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 tertiary structures shows that both leptins conform to the typical four α-helix bundle structure of the class-I α-helical cytokines. The zebrafish leptins are differentially expressed; the liver shows high leptin-a expression (in concordance with what we observed for carp leptins), while leptin-b is expressed at much lower levels, which are downregulated further upon fasting. The finding of duplicate leptin genes in teleosts adds to our understanding of the evolution of leptin physiology in the early vertebrate lineage.

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Introduction

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 α-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 leptin-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 and 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

Table 1 Primer sequences. Primer names that start with 'Q' indicate the primers used for qPCR

	Accession number	Primer	Sequence $5' \to 3'$
Gene			
Leptin-a	AM920658	zf.leptin-a.fw	ATG CGT TTT CCA GCT CTC
		zf.leptin-a.rv	TCA GCA GAT TTT CAG CTG GTC
		Q-zf.leptin-a.fw	GAC TGC ACA CTG AAG GAA TC
		Q-zf.leptin.a.rv	GCA CTG TCC TCT AGA AAA GC
Leptin-b	AM901009	zf.leptin-b.fw	ATG AAG TCT TCA ATG ATT TTT TGC
		zf.leptin-b.rv	CAG AGA ATG AAT GTC TCA GCC
		Q-zf.leptin-b.fw	ATT GCT CGA ACC ACC ATC AG
		Q-zf.leptin-b.rv	GAT GTC AGG GCC GAA ATC AA
40S ribosomal protein S11	CA472846	Q-40S.fw	AAA CAG CCC ACC ATC TTC CA
		Q-40S.rv	CTG TGA TAA CGA GGG AGC TTT TC
β-actin	AF025305	Q-BACT.fw	CAA CAG GGA AAA GAT GAC ACA GAT
		Q-BACT.rv	CAG CCT GGA TGG CAA CGT

expression is the liver (Kurokawa *et al.* 2005, Huising *et al.* 2006*a*), 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.* 2006*a*).

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.* 2006*a*). 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 2 List of BLAST hits following comparison between zebrafish leptin-a (A) and leptin-b (B) to the non-redundant protein database (nr). BLAST hits are scored by an 'E value', which applies statistical probability that the similarity between two sequences is based on stochastic events

	Species	Description	E value
Accession number			
A			
BN000380	Zebrafish	Leptin-a	5×10^{-89}
AJ868357	Common carp	Leptin-a-I	7×10^{-49}
AJ868356	Common carp	Leptin-a-II	2×10^{-48}
ABV57772	Goldfish	Leptin-a-II	2×10^{-32}
AAZ66785	Channel catfish	Leptin	2×10^{-14}
AY884210	*Xenopus laevis*	Leptin	6×10^{-9}
AAY68394	Tiger salamander	Leptin	9×10^{-6}
AM901009	Zebrafish	Leptin-b	2×10^{-5}
B			
AM901009	Zebrafish	Leptin-b	7×10^{-58}
AJ868356	Common carp	Leptin-a-II	5×10^{-8}
AJ868357	Common carp	Leptin-a-I	2×10^{-7}
ABV57772	Goldfish	Leptin-a-II	9×10^{-7}
BN000380	Zebrafish	Leptin-a	9×10^{-7}
AAZ66785	Channel catfish	Leptin	1×10^{-4}

party annotation accession number: BN000830; Huising *et al.* 2006*a*). 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

```
A    1 M  R  F  P  A  L  R  S  T  C  I  L  S  M  L  S  L  I  H  C
     1 atgcgttttccagctctccgctcaacctgtattttgagcatgctcagtttgattcattgc
    21 I  P  V  H  Q  H  D  R  K  N  V  K  L  Q  A  K  T  I  I  V
    61 attcccgttcatcagcatgaccggaaaaatgtcaaactgcaggcaaagaccatcatcgtc
   121 R  I  R  E  H  I  D  G  Q  N  L  L  P  T  L  I  I  G  D  P
   121 agaatcagggaacacattgacgggcaaaatttacttccaacgctcatcattggggatcca
    61 G  H  Y  P  E  I  P  A  D  K  P  I  Q  G  L  G  S  I  M  E
   181 ggacattatccagagattcccgctgacaaacccatccaagggctcggctccatcatggaa
    81 T  I  N  T  F  H  K  V  L  Q  K  L  P  N  K  H  V  D  Q  I
   241 accattaataccttccacaaggttcttcagaagcttccaaataagcatgttgaccagata
   101 R  R  D  L  S  T  L  L  G  Y  L  E  G  M  D  C  T  L  K  E
   301 cgccgagatctatccacacttctgggttacctggaaggcatggactgcacactgaaggaa
   121 S  T  N  G  K  A  L  D  A  F  L  E  D  S  A  S  Y  P  F  T
   361 tcaacaaatgggaaagcgctggacgcttttctagaggacagtgcttcatatccctttact
   141 L  E  Y  M  T  L  N  R  L  K  Q  F  M  Q  K  L  I  D  N  L
   421 ttagagtacatgactttaaacagactgaaacagtttatgcaaaagctgatcgataatctg
   161 D  Q  L  K  I  C  *
   481 gaccagctgaaaatctgctga

B    1 M  K  S  S  M  I  F  C  L  L  I  S  S  L  V  A  V  S  I  S
     1 atgaagtcttcaatgattttttgcttgttaatatcatccctggtggccgtgagcatcagt
    21 R  P  T  A  P  E  D  R  I  R  I  A  R  T  T  I  S  R  I
    61 cgacccacggctcccgaagacaggatacgaatcattgctcgaaccaccatcagccgaatt
   121 K  K  I  K  D  E  H  F  Q  M  S  P  E  I  D  F  G  P  D  I
   121 aaaaaaatcaaagatgagcacttccagatgtctccagagattgatttcggccctgacatc
    61 D  N  P  I  D  G  L  S  S  V  L  S  Y  L  S  Y  L  Q  L  R
   181 gacaaccccattgatggtctcagttctgtcttgagttacttgagttacctgcagttgcgg
    81 L  H  V  P  P  A  Q  H  L  Q  Q  V  Q  I  D  L  E  T  L  L
   241 ttgcatgttcctccagctcagcacctacagcaggtccagatagacttagagactctcctg
   101 R  T  L  E  E  L  A  V  S  Q  G  C  P  L  P  N  P  E  T  P
   301 aggacactggaggaactggccgtctcacagggatgccctctacccaatcccgagaccccg
   121 V  H  K  E  E  T  A  F  P  V  T  S  N  Y  L  H  L  L  E  L
   361 gtgcataaagaagaaacagccttccccgtcacctccaactacctgcacctcctggagctc
   141 Q  R  F  L  E  K  L  C  L  N  I  D  K  L  K  Y  C  K  D  T
   421 cagaggttcctggagaagctctgcctcaacatagacaaactgaaatactgcaaagataca
   161 D  V  A  E  T  F  I  L  *
   481 gatgtggctgagacattcattctctga
```

Figure 1 cDNA and deduced amino acid sequence of the coding sequence of zebrafish leptin-a and leptin-b. Accession numbers are AM920658 and AM901009 respectively.

```
                                                          Helix-A
Zebrafish leptin-a    MRFPALR-STCILSMLSLIHCIPVHQHDRKN-VKLQAKTIIVRIREHIDG-QNLLPTLII  57
Zebrafish leptin-b    MKSSMIF-CLLISSLVAVSISRPTAPE---DRIRIIARTTISRIKKIKDEHFQMSPEIDF  56
Carp leptin-a-I       MYFSALL-YPCILAMLSLVHGIPIHSDSLKNLVKLQADTIIIRIKDHNAE-LKLYPKLLI  58
Carp leptin-a-II      MYFSVLL-YPCILGMLSLVHAIPVHPDSLKNLVKLQADTIILRIKDHNEK-LKLSPKLLI  58
Human leptin          MHWGTLCGFLWLWPYLFYVQAVPIQKVQ--DDTKTLIKTIVTRINDISHTQSVSSKQKVT  58
                      *       :    :   :     *         :   :    * : **. :
                                             Helix-B                    Helix-C
Zebrafish leptin-a    GDPGHYPEIPADKPIQGLGSIMETINTFHKVLQKLPNKHVDQIRRDLSTLLGYLEG----  113
Zebrafish leptin-b    G-------PDIDNPIDGLSSVLSYLSYLQLRLHVPPAQHLQQVQIDLETLLRTLEELAVS  109
Carp leptin-a-I       GDPELYPEVPADKPIQGLGSIMDTITTFQKVLQRLPKGRVSQIHIDLSTLLGHLKERMTS  118
Carp leptin-a-II      GDPELYPEVPANKPIQGLGSIVETLSTFHKVLQRLPKGHVSQIRNDLFTLLGYLKDRMTS  118
Human leptin          G----LDFIPGLHPILTLSKMDQTLAVYQQILTSMPSRNVIQISNDLENLRDLLHVLAFS  114
                      *           :**   *..: . :   : *   *   .: *: ** .*   *.
                                                      Helix-D
Zebrafish leptin-a    MDCTLKESTNGKALDAFLEDSASYPFTLEYMTLNRLKQFMQKLIDNLDQLKIC-------  166
Zebrafish leptin-b    QGCPLPNPE-----TPVHKEETAFPVTSNYLHLLELQRFLEKLCLNIDKLYCKDTDVAE   164
Carp leptin-a-I       MHCTSKEPANGRALDAFLEDNATHHITVRYLALDRLKQFMQKLLVNLDQLKSC-------  171
Carp leptin-a-II      MRCTLKEPANERSLDAFLENNATHHITFGFLALDRLKQFMQKLIVNLDHLKSC------  171
Human leptin          KSCHLPWASGLETLDSLGGVLEASGYSTEVVALSRLQGSLQDMLWQLDLSPGC-------  167
                        *   .        ..      :    :     : * .*:  ::.:  ::*     *

Zebrafish leptin-a    ----
Zebrafish leptin-b    TFIL 168
Carp leptin-a-I       ----
Carp leptin-a-II      ----
Human leptin          ----
```

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.

(Canutescu *et al.* 2003). Both models were refined in YASARA using the YAMBER2 forcefield (Krieger *et al.* 2004). Coordinate files are available from the authors on request.

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 H_2O. 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

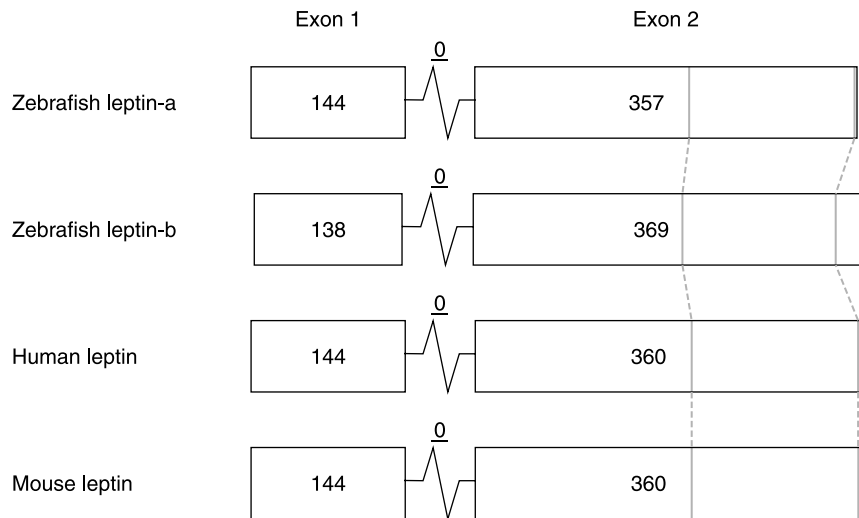|	Zebrafish leptin-a	Zebrafish leptin-b	Carp leptin-a-I	Carp leptin-a-II	Clawed frog	Human	Mouse	Dog	Cow
Zebrafish leptin-a	100								
Zebrafish leptin-b	24	100							
Carp leptin-a-I	64	25	100						
Carp leptin-a-II	63	25	81	100					
Clawed frog	24	13	27	27	100				
Human	19	19	21	23	35	100			
Mouse	21	19	23	25	34	83	100		
Dog	19	18	22	23	33	80	78	100	
Cow	20	19	23	25	34	84	83	88	100

Exon 1   Exon 2

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.

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.* 2006*a*), 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.* 2006*a*) 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
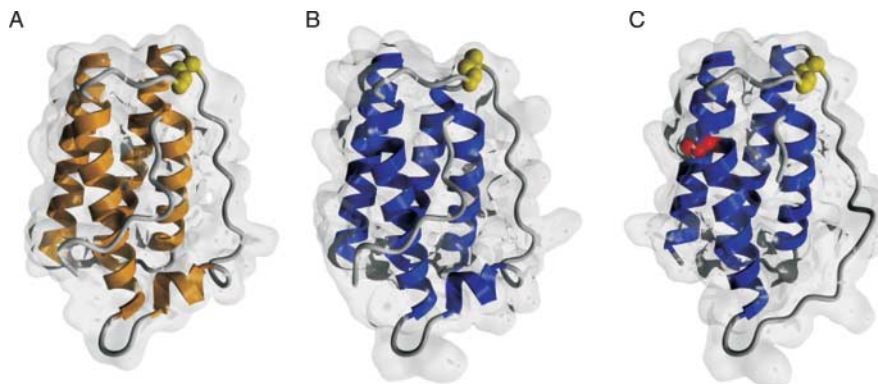
Figure 4 Protein models of the duplicate zebrafish leptins and human leptin. Zebrafish leptin-a (B), leptin-b (C) and human leptin (A) were modeled on the human leptin crystal structure and conform to the four α-helix bundle adopted by human leptin. In yellow the single disulphide bridge that stabilizes leptin's tertiary structure, in red the third cysteine of zebrafish leptin-b.
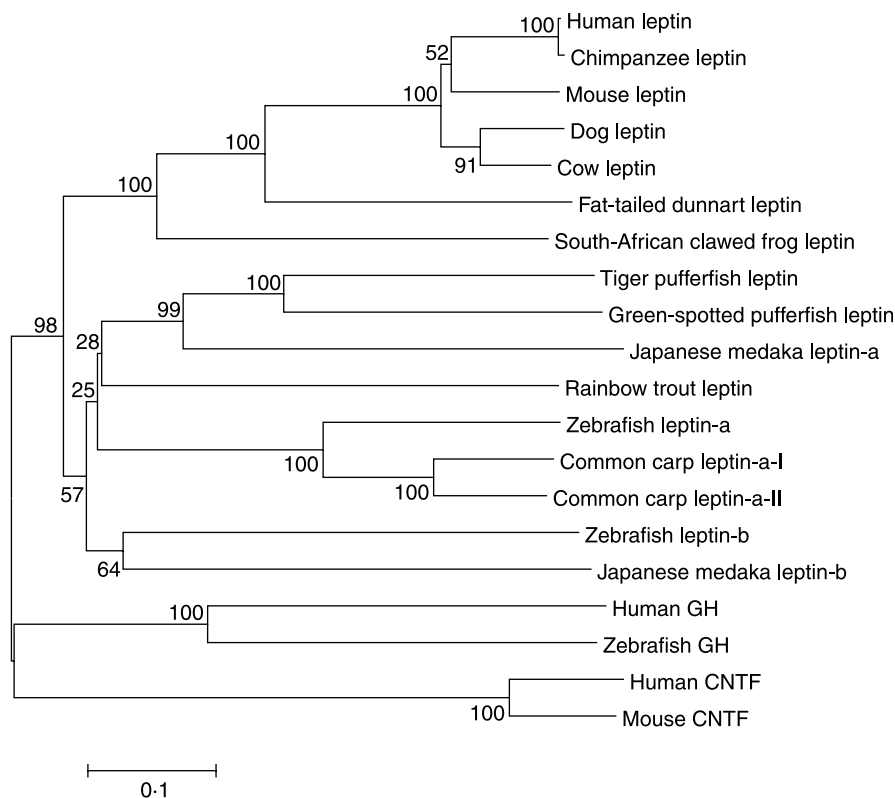
Figure 5 Phylogenetic tree of vertebrate leptins. Numbers at the branches reflect the confidence level as obtained by bootstrapping (1000 replications). GH and ciliary neurotrophic factor (CNTF; both class-I helical cytokines) were included as out-group. Only full length sequences were used for phylogenetic analysis. Accession numbers are as follows: chimpanzee leptin: O02750, human leptin: P41159, mouse leptin: P41160, dog leptin: O02720, cow leptin: P50595, fat-tailed dunnart leptin: AF159713, South-African clawed frog leptin: AY884210, carp leptin-a-I: AJ836745, carp leptin-a-II: AJ836744, zebrafish leptin-a: AM920658, rainbow trout leptin: AB354909, zebrafish leptin-b: AM901009, medaka leptin-a: AB193548, medaka leptin-b: BN001183, tiger pufferfish leptin: AB193547, green-spotted pufferfish leptin: AB193549, human GH: P01241, zebrafish GH: Q1JQ34, human CNTF: P26441, mouse CNTF: P51642.

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.* 2006*a*).

Characteristics of zebrafish leptins

The amino acid identity between zebrafish leptin–a and leptin–b 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. From the position of
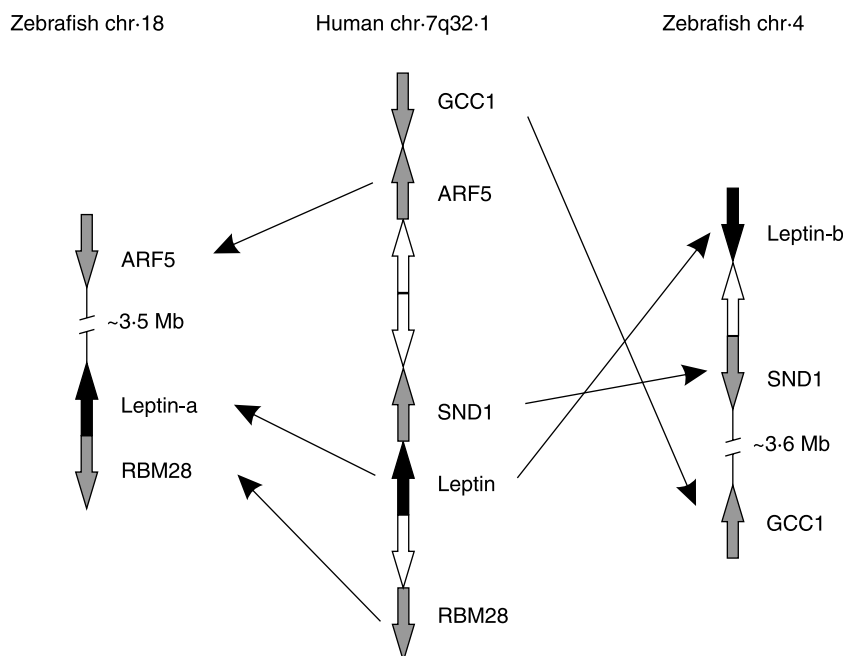
Figure 6 The synteny between the human leptin locus and both zebrafish leptin loci is conserved. A comparison between the human leptin locus (7q32.1) and the zebrafish leptin loci (located on chromosome 18 and 4 respectively) reveals that adjacent to both zebrafish leptins there are multiple genes that lie adjacent to human leptin. Arrows reflect genes, the direction of the arrow the orientation of the gene. Black arrows represent leptin orthologues, grey arrows represent genes in synteny in the human and zebrafish leptin loci. Genes are not drawn to scale, nor is intergenic space included. ARF5, ADP-ribosylation factor 5; RBM28, RNA binding protein motif 28; SND1, staphylococcal nuclease domain-containing protein 1; GCC1, GRIP and coiled-coil domain-containing 1.

this cysteine (indicated in red in Fig. 4) we cannot draw firm conclusions regarding the availability of this cysteine to form intermolecular disulphide bridges.

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.

Both zebrafish leptin genes share synteny with a human leptin

To further substantiate the orthology of the zebrafish leptin paralogues to mammalian leptins, we compared the synteny of both zebrafish leptins with human leptin. Synteny refers to the order and orientation of the genes of chromosomes and tends to be a conserved feature across species. For each zebrafish leptin, several genes are found in synteny with mammalian leptin (Fig. 6). The leptin-a gene of zebrafish is located next to RNA binding motif 28 (RBM28), as is the human leptin gene. In close proximity to leptin-b, Staphylococcal nuclease domain-containing protein 1 and GRIP and coiled-coil domain-containing 1 are found – again, these are also found in close proximity to human leptin.

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.
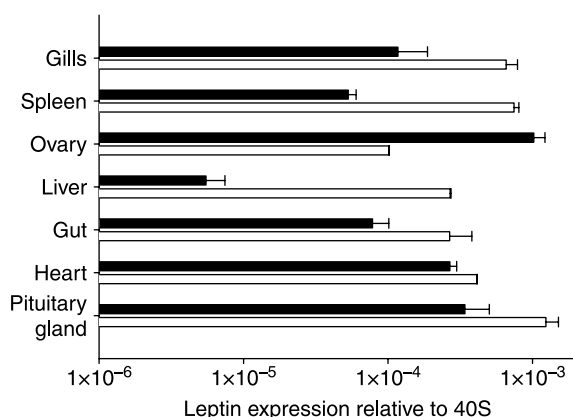
Figure 7 Basal expression of leptin-a (open bars) and leptin-b (closed bars). Leptin-a and leptin-b are constitutively expressed in all organs investigated. Bars represent the mean value of four individual zebrafish. Error bars indicate s.e.m. Note the logarithmic scale of the x-axis.

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.* 2006*a*) 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 distantly related fish substantiates the view that more bony fishes express orthologues of leptin-b. Gene duplications, and genome duplications in particular, are considered the main thrust contributing to the expansion of an organism's gene repertoire, as the presence of newly duplicated paralogues allows one of the two paralogues of a pair to drift and on occasion acquire a novel function 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.* 2006*b*), CXCL12 (Huising *et al.* 2004), and cytokine receptor (IL12p40; Huising *et al.* 2006*c*) 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 *Tetraodontoformes* 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.

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 (AY497007, 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
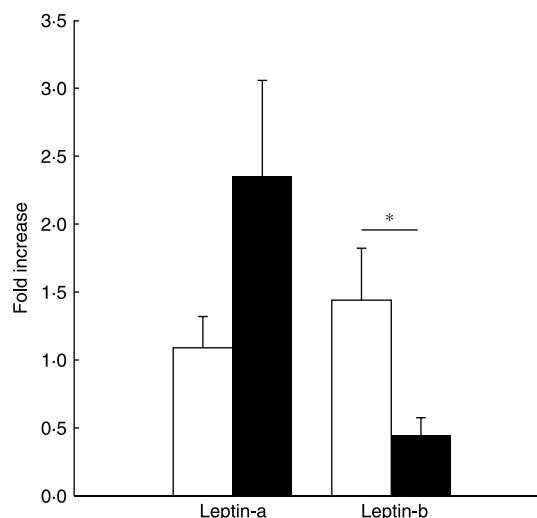

Figure 8 Leptin-a and leptin-b mRNA expression after 1 week fasted (closed bars) and fed (control; open bars) zebrafish. Leptin-b mRNA decreases significantly (*: $P<0.05$) after fasting for 1 week. Bars represent the mean value, error bars indicate s.e.m.
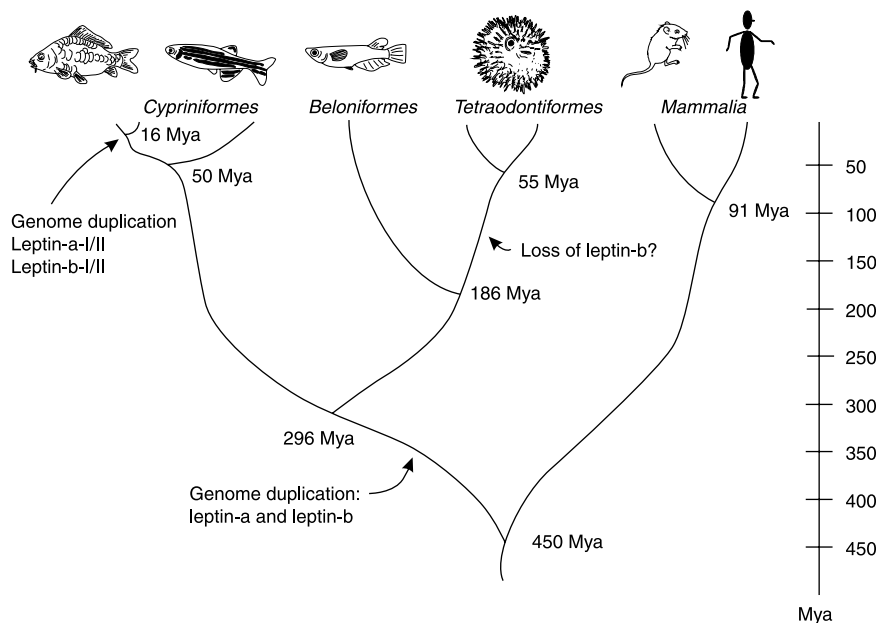
Figure 9 General phylogenetic tree of vertebrate evolution. Mammals and teleosts shared their last common ancestor ∼450 Mya. The finding of duplicate leptin paralogues in the medaka (*Beloniformes*) and zebrafish (*Cypriniformes*) dates the duplication event that gave rise to the duplicated leptins to ∼296 Mya, as these species shared their last common ancestor at that time point. The tetraploidization of the carp genome (∼16 Mya) is likely the event that gave rise to paralogous leptin-a-I and leptin-a-II genes in carp. Divergence estimates are based on: (Zardoya & Doadrio 1999, Hedges 2002, Hoegg & Meyer 2005, Volff 2005).

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.* 2006*b*, 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.* 2006*b*), 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.* 2006*b*). 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 (21%), 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 fishes. The future challenge will be to unravel the physiological function of both leptin genes. In fact, the presence of two highly divergent orthologues of mammalian leptin in bony fish is testimony to the dynamic evolutionary history of leptin as it suggests the possibility of a redundant leptin network in teleosts. Furthermore, it adds fuel to the proposition that fish leptins, acting redundantly or independently, have acquired fundamentally different roles compared with mammalian leptins.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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