neuropeptide Y (NPY), a potent appetite stimulant that is commonly suppressed by leptin, was also temporarily elevated in the telencephalon during fasting. The rise in NPY gene expression may therefore be part of a feeding mechanism by which periods of negative energy lead to an increase in appetite in order to replenish depleted energy reserves. The co-presence of NPY and OBR mRNAs in the telencephalon, along with their regulation by feeding status, suggests that this brain region plays a role in the central regulation of appetite.

Because hepatic leptin gene expression is indicative of anabolism, it was considered a candidate for regulating the endocrine growth axis. In this axis, circulating growth hormone (GH) stimulates production of insulin-like growth factors (IGF-I and IGF-II), mitogenic
hormones that drive somatic growth, through GH receptor (GHR1 and GHR2) signaling in the liver. Growth occurs during positive energy states, whereas fasting leads to hepatic GH resistance and decreased IGF production. We show for the first time in vertebrates that leptin upregulates gene expression of both GHRs, as well as that of IGFs, suggesting a previously unrecognized function of leptin in coordinating growth with nutritional state, when energy and resources are available.
Cloning and Characterization of Leptin in a Teleost Fish and its Role in Mediating Appetite and Growth

by
Eugene Thome Won

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Zoology

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2013

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Eugene Thome Won was born in New York City in 1973 and soon after moved to Raleigh, NC. He attended grade school in Raleigh and spent a year abroad in Hamburg, Germany before receiving a BA in English from Reed College in Portland, OR in 1996. Although he worked in NYC as a construction worker and commercial model maker between music industry jobs for several years after college, he spent many nights casting the beaches for striped bass, which planted the seeds of a career involving fish. After finding transitional work building exhibits in the Hall of Ocean Life (his favorite room in NYC) and volunteering in the fish collections depository at the American Museum of Natural History, he hastily assimilated a core biology curriculum and applied to graduate school. He deferred from a fisheries policy program at Duke University in favor of aquaculture research at NCSU. Eugene hopes to participate in the development of domestic aquaculture in order to relieve fishing pressure on wild fish stocks and reduce U.S. dependence on imported seafood.
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CHAPTER I

Cloning and Characterization of Leptin in a Perciform Fish, the Striped Bass (*Morone saxatilis*): Control of Feeding and Regulation by Nutritional State

1\footnote{Published in *General and Comparative Endocrinology*. 2012. 178, pp. 98–107 by Eugene T. Won, David A. Baltzegar, Matthew E. Picha and Russell J. Borski}
Abstract

In mammals, leptin is an anorexigenic peptide hormone that regulates energy homeostasis. It is produced predominantly by white adipose tissue and circulates as an endocrine indicator of energy reserves. Teleost leptin has been characterized in a few fish species, but its regulation is not well understood, particularly in response to nutritional status. In this study, we cloned a putative leptin in striped bass (*Morone saxatilis*) and report the first characterization of leptin in Perciformes, the largest and most diverse order of fish. The striped bass leptin coding sequence was 65% homologous with pufferfish, 52% with Atlantic salmon, and 46% with human. PCR showed that leptin mRNA was exclusively expressed in the liver, and not adipose or other tissues. The leptin coding sequence of striped bass and the more widely cultured hybrid striped bass variety (HSB; *Morone chrysops*, white bass × *M. saxatilis*) were identical. We then evaluated whether the metabolic status of HSB might alter leptin gene expression. Juvenile HSB were subjected to 3 weeks feed deprivation followed by 3 weeks of refeeding. Quantitative PCR showed that fasting for 3 weeks reduced hepatic leptin mRNA levels relative to fed controls. Leptin mRNA levels then increased upon refeeding, albeit levels were not completely restored to those seen in control fish fed throughout the experiment. Intraperitoneal injection of human leptin suppressed appetite in HSB. In as much as hepatic HSB leptin mRNA is regulated by nutritional state and has a corresponding anorexigenic effect, our results suggest that leptin may play a role in energy homeostasis in these advanced Perciformes.
1. Introduction:

Leptin is the 167 amino acid (aa), 16 kDa product of the *obese* gene. It consists of a 21 aa signal peptide and a 146 aa functional hormone belonging to the cytokine class of peptides. Although a wide range of biological activities has been associated with leptin in mammals, including reproduction, bone development and immune response, a predominant function is the maintenance of energy homeostasis [3, 8 for review]. Circulating leptin levels tend to correlate with adiposity [20, 30, 37]. As such, leptin serves as a critical endocrine signal from adipose tissue to the brain and peripheral tissue, relaying information about energy stores and modulating food consumption and energy expenditure accordingly [6]. In mammals, leptin is therefore referred to as an “adipostatic” signal, whose main function is to balance energy storage and usage.

Leptin centrally regulates food intake through activation of appetite suppressing neurons and inhibition of appetite stimulating neurons in the mammalian hypothalamic feeding center [1, 18]. It also promotes energy utilization through fatty acid metabolism in the periphery, as has been observed in skeletal muscle, pancreas and liver [10, 38, 39, 50, 57]. In mammals, periods of negative energy balance and diminished energy reserves result in decreased leptin production, a drop which opens neural pathways that increase food consumption until energy reserves are restored and signaling is consequently reestablished [2, 5, 59]. Alternately, during periods of positive energy status, increased concentrations of circulating leptin promote lipolysis and energy expenditure [3]. At the same time, leptin decreases appetite by upregulating hypothalamic anorexigens and downregulating orexigens [4, 6, 18]. While leptin has been studied extensively in mammals, relatively little is known
about its function and regulation in lower vertebrates, including teleosts. Although the leptin gene sequence is poorly conserved between different vertebrates, the deduced amino acid sequences of amphibian \textit{Xenopus laevis, 13} and fish leptins, including carp, pufferfish and trout \cite{24, 33, 40}, appear to be structurally similar to the mammalian class-I cytokine. Studies suggest that leptin may regulate food intake and energy balance in teleosts. Murine leptin injected intraperitoneally (IP) into green sunfish elicited an increase in intracellular fatty acid binding proteins \cite{34}, suggesting a potential role in fatty acid metabolism. Feeding was reduced by IP and ICV injection of murine leptin in goldfish \cite{58} and IP injection of homologous leptin in rainbow trout \cite{40}. Single IP injection of human leptin also acutely reduced feeding in goldfish, while chronic administration of the hormone additionally reduced food conversion efficiency and growth, and increased lipid mobilization and glycogenesis \cite{16}. However, human leptin was ineffective in altering body weight, energy stores (total lipid content, liver glycogen, hepatosomatic index), or plasma concentrations of insulin, IGF-1, T4 or GH in coho salmon \cite{7}. Nevertheless, IP administration of homologous recombinant leptin to rainbow trout decreased food intake within several hours, reduced mRNA expression of the orexigen neuropeptide Y (NPY) and increased gene expression of proopiomelanocortin (POMC), the precursor to the anorexigen, $\alpha$-MSH \cite{40}.

Studies on the nutritional regulation of leptin in fish are limited and equivocal. Carp hepatic leptin mRNA exhibited an acute postprandial increase but no long-term response to fasting or \textit{ad libitum} feeding \cite{24}. Two paralogs of leptin were found in Atlantic salmon, sLEPA1 and sLEPA2, and their expression in liver and adipose tissue showed discordant responses to satiation and 60\% subsatiation feeding \cite{47}. In visceral fat, the mRNA level of
sLEPA1 was enhanced in fish fed to satiety, while liver and belly flap muscle sLEPA2 mRNA levels increased in fish that ate less. Heterologous assays to measure circulating leptin concentrations show the hormone declines in starved catfish, trout and several centrarchids [28]. However levels of circulating leptin measured by homologous radioimmunoassay increased in trout that were fasted for three weeks, although it is uncertain whether the assay is specific for the sLEPA1 type, against which the antibody was raised, or both forms of leptin [30].

The purpose of this study was to clone and characterize leptin in striped bass (*Morone saxatilis*), representative of the largest order of fish, Perciformes, for which no leptin sequences have been reported to date. We show that leptin is produced by the liver and that hepatic leptin gene expression is sensitive to nutritional state in the cultured hybrid striped bass (HSB: *M. chrysops*, white bass x *M. saxatilis*), declining during catabolism and increasing during states of anabolism. We also show that recombinant human leptin suppresses food intake in these fish, which is consistent with its putative role as an anorexigenic hormone in teleosts.

2. Materials and Methods:

2.1 Nucleic acid isolation and cDNA synthesis

Liver from juvenile and adult striped bass and HSB maintained in fresh water under 12L:12D photoperiod were used for cloning of the *Morone* leptin gene. Total RNA and genomic DNA were isolated from frozen tissue samples using TRI Reagent® (Molecular Research Center; Cincinnati, OH) and the manufacturer’s procedure. RNA purity and
concentration were determined by spectrophotometry using a Nanodrop ND-1000 (Nanodrop Technologies; Wilmington DE) before and after DNase treatment (DNA-free®; Ambion; Austin, TX). Absorbance ratios at 260:280 nm ($A_{260/280}$) of all samples ranged from 1.8–2.0. The quality of the RNA isolations was confirmed by gel electrophoresis (1% agarose, 0.6 μg/ml ethidium bromide). Complementary DNA (cDNA) was synthesized from 1 μg of DNase-treated total RNA using a High Capacity cDNA Reverse Transcription kit and the manufacturer’s protocol (Applied Biosystems, Foster City, CA).

The RNA isolation procedure described above was performed for quantitative RT-PCR (qRT-PCR) assay of leptin mRNA abundance in the liver with the following modifications. After RNA extraction using TRI Reagent®, high salt and LiCl precipitation steps were added, followed by a Plant RNA Isolation Aid® (Ambion) treatment to remove the glycogen present in liver tissue. Large amounts of glycogen seen with fed fish have been previously shown to bias RNA quantification due to absorbance at 260 nm [46]. The $A_{260/280}$ ratio for liver samples ranged from 1.9-2.0.

2.2 Molecular cloning and sequencing

A partial coding sequence for HSB leptin was obtained by PCR of genomic DNA using degenerate primers (Forward: 5’-ATC AAG RYY GAM ATC TCN TCN CTG-3’; Reverse: 5’-RTT NTK YWG CAG CAG NWN GAG GAA STS CYT-3’), deduced from a translated amino acid alignment of Takifugu rubripes (GenBank accession no. NM001032725), Tetraodon nigroviridis (GenBank accession no. AB193549), and Oryzias latipes A (GenBank accession no. NM001104720) mRNA coding sequences. Alignment of
teleost sequences was performed using the Invitrogen Vector NTI® software suite (Invitrogen, Carlsbad, CA) [36].

All PCR reactions were carried out using GoTaq® polymerase and buffers, 10 mM dNTP mix and 10 μM primer concentrations (Promega; Madison, WI). The PCR cycling parameters for the partial HSB leptin genomic DNA sequence were: 1 cycle at 95°C for 2 min; 35 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and 1 cycle of final extension at 72°C for 5 min. Isolated amplicons were cloned into pCR II® cloning vector (Invitrogen) and transformed into JM109 series competent cells (Promega). Two selected clones from each amplicon were submitted to the University of Chicago Cancer Research Center for forward and reverse sequencing with universal M13 primers.

Gene specific primers for two rounds of 5' and 3' RACE-PCR were developed from the partial HSB leptin sequence, using the First Choice® RNA-ligase mediated RACE kit (Ambion). This method promotes the capture of full 5' sequence by 5' monophosphate degradation of the unprotected (methyl cap) mRNA template. Primer design, RNA treatment, and PCR amplification were performed in accordance with manufacturer's protocol. The following primers were used in conjunction with kit provided adaptor primers: 5'RACE-PCR – (primary) 5'-TGG GTC GCT GCT CAC TGC A-3' and (nested) 5' – TTG CCC TTG CCT CCA CTG AC – 3'; 3' RACE-PCR – (primary) 5'-GGG GGT GCT GCA AGA GCT AC-3' and (nested) 5'-GAA AAA GTT CAT TCA CAC CGT GAG C-3'.

Contiguous sequence analysis was performed for all obtained clones using Vector NTI® Contig Express software to generate a full-length HSB leptin cDNA sequence. To verify the in silico contig analysis, an additional primer pair for the full coding sequence
(CDS) was designed, which spans all sequences in the contig alignment. The CDS primers were: Forward 5′–ATG GAC TAC ACT CTG GCC ATC-3′ and Reverse 5′-TCA GCA AGT CTC GAG ATG AT–3′. The PCR cycling parameters for the leptin CDS were as follows: 1 cycle at 95°C for 3 minutes; 35 cycles at 95°C for 30 seconds, 54°C for 30 seconds, 72°C for 1 minute, 30 seconds; and 1 cycle final extension at 72°C for 10 minutes. Additional PCR was performed using these primers to amplify the leptin coding sequence of the striped bass, *M. saxatilis*, using identical PCR cycling parameters.

### 2.3 Protein model

The striped bass leptin amino acid sequence was translated from the nucleotide sequence using Vector NTI® software. Peptide structural features and tertiary configuration of mature striped bass leptin (residues 28–161) were predicted by SWISS-MODEL protein modeling and feature scanning software (http://swissmodel.expasy.org/SWISS-MODEL.html) [49]. Human leptin was automatically selected as the reference peptide structure (Protein Data Bank file 1AX8.pdb).

### 2.4 Phylogenetic analysis and genomic synteny comparisons of teleost leptin:

Bayesian phylogenetic analysis of leptin sequences was performed using MrBayes v3.1.2 [23] via an online teragrid network available through CIPRES Portal (URL: http://www.phylo.org/sub_sections/portal). The following leptin peptide sequences were used in the analysis: *Homo sapiens* (NP_000221.1), *Xenopus laevis* (NP_001089183.1), *Mus musculus* (NP_032519.1), *Tetraodon nigroviridis* (BAD94451.1), *Takifugu rubripes*
(NP_001027897.1), *Oryzias latipes* leptin a (NP_001098190.2) and leptin b (NP_001153914.1), *Danio rerio* leptin a (NP_001122048.1) and leptin b (NP_001025357.2), *Cyprinus carpio* leptin I (CAH33828.3) and leptin II (CAH33827.2), *Salmo salar* leptin A1 (ACZ02412.1) and leptin A2 (ADI77098.1), *Ictalurus punctatus* (AAZ66785.1, partial sequence), *Morone saxatilis* (JF919618) and *Gasterosteus aculeatus*. The *G. aculeatus* sequence was obtained by BLAST searching the sequenced genome (available at http://www.ensembl.org) and the coding sequence was estimated by aligning the genomic sequence with the coding sequences of known fish leptins. Three neuropeptide Y sequences were used for outgroup comparison: *M. musculus* (NP_075945.1), *D. rerio* (NP_571149.1), and *X. laevis* (NP_001081300.1). Bayesian analysis was performed with the General Time Reversible amino acid model and equal among-site rate variation with tree-sampling every 1000th generation (burn-in = 500) [23]. The two cold-chain runs reached 2.2 million generations before terminating when the standard deviation of split-frequencies fell below 0.01. Sampled trees (3,440 from both runs) were used to create a consensus tree file with 50% or greater clade credibility (support for each node). The taxon names and clade support values were modified *post hoc* for illustrative purposes only using TreeView [44] and CorelDraw (v12). No other modifications were performed.

Genomic synteny of teleost leptins was compared with our phylogenetic analysis to assess the evolutionary history of leptin(s) across the ray-finned fishes. Homologous loci were examined in the five fish genome assemblies currently available in *Ensembl* (Wellcome Trust Sanger Institute; URL: www.ensembl.org): Zebrafish (*Danio rerio*; v9) - chromosome 18: nucleotide position 9,756,856 to 10,161,450 and chromosome 4: 18,029,388 to
18,639,046 reverse complement (rc); Medaka (*Oryzias latipes*, v1) – chromosome 6: 14,886,924 to 15,029,716 and chromosome 23: 16,155,042 to 16,656,798; Stickleback (*Gasterosteus aculeatus*, v1) – group IV: 30,113, 540 to 30, 419, 505 and group XIX: 16,063,076 to 16,180,741 (rc); Fugu (*Takifugu rubripes*, v4) – scaffold 30: 1,146,574 to 1,249,241 (rc) and scaffold 8: 538,732 to 767,856 (rc); *Tetraodon nigroviridis*, v8 – chromosome 19: 2,363,782 to 2,576,018 (rc) and chromosome 13: 4,249,630 to 4,297,552.

Discrete genes were identified by the provided genome annotation and verified by BLASTX analysis to Genbank sequences (NCBI). The hypothetical ancestor was surmised by comparing the shared arrangement between fish and a representative tetrapod: Mouse (*Mus musculus*, v37) – chromosome 6: 28,392,359 to 29,166,364.

### 2.5 Tissue distribution

HSB leptin mRNA expression was evaluated in multiple tissues using RT-PCR. Fifty nanogram of cDNA derived from brain, pituitary, fat, liver, muscle, gut, gonad, kidney, heart and gill total RNA were analyzed using GoTaq® (Promega, Madison, WI) polymerase and buffers, with 10 mM dNTP mix and 10 μM primer concentrations. The tissue distribution RT-PCR primers were designed from the putative leptin coding sequence (forward: 5’-ATG GAC TAC ACT CTG GCC ATC-3'; Reverse: 5’-TCA GCA AGT CTC GAG ATG AT-3’). The housekeeping gene β-actin was screened by PCR in tandem on the same samples to verify the integrity of cDNA template across tissues (Forward: 5’-AGC CAA CAG GCC ATC-3’; Reverse: 5’-TGG GCC AAT GAT CTT GAT CT-3’). The PCR cycling conditions for tissue distribution analysis were the same as for the leptin coding sequence,
with the following modifications: 40 cycles were run with leptin primers at 54° C and β-actin at 57° C for 1 minute of extension time per cycle and a 5 minute final extension period. PCR products of both genes were visualized together by gel electrophoresis (3% agarose, 0.6 ug/ml ethidium bromide).

2.6 Leptin regulation by nutritional state

Juvenile (71.4g ± 0.8g; mean body weight ± SEM) HSB were obtained from the Tidewater Research Station (Plymouth, NC) and housed in two identical recirculating systems each with four 650-liter tanks in fresh water (hardness = 170mg/L; alkalinity = 250mg/L; temperature = 24.4° C ± 0.04° C; mean ± SEM) at North Carolina State University (Raleigh, NC). Fish were fed daily prior to the start of the experiment. Two tanks from each of the recirculating systems were randomly assigned as treatment and control groups (N = 4 tanks/group at 50 HSB/tank). Control fish were fed ad libitum twice daily with floating feed pellets (Ziegler Silver, Gardners, PA; 40% protein, 10% fat) over 42 days. Treatment fish were starved for 21 days and then refed ad libitum for 21 days. This and the feed intake procedure outlined below were approved by the NCSU Institutional Animal Care and Use Committee.

Control and treatment fish were sampled at time 0, day 21 (after 21 days of fasting), and at day 29 and 42 (after 8 and 21 days refeeding). The first 21 days of fasting reflect a catabolic state sufficient for inducing compensatory growth in Morone upon refeeding, while 8 and 21 days of refeeding encompass anabolic states in which growth rate and feed consumption (compensatory growth) exceeds that of a typical animal fed continuously [46].
Fish were anesthetized (tricaine methanesulfonate, MS 222; Argent Chemical Laboratories, Redmond, WA), individually weighed and subsequently killed by decapitation. Whole liver weight was determined and liver samples for quantitative RT-PCR measurement of mRNA were immediately frozen in liquid nitrogen and then stored at –70° C. Fish were not fed the day prior to sampling. Correlation between hepatic leptin gene expression and hepatosomatic index (HSI) was calculated using measurements from individual fish at all four time points (0, 21, 29 and 42 days). The group mean HSI, specific growth rate (SGR), feed consumption, and feed conversion ratio (FCR) data for this study was previously reported in an independent investigation evaluating nutritional regulation of insulin like growth factors and growth hormone during compensatory growth [46].

2.7 Quantitative RT-PCR of leptin mRNA abundance

Leptin mRNA expression was determined by quantitative RT-PCR according to our previously established protocols (Picha et al., 2006; Picha et al. 2008). Gene-specific quantitative RT-PCR (qRT-PCR) primers for HSB leptin (forward: 5’-CAG CGA CCC AAG CTT TCA GT -3’; reverse 5’- CAC GGT GTG AAT GAA CTT TTT CC -3’) were designed using Primer Express® (Applied Biosystems; Foster City, CA) gene detection software. Real-time qRT-PCR analysis was performed on an ABI 7900 HT Sequence Detection System, using Brilliant® SYBR Green master mix (Stratagene; La Jolla, CA), 1.5 μM primers, and 10 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 assessed using two
negative controls: sterile water used as template (No-Template Control; NTC) and DNase-treated RNA used as template (No-Amplification Control, NAC). Leptin primer specificity was verified by melting curve analysis, as well as visualization (4% agarose gel, 0.6 μg/ml ethidium bromide) and direct sequencing of a subset of qPCR amplicons.

Cycle threshold (Ct) values for experimental samples were transformed using a standard curve of serially diluted cDNA versus Ct values (R² = 0.99) and normalized to reflect the amount of cDNA template per ng total RNA. Since liver mass and total hepatic RNA values fluctuate considerably depending on the metabolic state of the fish, data was further normalized to total liver RNA production as a function of body weight. Total hepatic leptin mRNA levels relative to body mass (ng cDNA / g BW) was calculated by the following equation: [(ng total liver RNA) × (ng cDNA per ng RNA) / (g body weight)] [46].

2.8 Regulation of feed intake by human recombinant leptin

Juvenile HSB (30.83 g ± 0.67 g; mean body weight ± SEM) were obtained from Pungo Fisheries (Pinetown, NC) and housed in two identical recirculating systems, each with eight 75-liter tanks (salinity = 7 ppt; hardness = 200 mg/L; alkalinity = 250 mg/L; temperature = 22° C) at North Carolina State University (Raleigh, NC). For two months prior to the experiment, fish were fed daily at 10:30 a.m. to entrain them on a regular schedule. Feed was withheld from experimental fish for four days prior to the start of the experiment. On the day of the trial, fish were injected at 9 a.m. so that the first sampling point would coincide closely with the entrained feeding time. Five tanks between the recirculating systems were randomly assigned as control and treatment groups (N = 5
tanks/group with 4 fish/tank). Human recombinant leptin (Harbor-UCLA Medical Center, Torrence, CA) was dissolved in 0.1 mM PBS (pH 8.1) and diluted with biological saline. Fish were anesthetized with MS222 and injected intraperitoneally with 50 µL saline (controls) or leptin (treatments) at doses of 100 ng and 1 µg /g body weight using a 25-gauge needle. Fish were returned to tanks and allowed to recover for 2 hours before the first satiation feeding. Feeding was measured at 2 and 8 hours post-injection, as this time series incorporates the reported 3-hour half-life of leptin in rodents [5] and represents similar time-course feeding trials conducted in trout and goldfish [16, 40]. Feed (Ziegler Gold 3mm, Gardners, PA; 40% protein, 10% fat) was administered blind by an unbiased third party participant and ended when feeding had ceased for 5 minutes with at least 4 pellets remaining in the water. Uneaten feed pellets were counted, multiplied by mean dry pellet weight and subtracted from the weight of feed administered during the trial. Group feeding at each time point was quantified as a percentage of body weight (BW), then normalized as a percentage of the mean feed intake of the control group [(feed intake / BW) / (mean feed intake of controls / mean BW) * 100; mean ± SEM]. The experiment was repeated and feeding responses were similar in the two experiments.

2.9 Statistics:

Hepatic leptin mRNA and hepatosomatic index data was analyzed by two-way factorial ANOVA (treatment × time) followed by Fisher’s LSD test. Leptin gene expression data was log-transformed for statistical analysis in order to satisfy the assumption of homogeneity of variance. Correlation was analyzed by regression analysis. The effect of
leptin injection on feed intake (% body weight) was analyzed by three-way ANOVA (treatment × time × date). No significant main effect by date or between the repeated experiments was found ($P > 0.05$). The treatment and time main effects were both significant in the experiments. Feed intake data from the two experiments was therefore pooled (N =10). Leptin dosage effects on feed intake were compared by Fisher's LSD test. ANOVA analyses were performed using Statistica 7.0 software (Stat Soft; Tulsa, OK). Data are expressed as mean ± SEM. The threshold for statistical significance was set at $P < 0.05$.

3. Results:

3.1 Striped bass leptin sequence characterization and phylogeny

A full-length cDNA sequence of HSB leptin was characterized by RACE-PCR (Fig. 1). Sequence contig analyses of HSB leptin clones with 4X or greater coverage yielded a single 677 base pair (bp) cDNA sequence. An additional PCR reaction spanning all sequenced RACE clones was performed, which confirmed our in silico contig analysis. The 486 bp coding sequences of striped bass and HSB were identical. Sequence comparison with amplicons derived from PCR of genomic DNA suggests that a single 88 bp intron with a canonical AG-GT splice site is embedded within the leptin open reading frame.

The translated striped bass leptin contains 161 amino acids. Based on a SWISS-MODEL rendering (Fig. 2B), it has four antiparallel α-helices typical of class-I cytokines and other known leptins, including two cysteine residues that likely form a disulphide bond between the terminal ends of the C and D helices. Putative components of binding site II on the A and C helices and site IIIb on the A-B loop of striped bass leptin are inferred from the proposed
binding regions of tetrapod leptins [13, 42, 45] (Fig. 2A). The open reading frame shares the highest amino acid sequence similarity (identity/consensus) with the pufferfish, *Tetrododon nigroviridis* (52%/68%; not shown) and *Takifugu rubripes* (45%/58%), *Oryzias latipes A* (39%/49%) and *Gasterosteus aculeatus* (38%/50%), and lowest similarity to human (15%/29%) (Fig. 2A). Despite the low amino acid sequence identity to human, the projected tertiary structure of striped bass leptin is similar to the human form.

To confirm the homology of the striped bass sequence with other teleost leptins, a Bayesian phylogenetic analysis was performed from a survey of teleost and tetrapod leptin peptide sequences. *M. saxatilis* leptin grouped with pufferfish (*T. rubripes, T. nigroviridis*) leptin, stickleback (*G. aculeatus*) leptin, and medaka (*O. latipes*) leptin A in a monophyletic clade containing 96% support (Fig 3A). All other leptins, including the tetrapods (*H. sapiens, M. musculus, X. laevis*) and the remaining teleost sequences (*D. rerio* leptin A and B, *C. carpio* leptin I and II, *I. punctatus, S. salar* leptin A1 and A2, and *O. latipes* leptin B) formed a separate monophyletic group (98% support).

As exhaustive degenerate PCR screens failed to identify a second leptin homolog in the striped bass, we compared the five teleost genomes currently available in *Ensembl* (zebrafish, medaka, stickleback, fugu, and *Tetrododon nigroviridis*) to the leptin locus of the mouse (*M. musculus*). In mouse, leptin is enclosed by five genes syntenically conserved in the teleost genome: *Snd1* – Staphylococcal nuclease and tudor domain containing 1, *Pax4* – paired box 4, *Lrrc4* – leucine rich repeat containing 4, *Rbm28* – RNA-binding protein 28, and *Impdh1* – inosine monophosphate dehydrogenase 1. In all fishes, two homologous chromosomal regions were identified. As in mouse, a teleost leptin homolog is present
between two genes in the reverse orientation: *lrrc4* and *rbm28* (Fig 3B). In zebrafish and medaka, an additional leptin is observed between *snd1* (forward) and *imphd1* (reverse). This positional placement is conserved in mouse, and therefore likely represents the vertebrate ancestral state. In the stickleback and puffers (*T. rubripes* and *T. nigroviridis*), *snd1* and *imphd1* are retained on another chromosome; however no second leptin sequence is identifiable in the region between these genes, and was presumably lost in the common ancestor to these advanced groups of fishes (Fig 3B).

3.2 Tissue distribution and effects of nutritional status on leptin mRNA expression

Tissue distribution was analyzed by reverse transcription PCR using the housekeeping gene β-actin to control for gel electrophoresis loading and integrity of the cDNA template in each tissue sample. Leptin mRNA expression was restricted to the liver (Fig. 4) and showed no detectable expression in other tissues examined including intraperitoneal fat, brain, pituitary, muscle, intestine, ovaries, testes, kidney, heart and gill. We evaluated the responsiveness of hepatic leptin mRNA during altered metabolic states after 21 days of fasting and during refeeding. Relative to the onset of the experiment, total hepatic leptin mRNA levels (Fig. 5) in fed control fish increased significantly over the first 21 days of the experiment (206.8 ± 26.7 to 490.4 ± 48.2 ng cDNA/g BW; *P* < 0.01), and again from day 21 to day 42 (490.4 ± 48.2 to 1950.9 ± 559.9 ng cDNA/g BW; *P* < 0.001). Leptin mRNA levels declined by 73% in fasting fish over the 21 day period of food deprivation (206.8 ± 26.7 to 55.3 ± 5.2 ng cDNA/g BW; *P* < 0.001), then significantly increased 8 (day 29 = 290.4 ± 23.1 ng cDNA/g BW; *P* < 0.001) and 21 (day 42 = 845.1 ±
123.6 ng cDNA/g BW; $P<0.001$) days into the refeeding period, exceeding levels seen at the onset of the experiment, but remaining below levels of control fish on day 42 (treatments = $845.1 \pm 123.6$; controls = $1950.9 \pm 559.9$ ng cDNA/g BW; $P<0.01$).

The mean group HSI, SGR, feed consumption and FCR data from the fasting-refeeding study was previously reported by our group and the results are summarized here [46]. During the 21-day food deprivation phase of the experiment, treatment fish experienced a significant drop in HSI (from $3.85 \pm 0.11$ at the start to $1.11 \pm 0.11$ on day 21; mean $\pm$ SEM), followed by a steady rebound after 21 days of realimentation (up to $4.60 \pm 0.16$ on day 42), ultimately exceeding that of controls by 30% ($P<0.001$). Control HSI was stable, ranging between $3.53\pm 0.09$ and $3.96 \pm 0.09$ throughout the experiment. A weak correlation between hepatic leptin mRNA levels and HSI ($R^2 = 0.12$; $P<0.01$) was observed in fish representative of all groups and time points.

Fasting for 21 days resulted in a negative SGR (treatment = $-0.38 \pm 0.05$ %/d, control = $1.7 \pm 0.09$ %/d; $P<0.001$). During 21 days of refeeding, the SGR of treatment fish exceeded that of similar sized control fish fed throughout (treatment = $2.52 \pm 0.09$ %/d, control = $1.27 \pm 0.13$ %/d; $P<0.01$). Feed consumption in treatments exceeded control levels by 66% during 21 days of refeeding ($2.46 \pm 0.05$% BW/d for treatments, $1.48 \pm 0.05$% BW/d for controls; $P<0.01$). The FCR of refed treatment fish was also improved during 21 days of realimentation relative to controls (treatments = $0.87 \pm 0.03$, controls = $1.14 \pm 0.07$; $P<0.01$). These results demonstrate that *Morone* undergo a robust compensatory growth response or exhibit a supra-anabolic state during refeeding that follows a prolonged fast. Because SGR,
total feed consumption, and FCR were calculated by group rather than individually, determining correlations of these with leptin gene expression data was not possible.

3.3 Effect of recombinant human leptin on appetite

Prior to examining the effects of exogenous leptin on appetite, fish were initially fasted for four days to enhance feed consumption responsiveness. Two hours after intraperitoneal injection at dosages of 100 ng and 1 µg/g BW, recombinant human leptin significantly suppressed feed intake by 23.9% and 15.2% respectively relative to saline-injected controls (Fig. 6). All groups showed increased feed consumption (3.12, 2.38, and 2.65 % BW for saline, 100 ng, and 1 µg/g BW leptin, respectively) relative to the baseline feeding observed prior to the brief fasting period (1.4% BW). By 8 hours post-injection, feed intake between vehicle and leptin treated groups did not differ from each other or relative to basal feeding levels observed prior to the initial fasting.

4. Discussion:

This study reports the first characterization of a putative leptin in Perciformes, the largest and most diverse order of teleosts (9,300 extant species) [22]. The striped bass leptin coding sequence is most closely related to other more advanced teleosts, the Tetraodontiformes, Beloniformes and Gasterosteiformes, and its projected tertiary peptide structure shows conformational similarity to human leptin. We show that the leptin gene is expressed exclusively in the liver, is downregulated during fasting and upregulated during anabolism, and that exogenous treatment with the peptide suppresses appetite.
Identical 486 bp full-length coding sequences were characterized for striped bass and hybrid striped bass leptin. Based on sequence comparison of amplicons derived from PCR of cDNA and genomic DNA, a single 88 bp intron with a canonical AG-GT splice site embedded within the leptin open reading frame was found, indicating that the coding sequence of the putative Morone leptin is contained on two exons (Fig. 1A). The possible existence of a third or additional exons outside the coding region, as has been found in the 5’ UTR of pufferfish [33], medaka [32] and human [27] leptins, could not be assessed without additional striped bass genomic sequence for comparison.

The striped bass leptin coding and deduced amino acid sequences have overall poor similarity with other leptins (Fig. 2A), although several important structural elements are conserved. Striped bass leptin is composed of 161 amino acid residues, which form an antiparallel four-helix structure and tertiary fold characteristic of class-I helical cytokines, including leptins (Fig. 2A, B) [6, 25]. As in other leptins, two cysteine residues likely form a disulphide bond between the terminal ends of the C and D α-helices which is necessary for normal processing and secretion [9, 61], but not for receptor binding [26]. Of the three binding sites (I-III) found in similar cytokines, putative analogs for II and III may be of significant importance to leptin binding and activity based on mutagenesis studies in mammals. Leptin binding site I does not appear to be absolutely necessary for binding or biological activity, and is poorly conserved [45]. Site II is located on the surface of the A and C helices, and may involve an interaction between conformationally adjacent arginine and glutamine residues (R20 and Q75 in humans; R17 and Q63 in bass) which are well conserved across taxa [45] (Fig. 2A). A discontinuous leptin binding domain III in mouse is speculated
to involve consecutive serine and threonine residues (site IIIa) on helix D that interact with a hydrophobic section of β–strand (site IIIb; VTGLDFI) on the A-B loop [42, 48].

Conservation of the amino acid sequence (GLDFIP) on this loop of amphibian and mammalian leptins further suggests a role for this region as a possible binding domain [13]. Of the known teleost leptins, that of striped bass contains a hydrophobic sequence between the A and B helices that is most similar to the putative site IIIb tetrapod binding domain (Fig. 2A: GLTLSP), retaining three of the flanking residues (GL---P) while substituting a leucine for phenylalanine, both of which are hydrophobic residues. Regions of striped bass leptin may therefore be analogous to sites II and III of the tetrapod leptin binding domain, although functional analyses of these regions has yet to be performed in teleosts. Interestingly and despite its low nucleotide and amino acid homology to human leptin, the striped bass peptide has a remarkably similar tertiary structure to the human peptide (Fig. 2B).

Our evolutionary analysis suggests the order Perciformes, which includes M. saxatilis, may possess only one leptin. In our phylogeny, striped bass, pufferfish (T. rubripes, T. nigroviridis), and stickleback (G. aculeatus) leptin are orthologous to medaka (O. latipes) leptin type A (Fig 3A). Genomic synteny comparison of these fishes support this finding, but also suggests the loss of the leptin B type in the common ancestor of Gasterosteiformes and Tetraodontiformes, as well as the Perciformes (Fig 3B). Additionally, the leptin of percomorph fishes (Acanthopterygii) is quite divergent from that of more basal teleosts, including zebrafish (D. rerio) leptin A (Fig 3A). This could be explained by subfunctionalization or neofunctionalization of leptin paralogs following gene duplication [12, 35, 43]. Leptin A and B may have arisen from a putative whole-genome duplication
event which is thought to have occurred in the common ancestor of all teleosts [14, 19, 54, 55]; however the phylogenetic grouping of zebrafish leptin A with more ancestral groups suggests the rapid sequence divergence of leptin (type A) occurred after the split of the Ostariophysii (Fig 3A, B). Combined, our evolutionary analysis suggests two distinct events occurred in the evolutionary lineage of the Percomorpha: (1) duplication of the leptin gene, possibly through a whole-genome duplication event in earlier teleosts, and (2) the subsequent loss of one leptin paralog (leptin B type) at some point after the divergence of the Atherinomorpha (silversides, topminnows, and livebearers) [22].

Consistent with its function as an endocrine indicator of adipose energy reserves, leptin is produced predominantly by fat in mammals [20, 37]. The two leptin genes found in salmon, medaka, carp and zebrafish are expressed in various tissues, with one of the leptins highly expressed in liver [21, 24, 32, 47]. We found that leptin mRNA expression was detected only in the liver of HSB (Fig. 4), which matches the expression profile for the single leptin in fugu [33]. Collectively, it appears that the liver is a major site of leptin production in Morone and other teleosts. Like adipose tissue, the liver also represents a major lipid storage site in fish, including the striped bass.

Long-term regulation of leptin by nutritional state in teleosts has not been extensively examined. Carp exhibit an acute, post-prandial increase in hepatic leptin I and II mRNA expression, but no long-term regulation associated with nutritional state [24]. In Atlantic salmon, sLEPA1 mRNA levels in visceral adipose tissue were higher in fish fed ad libitum than in fish fed to only 60% satiation, while its paralog, sLEPA2, was relatively higher in liver and belly flap muscle of the fish on the lighter regimen [47]. We found that hepatic
leptin mRNA expression is sensitive to nutritional state, declining during catabolic states of fasting and increasing under anabolic conditions in HSB (Fig. 5), including during the refeeding period when growth compensation occurs and growth rates, feed consumption, and feed conversion efficiency exceed levels of control animals fed throughout [46]. After three weeks, fed fish had almost nine times higher hepatic leptin mRNA levels than fasted fish, substantiating a preliminary experiment in which larger (200 g) fish than those evaluated here showed a similar response to fasting (data not shown). Similarly in mammals, circulating leptin decreases during short-term [5, 29] and chronic [17, 29, 60] energy restriction, while refeeding after fasting returns leptin to basal levels [31, 59]. Regulation of hepatic leptin mRNA levels by nutritional state in HSB therefore seems to follow a pattern that generally resembles that observed in adipose of mammals and with that of LEPA1 expression in salmon adipose tissue [47].

The role of leptin in energy balance remains unclear in teleosts. In mammals, leptin is produced and secreted in proportion to adipose mass and stimulates lipolysis, metabolic rate and weight loss [3]. Londraville and Duvall (2002) demonstrate that daily injection of murine leptin for 14 days increases intracellular fatty acid binding protein in heart muscle, suggesting actions on fat metabolism in green sunfish. However, there was little affect of leptin on body weight or body fat in this study or with coho salmon treated peripherally with human leptin [7]. The liver of fishes store both glycogen and lipids with many species, including striped bass, having a higher capacity for lipid storage relative to others [52, 53]. Fed control HSB in the current study maintained a stable HSI ranging from 3.5 to 4.0 throughout the trial period, while leptin gene expression in these fish increased steadily (Fig.
5). Whether leptin is produced or secreted in proportion to hepatic lipid content or adipose mass in fish is unknown. We found that HSB leptin mRNA levels tend to fluctuate with HSI, declining in fasted and rising in refed fish, although the degree of correlation is reduced when comparing refed with fed control fish. Hepatic leptin gene expression did not increase proportionally to HSI after three weeks of refeeding; the HSI of refed fish ultimately exceeded that of continuously fed controls (day 42; HSI = 4.6 and 3.5 respectively) while their leptin mRNA levels only reached 43% of controls. A previous study in HSB shows that hepatic glycogen levels of fish during refeeding surpassed levels of continuously fed fish [15], suggesting that the overcompensation of HSI seen in the current study may be due to rapid glycogen accumulation. In golden perch, hepatic lipid accumulation is delayed relative to glycogen deposition during refeeding [11]. It is possible therefore, that a lower accumulation of lipid-rich hepatocytes might have resulted in the lower leptin mRNA levels in refed HSB relative to fed controls. Although liver composition was not analyzed at the time of this report, future studies are clearly required to investigate the relationship of stored energy, e.g. hepatic lipid content, to leptin gene expression in HSB and other fishes.

Nevertheless, the upregulation of leptin gene expression with anabolism suggests the hormone may function to maintain energy homeostasis in HSB possibly by promoting a shift away from eating and toward energy usage as has been demonstrated for mammals [3].

Considering that leptin gene expression increased with feeding, we evaluated if leptin might suppress appetite in HSB. Elevated leptin production during surfeit suppresses appetite in mammals. Exogenous leptin has also been demonstrated to suppress feeding in goldfish [16, 58] and rainbow trout [40], as well as African clawed frog [13], which seems to
underscore a conserved role for the peptide in appetite regulation over the course of vertebrate evolution. In this study, a single IP injection of human leptin in previously fasted HSB suppressed appetite by around 25% relative to controls in 2 hours (Fig. 6). Albeit less robust, the response is similar to that observed in trout injected with homologous leptin (80% decrease) or goldfish injected with murine leptin (30-60% decrease). The anorexigenic effect subsided by 8 hours, which is similar to the time course observed in goldfish and trout [16, 40]. The half-life of leptin in mouse is about three hours [5]. It is possible that the lack of effect by exogenous leptin at 8 hours may have resulted from clearance or degradation of leptin, internalization of its receptor, or convergence of appetite between groups due to circadian influence of endogenous leptin [51] or other factors, although it is unknown if a circadian component to leptin regulation exists in fish. The anorexigenic effect was elicited in HSB with a lower dosage than needed in goldfish (injected with murine leptin; 300 ng – 1 µg/g BW) or trout (injected with homologous leptin; 720 ng/g BW), although the lower threshold at which leptin diminishes appetite in trout was not determined. On the other hand, the anorexigenic property of leptin in HSB mildly diminishes at what might approach an excessively high dose, also seen in goldfish [16], suggesting an upper limit, above which signaling may be disrupted. Variability in the efficiency of heterologous leptins, along with inherent interspecific differences in leptin dynamics, could possibly contribute to dose discrepancies between these studies. However, the anorexigenic effect on HSB and stimulation of prolactin secretion in tilapia [56] by low dosages of human leptin illustrates that the peptide is bioactive in these Perciformes and may possess a conserved conformation to that of the endogenous ligand.
In summary, we have cloned leptin in striped bass, representative of Perciformes, and genetic synteny analysis suggests this order of fishes may possess only one form of leptin. Leptin is expressed exclusively in the liver in HSB and its gene expression is downregulated during catabolism and upregulated under anabolic states. This, along with the anorexigenic response to exogenous leptin, suggests the hormone may regulate energy homeostasis in *Morone* by suppressing appetite during periods of anabolism when hepatic energy stores accumulate. The crux of the mammalian adipostatic model, in which leptin reflects endogenous energy availability and consequently regulates food intake, may therefore apply to HSB despite class differences in metabolic demand, capacity for growth and energy partitioning. The fact that leptin is expressed in liver in these fish rather than in fat may perhaps be reconciled by considering both organs as energy depots.

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References


**Figure 1.** DNA coding and translated amino acid sequences of *M. saxatilis* leptin. Signal peptide and α-helices are indicated by boxes. Cysteine residues at terminal ends of C and D helices are underlined. Two exons containing the coding sequence are marked by hollow arrows.
<Figure 2. (A) Alignment of amino acid sequences of representative fish and tetrapod leptins. Species-specific signal peptide regions are bolded (G. aculeatus is undocumented). Helices A-D pertaining specifically to M. saxatilis are marked by solid bars. Putative binding domains (II, IIIa, IIIb) and conserved cysteine residues are boxed. Gray regions indicate conserved residues. Dashes are place holders. Conservation of peptide sequence identity (aa%), peptide sequence consensus (aA%) and coding sequence identity (nt%) is noted in relation to the striped bass leptin coding and derived amino acid sequences. Sequences used in the alignment were the same sequences used for the phylogenetic analysis. (B) Projected tertiary structure of the striped bass mature leptin peptide (residues 28-163) from translated amino acid sequence modeled by SWISS MODEL using human leptin (1AX8.pdb) as a reference. The striped bass leptin model has four antiparallel helices, similar to human leptin.>
Figure 3. (A) Unrooted consensus tree of leptin peptide sequences using Bayesian phylogenetic analysis. For teleosts possessing more than one leptin, the paralog identity is given in parentheses. Numerical values represent support values for each node (clade credibility values). Outgroup sequences, neuropeptide Y (NPY), are enclosed in the shaded circle. Symbols: partial – only partial sequence is available; asterisk – node support is < 50%. Branch length scale = 0.1 expected changes per site. (B) Genomic synteny of teleost leptin(s). Homologous leptin loci of the five teleost species with sequenced genomes mapped to a phylogeny of the Euteleostei [22, 41]. The light shaded circles represent proposed events in the teleost lineage, whole-genome duplication and subsequent loss of one leptin paralog in advanced percomorphs. White boxes – leptin homologs; light-shaded boxes – genes syntenically conserved to a hypothetical common ancestor with the tetrapods. The dark shaded boxes represent novel genes. Arrows indicate the 5' to 3' orientation. Symbols: Chr – chromosome, Grp – group, Impdh1 – Inosine monophosphate dehydrogenase 1, Lrrc4 – Leucine rich repeat containing 4, Pax4 – Paired box 4, Rbm28 – RNA-binding protein 28, Scf – scaffold, Snd1 – Staphylococcal nuclease and tudor domain containing 1. The distances between genes are not to scale.
Figure 4. HSB tissue distribution of leptin expression by reverse transcription PCR: brain (B), pituitary (P), fat (F), liver (L), muscle (M), small intestine (I), ovaries (Gf), testes (Gm), kidney (K), heart (H), gill (Gi) and no-template control (-). Only liver tissue produced an amplicon of expected size (486 bp). Expression of the housekeeping gene β-actin was also observed to ensure the integrity of the cDNA template of each tissue sample. PCR products were visualized by electrophoresis in a 3% agarose gel beside a Promega 1 kb DNA ladder (g5711).
Figure 5. Total hepatic leptin mRNA levels relative to body weight (ng cDNA / g BW) in HSB during fasting and refeeding (mean ± SEM.; n=7 per group at each time point). Controls (solid) were fed ad libitum for 42 days. Treatments (dashed) were fasted for the initial 21 days and then fed ad libitum for 21 days. Grey bar denotes period of feed restriction for treatment group. Letters denote differences between time points within groups. Asterisks denote significant differences between groups at each time point (**P<0.001; *P<0.05).
Figure 6. Effects of IP injected human recombinant leptin on feeding in HSB. All fish were fasted for 4 days, then injected with saline (control) or leptin and fed 2 and 8 hours later. Data is total feed intake expressed as a percentage of total body weight and then normalized to percentage of the mean feed intake of controls (mean ± SEM.; n=10 per group). Asterisks denote significant differences between the control group and treatment groups at each time point (**P<0.01; *P<0.05).
CHAPTER 2

Leptin stimulation of hepatic growth hormone receptor and insulin-like growth factor
gene expression in a teleost fish
ABSTRACT

Leptin is an anorexigenic peptide hormone that circulates as an endocrine indicator of adiposity in mammals, and functions to maintain energy homeostasis by balancing feeding and energy expenditure. In fish, leptin is produced predominantly by the liver, which is also an important energy storing tissue. The liver also produces the majority of circulating insulin-like growth factors (IGFs), which are the main mitogenic component of the growth hormone (GH)-IGF axis. Based on regulatory patterns and co-localization of leptin and IGFs in the liver, we hypothesized that leptin might regulate the growth axis and thereby help coordinate somatic growth with energy availability. Using a hybrid striped bass (*Morone saxatilis × M. chrysops*) hepatocyte culture system to simulate autocrine or paracrine exposure that might exist within the liver, this study examines the potential for leptin to promote growth through regulation of IGF gene expression, or indirectly through a stimulatory effect on the GH receptors (GHR) that mediate GH-induced IGF expression. First, we confirmed that bovine GH (50 nM) has a classical stimulatory effect on IGF-I as well as IGF-II transcription in hepatocytes. Leptin stimulated *in vitro* GHR1 and GHR2 as well as IGF-I and II gene expression at 24 hrs, and GHR2 gene expression as early as 8 hrs exposure. We then cultured cells with submaximal leptin and GH concentrations together to test if they had a synergistic effect on IGF gene expression, possibly through increased GH sensitivity due to GHR upregulation by leptin. In combination, however, the treatments only had an additive effect on stimulating IGF-I mRNA despite their capacity to increase GHR mRNA abundance. This suggests that leptin’s stimulation of GHRs may be limited to enhancing transcription or
mRNA stability and not to inducing full translation of functional receptors. Leptin was then injected IP (100 ng and 1 ug/g BW) to test the in vivo regulation of hepatic IGF-I and GHR1 gene expression. A 100 ng /g BW leptin dose significantly upregulated IGF-I mRNA levels relative to controls at 24 hrs, substantiating the in vitro results. Leptin was ineffective in regulating GHR1 gene expression in vivo. These studies suggest that stimulation of growth axis component transcripts by leptin may be an important mechanism for coordinating somatic growth with states of positive energy in these and perhaps other fish, and furthermore represent the first evidence for regulation of GHRs by leptin in vertebrates.

**Keywords:** Leptin, growth hormone receptor, insulin-like growth factor, growth axis, teleost
INTRODUCTION

The growth hormone (GH) / insulin-like growth factor (IGF) axis is central to regulating somatic and skeletal growth in vertebrates [15, 39, 50]. In this system, circulating GH secreted by the pituitary stimulates production of IGF-I, a potent mitogen that is largely responsible for somatic growth, and IGF-II, which is less studied but may elicit similar actions in teleosts [11, 19, 41, 58]. The systemic or endocrine source of IGFs is thought to be derived primarily from the liver [15, 51, 58]. Stimulation of IGF production by GH is mediated by GH receptors (GHR), two distinct forms of which have been identified in fish [25], although it is not clear to what extent the individual receptor types mediate the growth promoting versus metabolic effects of GH [27, 47]. Expression of hepatic GHRs provides sensitivity to GH-induced IGF production during anabolic states, whereas the liver is desensitized during negative energy states due to downregulation of GHRs [44, 53]. Hence, declines in hepatic GHR expression lead to reduced IGF-I production and somatic growth, this despite elevations in systemic GH seen with fasting-induced catabolism [38, 42, 44]. The endocrine mechanisms that mediate changes in the GH-IGF growth regulatory axis under differential metabolic or nutritional states are not fully understood.

Leptin is an anorexigenic peptide hormone that circulates as an endocrine indicator of adiposity in mammals [1]. Extensive mammalian and clinical research shows that circulating leptin levels communicate the status of endogenous energy reserves to the brain and periphery and function to balance appetite and energy expenditure based on fat deposition.
Positive energy states are accordingly characterized by elevated leptin production, decreased energy intake and increased energy utilization [4].

Aside from having a similar function to mammals in suppressing appetite [12, 36, 65], little is known about leptin actions in ectotherms, including teleost fishes. Leptin is produced predominantly by the liver in fish [22, 24, 30, 52], an important source of both lipid and glycogen stores, but its regulation by nutritional state appears to vary depending on species. In the liver, leptin-like immunoreactivity in European sea bass [21] and gene expression of one or both salmonid leptin types (types A1 and A2) rose during feed-restriction [18, 52, 60], possibly contributing to the rise in circulating peptide in certain salmonids [29, 60]. Conversely, transcription of A1-type leptin in Atlantic salmon was upregulated in the visceral fat of fish fed to satiety, suggesting a possible link to adiposity [52]. Fasting, on the other hand decreased gene expression of *danio* leptin-b [22]. In striped bass, which appear to have only one leptin gene, hepatic leptin mRNA levels declined with fasting and increased during refeeding [65], similar to the expression patterns observed in hepatic IGF-I, IGF-II and both GHRs [44]. The proximity of leptin, GHR and IGF expression in the liver, along with their concordant regulation by nutritional state, suggests a possible regulatory relationship between hepatic leptin and the endocrine growth axis.

Because leptin levels tend to be high during positive energy states and low during negative energy states, it might reasonably serve as an endocrine switch for opportunistic physiological processes, such as growth, that require surplus energy. Chronic leptin treatment increases circulating IGF-I in leptin-deficient women and in mice *in vivo*, the latter accompanied by increased muscle mass and bone formation (Chan et al. 2008; Bartell et al.)
2011), suggesting leptin may modulate IGF-I during states of increased energy deposition and tissue growth. These actions of leptin may occur, in part, through direct regulation of the liver, insofar as leptin was shown to increase in IGF-I mRNA levels from isolated porcine hepatocytes [3]. Whether leptin regulates IGF-I production directly or through alterations in GH sensitivity or GHR expression is unclear. A potential role for leptin in modulating GHR and IGFs in teleost, the most abundant and diverse group of vertebrates, has yet to be established.

The aim of this study, therefore, was to examine if leptin might act alone or in synergy with GH to regulate hepatic GHR1, GHR2, IGF-I and IGF-II gene expression in the hybrid striped bass (HSB: *Morone saxatilis × M. chrysops*). Hepatocyte incubations show that leptin is a direct stimulator of GHR and IGF gene expression at the cellular level, which may represent an autocrine/paracrine relationship within the liver of these fish. Hepatic IGF-I gene expression was also elevated *in vivo* by leptin injection relative to controls. This is the first demonstration of GHR regulation by leptin in any vertebrate class and, in conjunction with its stimulatory effect on both IGFs, suggests that leptin may serve as an anabolic signal for coordinating production of key elements involved in somatic growth.

**MATERIALS AND METHODS**

**Animals**

Experimental HSB were obtained from Pungo Fisheries (Pinetown, NC) and housed in fresh water (hardness = 200 mg/L; alkalinity = 250 mg/L; temperature = 22° C;
photoperiod = 12L:12D) recirculating systems at North Carolina State University (NCSU; Raleigh, NC). Fish were fed a maintenance diet of 1.5% BW / day (Ziegler Silver 5mm pellets, Gardners, PA; 40% protein, 10% lipid). This regimen, which is half of the typical commercial production feeding level, was chosen in order to maintain a mild anabolic state with GHR and IGF gene expression levels intermediate to the maximum observed with higher ration levels and the minimum seen with fasting in vivo [43].

Fish used for hepatocyte cultures (200 g) had an average condition factor [weight (g) * 100 / length^3 (cm)] of 1.05. Fish used in the in vivo leptin injection study (mean weight 44.9 g ± 0.80 g; mean body weight ± SEM; average condition factor of 0.99) were housed in six 75-liter freshwater tanks, which were randomly assigned as control or treatment groups (N = 8 fish/group). Fish were not fed during the 24 hr experimental period. Protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC).

**Hepatocyte Cultures**

*In vitro* experiments on HSB hepatocytes were conducted in three separate trials to test the regulatory effects of GH and leptin on GHR and IGF gene expression. In the first experiment, cells were incubated in growth medium with 5 or 50 nM bovine GH (National Hormone and Peptide Program) to validate cells were responsive to stimulation by IGF-I and hence were functioning appropriately. This study was also done to evaluate whether GH might regulate IGF-II synthesis in *Morone*. In the second experiment, hepatocytes were incubated with 5 and 50 nM human recombinant leptin (Harbor-UCLA Medical Center,
Torrance, CA) for 8 or 24 hrs to test for effects on hepatic GHR1/2 and IGF-I/II gene expression. A third experiment was conducted to evaluate if leptin and GH might have a synergistic effect in regulating IGF gene expression. Lyophilized leptin was first dissolved in 500 µL of 0.1 mM PBS (pH 8.3) and this stock was then diluted into RPMI growth media to desired concentrations. We have shown in previous publications that human recombinant leptin is bioactive in reducing feeding in HSB [65] and inducing prolactin secretion from tilapia pituitaries [59].

Livers from two fish were used for each in vitro experiment in order to yield enough cells for up to six replicates per group (N = 4-6). Hepatocytes were harvested as described previously [34]. In short, after fish were lethally dosed with buffered MS222, the posterior intestinal vein was cannulated and the liver flushed of blood with calcium-free Hank’s buffered salt solution (HBSS) for 5 minutes using a peristaltic pump (2 mL/min), then digested in situ with HBSS containing type IV collagenase (Sigma, St. Louis, MO) for 10-15 minutes. The bulbus of the heart was nicked to ease the flow-through of solutions. After partial digestion, the liver was removed, finely chopped with a razor blade in a glass petri dish and slowly agitated in collagenase solution for 10 minutes more at room temperature. The digested liver was mechanically forced through a 260 micron mesh filter to remove structural tissue, allowed to drip through a second 60 micron mesh, and collected in a beaker on ice. The harvested cells were washed, allowed to recover on ice for one hour, then resuspended in RPMI 1640 growth medium containing L-glutamine (Gibco, Carlsbad, CA; 1% streptomycin/penicillin added) and plated in 24-well Falcon primaria plates. Cell viability was determined to be > 95% by the Trypan Blue exclusion dye test. Each well was plated
with 1.5 X 10^5 cells in 1 mL media and allowed to settle overnight. Formation of a cell monolayer was visually confirmed by microscopy after the 24 hr recovery period (Figure 1). Cells were incubated at 18°C in an air atmosphere. After recovery, media was removed and replaced by experimental media. At termination of the incubations, media was removed and replaced with TRI Reagent. The cell suspension was removed by pipette for nucleic acid isolation.

Lyophilized leptin was first dissolved in 500 µL of 0.1 mM PBS (pH 8.3) and this stock was then diluted into RPMI growth media to desired concentrations. We have shown in a previous publications that human recombinant leptin is bioactive in reducing feeding in HSB [65] and inducing prolactin secretion from tilapia pituitaries [59]. Other studies show that mammalian GH, including bovine, is bioactive in stimulating IGFs in various teleosts [17, 55, 63]. The treatment concentrations of peptides used in these experiments were based on similar in vitro studies using fish [46, 48].

**In vivo Effects of Leptin on GHR1 and IGF-I gene expression**

Human recombinant leptin (Harbor-UCLA Medical Center, Torrence, CA) was first dissolved in 0.1 mM PBS (pH 8.1) and then diluted with biological saline for injection. Fish were anesthetized with buffered MS 222 (Western Chemical, Ferndale, WA) and injected intraperitoneally with 50 µL saline (control) or leptin at doses of 100 ng and 1 µg / g body weight using a half-inch 25 gauge syringe. As a positive control, animals were also injected with a single dose (5 ug/g body weight) of bGH to assure that IGF-I responsiveness was intact after 24 hrs. Fish were again anesthetized using MS 222 and liver samples were taken
6 and 24 hrs post-injection. Liver was placed in RNAlater (Qiagen, Valencia, CA) and tissues were subsequently processed for measurement of hepatic GHR1 and IGF-I mRNA. Nucleic acid samples from this experiment were lost due to a freezer malfunction prior to measuring GHR2 and IGF-II mRNA levels.

**Nucleic acid isolation and cDNA synthesis**

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

**Quantitative RT-PCR gene expression measurement**

Relative GHR1, GHR2, IGF-I and IGF-II mRNA expression was determined by quantitative RT-PCR (qRT-PCR) according to our previously established protocols [44]. Gene-specific primers (Table 1) were designed using Vector NTI software (Invitrogen). Analysis was performed on an ABI 7300 HT Sequence Detection System, using Brilliant SYBR Green II master mix (Stratagene; La Jolla, CA), 1.5 µM primers, and 6 ng of cDNA in a total reaction volume of 25 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 gel visualization of a single PCR amplicon (4% agarose gel, 0.6 μg/ml ethidium bromide) and by direct sequencing of the amplicon for each gene (University of Chicago Cancer Research Center), as well as by dissociation curve analysis following qRT-PCR cycles. For qRT-PCR analysis, cycle threshold (Ct) values for experimental samples were transformed using a standard curve of serially diluted cDNA versus Ct values ($R^2 > 0.97$) and then normalized to reflect the amount of template cDNA per ng total RNA loaded into each reaction (cDNA / ng total RNA). These results were comparable to data normalized to 18S RNA and the housekeeping gene, β–actin. Gene expression data for each group is presented as a percentage of the control mean (% control) ± SEM.

Statistics:

Quantitative real-time PCR data from hepatocyte culture experiments with a single time point was analyzed by one-way ANOVA followed by Fisher’s LSD test for significant differences using Statistica 7.0 software (Stat Soft; Tulsa, OK). Data from leptin assays with two time points (treatment × time) were analyzed by two-way ANOVA followed by Bonferroni’s post hoc test using GraphPad Prism 6 software (La Jolla, CA). Gene expression data for the in vivo study was log transformed for ANOVA to satisfy Bartlett's test for equal variances. The threshold for statistical significance was set at $P < 0.05$. 
RESULTS

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

In the first study (Figure 2), HSB primary hepatocyte cultures were incubated with two concentrations of bovine GH (5 and 50 nM) for 18 hrs. Growth hormone increased mRNA levels of IGF-I and IGF-II in a dose-dependent fashion. The 50 nM concentration increased IGF-I by 16-fold \( (P < 0.001) \) and IGF-II mRNA by 4.3-fold \( (P < 0.001) \). In as much as GH is a classic stimulator of IGF-I synthesis and secretion, this experiment validates that the hepatocytes were viable and functional following the isolation and incubation conditions utilized for these investigations.

Leptin regulation of GHR and IGF gene expression in hepatocytes

In the second experiment, HSB primary hepatocyte cultures were incubated with increasing concentrations of human recombinant leptin for 8 or 24 hrs to test whether leptin regulates hepatic mRNA expression of GHRs and IGFs. Leptin upregulated *in vitro* mRNA levels of both forms of GHR and IGF in hepatocytes (Figure 3). GHR2 gene expression was sensitive to both the low and high leptin concentrations by 8 hours, respectively rising 3.7 and 4.2-fold above control levels \( (P < 0.001) \). After 24 hrs, the GHR2 response to 50 nM leptin was maintained at a 3.4-fold higher level than controls \( (P < 0.001) \). The highest concentration of leptin also increased GHR1 mRNA levels by 5.4-fold during 24 hr incubations \( (P < 0.001) \). The high leptin concentration also directly upregulated IGF-I
mRNA by 3.3-fold \( (P < 0.001) \) and IGF-II by 2.2-fold \( (P < 0.001) \) after 24 hrs compared to untreated control cells.

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

In the third experiment, hepatocytes were incubated with GH, leptin, or both proteins for 24 hrs (Figure 4) to determine if the two peptides might work synergistically in stimulating IGF mRNA expression, namely through upregulated GHR expression by leptin, which would theoretically enhance responsiveness of IGF to GH stimulation. Leptin and GH were used at half the concentration of previous experiments (25 nM; Figures 2 and 3) to reduce maximal stimulation that could potentially mask synergistic or additive effect of the combined hormones.

There was no effect by GH alone on regulating mRNA levels of either GHR compared to controls. Leptin alone increased GHR1 (2-fold; \( P < 0.05 \)), but not GHR2 mRNA levels. Levels of GHR1 and GHR2 mRNA were elevated by about 2-fold (up 84% and 115% respectively; \( P < 0.05 \)) in the group co-incubated with leptin and GH. The level of IGF-I mRNA was moderately increased above controls by leptin alone (2-fold; \( P = 0.18 \)) and significantly by GH alone (3-fold; \( P < 0.05 \)), as well as by the combination of leptin and GH treatments (4.8-fold; \( P < 0.001 \)). The fold-increase in IGF-I gene expression elicited by leptin and GH together was approximately the sum of the fold changes elicited by the peptides individually. At the 25 nM dosage used no effect on IGF-II mRNA levels was detected \( (P > 0.05) \) by individual or combined hormone treatments.
In vivo effect of leptin on hepatic GHR1 and IGF-I gene expression

Hepatic GHR1 and IGF-I mRNA levels were measured at 6 and 24 hrs following 0 ng, 100 ng or 1 ug/g BW leptin injection (Figure 5). Hepatic IGF-I mRNA levels were significantly elevated by the low dose (up 10-fold; **P < 0.01), but not by the high dose of leptin (up 2.5-fold; P > 0.05) relative to controls at 24 hrs. Although the higher leptin dosage raised hepatic GHR1 mRNA levels by 2.3-fold at 6 hrs, the effect was not significant (P = 0.12). A single injection of bGH significantly increased hepatic IGF-I mRNA by 6-fold after 24 hrs (data not shown) confirming animals were responsive to hormone treatment in this experiment.

DISCUSSION

This study explored the possibility that leptin might modulate growth axis signaling through regulation of GHR and IGF gene expression in the liver. We show GH increases IGF-I and IGF-II mRNA in hepatocytes, but is ineffective in regulating either GHR1 or GHR2. Leptin enhanced gene expression of both forms of IGF and GHR in vitro. Evidence suggests this hormone may serve as an autocrine or paracrine stimulator of IGF-I and IGF-II production directly and via its ability to upregulate GHR transcription to enhance GH sensitivity.

In vivo and in vitro studies have clearly established that GH acts in its classic manner to stimulate growth through regulation of IGF-I synthesis and secretion in teleosts through both endocrine and paracrine mechanisms [31, 48, 54, 55]. Studies also suggest that IGF-II,
whose synthesis predominates in hepatic tissue throughout the life of teleosts [11, 19, 41, 44, 58] may be similarly regulated by GH in a range of fishes [9, 20, 35, 40, 45, 49, 55, 58, 61, 63]. The degree of effectiveness of GH in stimulating production of the two forms of IGF may vary, however. Native GH increased IGF-I mRNA and IGF-II mRNA levels by a similar magnitude in salmon hepatocytes [48]. By contrast, native GH (0.5-50 nM) stimulated IGF-II by around 8-fold while it increased IGF-I transcript by 2-fold in tilapia hepatocytes [45]. Similar to the response observed for IGF-I, we found that GH is also an effective stimulator of IGF-II gene expression in Morone. Evidence suggests, however, that the tissue IGF-I responsiveness to GH is greater than that for IGF-II in HSB. Indeed, a GH concentration as low as 25 nM was effective in stimulating IGF-I, but not IGF-II gene expression (Figure 4), while the potency with which 50 nM GH increased IGF-I mRNA levels was four times greater than that seen for IGF-II (Figure 1). Whether the discordance in in vitro responsiveness of the two IGFs to GH stimulation seen between Morone and other species reflects fundamental interspecies differences, variable effectiveness of heterologous and native peptides, or different experimental conditions is uncertain. Nevertheless, the present study demonstrates that hepatocytes used in these studies are functionally intact and responsive to GH following isolation, and that GH exerts direct effects at the level of the hepatocyte to stimulate production of IGF-II in Morone.

In fish, leptin is expressed predominantly in the liver where the majority of GH-mediated IGFs are produced. Hepatic leptin [65], GHR1, GHR2, IGF-I and IGF-II [44] show similar expression patterns under altered metabolic states in HSB, suggesting a possible interaction within the liver. Leptin was therefore considered as a possible candidate for
regulating these key elements of the hepatic endocrine growth axis. In order to test if leptin regulates canonical growth axis transcripts in this context, hepatocytes were incubated with 5 or 50 nM leptin and collected after 8 or 24 hrs. Interestingly, we found that leptin not only increased mRNA levels of both GHRs, but that transcription of both IGFs was also directly upregulated in the absence of exogenous GH (Figure 3). The response of GHR2 was more sensitive than GHR1 to leptin treatment, showing upregulated transcript levels at a lower leptin concentration and at an earlier time point, although the fold-change in GHR1 mRNA upregulation was nearly 2-fold greater. To our knowledge, these results represent the first account of leptin regulating GHRs in any vertebrate and it regulating IGFs in a teleost, suggesting leptin may have a previously unrecognized influence on somatic growth through upstream control of the hepatic growth axis.

In order to test whether the increase in GHR mRNA levels by leptin contributes to GH sensitivity, hepatocytes were incubated alone or in combination with submaximal leptin and GH concentrations (25 nM; Figure 4). The elevated mRNA levels observed of both GHRs in this trial were likely attributable to the leptin treatment component, as GH treatment alone did not affect mRNA levels of either receptor. While the reason for the lack of effect by leptin alone on GHR2 is uncertain, the combined treatment group did exhibit the anticipated elevation in GHR2 mRNA levels. Individual 25 nM leptin and GH treatments upregulated IGF-I transcript to lesser extents than the 50 nM concentrations used in the previous trial, while co-incubation with both treatment peptides together raised IGF-I mRNA levels by the sum of the marginal increases elicited by the hormones alone. This additive effect of the combined treatments on IGF-I gene expression suggests that the stimulatory
effect of leptin on GHRs may be limited to the transcriptional level, at least under the culture conditions utilized here. Had translation of new functional GHRs taken place, the presence of GH should theoretically have had a synergistic effect on IGF-I expression [32].

In contrast to IGF-I, IGF-II transcript levels were not altered by any of the treatments including the co-incubation. The unresponsiveness of IGF-II is likely a result of the lower, 25 nM-concentration used for the two peptides. It would appear that a minimal threshold concentration somewhere between 25 and 50 nM of both treatment peptides may be necessary to directly upregulate IGF-II mRNA, as 5 nM had already been shown to have no significant effect. The fact that IGF-II gene expression was not upregulated despite elevated GHR mRNA levels in the presence of GH further substantiates that leptin does not promote sensitivity of the cells to GH through enhanced production of GHR protein, but rather may be working to increase GHR transcription or mRNA stability. It is possible that additional anabolic factors present *in vivo* such as certain metabolic substrates or insulin [8, 23] may ultimately be needed in conjunction with leptin to induce the full translation of functional GHRs in liver.

In support of the *in vitro* evidence, 100 ng/g BW leptin injection upregulated hepatic IGF-I mRNA levels *in vivo* at 24 hrs relative to controls (Figure 5). Similar responses have been observed in mammals, in which chronic leptin treatment raised hepatic IGF-I mRNA levels in pigs [3], as well as circulating IGF-I levels in leptin deficient mice [7] and humans [10]. Control fish experienced a drop in IGF-I gene expression at 24 hrs compared to the earlier time point (Figure 5), likely due to feed restriction [50]. The supplemental leptin treatment may therefore have buffered the response to fasting and contributed to the
maintenance of hepatic IGF-I mRNA levels. It is not clear, however, why the lower leptin
dose had a greater effect in stimulating IGF-I mRNA, although a similar trend was
encountered when testing the effects of leptin on feeding in HSB with similar treatments, in
which the higher dose had a marginally weaker orexigenic effect [65]. Leptin also had a
biphasic effect in regulating its own receptor in vitro in rat hypothalamic explants, with
attenuated stimulation of OBR occurring at high concentrations until the effect was
ultimately reversed [14]. It is therefore possible that the range of dosages used in this trial
straddled an upper limit and became ineffective at the high dose.

Although the mechanism by which leptin stimulates IGF-I gene expression in vivo is
unknown, the hormone is likely to exert its effects, in part, through direct regulation of
hepatocytes, as demonstrated here, as well as indirectly, possibly through enhanced GH
signaling [6, 33, 57] or via other regulatory pathways. Exogenous leptin treatment did not
significantly alter hepatic GHR1 mRNA levels and data for GHR2 was not available.
Catabolic factors, such as somatostatins [62] and cortisol [56], which are known to inhibit
transcription and translation of GHRs in fish, may have dampened the response of growth
axis signaling in vivo, but would not likely have been convoluting factors in the in vitro
experiment using isolated cells.

The stimulation of hepatic GHR and IGF gene expression by leptin in these
experiments suggests that growth may be coordinated with nutritional status by leptin.
Transcript and plasma levels of both IGFs fluctuate with growth under disparate metabolic
states in a variety of fish [5, 19, 41, 58, 64]. In HSB, fasting downregulates gene expression
of both GHRs along with IGF-I and II in liver, while refeeding reversed the effects and was
coincident with rising IGF-I plasma levels and hyperanabolic growth [44]. Similar states of hepatic GH resistance during fasting [13, 53] and the return of sensitivity during refeeding [38, 56] is evident in other fish as well. Leptin’s role as an endocrine indicator of energy reserves and nutritional state is well documented in mammals [1, 4]. The hormone rises as energy deposition rises and declines during states of fasting [2, 16]. Our previous work in HSB, shows hepatic leptin mRNA levels also decrease during long-term fasting and rise during refeeding [65], which is similar to responses seen in hepatic GHRs and IGFs. Studies in other fish species support this finding [22, 26, 28, 37], while others show the opposite trend [18, 21, 29, 60] or no long-term relationship [24] between leptin and nutritional state. If leptin is reflective of nutritional state, then the current study suggests that leptin may function to maintain hepatic GHR and IGF gene expression during anabolism when leptin production is expected to be high. While it does not directly address the cause of hepatic GH resistance during fasting due to the potential active downregulation of GHRs or inhibition of GHR signaling by catabolic factors, it is possible that diminished leptin production during fasting contributes to reductions in GHR gene expression. Thus it would appear leptin might contribute to a coordinated growth response to nutritional state, namely by increasing IGFs directly or indirectly through enhanced GHR expression in the liver as energy resources become more available.

In summary, these studies show that leptin and GH exert direct effects at the level of the hepatocyte to stimulate IGF-I and II gene expression in HSB. In addition to upregulating IGF-I and IGF-II transcripts in vitro, leptin also stimulates accumulation of hepatic IGF-I mRNA in vivo, indicating for the first time in a teleost that leptin may influence expression
of these key growth regulatory hormones in the liver. The studies also establish for the first time in vertebrates, a novel function for leptin in increasing GHR1 and GHR2 gene expression and the potential to modulate hepatic GH sensitivity. Considering leptin is predominantly produced in the liver of these and other fish, we postulate that the hormone may serve as an important paracrine or autocrine modulator of hepatic GHR and IGF expression and ultimately somatic growth.

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FIGURES AND TABLES

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

<table>
<thead>
<tr>
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<th>5’-Forward-3’</th>
<th>5’-Reverse-3’</th>
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<tbody>
<tr>
<td>IGF-I</td>
<td>AGCCACACCCCTCTCCTACTACTGCTG</td>
<td>CAGGTGCAACGTACATCTCCAGGC</td>
</tr>
<tr>
<td>IGF-II</td>
<td>AAACAACAGACGGACCCAGAA</td>
<td>AGCAAGTTGAGGTCACAGCTA</td>
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<tr>
<td>GHR-1</td>
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<tr>
<td>GHR-2</td>
<td>TTGAGGAGCACAACGACAGACTGG</td>
<td>TGTTTCCTCAGGTGTCAGCAGCA</td>
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Figure 1. Hepatocyte culture monolayer at 20X and 40X (inset) magnification. Cells were plated at a concentration of $1.5 \times 10^5$ cells in 1 mL media per well.
Figure 2. Effect of 5 and 50 nM bovine GH on IGF-I (top) and IGF-II (bottom) mRNA levels in HSB hepatocytes incubated for 18 hrs. Gene expression levels are expressed as a percentage of control levels (mean ± SEM.; N = 5-6 wells/group). Asterisks denote significant differences between treatment groups and control group (**P < 0.001).
Figure 3. Effect of 5 and 50 nM human recombinant leptin on GHR and IGF mRNA levels in HSB hepatocytes incubated for 8 and 24 hrs. Gene expression levels are expressed as a percentage of control levels (mean ± SEM; N = 4-6 per group). Asterisks denote significant differences between treatment groups and the control group at each time point (**P < 0.001).
Figure 4. Effects of leptin and GH treatment (25 nM) on GHR and IGF mRNA levels in HSB hepatocytes incubated for 24 hrs. Gene expression levels are expressed as percent control (mean ± SEM; N = 4-5 per group). Different letters denote significant differences between groups ($P < 0.05$).
Figure 5. Effect of IP leptin injection (0, 100 and 1000 ng/g BW) on *in vivo* hepatic IGF-I (top) and GHR1 (bottom) mRNA levels. Samples were taken 6 and 24 hrs post-injection. Gene expression data are expressed as cDNA/ng total RNA (mean ± SEM; N=6-8 per group). Asterisks denote significant differences between treatment groups and control group (**P < 0.01).
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CHAPTER 3

Cloning of a leptin receptor in striped bass (*Morone saxatilis*) and its regulation by metabolic states in the brain
ABSTRACT

Circulating leptin concentrations indicate energy reserve levels in mammals and consequently regulate a diverse range of physiological activities, including feeding. Leptin’s actions are mediated peripherally and centrally by the leptin receptor (OBR), which is expressed in regions of the forebrain that produce neuropeptide Y (NPY), a potent orexigen that is suppressed by leptin. However, the OBR of only a few fish has been cloned and their regulation in these or other ectotherms is poorly understood. We have recently shown that hepatic leptin expression declines during catabolism and rises during anabolism and that the hormone exhibits anorexigenic properties in the hybrid striped bass (HSB, Morone chrysops x M. saxatilis). It is unknown, however, whether leptin sensitivity or expression of the receptor in the central nervous system might be subject to modification by nutritional state. This study evaluates the gene expression profile of central OBR under different metabolic states and assesses its potential corollary to NPY expression in regions of the brain that control appetite in fish. The putative long form OBR in striped bass was cloned and found to be ubiquitously expressed throughout the body, underscoring the systemic relevance of leptin. In order to test the hypothesis that metabolic state might affect central OBR expression, OBR mRNA in brain was measured by qRT-PCR in fish subjected to fasting (24 days) and refeeding (8 days). Levels of the OBR transcript in the hypothalamus were unaffected by feeding regimen in HSB. By contrast, OBR mRNA levels were elevated in the telencephalon on days 6 and 16 of fasting before returning to control levels on day 24, then rose again 8 days after refeeding. Hypothalamic NPY mRNA levels did not differ between
continuously fed and fasted/refed animals, while gene expression in the telencephalon increased transiently, peaking at 6 days of feed restriction and returning to levels of fed control fish by day 16. Although a concordant upregulation of both the NPY and OBR genes in the telencephalon occurred during the early phases of feed restriction, their relative abundance showed only a weak correlation when assessed over the entire course of the experiment ($R^2 = 0.20; P < 0.01$). These results show an increase in NPY gene expression in the telencephalon during fasting that is consistent with its potential function as an orexigen in HSB. Elevated OBR gene expression in the telencephalon was also found during fasting, when leptin levels are expected to be low, suggesting a mode of adjusting receptor sensitivity to low circulating ligand levels. The eventual decline in mRNA levels of these genes prior to refeeding suggests an adaptation to starvation that might prevent excessive energy expenditure toward foraging when reserves are limited, but that simultaneously suppresses anorexigenic signaling by leptin in anticipation of refeeding. The re-elevation in OBR gene expression during refeeding may then reinstate leptin regulation of appetite or other central anabolic functions of this pleiotropic hormone.

**Keywords**: Leptin, OBR, neuropeptide Y, *Morone*, teleost, catabolism
INTRODUCTION

Leptin is an anorexigenic hormone produced by energy storing tissues that circulates as a reflection of endogenous energy reserves, with high and low plasma levels of leptin representing positive and negative energy status, respectively [2]. This endocrine indication of energy availability, or lipostat, helps maintain homeostasis by centrally regulating energy uptake and expenditure [2]. Leptin’s actions are mediated by the leptin receptor (OBR), which is differentially expressed in regions of the brain and throughout peripheral tissues that require information about energy availability [3, 47].

The OBR is abundantly expressed in the mammalian hypothalamic feeding center located in the arcuate (ARC) nucleus, which houses neurons that produce central orexigens and anorexigens [19, 25, 34, 50], and that have afferent networks projecting into nuclei that drive or suppress appetite [10]. Although fish do not have an ARC, analogous regions in the hypothalamus and telencephalon may be involved in the regulation of feeding based on the expression patterns of OBR [26, 31, 48] and appetite-regulatory neuropeptides including neuropeptide Y [NPY: 14, 30, 36, 45], a potent appetite stimulant in vertebrates including fish, and whose expression increases in response to fasting [36, 43, 45].

In accord with its anorexigenic property, leptin inhibits NPY’s stimulatory effects on feeding in mammals [7, 41, 46], acting through centrally expressed OBRs co-located on NPY-producing neurons [19, 22, 34]. Exogenous leptin also reduces feeding in a variety of fish [35, 52, 54] and clawed frog [16], supporting a conserved function in regulating energy homeostasis for lower vertebrates. In goldfish, leptin treatment negates the orexigenic effect
of exogenous NPY, likely by downregulating NPY gene expression in the hypothalamus and
telencephalon, as shown by ICV leptin injection [52] and in vitro [1], with central sensitivity
to leptin suggesting the presence of active OBRs in these brain regions. Due to the limited
number of fish OBR genes that have been cloned, however, little is known about how it may
be involved in mediating leptin signaling in the brain, and particularly how its expression
pattern relates to metabolic state.

The gene encoding the receptor produces several splice variants in mammals
including the functional “long” form (OBRb) as well as multiple “short” forms, which
include the extracellular binding domain of OBRb, but not the intracellular motifs necessary
for signaling [2]. Truncated receptors may function as transports across the blood-brain
barrier [8, 12], while the shortest form, which lacks the transmembrane domain, is thought to
be secreted as a binding protein that alters ligand half-life and tissue availability [28, 29, 32].
Intracellular signal transduction by leptin through the long form of its receptor utilizes a
JAK-STAT pathway common to class-I cytokine receptors [3, 21].

The teleost homolog of the mammalian OBR has been cloned in medaka [55],
takifugu [26], danio [31], carp [13] and Atlantic salmon [40]. Similar to its ligand, fish
OBRs share low peptide sequence homology and have consequently been difficult to clone
and characterize. Further impeding comparisons with the better-known mammalian leptin
system is the existence of multiple, differentially regulated leptin genes in some basal fish
lineages [24, 40, 48] and the apparent lack of short receptor splice variants in medaka,
takifugu and danio, although truncated OBRs have been found in salmon and carp. Although
hypothalamic expression of OBR increases during fasting in mammals [9], no discernable
changes in central receptor gene expression were found in response to nutritional state in salmon [40] or goldfish [48], the only teleosts examined to date. Nutritional state nonetheless appears to regulate its ligand, leptin, or at least one form in cases where paralogs have been found in fish [20, 23, 40]. In the hybrid striped bass (HSB, *Morone chrysops x M. saxatilis*), leptin suppresses appetite and its expression declines with fasting and increases during anabolism in the liver, the primary site of hormone production (Won et al. 2012). This response suggests that receptor expression might be sensitive to nutritional state in the central feeding regions of the brain. The present studies were therefore undertaken to clone and characterize the OBR in striped bass, a representative perciform of the largest and most diverse order of vertebrates, and assess whether its expression levels, along with those of NPY, are altered in the hypothalamus and telencephalon under catabolic and anabolic states induced by fasting and feeding, respectively.

**MATERIALS AND METHODS:**

**Nucleic acid isolation and cDNA synthesis**

For cloning and quantitative RT-PCR (qRT-PCR), total RNA was isolated from tissue samples using TRI Reagent (Molecular Research Center; Cincinnati, OH) and the manufacturer’s procedure. RNA purity and concentration were determined by spectrophotometry using a Nanodrop ND-1000 (Nanodrop Technologies; Wilmington DE) before and after DNase treatment (DNA-free®; Ambion; Austin, TX). Absorbance ratios at 260:280 nm (A_{260/280}) of all samples ranged from 1.8–2.0. The quality of the RNA isolations
was confirmed by gel electrophoresis (1% agarose, 0.6 μg/ml ethidium bromide).

Complementary DNA (cDNA) was synthesized from 1 μg of DNase-treated total RNA using a High Capacity cDNA Reverse Transcription kit and the manufacturer’s protocol (Applied Biosystems, Foster City, CA).

**Molecular cloning and sequencing**

A partial coding sequence for *Morone* OBR was obtained by PCR using pooled striped bass liver and brain cDNA as template and degenerate primers designed from teleost (*takifugu*, medaka, Atlantic salmon and *danio*) OBR mRNA coding sequences (CDS). Isolated amplicons were cloned into a pGEM cloning vector (Invitrogen) and transformed into JM109 series competent cells (Promega). The full coding sequence was determined by expanding the partial OBR sequence using RACE-PCR (First Choice RNA-ligase mediated RACE kit; Ambion) and the manufacturer's protocol. Contiguous sequence analysis was performed for all obtained clones using Vector NTI Contig Express software to generate a full-length leptin cDNA sequence. Two primer sets were designed to confirm the *in silico* contig analysis of the full CDS sequence: FP1 5’–GATGCATATTTTCTGGTGCCC-3' and RP1 5’- CTCCCATGTGACCGTCAGGA–3'; FP2 5’–CAGAAACCTGCACCATCCAGC-3' and RP2 5’-CCTCTGCACGATAGTCCATTCC–3'. The PCR cycling conditions for the OBR sequence analysis were: 40 cycles at 56°C (primer set FP/RP1) or 58°C (FP/RP2) for 1 minute of extension time per cycle and a 5 minute final extension period. Two clones from each amplicon were submitted to the University of Chicago Cancer Research Center for forward and reverse sequencing from pGEM cloning vectors using universal M13 primers.
Feature map, phylogeny and vertebrate alignment

The translated *Morone* OBR peptide sequence was compared to sequences representing the long form OBR of known fish and other vertebrate classes found in the GenBank protein database (Japanese puffer fish, *Takifugu rubripes*: GenBank accession no. BAG67079.1; marine medaka, *Oryzias melastigma*: ABC86922.1; zebrafish, *Danio rerio*: AAY16198.1; Atlantic salmon, *Salmo salar*: BAI23197.1; chicken, *Gallus gallus*: AAF31355.2; clawed frog, *Xenopus tropicalis*: ABD63000.2 and human, *Homo sapiens*: AAB09673.1). Alignment and analysis of vertebrate peptide sequence homology was performed using the ClustalW2 algorithm (Larkin et al., 07; http://www.ebi.ac.uk/Tools/msa/clustalw2). The signal peptide was predicted by SignalP 3.0 software (http://www.cbs.dtu.dk/services/SignalP/). Fibronectin and immunoglobulin domains and the glutamic acid-rich region were identified by PROSITE motif recognition software [42] and the blastp 2.2 algorithm [6]. The cytokine receptor homology region (CRH), transmembrane domain and JAK/STAT boxes were identified or estimated through alignment comparison with the documented *takifugu* sequence [27], which had the closest sequence identity to striped bass OBR. The phylogenetic tree of known teleost and representative tetrapod class OBR peptide sequences was constructed using maximum likelihood inference with Phylogeny.fr software [18; http://www.phylogeny.fr/version2_cgi/index.cgi]. The phylogenetic analysis used the same peptide sequences as the alignment analysis with the addition of two more cyprinids (Crucian carp, *Carassius carassius*: ADZ75460.1; goldfish, *Carassius auratus*: HQ993048.1) as well
as human growth hormone receptor (GHR; NM_000163.4) as an outgroup. Node strength is indicated by a bootstrap value out of a possible 100.

Tissue distribution

Using RT-PCR, HSB leptin mRNA expression was evaluated in multiple tissues; brain, pituitary, fat, liver, muscle, gut, gonad, kidney, heart and gill. Fifty nanograms of cDNA derived from total RNA were amplified using GoTaq® (Promega, Madison, WI) polymerase and buffers, with 10 mM dNTP mix and 10 μM primer concentrations. For tissue distribution, PCR primers were designed from the putative leptin receptor coding sequence (forward: 5’- AACCCATCTACTTGTCCTCCTACA -3’; Reverse: 5’-AGTGGTACCAGAACTGACATTGG -3’). The PCR cycling conditions for the OBR tissue distribution analysis were: 40 cycles at 57°C for 1 minute of extension time per cycle and a 5 minute final extension period. The housekeeping gene β-actin was screened by PCR in tandem on the same samples to verify the integrity of cDNA template across tissues (Forward: 5’-AGC CAA CAG GGA GAA GAT GA–3’; Reverse: 5’-TGG GGC AAT GAT CT TT GAT CT-3’). The PCR for β-actin was run at 54°C. Amplicons from both genes were visualized by gel electrophoresis (3% agarose, 0.6 ug/ml ethidium bromide).

Leptin receptor and NPY regulation by nutritional state

Juvenile (68.5 g ± 2.6 g; mean starting body weight ± SEM) HSB were obtained from the Tidewater Research Station (Plymouth, NC) and housed in two identical recirculating systems, each with eight 40 L tanks in fresh water (hardness = 170 mg/L; alkalinity = 250
mg/L; temperature = 22°C) at North Carolina State University (Raleigh, NC). Fish were fed 3% BW ration daily (Ziegler Silver, Gardners, PA; 40% protein, 10% fat) for 14 days prior to the start of the experiment. During the experiment, control fish were fed for 32 days and treatment fish were fasted for 24 days and then refed ad libitum for 8 days. The control and treatment groups were evenly distributed between the systems and one tank per group at random was sampled at each time point (N = 7 fish/group). Procedures followed NCSU Institutional Animal Care and Use Committee guidelines.

Fish were sampled at time 0, day 6, day 16 and day 24 (representing fasting in treatments fish), and at day 32 (8 days of refeeding). Fish were anesthetized (tricaine methanesulfonate, MS 222; Argent Chemical Laboratories, Redmond, WA), individually weighed and subsequently killed by decapitation. Visceral adipose tissue and liver were individually weighed for calculation of hepatosomatic (HSI) and adiposomatic (ASI) indices [(organ weight / body weight) × 100]. Hypothalamus and telencephalon samples for quantitative RT-PCR measurement of cDNA converted mRNA were dissected and preserved in RNAlater (Qiagen), held at room temperature for four hours and then refrigerated overnight to allow thorough permeation into tissue prior to freezing. Using anatomical features mapped from the closely related European sea bass for reference [14], the hypothalamus was excised to include the preoptic region associated with NPY production, the nucleus lateralis tuberis, and telencephalon was sampled in its entirety.
Quantitative RT-PCR of leptin mRNA abundance

Central OBR and NPY mRNA expression was determined by quantitative RT-PCR according to our previously established protocols [38, 39]. Gene-specific qRT-PCR primers for HSB leptin receptor (forward: 5’- AAC CCA TCT ACT TGT CCC CCA TA -3’; reverse 5’- AGT GGT ACC GAA ACT GAC ATT GG -3’) and NPY (forward: 5’- CAC TAC TCT GCC TGG GCG CC -3’; reverse 5’- GAC TGT GGA AGC TGG TCT GTG CTT -3’) were designed using Primer Express (Applied Biosystems; Foster City, CA) software. Real-time PCR analysis was performed on an ABI 7900 HT Sequence Detection System, using Brilliant SYBR Green II master mix (Stratagene; La Jolla, CA), 1.5 µM primers, and 4 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 assessed using two negative controls: sterile water used (No-Template Control; NTC) and DNase-treated RNA used as template (No-Amplification Control, NAC). Primer specificity was verified by direct sequencing of PCR amplicons, as well as by melting curve analysis during qRT-PCR and amplicon visualization (4% agarose gel, 0.6 µg/ml ethidium bromide). Cycle threshold (Ct) values for experimental samples were transformed using a standard curve of serially diluted cDNA versus Ct values ($R^2 = 0.97$) and normalized to reflect the amount of cDNA target template per ng total RNA (ng cDNA/ng total RNA). Gene expression data was adjusted to reflect the fold change compared to control levels at the earliest time point of the experiment.
Statistics

OBR and NPY mRNA qRT-PCR data was analyzed by two-way factorial ANOVA (treatment × time) followed by Fisher’s LSD test for significant differences. Energy indices were compared by one-way ANOVA. Correlations between gene expression data and energy indices were analyzed by regression analysis. Statistical analyses were performed using Statistica 7.0 software (Stat Soft; Tulsa, OK). The threshold for statistical significance was set at $P < 0.05$.

RESULTS

Striped bass leptin sequence characterization and comparative analysis

A full-length cDNA sequence of Morone leptin was determined by RACE-PCR, yielding a 3459 base pair (bp) cDNA coding sequence. It was not possible to determine the intron sites without comparing the coding sequence to a genomic sequence, which is not available for this species. No transcript splice variants were evident from RACE, PCR or melting curve analysis. The translated striped bass OBR protein has 1152 amino acids (aa), which is similar to the size of the long form of the receptor homolog in other vertebrate classes (~1100 aa). All functional features found in tetrapod and other fish OBRs were identified in the striped bass sequence (Figure 1), including an N-terminus signal peptide, three fibronectin type 3 domains (FN3.1-3.3), a ligand-binding immunoglobulin (Ig) C2-like domain and at least one of two repeated tryptophan-serine (WSXWS) motifs flanking the cytokine receptor homology (CRH) domain in the extracellular portion of the receptor, as
well as JAK/STAT boxes in the intracellular domain, indicating the functional long form which has signal transducing capability.

The full striped bass OBR peptide sequence had higher homology with fish (takifugu = 63%, medaka 50%, Atlantic salmon = 46% and danio = 40%) and relatively lower homology with more evolutionarily distant tetrapods (human = 23%, chicken = 23% and clawed frog = 20%). The sequence for the putative Morone CRH domain, or putative leptin binding region, was estimated from an alignment with the full takifugu peptide sequence [27] and had similar amino acid homology with other vertebrates as that of the full peptide (Figure 2).

Phylogenetic analysis (Figure 3) shows that the full perciform Morone OBR peptide sequence groups among those of other teleosts, with the closest relationship to takifugu (tetraodontiform) and medaka (beloniform), followed by Atlantic salmon (salmonid). A separate clade was formed by the cyprinids among the teleost groupings. The analysis reflects the evolutionary relationship between taxa, with teleosts grouping separately from tetrapods (amphibian, bird and mammal).

**Tissue distribution and effects of feeding status on OBR mRNA expression**

Tissue distribution (Figure 4) of OBR transcript was analyzed by reverse transcription PCR using the housekeeping gene β-actin as a control for loading of the cDNA template in each tissue sample. Leptin receptor mRNA expression was detected in telencephalon (T), hypothalamus (Hy), pituitary (P), fat (F), liver (L), muscle (M), testis (Te), ovary (O), kidney (K), heart (He), Pyloric gut (Gu), skin (Sk), spleen (Sp) and gill (Gi).
The regulation of OBR mRNA in the hypothalamus and telencephalon was examined during 24 days of fasting and 8 days of refeeding (Figure 5) to determine if gene expression was regulated by metabolic state, possibly in conjunction with feeding behavior influenced by leptin in these central regions [54]. In the hypothalamus, OBR gene expression did not differ between control and treatment groups at any time point. In telencephalon, OBR mRNA in treatment groups was significantly elevated over controls after 6 (treatments = 2.21 ± 0.25, controls = 1.15 ± 0.41 ng cDNA/ng total RNA; P < 0.05) and 16 days (treatments = 2.49 ± 0.24, controls = 1.54 ± 0.35 ng cDNA/ng total RNA; P < 0.05) of fasting and again a week after refeeding (treatments = 1.54 ± 0.40, controls = 1.09 ± 0.27 ng cDNA/ng total RNA; P < 0.05).

In the hypothalamus, NPY gene expression was not significantly affected by fasting. In the telencephalon, NPY mRNA levels in the treatment group on day 6 of fasting were elevated 1.5 fold over fed controls (treatments = 1.71 ± 0.08, controls = 1.17 ± 0.09 ng cDNA/ng total RNA; P < 0.05), but returned to control levels thereafter during fasting and refeeding. NPY and OBR mRNA levels showed a weak, albeit significant correlation in both brain regions (Figure 5B; R² = 0.20 in telencephalon and 0.09 in hypothalamus) over the course of the feed study.

Energy indices dropped during feed restriction (Figure 6) in treatments, indicating catabolic processes on both adipose and liver stores. ASI in fasted fish dropped to about a quarter of control levels after 24 days (treatments = 0.51 ± 0.10, controls = 2.14 ± 0.15; P < 0.05) and did not rebound after refeeding (treatments = 0.62 ± 0.12, controls = 2.22 ± 0.20; P < 0.05). HSI of treatment fish continuously declined and was lower than that of control
levels throughout the fasting period [day 6 (treatments = 2.11 ± 0.09, controls = 2.97 ± 0.07; 
P < 0.05), day 16 (treatments = 0.73 ± 0.06, controls = 3.54 ± 0.13; P < 0.05) and day 24 
(treatments = 0.61 ± 0.09; controls = 2.84 ± 0.15; P < 0.05)]. The HSI subsequently 
rebounded after refeeding to levels that exceeded that of control fish fed throughout 
(treatments = 2.98 ± 0.18, controls = 2.32 ± 0.17; P < 0.05). There were no significant 
correlations between energy indices and OBR gene expression in either hypothalamus or 
telencephalon (data not shown).

**DISCUSSION**

In this study, a single, long-form OBR gene was cloned in striped bass and its 
transcript measured in the hypothalamus and telencephalon during fasted and fed conditions 
to determine how metabolic state might effect receptor gene expression, possibly as a means 
of modulating central leptin sensitivity during metabolic shifts. Currently, little is known 
about teleostean leptin or its role in lower vertebrates. Similar to mammals, leptin expression 
increases with anabolism and exogenous leptin suppresses appetite in HSB [HSB: 54] and 
other fish [17, 35]. Because the orexigenic peptide hormone, NPY, may be regulated by 
leptin in fish [52], its gene expression was also measured to determine if a relationship with 
OBR might exist in regions of the brain associated with appetite regulation. Findings support 
that characteristic features of the ubiquitously expressed OBR transcript and its translated 
peptide sequence are conserved between striped bass and other fish as well as tetrapods. 
Leptin receptor mRNA levels were upregulated during fasting in the telencephalon, and
again following refeeding, but were unaffected in the hypothalamus. NPY transcript was transiently elevated only in the telencephalon on day 6 of fasting before returning to control levels. These results indicate that the transient upregulation of NPY gene expression during fasting corresponds to its function as an orexigen, and that the telencephalon rather than the hypothalamus might be relevant to regulating appetite by this peptide in HSB. Leptin receptor gene expression was also elevated in the telencephalon during fasting, when leptin levels are expected to be low, suggesting a mode of enhancing sensitivity to low levels of circulating ligand. The eventual decline in mRNA levels of these genes prior to refeeding suggests an adaptation to starvation that might prevent expenditure of energy toward foraging when reserves are diminished, but that simultaneously limits anorexigenic signaling by leptin in anticipation of improved feeding opportunity. The resurgence in OBR mRNA levels following refeeding may then reinstate leptin regulation of appetite or other central anabolic functions of this pleiotropic hormone.

Secondary structural analysis shows that the domain and motif features of tetrapod OBRs are conserved with various degrees of amino acid identity in their fish analogs [26, 40, 55] including Morone. This includes the presence of a signal peptide at the N-terminus (cleaved after translation), three fibronectin domains, an immunoglobulin C2-like domain and two WSXWS repeated tryptophan-serine motifs in the extracellular domain, as well as an intracellular glutamic acid-rich region and JAK/STAT boxes that comprise key signaling elements for leptin (Figure 1). Unique to Morone in comparison with any of the fish or tetrapod sequences in this analysis, the first tryptophan is substituted by leucine in the second occurrence of the WSXWS motif, although it is not known to what extent this might affect
binding or receptor conformation since both are hydrophobic residues. Similar to the overall homology of the full amino acid sequence among higher vertebrate classes and other teleosts (alignment not included due to sequence length), the putative *Morone* leptin binding region (Figure 2) showed highest identity with *takifugu* (68%), its closest relative within the alignment analysis, and was least similar to human OBRb (28%), exhibiting an expected decrease in sequence similarity with evolutionary distance. The specific residues within the putative CRH domain that are necessary for ligand binding in any fish OBRs are yet to be determined, but all include an embedded fibronectin domain. Retention of key features and motifs along with their relative positions to one another suggests that the long form OBR structure remains conserved across vertebrate classes, and may allude to conserved function despite differences in the actual peptide sequence.

The human OBR transcript encodes the functional long form of the receptor, which possesses the transmembrane region and intracellular signaling components, as well as multiple truncated splice variants of the receptor that lack the intracellular JAK/STAT signaling mechanism, one form of which also lacks the transmembrane component and serves as a plasma binding protein [11]. No short OBR splice variants were detected in *Morone*, nor in tilapia (unpublished data), *takifugu* [26], *danio* [31] or medaka [55]; however, five splice variants were found in Atlantic salmon [40] and three in carp [13]. Northern blotting with probes against the transcript region coding the extracellular portion of the receptor protein, which appears to be common to all splice variants, should ultimately be performed to verify if only a single receptor form indeed exists in these species. If striped bass do in fact lack truncated forms of OBR, some of which are purported to transport leptin
across the blood-brain barrier, then the means by which circulating hormone executes central actions warrants further investigation.

Hybrid striped bass were fasted for 24 days and then refed for 8 days (Figure 5A) to determine how metabolic state affects central gene expression of OBR in the hypothalamus and telencephalon, which are associated with feeding and NPY production in fish [14]. Decreased hepatosomatic and adiposomatic indices in fasted fish (Figure 6) gave clear evidence of catabolism as endogenous energy reserves were tapped, however neither fasting nor refeeding significantly altered hypothalamic OBR or NPY mRNA levels between groups. Effects of fasting on these genes were evident in the telencephalon instead, with NPY gene expression becoming elevated by 1.8-fold after 6 days. Increased NPY expression during fasting supports its function as an orexigen [37, 44, 52, 53, 56], and suggests that NPY may stimulate appetite in response to fasting in Morone. Similar responses to feed restriction, although over different durations, were also observed in the telencephalon of salmon [2-fold over 3 weeks; 43] and goldfish [4-fold over 3 days; 36], more so than in the hypothalamus (2.5-fold) of the latter. It is not known if the response in these fish was prone to attenuation over time as seen in HSB, although the orexigenic role of NPY may be temporal, possibly transitioning appetite regulation to other central peptides that share this function over time [51]. It is also possible that NPY is produced during fasting, as suggested by elevated gene expression levels, and then sequestered in neurons for subsequent secretion [15]. The catabolic elevation of NPY gene expression suggests that the hyperphagic [5, 39, 49] response that ensues upon refeeding in HSB and other fish may be attributable at least in part to NPY’s actions in stimulating appetite.
In mammals, leptin receptors are co-located on NPY-producing neurons in the brain, and may mediate leptin regulation of NPY [19, 22, 34]. In HSB, OBR gene expression rose in tandem with that of NPY in the telencephalon during fasting, but remained elevated longer, until day 16. We have shown in previous work under a similar time course that hepatic leptin transcript decreases in HSB during fasting-induced catabolism [54] and, assuming a coincident drop in plasma leptin, the upregulated OBR transcript in the telencephalon may therefore represent a mode of increasing leptin sensitivity in order to compensate for a decline in circulating ligand [9, 33]. The reason for the attenuation of elevated OBR gene expression on day 24 of fasting in HSB is not known and has not been previously documented. No difference in OBR gene expression was detected in telencephalon or hypothalamus of goldfish fasted for two weeks [48], but no intermediary measurements were taken to provide comparison with the current study or suggest a similar dynamic in mRNA levels over time. As the fish enter catabolic extremities due to long-term fasting, we theorize that the decrease in OBR gene expression may be necessary to reduce anorexigenic signaling that even low amounts of leptin might trigger, thereby facilitating feeding behavior when food once again becomes available.

A re-elevation of OBR gene expression in the telencephalon occurred after 8 days of refeeding, which marked the return of previously fasted fish to anabolic status. Similar to that seen during the first two time points of the fasting period, elevated OBR gene expression following refeeding may be related to the persistence of depressed leptin levels that are likely only in the early stages of a rebound, as the anabolic rise in hepatic leptin mRNA levels after refeeding in these fish is gradual [54]. The resurgence of OBR expression during refeeding

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might nevertheless contribute to mediating leptin’s anorexigenic properties and to the eventual decline in hyperphagia following extended catabolism that has been documented in these and other fish [5, 49]. Enhancement of leptin sensitivity during the transition from catabolism to anabolism could also mediate different functions ascribed to leptin, independent of its role in energy homeostasis [4], which might explain the resurgence in OBR transcript following a complete swing in metabolic state, but while circulating leptin levels are likely to still be low.

In summary, we have cloned a putative long form OBR gene in striped bass, a representative perciform fish, and show conservation of key structural features and motifs that are common to other fish and tetrapod OBRs. The receptor is ubiquitously expressed, including in the hypothalamus and telencephalon regions of the brain, which are thought to control appetite. Leptin receptor gene expression showed tissue specific regulation, increasing during fasting in the telencephalon, but not the hypothalamus, and resurging upon refeeding when energy reserves accumulate. Gene expression of the orexigen, NPY, was also transiently upregulated during fasting in the telencephalon, albeit for a shorter duration than that of OBR. The elevated response in NPY with fasting supports its role in stimulating appetite in HSB in preparation for resumed feeding. The more sustained rise in OBR expression during fasting in the telencephalon may represent mode of enhancing central leptin sensitivity to leptin when ligand levels are expected to be catabolically depressed, while the rise during refeeding may reflect the need for sensitivity to leptin signaling in mediating feeding or various anabolic processes, such as growth or reproduction.
Figure 1. Putative *Morone* OBR nucleotide and translated amino acid sequences showing motifs and structural features. The receptor exhibits features common to long form OBRs; N-terminus signal peptide (dashed bar), three fibronectin type 3 (FN3) domains, an immunoglobulin (Ig)-like C2 type domain, the cytokine receptor homology domain/leptin binding region (shaded) and at least one tryptophan/serine repeat motif (WSXWS; boxed) in the extracellular portion; JAK/STAT boxes and a glutamic acid-rich region are located in the intracellular domain.
Figure 2. Amino acid sequence alignment of the cytokine homology domain (putative leptin binding region) of fish and tetrapod OBRs (human, *Homo sapiens*; chicken, *Gallus gallus*; clawed frog, *Xenopus tropicalis*; Japanese puffer fish, *Takifugu rubripes*; marine medaka, *Oryzias melastigma*; zebrafish, *Danio rerio*; Atlantic salmon, *Salmo salar*). Conserved residues between species are marked by grey (≥ 50% homology) or black (100% homology) shading. Dashes (-) are place holders. The percent similarity between a given sequence and that of striped bass (*Morone saxatilis*) is provided in parentheses.
Figure 3. Phylogeny representing the evolutionary relationship of *M. saxatilis* OBR to representatives of other teleost order and tetrapod class long form OBRs. The same sequences were used as in the binding domain alignment with the addition of two cyprinids (Crucian carp, *Carassius carassius* and goldfish, *Carassius auratus*). Human growth hormone receptor (GHR) was used as an outgroup. Node support is indicated by a bootstrap value out of a possible 100.
Figure 4. Tissue distribution profile of OBR expression in HSB measured by reverse transcription PCR. Tissue expression was measured in the telencephalon (T), hypothalamus (Hy), pituitary (P), fat (F), liver (L), muscle (M), testes (Te), ovary (O), kidney (K), heart (He), Pyloric gut (Gu), skin (Sk), spleen (Sp) and gill (Gi). Expression of the housekeeping gene β-actin was also amplified to ensure loading of cDNA template of each tissue sample. PCR products were visualized by electrophoresis in a 3% agarose gel.
Figure 5. (A) Regulation of OBR and NPY gene expression in the telencephalon (TEL) and hypothalamus (HYP) in fish fasted for 24 days and refed for 8 days (dashed line) compared to controls (solid) that were fed throughout (mean ± SEM; N = 7). Hatched bar on X-axis denotes period of refeeding from days 24-32 for the treatment group. No significant differences in expression of OBR and NYP were observed in the hypothalamus between groups. OBR gene expression in the telencephalon was elevated through days 16 of fasting before returning to control levels on day 24, then rising again after 8 days of refeeding. NPY gene expression in telencephalon was elevated after 6 days of fasting before converging with control levels for the rest of the trial. Asterisks denote significant differences between groups at each time point (*P < 0.05). (B) Correlation between OBR and NPY mRNA expression in telencephalon and hypothalamus. No correlations were observed.
Figure 6. Hepatosomatic (HSI) and adiposomatic (ASI) indices in fish fasted for 24 days and refed for 8 days (dashed) compared to controls (solid) that were fed throughout (mean ± SEM; n = 7). Both energy indices exhibited declines during the catabolic phase. After 8 days of refeeding, ASI remained depressed whereas HSI returned and exceeded initial levels. Hatched bar denotes period of feed restriction for treatment group. Asterisks denote significant differences between groups at each time point (*$P < 0.05$, **$P < 0.01$).
REFERENCES


CHAPTER 4

Endocrine Regulation of Compensatory Growth in Fish
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2This chapter is an invited review in preparation for submission to Frontiers in Endocrinology as Won, E.T. and R.J. Borski. “Endocrine Regulation of Compensatory Growth in Fish.”

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ABSTRACT

Compensatory growth is a period of accelerated growth that occurs following the alleviation of growth-stunting conditions, usually fasting, but also sub-optimal temperatures, crowding or other stressful conditions, during which an organism can make up for lost growth opportunity and potentially catch up in size to cohorts that were not subjected to adverse conditions. While compensatory growth has been observed in all vertebrates including in humans, fish show a particularly robust capacity for the response and have been the focus of numerous studies that demonstrate their ability to compensate for periods of fasting and depressed growth when food is again made available. This phenomenon is characterized by an accelerated growth trajectory compared to that of unstressed cohorts and is the result of catabolic alterations in physiology that ultimately contributes to enhanced mitogen production, growth rate, hyperphagia (increased feeding) and improvements in metabolic substrate assimilation efficiency (feed conversion) during refeeding. Because little is known about the underlying mechanisms that drive the response, this review is intended to provide a description of sequential endocrine adaptations during altered metabolic states that lead to compensatory growth; namely during the precedent catabolic phase (fasting) that taps into endogenous energy reserves, and the ensuing hyperanabolic phase (refeeding), during which accelerated growth occurs. During the catabolic phase, elevated orexigen and growth hormone (GH) production increase appetite and protein-sparing lipolysis, while the GH/IGF growth axis is suppressed, primarily as the result of hepatic GH resistance. In order to elicit a compensatory growth response, endogenous energy reserves must be moderately depleted by
catabolic processes, which alter endocrine profiles that enhance appetite and growth potential. Once feeding is reinstated, temporal hyperphagia provides an influx of energy and metabolic substrates that are allocated to somatic growth under the influence of resumed growth axis signaling. Under the right conditions, refeeding results in hyperanabolism and a steepened growth trajectory relative to constantly fed controls. The response then wanes as energy reserves are reaccumulated and energy homeostasis is restored. We ascribe possible roles for select appetite and growth regulatory hormones in the context of these catabolic and (hyper)anabolic phases of the compensatory growth response in teleosts, with emphasis on growth hormone (GH), insulin-like growth factors (IGFs), cortisol, neuropeptide Y (NPY), ghrelin and leptin.

**Keywords**: Compensatory growth, fish, aquaculture, growth hormone, ghrelin, NPY, leptin, Insulin-like growth factor, IGF
INTRODUCTION

Compensatory growth overview

Compensatory growth (CG) is a period of accelerated somatic growth following the alleviation of growth stunting conditions, that temporarily induces a steeper growth trajectory than that of cohorts not previously exposed to adverse conditions (Figure 1). This phenomenon was seminally documented nearly a century ago [114], and the term “compensatory growth” coined forty years later [18]. The CG phenomenon has been documented in all vertebrate classes; humans [17, 128, 137], other mammals [18, 97, 135, 177], birds [177], reptiles [16, 129] and amphibians [7, 172], but most extensively in fish [8 for review]. Despite the diversity of animals and plants that can exhibit CG, the underlying mechanisms governing the response are still poorly understood. Considering that CG results in enhanced growth rate and feed efficiency, it is not surprising that commercial production appears to be the driving impetus behind investigations into CG in fish, as the majority of studies to date involve cultivated species. Compared to conventional methods of fish farming that deploy a constant regimen, incorporation of rearing protocols that induce CG shows promise of reducing the amount of feed needed to grow at least some species of fish commercially. Also, an increased understanding of mechanisms controlling CG could provide the fundamental knowledge to assess and improve the recovery potential of neonates and children exhibiting growth stunting conditions associated with poor rearing conditions or nutrient deficiency.
A broad range of teleosts are capable of undergoing CG responses following alleviation of various growth stunting conditions or their combination, including suboptimal temperature, crowding or other stressful environments, and feed restriction, the latter reflecting the condition most often studied [8, 17, 68]. A CG response has been reported in salmonids [38, 69, 72, 94, 102, 111], cyprinids [134, 176], striped bass [120, 124, 158, 159], European seabass [45], flatfish [29, 65], stickleback [182], tilapia [174], catfish [59], centrarchids [64] and cod [70]. While the degree of growth compensation achieved depends on species, it is nonetheless typically characterized by hyperphagia, improved feed efficiency and elevated specific growth rate (SGR). Although not often assessed, a critical feature of CG is that the SGR of individuals is higher relative to similar sized cohorts (i.e. SGR normalized to body mass) that were never subjected to conditions that inhibit growth or that elicit a CG response [120, 143].

Because most fish show indeterminate growth and many are susceptible to seasonal changes in growth rate associated with natural variations in temperature and prey availability, they tend to exhibit a robust capacity for CG [100]. Hence, they can ostensibly serve as valuable subjects for evaluating the metabolic and endocrine mechanisms that may contribute to anabolic processes generally, and hyperanabolism specifically. Acknowledging the diversity of fish in which CG has been documented and the complexity of the response itself, few attempts have been made to consolidate what is known about the endocrine mechanisms that underlie the response; however, the cumulative research on isolated components of CG provides insightful information from which to extrapolate a fundamental framework. In particular, the CG response can be divided into catabolic (e.g. during fasting, stress, low
temperature) and anabolic (during realimentation or a return to more favorable conditions) phases, which elicit distinct and sequential endocrine responses (Figure 2, Table 1A). The purpose of this review is to ascribe possible roles to select appetite and growth regulatory hormones in the context of the catabolic and (hyper)anabolic phases of the CG response in teleosts, with emphasis on growth hormone (GH), insulin-like growth factors (IGFs), cortisol, neuropeptide Y (NPY), ghrelin and leptin.

I. Catabolic state: Priming the compensatory growth response

In order to induce CG, a preceding catabolic period is necessary, the degree of which affects the capacity or overall magnitude of the response [134, 174, 176]. This negative energy period depletes endogenous energy reserves and alters endocrine profiles that modulate appetite and growth potential when feeding is reinstated (Figure 2B). Brief periods of feed restriction do not sufficiently deplete stored energy or result in stunting, and can be countered with behavioral compensation such as decreasing energy expenditure [8]. Excessively long periods of fasting, on the other hand, lead to an irrecoverable lapse in growth that prevents full catch-up to fed cohorts [15, 59, 159]. Nonetheless, moderate catabolism that taps expendable energy-storing tissues physiologically primes the CG response by opening pathways that elevate circulating GH and stimulate orexigens such as ghrelin and NPY. Studies in striped bass suggest that a prerequisite drop in the hepatosomatic index (HSI) to about 1.5 is necessary in order to elicit the response [120, 158]. The rise in appetite and alterations in physiology that occur during the catabolic phase preceding CG thereby potentiate hyperphagia and accelerated growth when feeding is reestablished.
Hence, an adequate, but not excessive level of catabolism is essential to elevating the capacity of an animal to undergo CG and possibly reach full catch-up growth when conditions improve.

**Growth Hormone: Function and regulation during catabolism**

Growth hormone is pivotal in leading up to and during CG. Under differential regulation by a host of neuroendocrine regulatory factors, GH serves dual roles depending on metabolic state, mobilizing lipids during catabolism and promoting somatic growth during anabolism [Reviewed in 21]. During fasting, rising plasma GH [93], along with the related somatolactin in fish [98], protects nonexpendable tissue such as muscle and vital organs from being catabolized by preferentially metabolizing fat over protein. This lipolytic function has been demonstrated in fish with exogenous GH treatment *in vivo* in coho salmon [140] and *in vitro* in gilthead sea bream adipocytes [5], and is a critical adaptation to surviving negative energy periods.

Catabolically elevated GH secretion is mediated by reductions in metabolite levels (glucose/amino acids) and is stimulated *in vitro* and *in vivo* by orexigens, including ghrelin [striped bass: 121] and NPY [goldfish: 115], as well as by the lack of negative feedback inhibition from IGF-I. In striped bass pituitaries, IGF-I potently suppresses *in vitro* GH synthesis and release [51, 122], which likely contributes to elevated GH production during fasting when IGF-I levels are depressed. Cortisol, the dominant stress corticosteroid in fish [42, 101], also stimulates *in vivo* GH transcription in channel catfish [145] and *in vitro* release from somatotrophs in tilapia [112]. Plasma GH levels are elevated by as much as two
fold in trout [44, 113, 147], tilapia [153], channel catfish [145] and striped bass [157] during fasting when exogenous metabolic substrates are limited and fat reserves are needed for energy. Elevated GH is therefore able to mobilize lipids for maintenance of basal metabolism during food deprivation, but without directing limited energy resources toward growth due to catabolic GH resistance in the liver.

The actions of GH are mediated by GH receptors (GHRs), for which two distinct gene lineages exist in fish and which operate via different signaling pathways [83]. The teleost GHR-1 is homologous to the single mammalian GHR [56] and has a shared affinity for both GH and somatolactin [54] whereas the type 2 GHR is specific to GH activation [55, 67, 127]. The degree to which the individual receptor types mediate lipolysis or regulate growth is not clear, but GH actions in various tissues are likely contingent on the differential expression of these receptors depending on whether catabolic or anabolic processes are required [34, 67, 83, 136, 166]. During negative energy states, hepatic resistance to elevated plasma GH is evident as decreased IGF-I production [40, 124] due to catabolic suppression of ligand binding to GHRs [39, 61, 104]. This type of hepatic GH resistance during catabolism is characterized as downregulated GHR-1/2 transcripts in striped bass [124, 157] and gilthead seabream [136], decreased hepatic GH-binding in gilthead sea bream [118] and both reduced hepatic GHR-1/2 transcript and GH-binding in rainbow trout [113]; alleviated in all cases by refeeding.

Hepatic GH resistance is likely mediated, in part, by cortisol and somatostatins during fasting. While stimulating GH synthesis, cortisol simultaneously suppresses hepatic IGF-I production through either direct downregulation of IGF-I transcription and synthesis in sea
bream and tilapia [79, 90, 126], or in conjunction with suppression of GHR transcript in channel catfish [144]. The role of somatostatins during catabolism is somewhat paradoxical in as much as they are associated with reduced GH synthesis and secretion to inhibit growth; however, they also reduce GH binding capacity in the liver and suppress hepatic IGF-I gene expression [141, 167]. Hepatic resistance to GH through reduced receptor expression and signaling, despite elevated levels of circulating ligand, signifies an uncoupling of the lipolytic and growth regulatory functions of GH during negative energy states. The carry over of catabolically elevated GH, in turn, helps drive enhanced IGF-I production and may ultimately potentiate hyperanabolic growth pending the return to a positive energy state when hepatic GHR signaling resumes.

**Ghrelin: Peripheral modulator of GH secretion and appetite during catabolism**

As discussed, the elevation of lipolytic GH during fasting is fundamentally imperative for sparing muscle and organs by deferring catabolism to fat stores. Upstream regulation of enhanced GH production during fasting is therefore dependent on an indicator of nutritional state to coordinate catabolic lipolysis with negative energy states. The orexigenic peptide, ghrelin, responds to fasting and is a potent GH secretogogue, comparable in effect to growth hormone releasing hormone (GHRH) [63]. Ghrelin’s actions are mediated by the growth hormone secretogogue receptor (GHSR), which is distinct from the GHRH receptor [9, 27, 86]. In fish, ghrelin mRNA is expressed predominantly in the stomach [165] and, interestingly, is the only known peripheral orexigen originating from the gut considering proximity to nutrient uptake. To a lesser extent than in the stomach, central ghrelin gene
expression has also been detected in rainbow trout [75], eel [76], Mozambique tilapia [77] and goldfish [163], as has GHSR transcript [27]. Functionally, exogenous ghrelin stimulates GH secretion in vitro in cultured orange spotted grouper pituitaries [130] and in vivo and in vitro in tilapia [48], rainbow trout [75], goldfish [164] and striped bass [121]. Besides actively stimulating GH secretion, in mammals ghrelin also independently acts as a functional antagonist to somatostatin [11, 33, 149, 150], itself an inhibitor of GH secretion. Ghrelin is therefore capable of stimulating GH secretion either peripherally through vagal afferents originating near the stomach and by acting directly on the pituitary, or centrally through modulation of GH release factors.

Ghrelin is also a potent appetite stimulant. The orexigenic properties of ghrelin have been reviewed in mammals [9, 161] and fish [165]. Gastric ghrelin is produced in response to hunger, serving as a peripheral signal to the brain to initiate feeding [13, 32] during periods of negative energy balance. Orexigenic ghrelin signaling operates via vagal afferent as well as central pathways paralleling those of GH regulation, although appetite and GH stimulatory pathways are independent [33, 108, 155, 179]. Appetite is stimulated by a single IP/ICV injection of ghrelin in goldfish [96, 99, 165] and chronically by osmotic pump infusion in tilapia [132].

In accord with its function as an appetite stimulant, ghrelin production is elevated during catabolism, a necessary metabolic pre-condition to inducing CG [78, 121]. Conversely, high caloric intake and positive energy balance inhibit ghrelin production [156], qualifying ghrelin as an important peripheral signal for regulating GH secretion and food consumption based on nutritional status in fish. Differential regulation of appetite and GH
secretion by ghrelin in fish may rely on specific Ser3 modifications [74, 132, 163]. The response of ghrelin production to fasting and the lag time between refeeding and the ensuing return of ghrelin to basal levels may depend on the degree to which the catabolic period uses up energy reserves. In the short term, gut and hypothalamic preproghrelin mRNA as well as circulating ghrelin increase on the order of days in fasted goldfish, then go down within several hours after refeeding [162]. European seabass stomach ghrelin mRNA levels increase over 35 days of fasting, then drop back to basal levels after 10 days of refeeding [151]. Striped bass exhibit elevated plasma ghrelin levels after three weeks of fasting and a 43-fold increase relative to fed controls after continued fasting and cold-banking for 90 days, followed by a return to baseline within 21 days of refeeding and temperature warmup [121]. The plasma GH levels in these fish accordingly remained elevated during fasting and then returned to control levels after refeeding. Refeeding in these fish, at a point when ghrelin levels were elevated, was additionally marked by hyperphagia and full catch up growth. Interestingly, Picha et al. (2009a) show that ghrelin stimulation of GH in vitro occurs in somatotrophs derived from both continually fed and fasted fish, but not from refed fish, suggesting the hypophyseal GHSR may be downregulated during CG, which would contribute to the eventual decline in GH seen during refeeding of fasted fish. Taken together, the trends in ghrelin regulation and function observed in fish suggest that catabolically elevated ghrelin simultaneously raises lipolytic plasma GH while priming fish for a hyperphagic response once feeding is reestablished. The coincidence of these responses likely contributes significantly to the CG response, and may in part explain why a catabolic phase is needed to precede hyperanabolism.
Central catabolic modulators of appetite

Appetite is increased during fasting through the upregulation of central orexigenic neuropeptides located in the hypothalamus [80], with similar components existing in mammals and fish. Orexigens, including NPY, Agouti-related peptide (AgRP), galanin and the orexins A/B are expressed in the pre-optic hypothalamic region, which appears to be the teleost analog to the mammalian feeding center. Central injections of NPY [6, 23, 82, 92, 99, 109], galanin [35, 171] and orexins [168] accordingly increase appetite in teleosts. While NPY is considered the most potent orexigen in fish and has garnered the most research, these other central peptides interact with NPY to augment appetite in response to negative energy status [169].

Central NPY [25, 88, 89, 116, 142] and AgRP [26] mRNA is regulated by nutritional state and increases during negative energy states to promote energy intake. Ghrelin stimulates central gene expression of NPY, which has been shown to mediate the orexigenic effects of ghrelin in goldfish [99]. As in mammals [138, 146], peripheral injection of the anorexigen, leptin (discussed separately below), suppresses the effects of exogenous NPY on appetite in fish [91, 170]. Stimulation of appetite by NPY therefore occurs when ghrelin levels are high and leptin low [12], or during periods of negative energy balance, although leptin’s regulatory pattern in fish is under debate (discussed below). The upregulation and interaction of central orexigenic neuropeptides during fasting likely culminates in the hyperphagic response observed when feeding resumes, thus providing the substrate and energy necessary for a CG response to occur.
Summary of catabolic state preceding compensatory growth

An antecedent fasting, or catabolic period is necessary for a CG response to occur when feeding is reinstated. Fasting elevates circulating GH and stimulates orexigens that, together, preserve structural protein during catabolism and anticipate the recovery of lost energy stores by elevating appetite (Figure 2B; Table 1). Growth axis signaling is suppressed, however, largely due to downregulation of hepatic GHRs and desensitization to GH-induced IGF production. The endocrine responses to catabolism are influenced by the negative energy state and represent a physiological adjustment to diminished energy reserves. A catabolic precedent to CG is therefore needed in order to achieve elevated GH and orexigen levels that, in turn, anticipate the hyperanabolic response by promoting hyperphagia and potentiating the GH/IGF growth axis once feeding is reestablished and GH resistance is alleviated.

II. Refeeding: Hyperphagia and hyperanabolism (compensatory growth)

Hyperanabolism, or the accelerated growth phase that characterizes CG, is the result of hyperphagia and heightened growth axis activity during refeeding, particularly in the rapid rise in IGF-I production that occurs when hepatic sensitivity to GH returns. As discussed in the first half of this review, CG is preceded by a catabolic phase that primes an organism for hyperanabolism. If the endurance of the catabolic state is sufficient, and food is ample when feeding resumes, then a temporal hyperphagic response is elicited and a net positive energy state achieved through the reintroduction of exogenous energy and metabolic substrates. The duration of catabolism determines the period needed to recover endogenous energy reserves,
which may ultimately determine the duration of hyperphagia. When energy stores are regained, orexigenic signaling decreases. Under these terms, an organism exhibits lipostatic regulation of energy homeostasis [68], a system in which energy reserves are maintained within a certain range by endocrine signals derived from energy storing tissues themselves that regulate feeding and energy expenditure. Compensatory growth seems to occur during the lag time between refeeding and the lipostatic abatement of hyperphagia and enhanced growth axis activity.

**Hyperphagia and assimilation efficiency**

Hyperphagia is an integral component of CG [8] and is a common response to energy deficit in a variety of fish; European minnow [134], Atlantic salmon [19], centrarchid sunfish [64], Nile tilapia [174], striped bass [120, 124, 158, 159] and stickleback [182]. The magnitude of the hyperphagic response depends on the duration of fasting in salmon, and appears to be largely influenced by the degree to which lipid reserves are depleted by catabolic processes [20]. Hyperphagia during CG is attributable to catabolically elevated levels of orexigens that are upregulated during negative energy states.

Hyperanabolism during refeeding is fueled by an influx of metabolic substrates that are rapidly allocated to somatic growth through heightened mitogenic activity of the growth axis; however, hyperphagia alone may not account for the accelerated growth rate experienced during CG. Gurney et al (2003) propose, through energetics modeling, that high substrate assimilation rates during hyperphagia drive CG by partitioning resources specifically to skeletal growth rather than to energy reserve deposition. Skalski et al (2005)
elaborate on the energetics model of hyperanabolism, suggesting that physiological changes, including increased assimilation efficiency during feeding and reduced mass-specific maintenance costs during fasting, work in conjunction with hyperphagia to drive CG in striped bass. A subsequent study that normalizes SGR to body size supports that the growth rate is significantly higher in fasted/refed hybrid striped bass relative to controls [124], and is not merely an allometric artifact of smaller, stunted fish compared to larger, fed cohorts.

The mechanism that adjusts energy allocation around assimilation rate is undefined, although the lipolytic and growth promoting functions of GH, along with its regulatory profile under variable metabolic conditions, suggests an influential role in optimizing substrate conversion to skeletal growth. Long-term GH treatment using sustained-releasing implants in rainbow trout improved feed conversion by 60% [84]. Exogenous GH treatment enhanced amino acid uptake [30] as well as growth rate, appetite, and food conversion in coho salmon [95]. In fed striped bass, weekly bovine GH injection increased the number of intestinal amino acid transporters and intestinal mass [148], suggesting improved protein uptake capacity. Similarly in carp [46] and rainbow trout [47], protein assimilation and feed conversion were improved by chronic GH treatment. The carry over of catabolically elevated GH after refeeding, in addition to driving somatic growth through stimulation of IGFs, may therefore also improve protein assimilation at a time when substrate is in abundance due to elevated feeding [93, 119].
Hyperanabolism: Augmentation of the GH/IGF growth axis

Compensatory growth ultimately refers to the rapid growth, or hyperanabolic, response that occurs during the feeding of previously fasted animals, and which allows them to recover lost growth opportunity. During positive energy states, circulating GH binds hepatic GHRs to induce production and secretion of IGF-I, the prominent mitogen responsible for somatic growth in vertebrates [49, 117, 123]. Like IGF-I, hepatic IGF-II transcription is also stimulated by GH in a broad range of fish [22, 41, 57, 105, 106, 125, 139, 152, 173] and remains responsive to GH into adulthood, suggesting that IGF-II may operate alongside IGF-I within the teleostean growth axis. Hepatic GHR transcription positively correlates with GH binding in gilthead sea bream [118] and circulating IGF-I levels in channel catfish [144] during fasting and refeeding, corroborating that metabolic state mediates GH signaling through changes in GHR expression in the liver where the majority of endocrine IGFs are produced [131, 152, 173]. The return of hepatic sensitivity to GH during refeeding, which reinstates IGF synthesis, is therefore critical to CG. Chronic GH treatment simulates CG in a diverse range of fish [1, 24, 95, 175], effectively mimicking the catabolically elevated GH levels that temporarily persist during refeeding of previously fasted fish. The duration of high plasma GH levels contributing to IGF production during CG may again depend on catabolic history, and appears to gradually decline back to normal levels over weeks, which is the approximate duration of the rapid growth phase following refeeding [113, 121, 157, 180]

Consistent with the mitogenic attributes of IGFs, plasma IGF-I [14, 160] and hepatic IGF-I and II mRNA [124] levels positively correlate with SGR in fasted and refed fish.
Interestingly, we show that the relative change in circulating IGF-I over a growth increment is an even better corollary to SGR in striped bass than absolute IGF-I levels [120], which may indicate enhancement of IGF receptor sensitivity during the transition from catabolic back into anabolic states as plasma IGF-I levels are in the process of rising. The steep rebound of depressed plasma IGF-I levels during the refeeding of fasted striped bass corresponds directly to the hyperanabolic phase of the growth curve [124]. Moreover, transcript levels of hepatic GHRs and IGFs in these fish during refeeding actually exceeded those of unfasted control fish, suggesting that overcompensation in expression of key growth regulatory hormones may be contributing significantly to the accelerated growth that occurs with CG. This overcompensation is further exacerbated in striped bass when the alleviation of a previous period of feed restriction is combined with cold-banking [122], similar to what wild fish experience during spring warm up when temperature and prey availability are more optimal for growth [98, 117]. Similarly, overcompensation in circulating IGF-I concomitant with enhanced activation of IGF-I signaling in muscle tissue was observed in fine flounder [52]. An elevation in muscle IGF-I transcript levels has been observed in a number of other fish [28, 84, 102, 103, 124], suggesting a parallel autocrine or paracrine mechanism within the skeletal tissue itself. Taken together, the coordinated dynamics of the GH/IGF growth axis appears key in eliciting CG, whereby the expression levels of and sensitivity to growth-regulatory hormones is increased relative to normal animals on a continuous regimen, subsequently resulting in a hyperanabolic state and enhanced skeletal and somatic growth characteristic of CG.
The lipostatic return to energy homeostasis

The CG response to fasting is finite, attenuating once lost energy resources re-accumulate and hyperphagia abates. The lipostatic model of energy homeostasis proposes that adiposity acts as a regulatory mechanism on appetite in order to maintain a threshold of energy deposition [81]. Kennedy observed that depletion of adipose stores in fasted rats stimulated feeding, which returned to normal levels when reserves returned to a critical mass. Studies support the presence of a teleost lipostat-like mechanism, as well, although lipid partitioning can vary considerably between adipose tissue, liver and muscle in different fishes [37, 50]. The hyperphagic response to long-term fasting in salmonids appears to be driven by a decrease in whole body lipid content and terminates, along with the CG response, when proximate composition is restored [71, 72]. Fluctuating gross lipid levels during fasting and refeeding similarly suggest lipostatic regulation of hyperphagia in three-spined stickleback [182] and striped bass [158] undergoing CG. The liver is a significant lipid storing and metabolizing tissue in some fish, and may also be involved in sensing and maintaining energy reserve levels. More so than visceral adipose mass, changes in the HSI during cycles of feed deprivation and refeeding in striped bass, in which liver is a major lipid storage organ, are indicative of metabolic state and the likelihood of achieving an elevated SGR, and hence CG, during refeeding [120, 159].

Is leptin the teleost lipostatic hormone?

In part because different fish partition stored energy in various locations, the mechanism that articulates overall energetic status, endocrine or otherwise, is not well
defined. In mammals, the peptide hormone leptin reflects fat deposition and modulates food consumption and energy expenditure according to endogenous energy availability, and is considered the primary lipostatic hormone [3, 10]. If leptin functions as a lipostat in teleosts as well, it would likewise need to positively reflect energy stores. Even though leptin is consistently anorexigenic in fish [36, 107, 178] as in mammals [3], evolutionarily isolated gene duplication events, physiological differences in energy storage and diverse life histories may underlie a teleost leptin system that shares only a partial resemblance to that of higher vertebrates.

Leptin centrally regulates feeding by stimulating appetite-suppressing neuropeptides and inhibiting appetite-stimulating neuropeptides in mammals [2, 4, 43, 181] and fish [107, 170]. Leptin injection accordingly reduces feeding in goldfish [36], rainbow trout [107] and striped bass [178]. While its anorexigenic property logically integrates into a system in which leptin serves as a lipostat, such as the mammalian paradigm, it fits less aptly into a system where leptin expression may not correlate with energy reserves, as may be the case for some teleosts.

Studies evaluating leptin responsiveness to metabolic state in fishes are equivocal. Circulating leptin [73, 110] or mRNA levels in liver tissues [60, 178] are depressed in some fish during fasting, or otherwise act in a lipostatic-like manner [84] as energy reserves are tapped. Conversely, other species, namely salmonids, exhibit rising plasma [53, 85, 154] or gene expression levels [50, 58, 133, 154] in lipid storing tissues under conditions of feed restriction, while others show no long-term regulation by feeding regimen [66]. Leptin gene expression profiles in different energy-storing tissues during altered metabolic states
therefore vary between studies, even among closely related species, and are not necessarily concomitant with plasma levels. This calls into question whether leptin functions as a lipostatic endocrine signal aimed at mobilizing surplus energy stores or might instead drive other catabolic processes [31]. It is also important to consider that some fish lineages possess multiple leptin genes [66, 87, 133] arising from different genome duplication events, and that these paralogs may have different functions. If a lipostatic function for leptin, or one form of leptin, is inherent in fish, then the replenishment of lost energy reserves may instigate the return of energy homeostasis in previously fasted animals through leptin signaling, thereby attenuating hyperphagia and marking the end of the CG response. If not, then further studies will be needed to reconcile the paradox of rising plasma leptin levels during fasting in some fish in light of its conserved anorexigenic property.

Summary: Sequence of events leading to CG during catabolic and anabolic states

Compensatory growth is a period of accelerated growth following the alleviation of growth stunting conditions, such as fasting, that potentially allows an organism to make up for lost growth opportunity (Figure 1). The CG response can be divided into a catabolic phase, during fasting, for instance, when growth is impeded and energy reserves are tapped, and an anabolic phase during refeeding when growth resumes at an elevated rate that exceeds that of individuals never subjected to a growth stunting event (Figure 2). This review chronologically describes physiological adjustments and endocrine activity during these metabolic phases that may ultimately make CG possible through enhanced feeding, substrate assimilation and growth.
First, a catabolic precedent is required that increases appetite as energy reserves are depleted. During this negative energy phase, elevations in peripheral ghrelin and central orexigens (Figure 2B) prime the organism for hyperphagia once feeding resumes. Increased ghrelin and cortisol production, as well as attenuation of negative feedback from declining IGF-I secretion, leads to elevations in GH. Growth hormone, in turn, induces lipolysis, expending energy reserves needed for maintenance during states of fasting. Hepatic GH resistance due to downregulation of GHRs leads to a reduction in IGF production under the pretext that conditions are unfavorable for growth. A critical period of catabolism thus primes the CG response to refeeding by elevating appetite and super-potentiating the growth axis with elevated GH.

When feeding resumes, CG (Figure 2C) is fueled by the hyperphagic influx of exogenous energy and metabolic substrates, which are taken up and assimilated with heightened efficiency as the result of modifications to metabolic substrate absorption, in part attributable to elevated GH levels. Elevated GH levels also resume growth axis signaling when hepatic GHRs are reinstated and GH sensitivity returns, thereby stimulating a steep rise or overcompensation in IGF-I production and a correspondingly accelerated growth rate. Production of IGF-I is also influenced by the decline in the growth-inhibitors, cortisol and somatostatin, during refeeding. Substrate and energy availability, enhanced substrate assimilation efficiency, and augmentation of the growth axis culminate in a hyperanabolic, rapid growth phase until a lipostat-like mechanism, possibly leptin in some fish, initiates the return to basal appetite and a growth axis profile representative of a normal growth trajectory.
(Figure 2A). Restoration of energy reserves can take several weeks depending on the duration of catabolism experienced.

Compensatory growth is therefore mediated by sequential endocrine responses during distinct metabolic states (Table 1). While by no means inclusive, this theoretical composite of documented hormonal activity during catabolic and anabolic states is intended to provide a basic framework of the endocrine regulation of CG in fish, and perhaps higher vertebrates. Teleosts have been the subject of numerous CG studies; however, our understanding of their endocrine mechanisms during this phenomenon, or during different metabolic states, is commonly based on observations in a few model species or extrapolations from studies in higher vertebrates. The relevance of certain hormones, time frames and even the potential of the CG response itself are likely to be contingent on species, size or life stage. Nonetheless, the variables presented in this review are estimated to be of fundamental importance to CG in fish, the degree of their relevance in particular species notwithstanding.
Figure 1. Compensatory growth (CG) paradigm during fasting and refeeding (dashed line) compared to constant growth rate in fed controls (solid line). Normal growth (A) is disrupted by feed restriction (hatched bar), which results in a plateau in the growth trajectory (B) and a size disparity compared to control animals fed a constant regimen. When feeding resumes, hyperphagia and enhanced growth axis activity drive a hyperanabolic phase (C) marked by a steeper growth curve than that of constantly fed animals. In some cases, the CG response allows stunted animals to fully compensate for lost growth opportunity and re-converge in size with controls.
Figure 2. Endocrine regulation of growth and appetite during normal anabolism, catabolism and hyperanabolism resulting from feeding status. Growth is regulated by the GH/IGF axis; GH secreted into circulation by the pituitary binds its receptor (GHR) to stimulate hepatic IGF-I production, which systemically drives somatic growth. Lipolysis is an alternate function of GH. Peripheral signals from a lipostatic mechanism (anorexigenic), possibly leptin, and ghrelin (orexigenic) regulate energy intake by centrally modulating NPY and other neuropeptides in the central feeding center. Ghrelin also functions as a GH
secretagogue. Arrows show the direction of regulatory pathways; widening/narrowing of arrows represents a dynamic increase/decrease in a component over the duration of a particular metabolic state. A) During constant feed availability, energy homeostasis is maintained by matching energy intake and expenditure. Peripheral signals counter-regulate appetite centrally according to energy availability. Growth is mediated through IGF-I regulation by nominal circulating GH levels and hepatic GHR expression. GH is under negative feedback control by circulating IGF-I. B) Fasting necessitates catabolic processes that provide energy for basal metabolism. Diminishing energy reserves and increasing ghrelin production increase appetite and circulating GH levels. Elevated lipolytic GH exploits stored energy reserves, while reduced GH-R expression desensitizes the liver to GH-induced IGF-I production. C) Refeeding signifies the switch from catabolic to anabolic processes. The return to positive energy status is characterized by the resumption of hepatic GH sensitivity and a rise in circulating IGF-I levels, which drives accelerated growth. Temporally elevated orexigens carried over from fasting drive hyperphagia. Eventually, the repletion of energy reserves and negative feedback from IGF-I returns GH and appetite to nominal levels, marking the return to growth rates.
Table 1. Modulation of select endocrine component profiles due to fasting or refeeding. Symbols indicate increase (↑), decrease (↓), no change (↔) or transitional change during refeeding (…↑, …↓) of components relative to those of constantly fed controls.

<table>
<thead>
<tr>
<th>Component</th>
<th>Fasting (Catabolism)</th>
<th>Effect</th>
<th>Refeeding (CG)</th>
<th>Effect</th>
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<tr>
<td>GH</td>
<td>↑</td>
<td>Lipolysis (protein sparing), Diminished</td>
<td>↑…↓ Residually high, then moderate</td>
<td>Stimulation of IGF production; Enhanced</td>
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<td>energy reserves</td>
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<td>protein uptake</td>
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<td>GH-R (Liver)</td>
<td>↓</td>
<td>GH resistance</td>
<td>↑</td>
<td>GH-induction of IGF production</td>
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<td>IGF-I/II (Liver)</td>
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<td>Growth stasis</td>
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<td>Enhanced somatic growth</td>
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<tr>
<td>Ghrelin</td>
<td>↑</td>
<td>Increased appetite GH secretion</td>
<td>↑…↓ Residually high, then decreasing</td>
<td>Hyperphagia, Repleted energy reserves</td>
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<tr>
<td>NPY</td>
<td>↑</td>
<td>Increased appetite</td>
<td>↑…↓ Residually high, then decreasing</td>
<td>Hyperphagia, Repleted energy reserves</td>
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<td>Leptin</td>
<td>↓/↑ (mixed reports)</td>
<td>(if ↓) Increased appetite (if ↑) Lipolysis</td>
<td>…↓/…↑ (mixed reports)</td>
<td>(if …↑) Lipostatic signal, reduced appetite</td>
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<td>(if …↓) Decreased lipolysis</td>
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<td>Hepatic GH resistance</td>
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<td>↑</td>
<td>Hepatic GH resistance</td>
<td>↓</td>
<td>Hepatic GH sensitivity</td>
</tr>
</tbody>
</table>
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