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# Comparative endocrinology of leptin: Assessing function in a phylogenetic context

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## Abstract

As we approach the end of two decades of leptin research, the comparative biology of leptin is just beginning. We now have several leptin orthologs described from nearly every major clade among vertebrates, and are moving beyond gene descriptions to functional studies. Even at this early stage, it is clear that non-mammals display clear functional similarities and differences with their better-studied mammalian counterparts. This review assesses what we know about leptin function in mammals and non-mammals, and gives examples of how these data can inform leptin biology in humans.

## Introduction

Nine years have passed since the first leptin sequence was identified in a non-mammalian vertebrate (*Takifugu rubripes*; Kurokawa et al., 2005). Due largely to advances in genomic technology, leptin and leptin receptor genes now have been cloned from all the major vertebrate classes, with the possible exception of Aves (Figure 1). Although non-mammal leptin studies still comprise ~ 1% of all leptin studies (Web of Science returns ~29,000 leptin studies (non-reviews or editorials); <300 of those focus on non-mammals), there is now a sufficient body of literature to approach the question of whether leptin function is conserved among vertebrates (Figure 2). Comparative study of leptins has the goal of not only solving comparative questions, but also clarifying our understanding of leptin function in mammals (by uncovering the origin of leptin function). Thus in this review we describe recent advances of leptin biology in both comparative and biomedical contexts.

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## Leptin's Tertiary Structure is Conserved among Vertebrates

One aspect that both aided and hindered leptin research in non-mammalian systems was its structure. Early studies assumed conserved primary structure between mammalian and fish leptins, and thus interpreted fish immunoreactivity with anti-mouse leptin antibodies as evidence of leptin expression (e.g. Johnson et al., 2000). However, traditional redundant-primer strategies were unsuccessful at amplifying a fish leptin for over a decade. Kurokawa's insight of searching for leptin in a conserved pattern of genes, or gene synteny, finally resulted in a true non-mammal leptin gene from a fish genome (Kurokawa et al., 2005). With this breakthrough, the nonintuitive idea that tertiary structure was conserved even though primary sequence was not gained support with each new leptin clone (Crespi and Denver, 2006; Denver et al., 2011; Gorissen et al., 2009; He et al., 2013; Prokop et al., 2012). Leptin orthologues are now described for all major classes of vertebrates, (with the exception of birds (Figure 1)), and all are predicted to have a very similar, class I helical cytokine tertiary structure. It is important to note, however, that crystal structure has been determined for only a single leptin, and that structure was for a leptin modified to enhance crystal formation (human e-100 leptin; Zhang et al., 1997). All non-mammal leptin structures (and most mammal leptin structures) are inferred by modeling algorithms; an empirical determination of a non-mammal leptin structure is needed to validate the computationally derived models.

## Basal Vertebrates Express Multiple Leptin Orthologs

All mammals (e.g. Ball et al., 2013; Clarke et al., 2001; Comuzzie et al., 1997; Zhang et al., 1994) and amphibians (Boswell et al., 2006; Crespi and Denver, 2006) express a single ortholog of leptin (*lep*). Two *lep* orthologues are present in the green anole (lizard) genome, but only one may be expressed (Denver et al., 2011). The presence of multiple orthologs within a genome is generally attributed to genome and/or gene duplication (Gorissen et al., 2009; Kurokawa and Murashita, 2009; Ronnestad et al., 2010). Fish leptins are, by far, the best-studied among non-mammal leptins (Figure 2). Initially our group proposed that all fishes express two *lep* paralogs (reviewed by Copeland et al., 2011), with the possible exception of *Fugu rubripes* (Kurokawa et al., 2005). Now, more recent work indicates that some advanced fishes (including *Fugu* and other Percomorphs) lost the second *lep B* ortholog (striped bass *Morone saxatilis* and stickleback *Gasterosteus aculeatus;* Won et al., 2012, and chinese perch *Siniperca chuatsi;* He et al., 2013). The scenario of vertebrates expressing a single paralog after the Percomorph split (advanced by Won et al., 2012) is contradicted by two leptin paralogs in more-derived groups (orange-spotted grouper *Epinephelus coioides* (Zhang et al., 2013) and green anole (Denver et al., 2011)).

Leptin orthologs now are identified for many different vertebrate taxa separated by considerable evolutionary time (Figure 1). Given the caveat that the number of orthologs per species is often revised (up) as each genome is analyzed (e.g. initial estimates in salmonids did not recognize a leptin B; Angotzi et al., 2013), we can state that at least some advanced fish taxa express a single leptin ortholog (e.g. *Takifugu*, given that its genome was among the first to be completed and the extent to which it is annotated) and some advanced fish taxa express two leptins (grouper; Zhang et al., 2013). Adding to the confusion, the two

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leptins present in some fish lineages (carp, goldfish, and salmon) should not be considered homologous to the A and B leptins that appear to be the result of a deep divergence in bony fishes. Rather these are the result of much more recent duplications of leptin A in these lineages, hence the labels leptin a-1 (A1) and a-II (A2). Hereafter, A and B will refer to the presumed deep orthologs of leptin as represented by the two copies of leptins in zebrafish and Japanese Medaka. Similarly the green anole paralogs are the result of a recent gene duplication and thus not homologous to both A and B leptins of fish (Denver et al., 2011).

Are the ‘A’ and ‘B’ leptin paralogs functionally distinct? Although the tissue distribution pattern of A and B are discrete (suggesting functional differences, (Angotzi et al., 2013; Gorissen et al., 2009), a great majority of fish studies exclusively focus on leptin A (Dalman et al., 2013; Frøiland et al., 2010; Huising et al., 2006; Liu et al., 2012). To date, functional study of leptin B has been limited to expression studies (responds to food restriction- Gorissen et al., 2009; increases during early development- Angotzi et al., 2013), and all manipulations using species-specific recombinant leptins have used leptin A (Dalman et al., 2013; Liu et al., 2012; Lu et al., 2012; Murashita et al., 2011, 2008).

It may be that leptin B contributes little to leptin function. Relative qPCR cannot determine copy number among different mRNAs (e.g. leptin A vs. leptin B), only relative differences within a given mRNA (Ball et al., 2013). Thus studies using relative qPCR would not identify if a gene were expressed at very low mRNA levels *overall*, just relative differences among treatments. Using absolute qPCR, we estimate that leptin B copy number is at least 10x lower than leptin A in zebrafish (Ball and Londraville, unpublished). Similarly, leptin 2 in *Anolis* cannot be amplified by RT-PCR (Boorse and Libbon, J.V., 2010), although it is undetermined whether *lep 2* in *Anolis* is homologous to leptin B in fish. Finally, the binding energy of leptin interacting with its receptor (*in silico* simulations) is an order of magnitude higher for A vs. B in both zebrafish and *Medaka* (Prokop et al., 2012).

Given that all tetrapods express a single ortholog of leptin (n.b.-Aves may not express any leptin ortholog), and many more ancestral vertebrates express two or more leptins, which of the ancestral orthologs is the homolog to tetrapod leptin (particularly mammalian leptin)? Gorissen et al. (2009) argued for leptin B based on its exon structure and gene synteny. However, recent analyses clearly indicate that the synteny associated with mammalian leptin is parsed between A and B paralogs in ancestral vertebrates (with *rbm28* associating with *lep A* and *snd1*, *lrrc4*, and *impdh* genes associating with *lep B;* Denver et al., 2011; Won et al., 2012). Further, some phylogenetic analyses of leptin sequences favor A over B as the mammalian homolog, but with weak bootstrap support (He et al., 2013; Zhang et al., 2013). Our phylogeny cannot resolve which leptin ortholog is most similar to tetrapod leptin, and other approaches (Neighbor Joining, Maximum Likelihood, Maximum Parsimony, multiple assumptions of sequence evolution) return essentially the same tree. Even inclusion of the recently identified coelacanth leptin sequence (Figure 1) has yielded little resolution, as this sequence has very clear affinities to tetrapods rather than fish. Clearly, more leptin genes among more diverse taxa, and more functional work on the physiology of leptin B is needed to determine the origin of the tetrapod leptins.

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## The leptin/leptin receptor complex

LepRb is the only isoform that can activate Jak/STAT signaling through its intracellular domain (reviewed in Denver et al., 2011). A vast majority of the work done on non-mammal leptin receptors concentrates on the long-form of the leptin receptor (lepRb), although multiple leptin receptors are expressed throughout vertebrates (Cao et al., 2011; Ronnestad et al., 2010). Recent simulation (Prokop et al., 2012), crystal structure of the leptin receptor (Carpenter et al., 2012) and cryo-electron microscopy imaging (Mancour et al., 2012) studies identify the extracellular cytokine homology region (CHR2 domain) as the site of leptin/leptin receptor binding. Two lepRb molecules cross over each other, each binding a separate leptin molecule to form an "X" quaternary structure (Mancour et al., 2012). Mutations that disrupt this quaternary structure eliminate leptin signaling (Peelman et al., 2006). Prokop et al. (2012) did a comparative analysis of leptin and leptin receptor interaction across vertebrates. They identified several instances of potential coevolution between receptor and ligand within the CHR2 domain. In macaque, platypus, green anole, *Xenopus*, and guinea pig, amino acid substitutions (relative to human) in the binding site of the ligand were accompanied by compensatory changes in the receptor. The authors hypothesize that these substitutions served to stabilize the interaction and maintain predicted binding energy across taxa.

Given that the binding site for leptin to its receptor is well established, and that it is conserved across diverse taxa (Hammond et al., 2012; Prokop et al., 2012; Ronnestad et al., 2010) it can now be used as another character to evaluate new leptin clones. In the Prokop et al. (2012) *in-silico* evaluation of 35 taxa, only bird leptins did not form stable complexes with their homologous receptors. Leptin receptors are represented in many bird genomes (chicken CGNC:49091; mallard NW_004677703.1; zebra finch ENSTGUG00000010030.1; turkey NC_015020.1; rock pigeon LOC102098873; saker falcon LOC102049003; peregrine falcon 101921754; collared flycatcher NC_021680.1; medium ground finch LOC102041765; white-throated sparrow NW_005081684.1). However, the ligand that binds to these receptors has been notoriously difficult to find, even in the face of considerable effort. The original reports of chicken leptin (Taouis et al., 1998) cannot be independently verified (Friedman-Einat et al., 1999; Pitel et al., 2000; Sharp et al., 2008). In addition, many of the genes expected to be found in synteny with leptin are missing from current builds of the chicken genome, and from chicken EST libraries; in sum there are considerable and varied data that suggest that chickens do not express leptin (Pitel et al., 2010). Is leptin missing from all bird genomes? The zebra finch genome project has identified a partial leptin transcript (XM_004175791) that retains features of leptin primary structure consistent with predicted phylogenetic distance for birds (unlike the reported chicken leptin; Prokop et al. unpublished results). Binding analyses, expression studies, and functional data will be needed before we know if a true bird leptin has been cloned, and how (if) it differs from other vertebrate leptins.

## Leptin functional diversity

Given the diverse life histories, physiologies, and ecologies of the organisms with leptin clones, the functional contexts in which leptin is being studied has greatly expanded, and

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will continue to expand with new transcriptomes and genomes sequenced each year. Thus far, the overwhelming majority (47%) of studies conducted in non-model organisms have focused on leptin's function as an adipostat and anorexigen, followed by reproductive function (16%; Figure 3). This trend mirrors the research focus using biomedical models during the first years of leptin research. However, there is a disproportionately large community of researchers investigating leptin-immune system interactions in non-model organisms (12.6%) relative to biomedical models (1.7%), which highlights particular interests in this field of leptin research (see recent reviews by Carlton et al., 2012; French et al., 2011). In addition, because scientists studying these non-model organisms are following their own historical species-specific research interests, we see different foci of leptin research within taxa. For example, we see studies investigating leptin's role in thermogenesis in the high-elevation Plateau pika (Yang et al., 2011) or vole (Chen et al., 2012), seasonality in Siberian hamsters and bears (Adam and Mercer, 2004; Demas, 2004; Gardi et al., 2011), and in embryonic or larval development in zebrafish (Liu et al., 2012, 2010) and *Xenopus* (Crespi and Denver, 2006). In the following sections, we highlight what we currently know about leptin function across vertebrates, and we integrate these insights with recent findings within the biomedical literature to provide a prospectus of exciting, future research directions in this field.

## Leptin as an anorexigen and adipostat

Administration of exogenous leptin (either homologous or heterologous) reduces food intake in mammals, birds, amphibians, lizards, and fishes (Table 1A); as such leptin's function as an anorexigen may be the only function that is shared among vertebrates (without qualifying statements). These studies were some of the first done in a comparative context, as this question was arguably the foremost in a comparative scientist's mind (what motivates organisms to feed). In many cases these studies were done before homologous recombinant protein was available (e.g. Londraville and Duvall, 2002), although non-homologous leptins are still used in feeding studies (Table 1A). Given the fact that leptin primary structure is highly variable, many researchers rightly argued for caution in interpreting studies that use heterologous leptins (Denver et al., 2011; Murashita et al., 2008). One irony is that the artifactual chicken leptin has been used in feeding studies (Lõhmus et al., 2006) even though it does not form a stable complex with the chicken leptin receptor (Prokop et al., 2012); most leptin administration studies in birds use mammalian leptin (Cerasale et al., 2011; Song et al., 2009; Yang and Denbow, 2007). However, with recent structural studies indicating the leptin/leptin receptor binding interface is highly conserved (Prokop et al., 2012), and empirical studies that have shown leptin derived from diverse species can activate mammalian leptin receptors (Crespi and Denver, 2006), perhaps the 'older' studies should not be dismissed. Certainly homologous proteins, and species-specific antibodies should be used whenever possible, especially given that recombinant leptins from many vertebrates are now available.

The second element of leptin dogma known widely to the lay public is its role as an adipostat. In early models leptin was thought to faithfully report total lipid stores to the CNS, such that changes in stores can be sensed quickly and physiology adjusted to survive a starvation event (Ahima and Flier, 2000). However, regressions between BMI (body mass

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index) and leptin titer in humans contain significant scatter, such that individuals at a given BMI can vary in their leptin titer by an order of magnitude (Cnop et al., 2002). In hibernating woodchucks, leptin titer and total body mass are out of phase (Concannon et al., 2001). A majority of fish studies report an increase in leptin mRNA or circulating protein with fasting (Table 1B) and a rapid decrease upon refeeding (Fuentes et al., 2013), which does not support the adipostat model. Recently, a leptin receptor mutant was generated in medaka (Chisada et al., 2014) and these leptin receptor −/− fish have increased appetite and growth rate. Unexpectedly, these mutants do not have increased fat stores in liver or skeletal muscle, but do develop a visceral fat body as adults (controls do not). The authors did not report the response of leptin to the knockout, but it is curious that the one tissue that increases fat stores does not express leptin in fish. Clearly leptin does not faithfully report energy stores in fishes, and as such should not be considered an ‘adipostat’; other species need further study. Fuentes et al. (2013) argue that increased circulating leptin during fasting activates energy liberating pathways (through AMPK), while inhibiting anabolic pathways (through TOR).

## Leptin as a pleiotropic stress-responsive hormone

As described above, leptin has anorexigenic effects via its actions in the central nervous system in all vertebrate groups in which it has been tested, which strongly supports an ancient origin of this function. While this action has been primarily studied within the context of the regulation of meal size and frequency in biomedical models, leptin’s anorexigenic effects may be associated with adaptive responses to other physiological stressors. For example, hypoxia increases leptin mRNA expression in zebrafish (Chu et al., 2010) and carp (Bernier et al., 2012), as it does in the neonatal rat (Bruder et al., 2005), the mammalian placenta (Grosfeld et al., 2001), and in adipocytes of obese humans (Trayhurn, 2013). Functionally, this increase in leptin message is associated with the anorexia, but it also protects gill tissues or neurons from apoptotic effects of hypoxia (see Gavello et al., 2012; Shin et al., 2009). Similarly, increases in circulating leptin associated with sickness behavior have been proposed to induce anorexia (i.e. cachexia), but also stimulate pro-inflammatory immune responses and wound healing associated with illness or injury (Carlton et al., 2012). Leptin also has been proposed to be a cold stress protein, as it is expressed in brown adipose tissue and induces thermogenesis in cold-adapted mammals (Yang et al., 2011). These examples demonstrate that leptin has adaptive pleiotropic actions geared toward either the protection or recovery from these challenges as well as the alteration of feeding behavior.

In addition, interactions between leptin and glucocorticoids or corticotropin-releasing factor (CRF) have been established in mammals, which suggests that there may be direct links between the hypothalamo-pituitary-adrenal/interrenal axes (Roubos et al., 2012). These interactions have been implicated in the homeostatic controls of food intake (Ahima et al., 2000) but also been associated with stress responses. For example, leptin treatments blunt stress responses in mice (Glasow and Bornstein, 2000; Heiman et al., 1997), suggesting that leptin could mediate condition-dependent responses. Leptin also attenuated activation of the neuroendocrine stress axis in the common carp by blunting CRF-induced adrenocorticotropic (ACTH) hormone at the level of the pituitary, but it did not affect

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ACTH sensitivity of the interrenal gland (Gorissen et al. 2012). However, the interactions between leptin and both glucocorticoids and CRF are context-dependent and can vary with developmental stage (Ahima et al., 2000; Spencer, 2013). Surely, additional studies in novel contexts across all vertebrate species will yield a much greater mechanistic understanding of leptin's role as a stress-responsive hormone.

## Leptin as an immunomodulator

Given that leptin is an adipokine and its receptor is a class I helical cytokine receptor, it is not surprising that leptin signaling is involved in multiple arms of the immune system. While leptin has been studied primarily as an energy balance factor that also affects the immune system, many cytokines, such as interleukins and chemokines, are primarily studied as immune factors with effects on energy balance (Huising et al., 2006). Indeed, in mammals leptin has effects on innate and acquired immune responses (Dardeno et al., 2010; Lam and Lu, 2007). Leptin, itself proinflammatory, stimulates the secretion of other proinflammatory cytokines in the innate immune system such as IL-1, IL-6 and TNF-α (Loffreda et al., 1998), and increases phagocytic activity of macrophages (Mancuso et al., 2002). In relation to the acquired immune system, leptin treatment enhances proliferation and suppresses apoptosis of T-cells (Lord et al., 1998; Papathanassoglou et al., 2006). Leptin also enhances wound healing in rodents (Frank et al., 2000; Slavkovsky et al., 2011).

Leptin also has enhancing interactions with the immune system in different vertebrate classes (e.g. Dalman et al., 2013; Mariano et al., 2013 in fishes; Lõhmus et al., 2004 in birds; French et al., 2011 in lizards; Crespi et al., 2012; Hicks-Courant, M.L. and Crespi, E.J., 2006 in amphibians), but recent studies emphasize the role leptin plays in energetic tradeoffs between the immune system and other physiological systems (see Demas, 2004). For example, in lizards leptin treatment also accelerates wound healing (using murine leptin, French et al., 2011a), but enhancement only occurred in food-restricted conditions during the reproductive season when wounds typically heal more slowly. Leptin actions in this context suggests that leptin mediates resource-dependent trade-offs between reproduction and immunity (French et al., 2011). Changes in leptin levels also appear to mediate changes in immune function with seasonal changes in resource availability. In Siberian hamsters, stored fat, leptin levels, and humoral immune responses are reduced during the short-days of winter, but leptin treatment can restore humoral immunity along with increasing food intake and cortisol levels (Drazen et al., 2001). Although much more research needs to be conducted in this area, these studies suggest that leptin is an evolutionarily conserved immunomodulator that regulates immune responsiveness within the context of the life history stage, nutritional state, and energetic demands of the animal.

## Leptin as a growth factor during early development

Another exciting area of research that is emerging is on the role of leptin as a potential morphogen and growth factor during embryonic and larval development. Studies of leptin function in zebrafish and *Xenopus*, both primary models of early developmental processes in vertebrates, have shown a role for leptin. In zebrafish, leptin receptor is expressed first in the notochord area, but also in the gut, muscle, inner ear and hindbrain of early-staged larvae

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(Liu et al., 2010). Morpholino knockdown showed that leptin signaling is important for proper formation of the inner ear and dorsal brain, as well as retina and overall body size and curvature (Liu et al., 2012). In salmonids, the story is more complicated because of differential mRNA expression of leptin paralogs across development and in different tissues (Angotzi et al., 2013): Leptin A1 is highly expressed in embryos, and its expression increases until hatching, whereas Leptin B1 is the dominant leptin expressed in the brain and B2 is expressed in the gill through subsequent developmental stages. Leptin may also affect bone formation in zebrafish, as leptin knockdown dramatically reduces calcified bone (Figure 4; Liu and Londraville unpublished data). The field is very unsettled as to the effect of leptin on bone in mammals. There are proponents of central leptin control of bone growth exclusively through a hypothalamic relay, and the effects of increased leptin signaling as *inhibitory* to bone formation (Wei and Ducy, 2010), and other groups strongly advocating for the control of bone growth through peripheral leptin, and the effects of increased leptin signaling as *stimulatory* to bone formation (Turner et al., 2013). Again, a comparative approach can only contribute to solving this conundrum.

Studies of leptin function in *Xenopus* have resulted in additional roles of leptin during early development not described in mammalian species. Leptin administration also accelerated limb development in amphibian tadpoles *in vivo* and in culture, supporting the idea that leptin acts on leptin receptors (which are expressed in the developing limb) to stimulate proliferation (Crespi and Denver, 2006). Leptin also enhances lung development in *Xenopus* tadpoles (Torday et al., 2009), and an up-regulation in leptin mRNA expression at the site of tail excision links paracrine actions of leptin to regenerative processes in *Xenopus* tadpoles (Love et al., 2011). These studies have only scratched the surface of describing both paracrine and endocrine roles of leptin in early development.

## Future directions in leptin biomedical research

Leptin was the first identified hormone produced by adipose tissue in mammals and established fat as an endocrine organ (Zhang et al., 1994). The research that followed during the last 20 years shows that leptin orchestrates a complex physiological system that maintains homeostatic control of body weight, informing the brain about the status of the body's energy reserves (in the form of fat tissue), and at the same time regulates food intake and energy expenditure (Friedman, 2011). The leptin message is of greatest importance since it informs the central nervous system whether energy reserves are sufficient to maintain important functions such as reproduction and immune homeostasis (Friedman, 2011). Regardless of the actual amount of body fat stores, the leptin signal is essential to maintain glucose homeostasis, food intake, immune function, and reproduction. This is clearly observed in leptin- or leptin receptor-deficient rodents and humans, which are obese and have multiple neuroendocrine and immune alterations (e.g. lipodystrophy syndromes; Mantzoros, 2012).

## Leptin resistance

Obese individuals show high serum levels of leptin (R V Considine et al., 1996; Maffei et al., 1995), and leptin treatment has little effect on weight loss in humans (Fogteloo et al.,

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2003; Heymsfield et al., 1999). Understanding how leptin resistance or tolerance arises is one of the most important areas of biomedical research related not only to leptin biology, but most importantly, to the pathophysiology of obesity. Current findings support the notion that during obesity, leptin action can be impaired at the signaling level; inhibitor proteins (SOCS3, PTP1B, SHP2), which normally act as negative feedback regulators, may be over-activated during obesity and thus contribute to leptin resistance (Dardeno et al., 2010). Leptin transport across the blood brain barrier is also impaired, due to reduced transport activity and saturation of transporters by high leptin levels (Burguera et al., 2000; Vilà et al., 1998). The most recently proposed mechanisms of leptin resistance include: endoplasmic reticulum (ER) stress (Ozcan et al., 2009), decreased mitochondria-ER contacts (Schneeberger et al., 2013), and mitochondrial dysfunction in the hypothalamus (Kleinridders et al., 2013). Additionally, leptin resistance may be selective, in that some leptin signaling pathways are diminished while others are overactivated, leading (for example) to increased blood pressure and hypertension in obesity (Mark, 2013). A deeper understanding of the intricate mechanisms leading to selective leptin resistance and its interaction with glucose homeostasis and insulin signaling (Dardeno et al., 2010; Kim et al., 2000) will lead to the development of new therapies against obesity, diabetes, and insulin resistance syndromes.

Although it has received relatively little attention, the leptin receptor overlapping transcript (LEPROT) may be a key controller of leptin signaling, and an opportunity for comparative approaches to contribute to solving the problem of leptin resistance. In humans and other mammals, LEPROT is a product of the leptin receptor gene via an alternate start site; its mRNA is spliced from four exons not expressed in leptin receptors. Thus LEPROT shares no sequence identity with leptin receptors but is under control of the leptin receptor promoter (Bailleul et al., 1997). Work from the labs of Ralf Jockers and Yves Rouille, suggests that LEPROT (renamed endospanin) is a powerful regulator of leptin signaling (Couturier et al., 2007; Seron et al., 2011). LEPROT codes for a small (131 amino acid), 4-transmembrane spanning protein that localizes to endosomes and the trans-Golgi (Seron et al., 2011). LEPROT does not regulate total expression of the leptin receptor, but rather how many copies of leptin receptor are functional at the cell surface. Immunolabeling experiments suggest that LEPROT negatively regulates the post-translational Golgi processing of leptin receptor, such that when LEPROT expression is high, surface expression of the receptor is low. Further, knockout mice for LEPROT express maximal leptin receptors at the cell surface and are resistant to diet-induced obesity (Couturier et al., 2007). Jeon et al. found evidence of a similar mechanism in humans; they documented a negative correlation between exon 2 (present in LEPROT) copy number and leptin receptor expression, and positive correlation between exon 2 and LEPROT expression (Jeon et al., 2010). LEPROT control of leptin receptor expression may be specific to neuronal (vs. peripheral) leptin receptors (Satoh et al., 2009), and may also regulate growth hormone receptors through a similar mechanism (Touvier et al., 2009).

Unlike leptin or its receptor, LEPROT primary structure is highly conserved among vertebrates (86% identical between zebrafish and humans, vs. 22% for leptin; Londraville and Niewiarowski, 2010), and is structurally and functionally related to yeast VPS55p, a membrane-trafficking protein. Such sequence conservation across distant taxa usually means

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that the gene is essential. Interestingly, both *Medaka* and zebrafish LEPROT genes are *not* under control of the leptin receptor promoter, but instead are separate genes on separate chromosomes (Kurokawa and Murashita, 2009). Is the control of leptin signaling via LEPROT a mammalian innovation, and is control by a common promoter necessary for LEPROT to regulate leptin signaling? Is LEPROT tied to leptin resistance? A comparative approach can test this question.

## Cancer and stem cell biology

In addition to its prominent role in metabolic homeostasis, leptin is implicated in cancer development and progression; studies show a positive association between leptin serum levels and breast and thyroid cancer (Akinci et al., 2009; Niu et al., 2013). Proposed mechanisms include: increased estrogen production and decreased estrogen receptor degradation, upregulated expression of VEGF and VEGFR (Dutta et al., 2012), stimulation of cyclin D1 expression, and induction of cancer stem cell survival and pluripotency (Zheng et al., 2013, 2012) in breast cancer and colorectal cancer (Bartucci et al., 2010), and increased expression of leptin receptor leading to higher levels of the anti-apoptotic protein XIAP (Cheng et al., 2010) in thyroid cancer. Leptin receptor antagonists are being developed and could be useful tools for cancer therapeutics (Otvos and Surmacz, 2011).

Another exciting area for present and future leptin biomedical research is stem cell biology, not only in the context of cancer as mentioned previously, but also in maintenance of the hematopoietic stem cell pool in which bone marrow leptin-receptor-expressing perivascular stromal cells play an important role (Ding et al., 2012); in addition, leptin regulates mesenchymal progenitor cell differentiation (Scheller et al., 2010) and promotes mobilization of bone marrow progenitors and their differentiation into vascular cells (Schroeter et al., 2012). Leptin also seems to play an important role in neurogenesis; it induces fetal hypothalamic stem/progenitor cell differentiation into neurons *in vitro* (Desai et al., 2011), increases neurogenesis in the hippocampus of adult mice (Garza et al., 2008), stimulates neural stem cell proliferation, neurogenesis, gliogenesis, and angiogenesis after stroke (Avraham et al., 2011), and attenuates neurodegeneration via increased neural progenitor proliferation in rodent models of Alzheimer's disease (Pérez-González et al., 2011). Interestingly, neuronal leptin resistance has been suggested in Alzheimer's disease (Bonda et al., 2014), raising the question of whether leptin resistance in obesity and other pathologies may lead to previously unrecognized neurological alterations.

## Leptin as a therapeutic drug

Soon after the long-expected cloning of the *ob* gene in 1994 (Zhang et al., 1994), weight loss effects of leptin were evaluated in obese patients with and without congenital leptin deficiency. Treatment with recombinant methionyl human leptin proved to be a life-changing intervention for leptin-deficient patients, since it not only reduced body weight dramatically but also normalized most neuroendocrine and immune abnormalities observed in such patients (Farooqi et al., 2002, 1999). Leptin has also been used in partial leptin-deficiency states including lipodystrophy (congenital or related to highly active retroviral therapy for HIV) and hypothalamic amenorrhea. Lipodystrophy is characterized by

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drastically reduced subcutaneous adipose tissue, dyslipidemia, insulin resistance, hyperglycemia, and hepatic steatosis. Treatment of these patients with physiological doses of leptin reduces central fat mass, improves insulin and glucose levels, and increases insulin sensitivity (Chong et al., 2010; Lee et al., 2006). Hypothalamic amenorrhea is due to dysfunction of the hypothalamic-pituitary-gonadal axis, resulting in the absence of ovulatory menses, and it occurs secondary to stress, excessive exercise, and low food intake. Leptin treatment results in the recovery of menstrual cycles and normalization of neuroendocrine abnormalities in women with this condition (Chou et al., 2011; Welt et al., 2004).

In contrast to its clear therapeutic effects on leptin-deficient states, clinical trials using recombinant leptin in most obese subjects showed little or no weight-reduction (Fogteloo et al., 2003; Heymsfield, 1999), a result not completely unexpected knowing that, rather than a lack of leptin, most obese patients have elevated levels of serum leptin and are presumably tolerant or resistant to its effects (Considine et al., 1996; Maffei et al., 1995). Nonetheless, more recently, leptin regained promise as a therapeutic factor against obesity-related metabolic alterations. Combination therapy with metreleptin (human recombinant leptin) and pramlintide (an amylin analog) in overweight and obese subjects showed significantly greater weight loss effects than monotherapy-treated groups (Ravussin et al., 2009). Also, leptin treatment after weight loss prevents declines in energy expenditure, muscle work efficiency, and thyroid hormone levels that normally occur to compensate for reduced energy stores. Thus, leptin therapy is suggested to help maintain reduced body weight (Rosenbaum et al., 2005, 2002). Another new area for leptin therapeutics is its potential for achieving better glucose control with lower insulin dosage in type 1 diabetes patients; a clinical trial evaluating these effects is currently ongoing and is based on previous studies showing improved glucose metabolism in type 1 diabetes in rodents after combinatorial treatment with insulin and leptin. Several other clinical trials are assessing the safety and efficacy of metreleptin treatment on lipodystrophy, hypothalamic amenorrhea, obesity, and diabetes (Chou and Perry, 2013).

## How comparative models may inform biomedical research

Given the predominant effect of leptin on food intake, energy homeostasis, reproduction, immune function and development, comparative models represent excellent tools to understand leptin’s function, particularly in species showing extreme phenotypes of metabolic plasticity, such as hibernators, marine mammals, and birds.

Hibernation is an evolutionary adaptation allowing survival in conditions of prolonged low ambient temperature and scarce food. Mammalian hibernators increase their food intake and fat storage in the active season; however, food intake peaks and starts declining months before fat storage reaches its maximum, right before the start of the hibernation period. This apparent discrepancy is explained by a drastic reduction in metabolic rate favoring fat accumulation. In most hibernators, leptin levels correlate positively with adipose tissue mass (Concannon et al., 2001). Serum leptin is lowest at the end of hibernation in spring and starts rising during the summer along with fat reserves, reaching its maximum level around the time when food intake declines, suggesting that leptin is one of the anorexigenic drivers. Leptin levels and fat stores stay high during autumn until both start declining in winter

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(Florant and Healy, 2012; Florant et al., 2004). Several relevant issues regarding leptin function may be studied using this model of dramatic circannual changes of body mass and food intake; for example, is leptin involved in establishing the set point for peak body mass? Also, leptin levels and fat stores remain high during autumn, together with reduced feeding behavior; if leptin is responsible for this anorexigenic state, what mechanisms prevent leptin resistance in this particular context of obesity? The Tups laboratory (Tups, 2009; Tups et al., 2012) showed that in the siberian hamster (*Phodopus sungorus*), seasonal changes in leptin sensitivity may involve fluctuations in hypothalamic STAT3 activity, resulting from leptin receptor binding to the inhibitor SOCS3 and to its competitive negative modulators: SHP2/GRB2. A relevant question for biomedical research is, what is the mediator of the photoperiod-induced reversibility of leptin resistance?

Finally, what are the molecular underpinnings of why obese non-hibernating rodents and humans develop leptin resistance and metabolic disease, whereas hibernating mammals stay healthy? For instance, despite presenting obesity, hypercholesterolemia, reduced blood flow and inactivity, brown and black bears are resistant to atherosclerosis (Arinell et al., 2012) and immobility-induced bone loss (Seger et al., 2011), respectively. Another feature of hibernators is their ability to excessively reduce body temperature, along with changes in blood pressure and flow during short cycles of hibernation (torpor) and arousal. Such drastic alterations in non-hibernating mammals are deleterious for most organs, as seen in cases of heart attack and stroke. Therefore, understanding the mechanisms mediating organ protection in hibernators could lead to development of treatments against ischemia-reperfusion injury as well as improved preservation of organs for transplants. Recently identified mechanisms that distinguish hibernation ischemia-reperfusion are increased fatty acid metabolism (Xu et al., 2013) and cytoskeletal reorganization (Hindle and Martin, 2013).

Marine mammals and diving birds represent opportunities for how comparative leptin study may inform human health. Marine birds and mammals maintain large lipid reserves without the morbid obesity syndrome seen in humans, and these large lipid reserves express very high copy numbers of leptin transcripts (Ball et al., 2013). Is there some aspect of leptin signaling that allows these ‘obese’ vertebrates to avoid leptin resistance? Marine mammal leptins evolved more rapidly than those of terrestrial mammals (Hammond et al., 2012), but the structural changes do not map where leptin interacts with its receptor (Hammond et al., 2012; Prokop et al., 2012). Hammond et al. (2012) postulate that the structural changes to seal leptins result in a leptin that is a more effective lung surfactant, which is critical to reinflating the lungs after deep dives. If so, the seal leptin could serve as a model for modifying human leptin as a treatment for newborn humans with pulmonary challenges.

Another area of research that will greatly influence biomedical studies is the examination of leptin actions in early developmental processes. Recent findings showing that leptin has roles in early morphogenesis and organogenesis in zebrafish and *Xenopus* (see references above) are likely the first of many studies to reveal roles for leptin during this developmental window. Hoggard et al. (2000) showed that leptin mRNA and protein are expressed in early fetal development of mouse, but little has been resolved regarding the function of leptin at these stages. Leptin may act as a growth factor during embryonic development, much like

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IGF and related peptides; but then take on the role of a nutritional modulator of the timing of early developmental processes and thus, adjust the timing of early life history transitions according to available resources (similar to leptin's association with the timing of puberty, Cheung et al., 1997). This hypothesis is intriguing because adipose tissue is not present at these early developmental stages and the source of leptin as a hormone could be from the liver or placenta, or leptin can have effects through paracrine pathways, or both. Leptin regulation of development rate can happen in unexpected ways. For example, in the Indian short-nosed bat, the increase in leptin levels during gestation inhibits progesterone secretion, thereby delaying development rates (Banerjee et al., 2010). Whether leptin-progesterone interactions are important in humans is not yet resolved, but taking a comparative approach to investigating the many roles leptin may be playing in the fetus during early development, or in the placenta, is likely to generate novel hypotheses that can be applied to humans (Zhao et al., 2003).

## Comparative endocrinology of leptin: research prospectus

The field of comparative endocrinology of leptin is in its infancy, and this review only highlights several of the exciting research directions currently undertaken by this growing community. The sequencing of leptins across diverse vertebrate taxa have given some insight into the evolution of this gene, and as shown here, the duplication of leptin genes in fishes has increased diversity of leptin, and potentially its function in these lineages. There are many novel contexts of leptin function that have been described, especially in the field of environmental endocrinology, but it is still too early to make definitive conclusions about how leptin function has evolved in vertebrates or even more broadly within the animal kingdom, as a presumptive leptin homologue in *Drosophila* recently has been described (Unpaired 2 gene). While initial experiments have demonstrated the anorexigenic effects of leptin across vertebrates, the physiological roles of leptin in the regulation of energy balance and food intake has not yet been resolved in most species; nor can we make clear distinctions between the roles of leptin in endoderms vs. ectotherms at this time. However, the necessity to generate species-specific molecular tools to examine leptin's role in non-model organisms, given its tremendous amount of diversity in nucleic acid and amino acid sequence across vertebrates, continues to prevent the pace of research to keep up with the growing interest in working with leptins in non-model organisms. Furthermore, the association between leptin and obesity has been extremely stimulatory to the field, but is also a hurdle to overcome when trying to understand its 'holistic' function from an organismal and/or evolution point of view. It is clear that those working on leptin in non-model organisms need to steer clear of biases of leptin function as described in rodents and humans, but the more leptin is studied in novel phylogenetic groups and in animals with diverse life histories, the greater our understanding of the pleiotropic nature of this unique, adipokine hormone.

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## Highlights

- Leptin diversity within and among vertebrates is reviewed with a new phylogeny of leptins
- Leptin universally reduces appetite among all vertebrates tested, but does not reflect fat stores in fish
- Leptin is a stress-responsive hormone in multiple contexts and is an important immunomodulator throughout vertebrates
- Several comparative models offer opportunity for studying human disease

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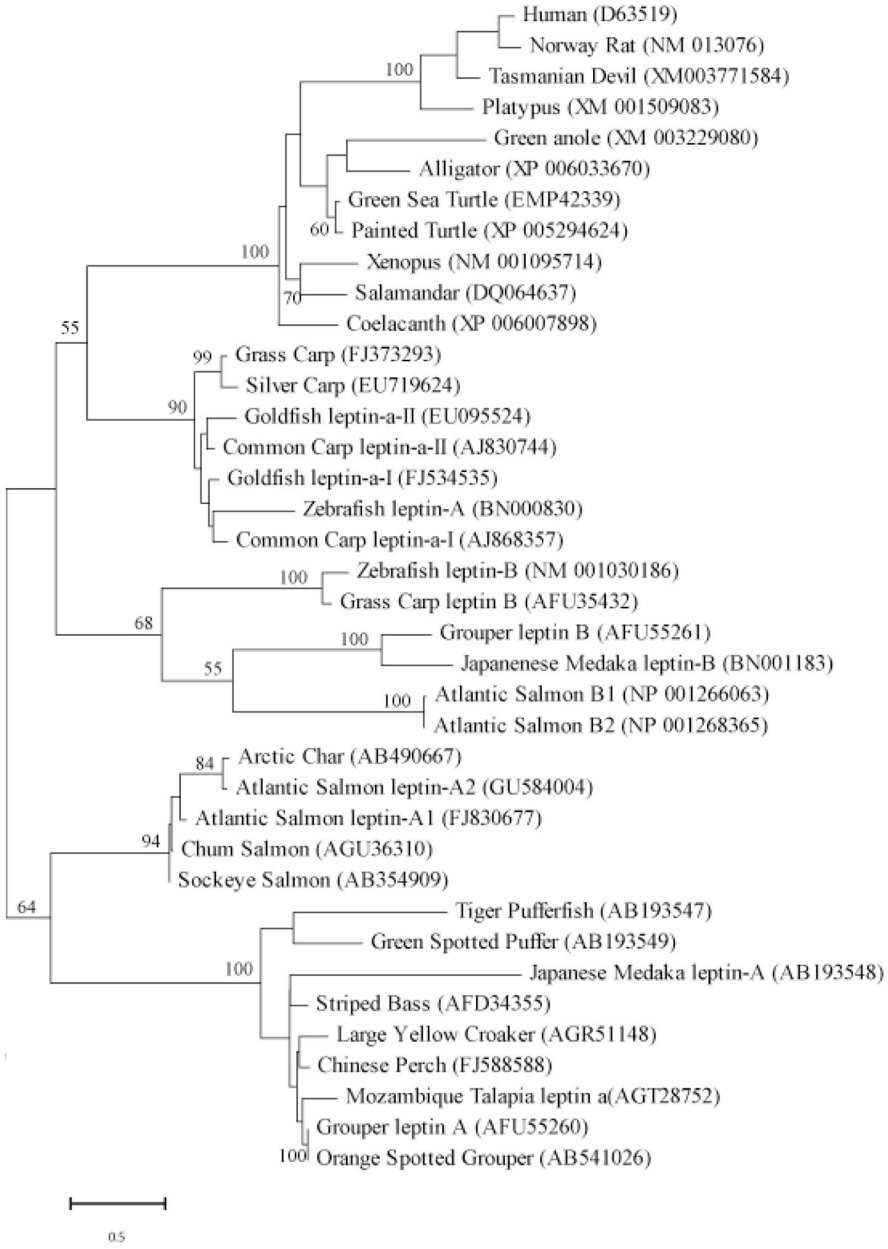


**Figure 1. Phylogenetic tree of 29 boney fish and representative tetrapod leptins**

The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992) as conducted in MEGA5 (Tamura et al., 2011). Numbers at nodes represent percentage of 100 bootstrap replicates. Notes with no number indicate bootstrap support of less than 50%. Inferred leptin amino acid sequences were manually aligned in MEGA5 informed by protein structural homologies. GenBank accession numbers in parentheses represent protein accessions.

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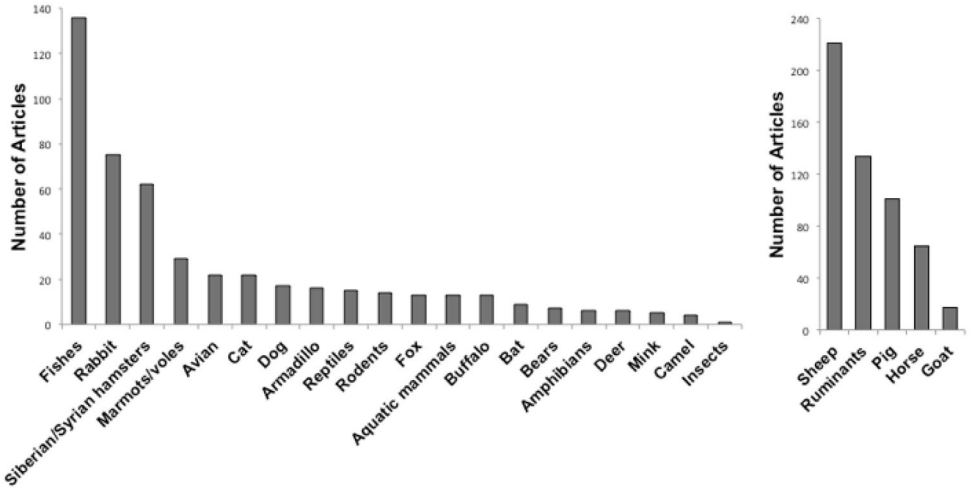


**Figure 2. Diversity of organisms in which leptin physiology has been studied from 1990s on-ward**

We resolved the number of published studies on leptin in non-model organisms and agricultural organisms resulting from searches conducted in Web of Science. More than 15,000 articles involving biomedical model organisms (rat, mouse, human, non-human primate). When searching for articles involving leptin in non-model and agricultural organisms, each article that was listed using various search terms was inspected to verify the primary organism(s) studied. We did not include zebrafish or *Xenopus* in biomedical models even though they are genetic and developmental model organisms, nor did we include chicken or fishes reared in fisheries or chicken as agricultural organisms because these organisms better reflect phylogenetic diversity of leptin in this context. For non-model organisms, we included studies that used both homologous and heterologous leptins or leptin probes in our lists.

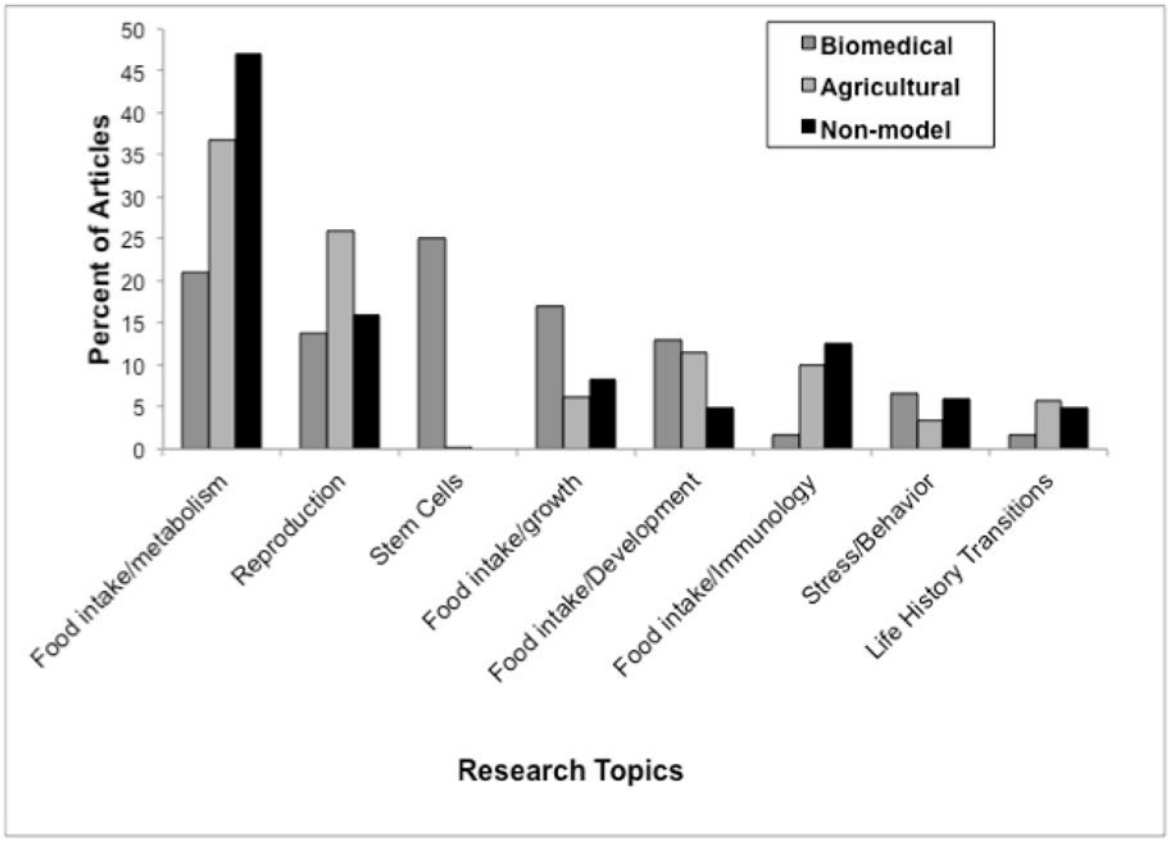


**Figure 3. Diversity of leptin functions studied in biomedical model organisms, agricultural species, and non-model organisms**

(see definition of categories in Figure 1).

Articles retrieved by Web of Science searches were saved to EndNote and categorized by their various general animal names as represented in the figures of the papers. For the non-model and agricultural organisms the articles were reviewed individually and organized into their specific research area. We included studies investigating the role of leptin in the timing of puberty in the Life History Transitions category. Due to the large numbers of biomedical studies, they were sorted into categories based on keywords associated with the article.



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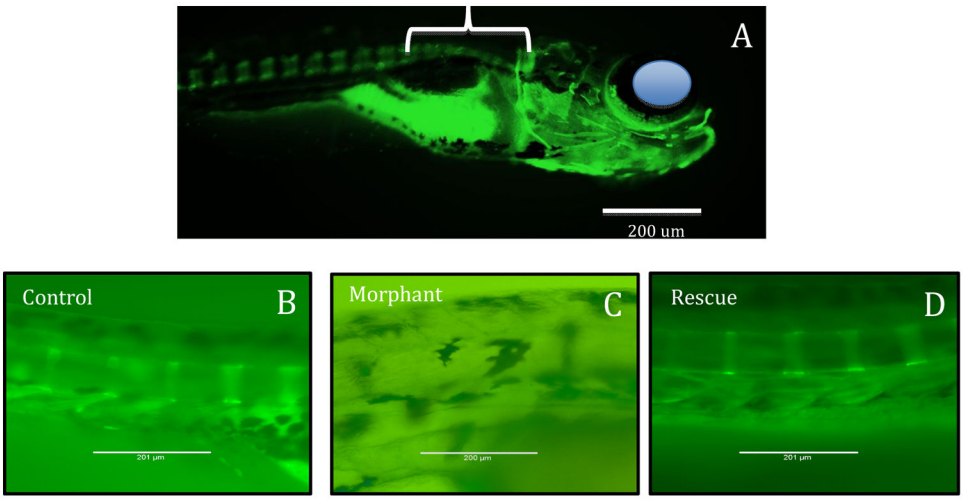


**Figure 4. Effect of leptin knockdown on bone mineralization in zebrafish**

A) Fourteen day post-fertilization (DPF) zebrafish stained with calcein, which stains mineralized bone (Du et al., 2001). Eye is depicted by blue circle for orientation, bracket indicates area magnified in B-D. Mineralized bone stains bright green (there is also a bright signal from ingested stain because the embryos are stained live). B) Control fish 9 DPF showing the first vertebrae to mineralize immediately caudal to the cranium C) 9 DPF fish treated with anti-leptin A morpholino oligonucleotide lepMO1 as in Liu et al., (2012). No mineralization is evident. D) 9DPF fish coinjected with anti-leptin A morpholino oligonucleotide lepMO1 and recombinant zebrafish leptin A as in Liu et al., (2012). Bone mineralization is rescued.

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## Table 1A

Effects of Leptin Manipulation on Food intake in Non-Mammalian Species

| Species | Leptin Source | Mode | Effects | Reference |
|---|---|---|---|---|
| *Xenopus* | *Xenopus* | ICV | ↓ food intake<br>↑ limb development | Crespi and Denver, 2006 |
| Fence lizard | Mouse | pellet | ↓ food intake<br>↓ activity<br>↑ metabolic rate | Niewiarowski et al., 2000 |
| Chicken | Human | ICV | ↓ food intake | Kuo et al., 2005 |
| Goldfish | Mouse | ICV, IP | ↓ food intake | Volkoff et al., 2003 |
| Goldfish | Human | ICV, IP | ≈ food intake (8 hrs.)<br>↓ weight gain | de Pedro et al., 2006 |
| Green sunfish | Mouse | IP | ↑ FABP<br>≈ total lipid stores | Londraville and Duvall, 2002 |
| Rainbow trout | Human | ICV | ↓ food intake<br>↑ glucose sensing | Aguilar et al., 2010 |
| Rainbow trout | Rainbow trout | IP | ↓ food intake (8 hrs.) | Murashita et al., 2008 |
| Rainbow trout | Rainbow trout | IP | ↓ growth | Murashita et al., 2011 |
| Grass carp | Grass carp | IP | ↓ food intake (short term) | Li et al., 2010 |
| Zebrafish | Zebrafish | Morpholino knockdown | ↓ metabolic rate | Dalman et al., 2013 |

ICV – intracerebroventricular injection; IP = intraperitoneal injection, pellet=slow release implant, morpholino knockdown= morpholino oligonucleotide knockdown of leptin A.

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## Table 1B

Leptin Response to Treatment

| Species | Treatment | Assay | Δ leptin | Reference |
|---|---|---|---|---|
| Common carp | Fasting | qPCR | ≈ 6d, 6 wks | Huising et al., 2006 |
| Zebrafish | Fasting | qPCR | ≈ leptin A<br>↓ leptin B | Gorissen et al., 2009 |
| Rainbow trout | Fasting | Salmon RIA | ↑ | Kling et al., 2009 |
| Atlantic salmon | Fasting | qPCR | ↑ | Trombley et al., 2012 |
| Fine flounder | Fasting | Salmon RIA | ↑ | Fuentes et al., 2012 |
| Fine flounder | Refeeding | Salmon RIA | ↓ | Fuentes et al., 2013 |
| Arctic charr | Seasonal cycle | qPCR | ↑ (during fasting months) | Frøiland et al., 2010 |
| Common carp | Hypoxia | qPCR | ↑ | Bernier et al., 2012 |
| Zebrafish | Hypoxia | qPCR | ↑ | Chu et al., 2010 |