Two divergent leptin paralogues in zebrafish (*Danio rerio*) that originate early in teleostean evolution

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Abstract

We describe duplicate leptin genes in zebrafish (*Danio rerio*) that share merely 24% amino acid identity with each other and only 18% with human leptin. We were also able to retrieve a second leptin gene in medaka (*Oryzias latipes*). The presence of duplicate leptin genes in these two distantly related teleosts suggests that duplicate leptin genes are a common feature of teleostean fishes. Despite low primary sequence conservation, we are confident in assigning orthology between mammalian and zebrafish leptins for several reasons. First, both zebrafish leptins share their characteristic gene structure and display key features of conserved synteny with mammalian leptin genes. Secondly, the cysteine residues that make up leptin’s single disulphide bridge are equally spaced in mammalian and zebrafish leptins and are unique among all members of the class-I helical cytokine family. Thirdly, the zebrafish leptins cluster with other fish leptins and mammalian leptins in phylogenetic analysis, supported by high bootstrap values. Within the leptin cluster, leptin-b forms a separate clade with the leptin-b orthologue from medaka. Finally, our prediction of the orthologue from medaka. Finally, our prediction of the positional cloning of the obese (*ob*) gene in 1994 (Zhang et al. 1994), identified the factor responsible for the morbid obesity of *ob*/ob mutant mice. This gene encodes a unique member of the class-I helical cytokine family, a 16 kDa protein named leptin after the Greek root *leptos* for lean. It is made up of a characteristic four \(\alpha\)-helix bundle conformation (Zhang et al. 1997). The key role of leptin in the regulation of body weight and energy homeostasis is well established (Schwartz et al. 2000, Morton et al. 2006). Leptin circulates in the bloodstream in proportion to the amount of body fat and signals to the brain. A major site of action is the arcuate nucleus (ARC), which contains two distinct populations of leptin-responsive neurons. One set co-expresses neuropeptide Y and agouti-related protein, is orexigenic and is inhibited by leptin (Broberger et al. 1998), while the other expresses pro-opiomelanocortin and cocaine and amphetamine regulated transcript, is anorexigenic and is stimulated by leptin (Elias et al. 1998).

Zhang et al. (1994) addressed the evolution of leptin by hybridizing genomic DNA of vertebrates that originated early in vertebrate evolution, including teleost fish, with a murine *ob* probe. Positive signals from teleost genomic DNA led them to conclude that leptin is highly conserved throughout the vertebrates. Despite the detection of lep-tin-like immunoreactivity in the blood and liver, it took more than a decade to characterize the first teleost leptin orthologue (Kurokawa et al. 2005, Huising et al. 2006a) or even amphibian leptin orthologues (Boswell et al. 2006, Crespi & Denver 2006). No bona fide avian or reptilian leptin genes have been described to date (Huising et al. 2006b). Both fish and *Xenopus* leptins show a low degree of primary sequence conservation compared with human leptins (varying from 13 to 30% amino acid identity respectively). Although the mere presence of a leptin orthologue in teleost fish supports the notion of leptin’s evolutionary conservation, leptin is among the class-I helical cytokines with the poorest sequence conservation throughout the vertebrate subphylum (Huising et al. 2006b). In fish, a major site of leptin...
expression is the liver (Kurokawa et al. 2005, Huising et al. 2006a), which is rich in fat droplets and has therefore been suggested as an appropriate site to monitor adipose stores. Yet, our understanding of the contribution of leptin to the regulation of energy metabolism in fish is scant and a key role of leptin in the regulation of body weight and energy homeostasis in non-mammalian vertebrates has not been established thus far (Volkoff et al. 2005, Gorissen et al. 2006). In carp, hepatic leptin mRNA increases post-prandially, but not after fasting or feeding to satiation for up to 6 weeks (Huising et al. 2006a).

It is well known that teleost fish possess duplicate copies for a number of genes (Taylor et al. 2003, Volff 2005). Therefore, we searched the zebrafish genome database to see if leptin too occurs in duplicate. Here, we demonstrate duplicate leptin genes in zebrafish (Danio rerio). An earlier systematic search of the zebrafish genome database revealed a predicted leptin gene with high (61—62%) amino acid identity to both carp leptin-a-I and leptin-a-II (accession number BN000830) now designated leptin-a (Huising et al. 2006a). We cloned this leptin gene, and a second substantially different and paralogous leptin gene in zebrafish. Both zebrafish leptin paralogues share 24% primary amino acid sequence identity with each other and 18% with mammalian leptins. Zebrafish leptin-a shares high primary sequence conservation with both carp leptins (61—62%); leptin-b, however, shares only 25% amino acid identity with both carp leptins. Despite these low identities, conservation of gene structure, tertiary structure, stable phylogenetic analysis, and synteny substantiate the unambiguous orthology of zebrafish leptin-a and leptin-b with mammalian leptins.

Materials and Methods

Animals

Zebrafish (D. rerio) were commercially obtained and reared in two liter tanks at 26 °C with recirculating, u.v. treated, Nijmegen tap water. Eight fish were kept and fed with 2.5% body weight Tetra-min (Tetra, Melle, Germany) each day. Eight other fish were not fed for 2 weeks. For the determination of leptin tissue distribution, fish were fed with 2.5% body weight daily and killed 1 h after feeding. All fish were euthanized in a 0.1% (w/v) 2-phenoxyethanol solution. Animal experiments were performed in accordance with national legislation and approved by the ethical committee of the Radboud University Nijmegen.

Identification of zebrafish leptin paralogues

We screened the ENSEMBL zebrafish genome (www.ensembl.org) with several teleost leptin sequences, using the BLAST algorithm (Altschul et al. 1997). The initial screen revealed two leptin-like sequences, one of which was already predicted in an earlier screen of the zebrafish genome (third

Table 1

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Table 2

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Divergent leptin paralogues in zebrafish

party annotation accession number: BN000830; Huising et al. 2006a). Using primers zf.leptin-a.fw, leptin-a.rv, and leptin-b.fw, leptin-b.rv (Table 1), based on these partial leptin sequences, two cDNA sequences were obtained from the liver and gonads respectively. RNA isolation, cDNA synthesis, cloning, and sequencing was carried out as previously described (Metz et al. 2005). Briefly, PCR products were ligated and cloned in TOP10 chemically competent Escherichia coli in the pCR4-TOPO vector (Invitrogen). Plasmid DNA was isolated with a Miniprep Kit (BioRad) and sequences were determined from both strands using the ABI prism big-dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

Multiple sequence alignments were carried out using ClustalW (http://www.ebi.ac.uk/Tools/clustalw/; Thompson et al. 1994). A phylogenetic tree was constructed based on amino acid difference (p-distance) with the neighbor-joining algorithm (pairwise deletion) in MEGA version 3.1 (Kumar et al. 2004). The reliability of the tree was assessed by bootstrapping, using 1000 replications. Only full-length coding sequences were used for analysis.

In order to determine synteny between the zebrafish leptin paralogues and human leptin, we mapped the upstream and downstream genes of leptin on the respective chromosomes of zebrafish and human using the ENSEMBL genome browser (www.ensembl.org).

Modeling of tertiary structures

The structure of human leptin (PDB entry 1AX8), which was resolved at 2.4 Å resolution (Zhang et al. 1997), was used as a template to build models of zebrafish leptin-a and leptin-b. Initial alignments were obtained from the PSIPRED fold recognition server (McGuffin & Jones 2003). Side-chain rotamers were modeled using SCWRL3.0
Divergent leptin paralogues in zebrafish

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Helix-A

Zebrfish leptin-a  MRFPALR-STCILSMLSLHCTPFVHQHDKRN-VKLQACTITIRERHIDG-QNLPLTLL  57
Zebrfish leptin-b  MKSSMIF-CLLISSLVAVSISMEPPE--DRIRITAARTISRIKIKDEHFOMSEPIDE  56
Carp leptin-a-I  MYFSALE-YPCILSMLSVHGIPKSHDLKLNVLQADTTIIIRIKDNNAE-RLLYPKLLI  58
Carp leptin-a-II  MYFSSVLL-YPCILMGLSILVHPDLSVKLNLQADTTILIRKIDHEKLK-RLKSPKLLI  58
Human leptin  MHHWIICFGFLWPLFYPYVQPMPIQPQVQ-DAETKLIRKTVIRINDSHFTVSNSKQKV  58

Helix-B

Zebrfish leptin-a  GDPGHYFPETPAPDPIQCLSMETIINTTHFKLVQRLPKHVMQQRSDLSTLLGLSYLEG-  133
Zebrfish leptin-b  G-----PDIDNIPDGLSSLYLQRLHVVPAHLQOQVDIINTLRTLLELA  109
Carp leptin-a-I  GDPELYEPVFAPDPIQCLSMETIINTTHFKLVQRLPKHVMQQRSDLSTLLGLSYL  118
Carp leptin-a-II  GDPELYEPVFAPDPIQCLSMETIINTTHFKLVQRLPKHVMQQRSDLSTLLGLSYL  118
Human leptin  G-----LDFIPEGPHILISATMOTAVNYQOIQILSDMPVRNVTQISNDLENRLDLHLYAFS  114

Helix-C

Zebrfish leptin-a  MDCRTKLESTNGKALDAFLEDSASYPFQGCPLPNPE-DIV  164
Zebrfish leptin-b  QGCPLPNPE-----TPVHELTETTFVTSNYLHHLEQRFLEKLCLNIDKLYKYKCTDVAE  164
Carp leptin-a-I  MHCTKEPANGRALDIFEDNATHTTIVRVLADRIRLQKMFQRLVNLQDSC  171
Carp leptin-a-II  MRCTKEPANERSLDAFLENNATHTTIVRVLADRIRLQKMFQRLVNLQDSC  171
Human leptin  KSCHLPWASGLETLSLGVLEASCYTVALSRLGSLQDOMLDWLDSPGC  167

Helix-D

Zebrfish leptin-a  ----  168
Zebrfish leptin-b  TFIL  168
Carp leptin-a-I  ----  168
Carp leptin-a-II  ----  168
Human leptin  ----  168

Figure 2 Multiple sequence alignment of zebrafish leptins, carp leptins and human leptin. Asterisks indicate amino acids that are conserved in all sequences, whereas colons and dots reflect decreasing levels of amino acid similarity. The four α-helices (A–D) were inferred from human leptin and are boxed in the alignment. The cysteine residues that form leptin’s single disulphide bridge are shaded. Accession numbers: zebrafish leptin-a: AM920658, zebrafish leptin-b: AM901009, carp leptin-a-I: AJ868357, carp leptin-a-II: AJ868356, human leptin: P41159.

Expression of zebrafish leptins

Relative expression of zebrafish leptin paralogues was assessed by real-time qPCR. We designed primers using primer express software (Table 1; Applied Biosystems). Five microliters cDNA and 300 nM forward and reverse primers were added to 12.5 μl SYBR Green Mastermix (Applied Biosystems). The total volume was adjusted to 25 μl with deionized H₂O. qPCR (10 min 95 °C, 40 cycles of 15 s 95 °C and 1 min 60 °C) was carried out using a GeneAmp 7500 sequence detection system (Applied Biosystems). Different samples were run on a single plate. Dual internal standards (40S ribosomal protein S11 and β-actin) were incorporated in all measurements and results were confirmed to be very similar following standardization to either gene. Only results relative to 40S are shown. Constitutive expression of leptin in zebrafish organs and tissues was corrected for primer efficiency and plotted as a ratio between target gene versus

Table 3 Percentages for amino acid sequence identities between vertebrate leptin sequences

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<th>Zebrafish leptin-b</th>
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<th>Carp leptin-a-II</th>
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<th>Human</th>
<th>Mouse</th>
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reference gene. Relative expression of leptin paralogues in the liver following fasting was corrected for primer efficiency and reference gene, and plotted relative to controls.

Results

Zebrafish expresses duplicate and divergent leptin genes

A systematic BLAST search of the ENSEMBL zebrafish genome database with mammalian leptin sequences revealed two partial leptin sequences, one of which represented leptin-a (already described by Huising et al. 2006a), the other represented a new leptin-like orthologue that we named leptin-b. The (automated) genomic sequences were corrected by hand for correct splice sites and the obtained sequences were used in a homology cloning approach to identify both leptin cDNA sequences. Protein–protein BLAST (BLASTp) showed significant hits with other fish leptins (Table 2). The cDNA- and deduced amino acid sequences of zebrafish leptins are shown in Fig. 1. Both leptin-a and leptin-b are comparable in size, 166 and 168 amino acids respectively, both with a predicted signal peptide of 20 amino acids. Previously, we described two highly similar leptin genes in common carp (Huising et al. 2006a) which we designated leptin-I and leptin-II. These carp leptin paralogues are likely the result of the recent genome duplication ~16 Mya that led to the tetraploidization of the common carp genome (Larhammar & Risinger 1994). Our results suggest that zebrafish leptin-a and leptin-b are the result of the ancient genome duplication that teleost fish experienced (Taylor et al. 2003, Volff 2005). Therefore, we amend the names of the previously described carp leptins to

Figure 3 The gene structures of zebrafish leptins and mammalian leptins are conserved. Boxes represent coding exons only and are drawn to scale. Numbers inside the boxes reflect exon sizes in nucleotides. The intron phase is indicated with underlined numbers.

Figure 4 Protein models of the duplicate zebrafish leptins and human leptin. Zebrafish leptin-a (B), leptin-b (C) and human leptin (A) were modeled on the human leptin crystal structure and conform to the four α-helix bundle adopted by human leptin. In yellow the single disulphide bridge that stabilizes leptin's tertiary structure, in red the third cysteine of zebrafish leptin-b.
leptin-a-I and leptin-a-II. We want to stress that the low amino acid identity of the leptin proteins between fish and mammals serves as a reminder that we assign the name leptin solely based on the structural similarities described above. Orthologous proteins do not by default share analogous roles, particularly proteins that share so little of their primary amino acid sequences as teleostean and mammalian leptins do (Huising et al. 2006a).

Characteristics of zebrafish leptins
The amino acid identity between zebrafish leptin-a and leptin-a-II is 24%. Zebrafish leptin-a is more similar (60% primary amino acid sequence identity) to carp leptin-a-I and leptin-a-II. The identity between zebrafish leptin-b and carp leptins is, at only 25%, marginally higher than the identity between leptin-b and mammalian leptins (19%; Fig. 2 and Table 3). The cysteine residues that make up leptin's single disulphide bridge, connecting the carboxy-terminal ends of α-helices C and D are conserved. Both zebrafish leptin genes are encoded by two exons that are similar in size compared with mammalian leptins (Fig. 3). Zebrafish leptin genes possess a short intron, with consensus 5′ donor (gt) and 3′ acceptor (ag) splice sites. The intron phase indicates whether the intron is situated in between triplets (phase 0), or following the first or second base of a triplet (phase 1 or phase 2 respectively). The intron phase for both zebrafish leptins is identical to the intron phase of mammalian leptins: phase 0.

Our models of both zebrafish leptins conform to the typical four α-helix conformation (up-up-down-down) of human leptin (Fig. 4), indicating that the tertiary structures of zebrafish leptins are comparable with mammalian leptins. In contrast to all other leptin sequences, leptin-b contains an additional cysteine residue in helix-D.
**Phylogeny of zebrafish leptins**

The zebrafish leptin paralogues cluster together with other vertebrate leptin genes, supported by a high bootstrap value (98), supporting the orthology of both zebrafish leptins with mammalian leptins (Fig. 5). Within the leptin cluster, the overall topology of the phylogenetic tree adheres to the established pattern of evolution, as the teleost leptin cluster branches off before the separation of the amphibian and mammalian cluster. Within the mammalian leptin cluster, the only known sequence of a marsupial leptin (that of the fat-tailed dunnart) branches outside the leptin sequences of placental mammals. In the teleost leptin cluster, zebrafish leptin-a and the carp leptins form a separate clade. We also screened other fish databases in order to assess the presence of leptin-b orthologues in other teleost fish species. Using the zebrafish leptin-b sequence in a BLAST search of the ENSEMBL medaka (*Oryzias latipes*) genome, we retrieved a leptin-b orthologue with 28% amino acid identity to zebrafish leptin-b (BN001183). Zebrafish leptin-b and medaka leptin-b form a separate clade within the teleost leptin cluster.

**Constitutive expression of zebrafish leptins**

Zebrafish leptins (Fig. 7) show a differential expression pattern. Whereas leptin-a is prominently expressed in the liver, in accordance with previous observations of carp leptin-a-I and -II, leptin-b is not. Leptin-a is expressed at higher levels than leptin-b in most organs except the ovary, which is a major site of leptin-b mRNA expression.
Leptin mRNA expression after fasting for 1 week

To gain insight into possible physiological functions of the leptin paralogues, we investigated leptin mRNA expression after fasting for 1 week. Leptin-a mRNA levels show no significant response to fasting for 1 week (Fig. 8). By contrast, hepatic leptin-b expression is significantly downregulated (P<0.05) after 1 week of food deprivation.

Discussion

Zebrafish possesses duplicate leptin genes, coding for leptin-a and leptin-b, which differ substantially from each other (24% amino acid identity). It is possible that a major genome duplication that took place ~300 Mya in the early fish lineage (Taylor et al. 2003, Volff 2005) resulted in duplicated leptins. The discovery of a leptin-b orthologue in the Japanese medaka supports this view as zebrafish and medaka represent two distant teleost lineages, the Cypriniformes and the Beloniformes respectively, which shared their last common ancestor ~296 Mya (Hoegg & Meyer 2005; Fig. 9).

In contrast, from the primary sequence identity and phylogenetic analysis, it follows that the duplicate carp leptins that we described recently (Huising et al. 2006a) likely resulted from the more recent genome duplication in carp (~16 Mya; Larhammer & Risinger 1994) and represent the duplicated orthologues of zebrafish leptin-a. Therefore, we propose that these carp leptin sequences should be renamed leptin-a-I and leptin-a-II. This observation, combined with the identification of leptin-b in two distant teleost lineages, the Brachycephalia and the Teleostei respectively, represents strong support for the hypothesis that the pufferfish lineage does not possess duplicate leptin genes.

Recently, multiple entries have been submitted in the EMBL database for several fish leptin orthologues that all share 97–99% sequence similarity at the nucleotide level (AY47007, AY547279, AY547322, AY551335, AY551336, AY551337, AY551338, AY551339, AY551340, DQ784814, DQ784815, DQ784816). Non-synonymous substitutions are subject to selection as they result in differences in amino acid sequence, whereas synonymous substitutions are generally not. Therefore, the almost complete absence of synonymous substitutions (over 97% nucleotide identity) between these deposited ‘teleost’ leptin sequences and mammalian leptin sequences would represent an extraordinary and very unlikely

while the original function is maintained by the other. Gene duplications in the teleost lineage are common, and there are several well-documented examples of large scale (often referred to as whole) genome duplication events. A major genome duplication (Taylor et al. 2003, Volff 2005) is thought to have yielded several duplicate class-I helical cytokines, viz. duplicate interleukin-11 (Huising et al. 2005), IL-12p35 (Huising et al. 2006b), CXCL12 (Huising et al. 2004), and cytokine receptor (IL12p40; Huising et al. 2006c) genes. We could not retrieve a leptin-b orthologue from the available pufferfish genomes (tiger pufferfish; Takifugu rubripes, and the green spotted pufferfish; Tetraodon nigroviridis). While one reason for our inability to retrieve leptin-b orthologues from these species may be that their respective genomes are incomplete, it is also possible that the Tetraodontiformes may have lost leptin-b from their gene repertoire. In the genome of T nigroviridis, we found two regions with a conserved genomic neighborhood compared with human leptin. Indeed, only one of these loci carries a leptin orthologue, which is strong support for the hypothesis that the pufferfish lineage does not possess duplicate leptin genes.

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example of evolutionary convergence, as teleosts and mammals shared their last common ancestor over 450 million years ago. Instead, these sequences should be regarded as artifacts. A similar situation unfortunately has occurred for chicken leptin that was reported to be highly similar to mouse leptin by two independent groups (Taouis et al. 1998, Ashwell et al. 1999). Subsequent studies have raised concerns regarding the validity of these published chicken leptin sequences (Friedman-Einat et al. 1999, Doyon et al. 2001, Huising et al. 2006b, Sharp et al. 2008).

Despite the relatively low amino acid conservation that was previously noted for other teleost leptins, we are confident to assign orthology between zebrafish leptin-b and mammalian leptins, supported by several key features of zebrafish leptin-b. First, both zebrafish leptin genes are encoded by two exons of comparable size to the ones coding for mammalian leptins. Vertebrate class-I cytokines are typically encoded by three or more (usually five) exons. In fact, the only class-I helical cytokine other than a leptin composed of two exons is ciliary neurotrophic factor (Huising et al. 2006b), which differs substantially in primary sequence as well as gene structure from a leptin. Furthermore, the spacing of the two cysteine residues that make up leptin’s single disulphide bridge is unique among class-I helical cytokines (Huising et al. 2006b).

Thirdly, the stable phylogenetic clustering of the zebrafish leptin sequences with other fish leptins, as with the mammalian leptins, supports the unambiguous identity of the two zebrafish leptins. Finally, the predicted tertiary structure of zebrafish leptin-b, conforming to the human crystal structure of leptin, and the conservation of synteny between the mammalian leptin-locus and both zebrafish leptin loci further strengthens the assignment of orthology between zebrafish leptins and mammalian leptins.

An intriguing feature of the leptin-b sequence is the cysteine residue at the N-terminus of α-helix-D. We designed 3D models of leptin-b to address the spatial orientation of this additional cysteine residue to see if this free cysteine would potentially be surface-exposed — and thus available for disulphide bridging — or is buried within the leptin’s hydrophobic core. These models did not allow a firm prediction of the availability of this cysteine to form disulphide bridges, either within one leptin molecule or between two molecules because its position in the models is at the boundary of the protein surface and the protein core. It is possible that the residue is buried within the protein, and as a result not exposed to the environment and not available for disulphide interactions. The predicted mature leptin-b peptide contains no cysteine to form a disulphide bridge with the helix-D cysteine. A similar phenomenon has been observed for interleukin-11 genes in teleosts. Fish IL-11a and IL-11b both possess a single cysteine residue near the C-terminus, whereas mammalian IL-11 does not (Huising et al. 2005). Medaka leptin-b lacks an additional cysteine, indicating that this is not a universal feature among teleostean
leptin-b genes. The elucidation of additional teleost leptin-b sequences will shed light on the uniqueness of this characteristic of zebrafish leptin-b.

We observed substantial differences between the expression patterns of zebrafish leptin-a and leptin-b. It is now generally accepted that leptin, in addition to its ‘classical’ role, is truly pleiotropic (Popovic et al. 2001, De Rosa et al. 2007). Indeed, in zebrafish, leptin-a and leptin-b are expressed in considerable amounts in the pituitary gland. We do not know the exact nature of the pituitary cells that (co-) express leptin in fish, nor the exact function of this leptin; in mammals, it is known that leptin is expressed in around 70% of the corticotropes and to a lesser extent in somatotropes (2%), gonadotropes (29–33%), and thyrotropes (32%; Popovic et al. 2001). We propose that fish leptin produced in the pituitary gland must have additional, local (paracrine?) functions that allow zebrafish to maintain equilibrium in the face of challenges to homeostasis.

Whereas the high level of expression of leptin-a in the zebrafish liver conforms to the expression pattern observed for carp leptins, leptin-b is expressed at lower levels in the liver. Interestingly, it is this hepatic leptin-b mRNA level that decreases after fasting. The sheer size of the fish liver may guarantee a sufficient output of leptin-(b) protein, despite the relatively low leptin-b mRNA expression level.

Leptin-b shows the highest expression in the ovaries, which hardly express leptin-a. In mammals, leptin serves a function in the regulation of reproduction as ob/ob mice treated with leptin recover fertility (Caprio et al. 2001, Archanco et al. 2003). Given the high expression of leptin-b in zebrafish ovaries, the reproductive function of leptin in this species may be carried out by leptin-b.

In addition to the marked differences in leptin’s primary sequences between teleosts and mammals – which indicate potential differences in function – we now have demonstrated the existence of a second, equally divergent leptin in zebrafish and medaka that is likely a feature shared by more teleost species. We propose that fish leptin produced in the pituitary gland may be responsible for maintaining homeostasis, whereas leptin-b in zebrafish ovaries may function in the regulation of reproduction as suggested by the studies in rats (Caprio et al. 2001, De Rosa et al. 2007).

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Received in final form 4 March 2009
Accepted 13 March 2009
Made available online as an Accepted Preprint 16 March 2009