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Increased Leptin Expression in Common Carp (*Cyprinus carpio*) after Food Intake But Not after Fasting or Feeding to Satiation

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Leptin is a key factor in the regulation of food intake and is an important factor in the pathophysiology of obesity. However, more than a decade after the discovery of leptin in mouse, information regarding leptin in any nonmammalian species is still scant. We report the identification of duplicate leptin genes in common carp (*Cyprinus carpio*). The unique gene structure, the conservation of both cysteines that form leptin's single disulfide bridge, and stable clustering in phylogenetic analyses substantiate the unambiguous orthology of mammalian and carp leptins, despite low amino acid identity. The liver is a major yet not the only site of leptin expression. However, neither 6 d nor 6 wk of fasting nor subsequent refeeding affected hepatic leptin expression, although the carp predictably shifted from carbohydrate to lipid metabolism. Animals that were fed to satiation grew twice as fast as controls; however, they did not show increased leptin expression at the termination of the study. Hepatic leptin expression did, however, display an acute and transient postprandial increase that follows the postprandial plasma glucose peak. In summary, leptin mRNA expression in carp changes acutely after food intake, but involvement of leptin in the long-term regulation of food intake and energy metabolism was not evident from fasting for days or weeks or long-term feeding to satiation. These are the first data on the regulation of leptin expression in any nonmammalian species. (*Endocrinology* 147: 5786–5797, 2006)

THE POSITIONAL CLONING of the *obese* (*ob*) gene in mouse, more than a decade ago (1), identified the factor responsible for the profoundly obese and type II diabetic phenotype of the *obese* mouse mutant (2). The *ob* gene encodes a 167-amino acid soluble peptide that was named leptin after the Greek root *leptos*, meaning lean. The absence of a humoral factor as the cause for the obese phenotype was already established by parabiosis: a soluble factor in the blood of wild-type mice reduced food intake and weight gain of the obese parabiont and partially reverted the profoundly obese, diabetic, and hyperphagic phenotype (3).

Leptin is a member of the class I helical cytokine family, which includes IL-6 and GH; the protein is characterized by a typical four-helix bundle conformation (4). In mammals, it is secreted by adipocytes in response to feeding and reaches the brain to evoke satiety and terminate food intake (5–7). Leptin conveys information about the energy status of the body to the brain and is considered a major factor in appetite control and body weight regulation (8, 9). Within the brain, leptin activates the long form of its receptor that is found in several hypothalamic nuclei (10, 11), with the arcuate nucleus taking a central position in the control of feeding and energy metabolism. This nucleus contains two distinct and antagonistic sets of leptin-responsive neurons (12, 13). One set of neuropeptide Y (NPY)-positive neurons is inhibited by leptin. A high percentage of these NPY^+ neurons coexpresses agouti gene-related protein (14), which is an α-MSH antagonist at the level of the melanocortin-receptor 4 and melanocortin-receptor-3 (15). The other set of leptin-responsive neurons coexpresses the anorexigens α-MSH [derived from the precursor molecule proopiomelanocortin (POMC)] and cocaine- and amphetamine-regulated transcript (16). Thus, within the arcuate nucleus leptin targets antagonistic populations of anabolic (NPY^+, agouti gene-related $protein^+$) and catabolic ($POMC^+$, cocaine- and amphetamine-regulated $transcript^+$) neurons (12, 17). Many other hypothalamic neuropeptides, including TRH and the anorexigen CRH, participate in the complex neural network that controls food intake and metabolism (13).

Zhang *et al.* (1) addressed the evolutionary conservation of the *ob* gene by hybridizing genomic DNA of representative vertebrate species with a murine *ob* probe. Positive signals from genomic DNA of species that are distantly related to mouse, such as chicken and eel, led them to conclude that the *ob* gene is highly conserved among vertebrates (1). Indeed, leptin-like immuno-cross-reactivity was reported in blood, brain, and liver of several fish species (18–20); blood and liver of lizards (21); and the stomach of snakes and *Xenopus laevis* (22). Furthermore, intracerebroventricular injection of human recombinant leptin in goldfish inhibits food intake (23), suggesting the presence of a leptin-like molecule in bony fish.

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Abbreviations: CNTF, Ciliary neurotrophic factor; NEFA, nonesterified fatty acid; NPY, neuropeptide Y; ob, obese gene; POMC, proopiomelanocortin; ppTRH, prepro-TRH.

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The sequence of a leptin-like molecule in teleostean fish has been reported only very recently in pufferfish (*Takifugu rubripes*) (24). Information on the possible role(s) of leptin in the regulation of food intake and energy metabolism in ectothermic vertebrates lacks altogether at this point. Here we report the presence of duplicate *ob* genes in common carp (*Cyprinus carpio*) and, for the first time in any nonmammalian species, address the regulation of the expression of the *ob* gene product in response to different feeding regimes.

Materials and Methods

Animals

Common carp (*C. carpio*) of the R3×R8 line were reared at 23 C in recirculating UV-treated Nijmegen city tap water. R3×R8 are the inbred offspring of a cross between fish of Polish (R3 strain) and Hungarian (R8 strain) origin. Carp were fed dry food pellets (LDX Filia slow sinking; Trouw Nutrition International, Putten, The Netherlands) once daily. Animals were weighed weekly and the amount of was food adjusted accordingly. Fish were irreversibly anesthetized with 0.1% 2-phenoxyethanol before the collection of plasma and tissue samples. All animal experiments were performed in accordance with national legislation and institutional guidelines regarding the treatment of experimental animals.

Identification of carp ob genes

We screened the Ensembl zebrafish genome database with mammalian leptin sequences, using the BLAST algorithm (25). This initial screen revealed a partial zebrafish leptin-like sequence. Using primers leptin.fw3 and leptin.rv2 (Table 1), which were based on this partial zebrafish leptin-like sequence, two similar and partial cDNA sequences were obtained from the liver of common carp. The corresponding full-length cDNA sequences were obtained from carp liver by nested rapid amplification of 5′- and 3′-cDNA ends (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with primers leptin.fw3, leptin.fw4, leptin.rv2, and leptin.rv5. This approach led to the identification of duplicate carp *ob* genes, designated *ob1* (encoding leptin-I) and *ob2* (encoding leptin-II). The gene structure of both carp *ob* genes was determined by amplification of the coding sequences from genomic DNA. Cloning and sequencing was carried out as previously described (26). Briefly, PCR products were ligated and cloned into JM-109 cells using the pGEM-T-easy kit (Promega, Leiden, The Netherlands). Plasmid DNA was isolated with the QIAprep spin miniprep kit (QIAGEN, Leusden, The Netherlands), and sequences were determined from both strands using the ABI Prism BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA).

Phylogenetic analysis

Multiple sequence alignments were carried out with ClustalW. A phylogenetic tree was constructed on the basis of amino acid differences (p-distance) with the neighbor-joining algorithm (complete deletion) in MEGA version 3.0 (27). Reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replications.

Modeling of carp leptin

The structure of human leptin (PDB entry 1AX8), which was solved at 2.4 Å resolution (4), was used as a template to build models of carp leptin-I and leptin-II. Initial alignments were obtained from the PSIPRED fold recognition server (28). Side-chain rotamers were modeled using SCWRL3.0 (29). Both models were refined in YASARA using the YAMBER2 forcefield (30). Coordinate files are available from the authors on request.

Feeding paradigms

The effects of different feeding regimens on leptin expression were evaluated in different feeding paradigms. For the analysis of leptin expression in response to short-term fasting and refeeding, two groups of animals, reared from the same offspring, were acclimatized to a daily ration of 1.2% of their estimated body weight, once daily at 0900 h. Control animals were maintained on this feeding regimen throughout the 10-d experiment, whereas the experimental animals were fasted for 6 d (d 2–7) followed by refeeding in the final 3 d of the experiment (*ad libitum* at d 8, 1.2% at d 9 and 10). Five fish from both groups were sampled daily at 1 h after the (scheduled) feeding time. The effects of long-term fasting were determined in two groups of animals reared from the same offspring and acclimatized to a daily ration of 2% of their body weight fed once daily at 0900 h. Control animals were maintained at this regimen throughout the experiment; experimental animals were fasted for 6 wk. Eight animals from each group were sampled weekly at 1 h after the (scheduled) feeding time. The effects of long-term feeding to satiation were determined in two groups of animals, reared from the same offspring and acclimatized to a daily ration of 2% of their estimated body weight. Control animals were maintained on this regimen throughout the experiment. Experimental animals were fed to satiation for 6 wk during which the bulk of the feed was given at 0900 h, and the rest was fed on an automated feeder to prevent water fouling. The percentage of the estimated body weight consumed daily during these 6 wk of feeding to satiation was at 7.5% during the first week and

TABLE 1. Primer sequences and corresponding accession numbers

Gene	Accession no.	Primer	Sequence (5′→3′)
Leptin	AJ830744, AJ830745	leptin.fw3	AAACTGCAGGCAAAGACCATCAT
		leptin.rv2	ATCAGCTTTTGCATAAACTGTTT
		leptin.fw4	GCAAAGACCATCATCGTCAGAAT
		leptin.rv5	CTCCTTGGATGTGCAATGCAT
		qleptin-I.fw1	CATATTGATTTGTCCACCCTTCTG
		qleptin-I.rv1	CCATTAGCTGGCTCCTTGGAT
		qleptin-II.fw1	AGATACGCAACGATTTGTTCACA
		qleptin-II.rv1	GCGTTGTTCTCCAAGAAAGCA
CRH	AJ317955, AJ576243	qCRH.fw2	CATCCGGCTCGGTAACAGAA
		qCRH.rv2	CCAACAGACGCTGCGTTAACT
POMC	Y14618, Y14617	qPOMC.fw1	TTGGCTCTGGCTGTTCTGTGT
		qPOMC.rv1	TCATCTGTCAGATCAGACCTGCATA
NPY	AF287347	qNPY.fw1	CTCTAACGGAAGGGTATCCA
		qNPY.rv1	GCCGTGTTATGAGGTTGATG
ppTRH	AB179818	qTRH.fw1	TTACCGGTCGCTTTCACGTT
		qTRH.rv1	AGCTGTGTGCCAAACCAAAC
40S ribosomal Protein S11	AB012087	q40S.fw1	CCGTGGGTGACATCGTTACA
		q40S.rv1	TCAGGACATTGAACCTCACTGTCT
β-Actin	CCACTBA	qACT.fw1	CAACAGGGAAAAGATGACACAGATC
		qACT.rv1	GGGACAGCACAGCCTGGAT

FIG. 1. DNA and deduced amino acid sequences of carp *ob1* (encoding leptin-I; A) and *ob2* (encoding leptin-II; B). The start codons are indicated by *asterisks*. Potential instability motifs are *underlined*. Introns are in *italics*. Splice sites are indicated by *arrowheads* and 5′ donor (gt) and 3′ acceptor (ag) splice sites are in *bold*. Accession numbers are AJ868357 and AJ868356, respectively.

declined from 5.0 to 3.7% in wk 2–6. After 6 wk of feeding to satiation, animals (n = 8) were sampled at 1 h after feeding. For the determination of the kinetics of leptin expression during the course of a single day, fish from a single tank were fed at 0900 h and sampled before and at several times after feeding.

RNA isolation and cDNA synthesis

RNA was isolated using Trizol reagent (Invitrogen), following the manufacturer's instructions; RNA concentrations were measured by spectrophotometry. First-strand cDNA synthesis was carried out as previously described (26). Briefly, total RNA was DNase treated, followed by random hexamer primed cDNA synthesis (Invitrogen). A control containing no reverse transcriptase was included for each sample.

Real-time quantitative PCR

Primer Express software (Applied Biosystems) was used to design primers for analysis of gene expression by real-time quantitative PCR

TABLE 2. Percentage amino acid identity between vertebrate leptin amino acid sequences

	Carp leptin-I	Carp leptin-II	Zebrafish	Pufferfish	Green puffer	Medaka	*Xenopus*	Eastern tiger salamander	Fat-tailed dunnart	Mouse	Rat	Pig	Macaque	Human
Carp leptin-I	100													
Carp leptin-II	82	100												
Zebrafish	62	61	100											
Pufferfish	26	26	19	100										
Green puffer	28	26	26	50	100									
Medaka	25	25	25	30	31	100								
Xenopus	27	27	28	23	18	19	100							
Eastern tiger salamander	23	24	28	17	19	21	60	100						
Fat-tailed dunnart	20	22	21	14	16	17	33	36	100					
Mouse	24	24	23	16	18	19	34	31	67	100				
Rat	24	23	23	16	17	19	34	32	67	96	100			
Pig	24	24	23	16	18	19	34	35	71	82	83	100		
Macaque	23	24	22	16	15	17	34	33	69	81	80	84	100	
Human	24	25	22	18	16	16	36	34	68	83	82	85	90	100

(Table 1). Five microliters cDNA and forward and reverse primers (300 nM each) were added to 12.5 μl Sybr Green PCR master mix (Applied Biosystems), and the volume was adjusted to 25 μl with demineralized water. Real-time quantitative PCR (2 min at 48 C, 10 min at 95 C, 40 cycles of 15 sec at 95 C, and 1 min at 60 C) was carried out on a GeneAmp 5700 sequence detection system (Applied Biosystems). Data were analyzed with the ΔΔCt (cycle threshold) method. Dual internal standards (40S ribosomal protein S11 and β-actin) were incorporated in all real-time quantitative PCR experiments, and results were confirmed to be similar after standardization to either gene. Results standardized for 40S expression are shown.

Plasma parameters

Plasma glucose concentration was determined with a Stat Profile pHOx Plus analyzer with automated two-point calibration and equipped with an enzymatic glucose electrode (Nova Biomedical, Waltham, MA) (31). In the experiments investigating long-term fasting and feeding to satiation, glucose levels were determined with an enzymatic colorimetric kit (Instruchemie, Delfzijl, The Netherlands) according to the manufacturer's instructions. Plasma nonesterified fatty acids (NEFAs) were measured with the NEFA-C kit (Wako Chemicals, Neuss, Germany), according to the manufacturer's instructions. Plasma glucose and NEFA values provide insight into the nutritional status of the animals because plasma glucose will be high during the postprandial state, whereas increased NEFA values indicate energy mobilization via lipolysis in the absence of high glucose levels (32).

Statistics

Statistical analyses were carried out with SPSS software (version 12.0.1; SPSS, Chicago, IL). The level of significance of differences assessed by ANOVA was evaluated with a two-sided Student's *t* test. Homogeneity of variances was tested with Levene's test, and the correction for nonhomogeneous variances was applied where necessary.

Results

Characteristics of carp leptin

The nucleotide differences throughout the coding strands, 5′ and 3′ untranslated regions, and introns, added to the different size of the intron between both carp leptin sequences clearly illustrate that both carp leptin transcripts are derived from separate genes rather than from the same gene through alternative splicing (Fig. 1 and supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Both carp *ob* genes encode 171 amino acid leptin proteins that share 82% amino acid identity (Table 2). Carp leptins are equally similar (62 and 61%, respectively) to the single leptin of zebrafish (supplemental Fig. S2). Amino acid identity with mammalian leptins is markedly lower at 20–25%. Furthermore, the amino acid identities between cyprinid and puffer leptin sequences are only marginally higher at 26–28% (Table 2). The identity of the carp leptins with leptin proteins of *X. laevis* and the Eastern tiger salamander (*Ambystoma tigrinum tigrinum*) is also only marginally higher than the identity between carp and human. Identical amino acid residues in all vertebrate leptin sequences are distributed evenly through-

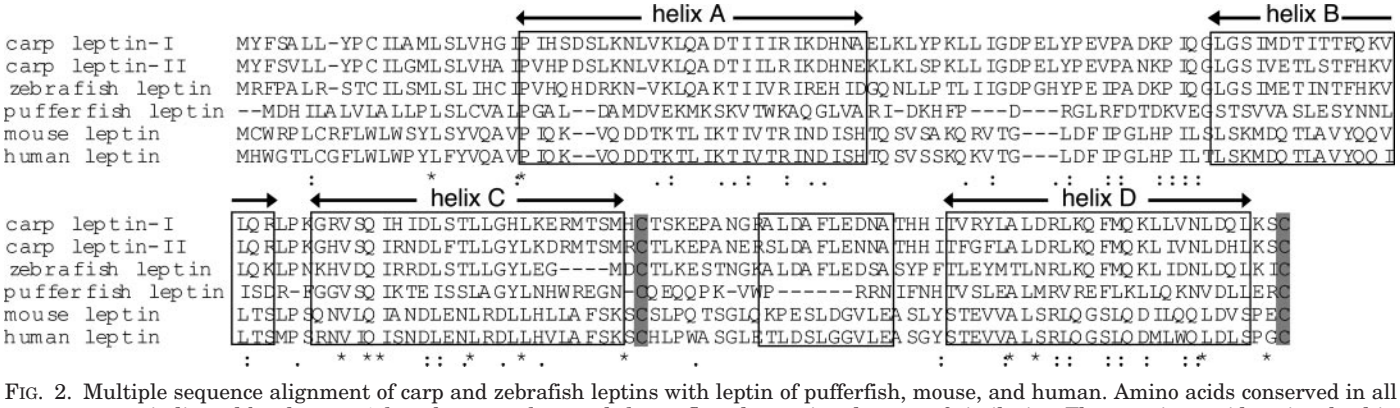

FIG. 2. Multiple sequence alignment of carp and zebrafish leptins with leptin of pufferfish, mouse, and human. Amino acids conserved in all sequences are indicated by the *asterisks*, whereas *colons* and *dots* reflect decreasing degrees of similarity. The cysteine residues involved in disulfide bridge formation are *shaded*, the α-helices, inferred from human leptin, are *boxed*. Accession numbers are as follows: carp leptin-I, AJ830745; carp leptin-II, AJ830744; zebrafish, BN000830; pufferfish leptin, AB193547; mouse, P41160; human, P41159.

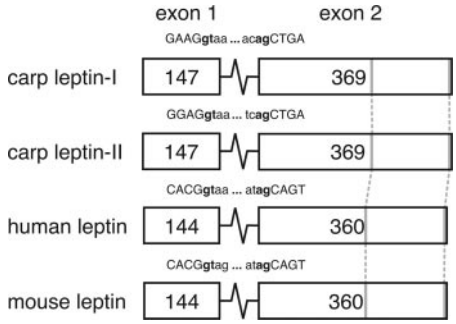

FIG. 3. The gene structure of vertebrate *ob* genes is conserved. *Boxes* represent coding exons and are drawn to scale, *numbers* indicate exon sizes in nucleotides. Conserved cysteine residues are *shaded*. The residues surrounding each splice site are given, coding residues are represented as *capitals*. The 5′ donor (gt) and 3′ acceptor (ag) splice sites are in *bold*. Accession numbers of the carp leptin-I and -II genes are AJ868357 and AJ868356, respectively. Accession numbers for human and mouse *ob* genes are AY996373 and U22421, respectively.

out the leptin protein and include both cysteine residues that together form a disulfide bridge connecting the carboxy-terminal ends of α-helices C and D (Fig. 2). The gene structure of the carp *ob* genes is identical, and both leptins are encoded by two exons, separated by a short intron with consensus 5′ donor (gt) and 3′ acceptor (ag) splice sites (Fig. 1). Both coding exons of the carp *ob* genes are very similar in length to the corresponding exons of the human and mouse *ob* genes and differ only one and three codons, respectively (Fig. 3).

Although information on the genomic context of the carp leptin genes is not available, the *ob* gene of the closely related zebrafish is positioned in the opposite orientation and in close proximity (<3 kb) to the gene encoding RNA binding motif protein 28 (RBM28) (not shown). RBM28 is also present in juxtaposition of and directly adjacent to the *ob* gene in the genomes of both human and mouse, and this information further strengthens the orthology of fish and mammalian leptins.

In phylogenetic analyses that include other members of the class I helical cytokine family, all vertebrate leptins cluster together (Fig. 4). The bootstrap value that anchors the vertebrate leptin cluster corroborates the *bona fide* identity of the carp leptins, although the branch lengths that separate mammalian and fish leptins are long, reflecting their considerable sequence dissimilarity. Furthermore, the branching pattern with the leptin cluster is in accordance with the established patterns of vertebrate evolution because the teleostean leptins branch off before the separation of the amphibian and mammalian leptin cluster. Within the mammalian leptin cluster, the sequence of the fat-tailed dunnart (*Sminthopsis crassicaudata*), the only marsupial leptin that is known, branches outside the leptin sequences of placental mammals.

Human leptin consists of four α-helices, arranged in a bundle with an up-up-down-down topology (4). Despite the relatively low overall amino acid identity with their mammalian orthologs, our models of carp leptin, which are based on the crystal structure of human leptin, indicate that both carp leptins conform well to the four-helix bundle topology that is characteristic of human leptin (Fig. 5) (4).

Constitutive expression of carp leptins

We investigated the expression of both carp *ob* genes in a panel of peripheral organs. The highest expression that we observed was that of leptin-II in the liver, which was more abundantly expressed than leptin-I. Expression of both leptin genes was also observed in thymus and kidney and at low levels in other organs and tissues including muscle and visceral adipose tissue (Fig. 6).

Leptin expression in response to short-term fasting and refeeding

After the establishment of the liver as an important site of leptin expression, we investigated liver leptin expression in response to short-term (*i.e.* 6 d) fasting, followed by refeeding. Plasma glucose and NEFA values differed clearly between fed and fasted animals and proved reliable indicators of nutritional status. Postprandial plasma glucose values in

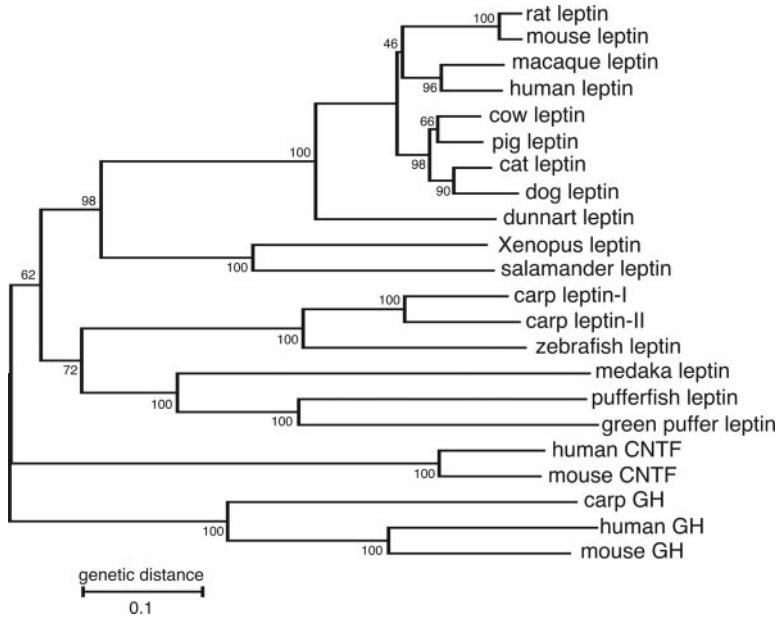

FIG. 4. Phylogenetic tree of vertebrate leptin amino acid sequences. Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. GH and CNTF are included as outgroup. Accession numbers are as follows: human leptin, P41159; rhesus macaque leptin, Q28504; mouse leptin, P41160; rat leptin, P50596; cow leptin, P50595; pig leptin, Q29406; dog leptin, O02720; cat leptin, AB041360; fat-tailed dunnart leptin, AF159713; *Xenopus* leptin, AY884210; Eastern tiger salamander leptin, CN054256; zebrafish leptin, BN000830; carp leptin-I, AJ830745; carp leptin-II, AJ830744; medaka leptin, AB193548; pufferfish leptin, AB193547; green puffer leptin, AB193549; human CNTF, P26441; mouse CNTF, P51642; human GH, P01241; mouse GH, P06880; carp GH, P10298.

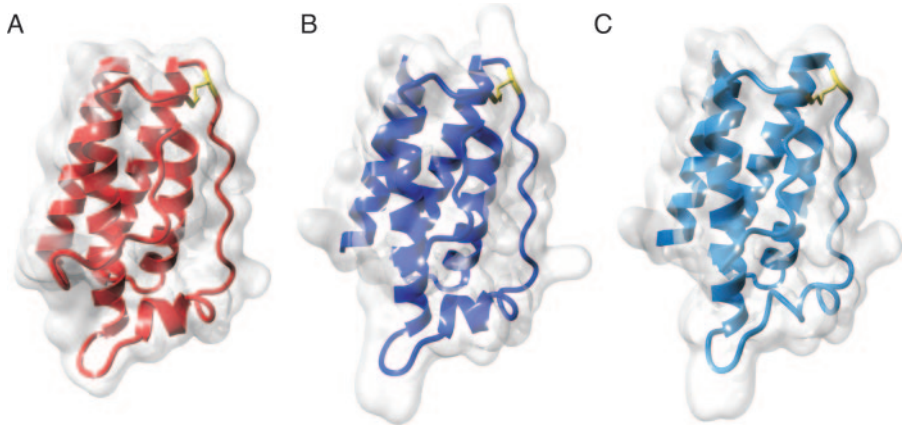

FIG. 5. Protein models of carp leptin. Carp leptin-I and -II, modeled over the human leptin crystal structure (A), illustrate the four-helix bundle conformation adopted by carp leptin-I (B) and leptin-II (C), stabilized by a single disulfide bridge (*yellow*).

the control animals consistently ranged from 8 to 11 mM. In contrast, plasma glucose values in the fasted animals were maintained at 2.5–3.0 mM (Fig. 7A). In contrast to glucose, plasma NEFA values rapidly rose in response to fasting from approximately 0.05 to 0.25–0.35 mM (Fig. 7B). Upon resumption of the normal feeding regimen, plasma glucose and NEFAs rapidly normalized, although glucose and NEFAs did exhibit a slight over- and undershoot, respectively, in the days after refeeding. Short-term fasting did not result in any changes in leptin-I expression in the fasted animals (Fig. 7C). The expression of leptin-I in the control group was significantly elevated at one point, 5 d into the experiment, which may be the result of variation in the postprandial expression of leptin-I in control animals (see below). The expression of leptin-II was also not affected by fasting (Fig. 7D). Furthermore, the expression of neither leptin changed in response to refeeding.

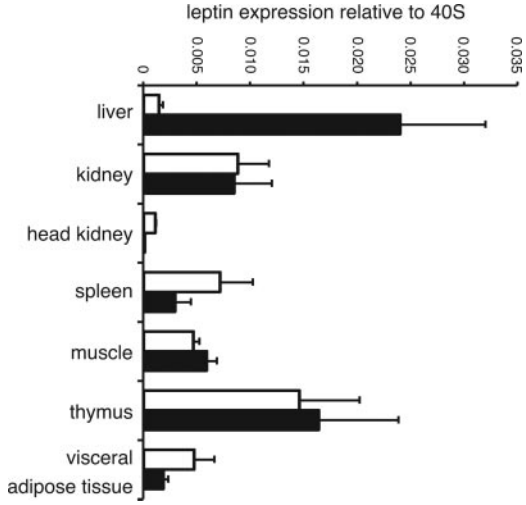

FIG. 6. Constitutive expression of carp leptin-I (*open bars*) and -II (*closed bars*) in selected carp organs detected by real-time quantitative PCR. Carp leptin is predominantly expressed in liver, with additional expression observed in the thymus, kidney, and spleen. Low-level constitutive expression was observed in most organs, including visceral adipose tissue. Expression was normalized to the expression of 40S ribosomal protein S11. *Error bars* indicate the SE of five replicate samples.

The effects of long-term fasting

Because short-term fasting did not produce an effect on leptin gene expression, we then fasted carp for 6 wk. Plasma glucose and NEFA levels were clearly affected by long-term fasting. Plasma glucose levels of fasted animals were consistently less than 2 mM and dropped to almost undetectable levels after 3 wk of fasting (Fig. 8A). Postprandial glucose levels of control animals ranged from 7 to 10 mM throughout the experiment. Long-term fasted animals show increased plasma NEFA concentrations, whereas NEFA values in fed animals are low (Fig. 8B). Because fasting is known to affect the gene expression of several of the pivotal factors in the regulation of food intake and metabolism, we assessed hypothalamic gene expression in fasted and control animals at 6 wk. The hypothalamic expression of the anorexigens CRH and POMC (the precursor for α-MSH) had significantly decreased in fasted animals, compared with controls, whereas the expression of the orexigen NPY was unaffected (Fig. 8C). The hypothalamic expression of prepro-TRH (ppTRH) decreased on fasting. The impact of fasting is illustrated by the differences in total body weight between control and fasted fish. Fasted animals lost more than 30% of their initial weight toward the end of the experiment and finally weighed 50% less than controls (Fig. 8D). Nevertheless and despite these paramount differences in plasma glucose and NEFA values, hypothalamic gene expressions, and body weight, we observed no effects of long-term fasting on the hepatic expression of leptin-I (Fig. 8E) and leptin-II (Fig. 8F).

Long-term feeding to satiation

In a reverse approach, we next tested the effects of feeding to satiation for a period of 6 wk on hepatic leptin expression. After 6 wk of feeding to satiation, postprandial plasma glucose values were high in both groups (>20 mM) and did not differ significantly (Fig. 9A). These high glucose values were accompanied by low (<0.05 mM) NEFA concentrations that did not differ between control animals and animals fed to satiation (Fig. 9B). Also, the postprandial hypothalamic gene expressions of CRH, POMC, NPY, and ppTRH were unaffected by 6 wk of feeding to satiation (Fig. 9C), compared with fed controls. Fish fed to satiation showed a 2.5-fold enhanced growth rate, compared with controls, and more than quadrupled their initial weight (51.8 g) over this 6-wk

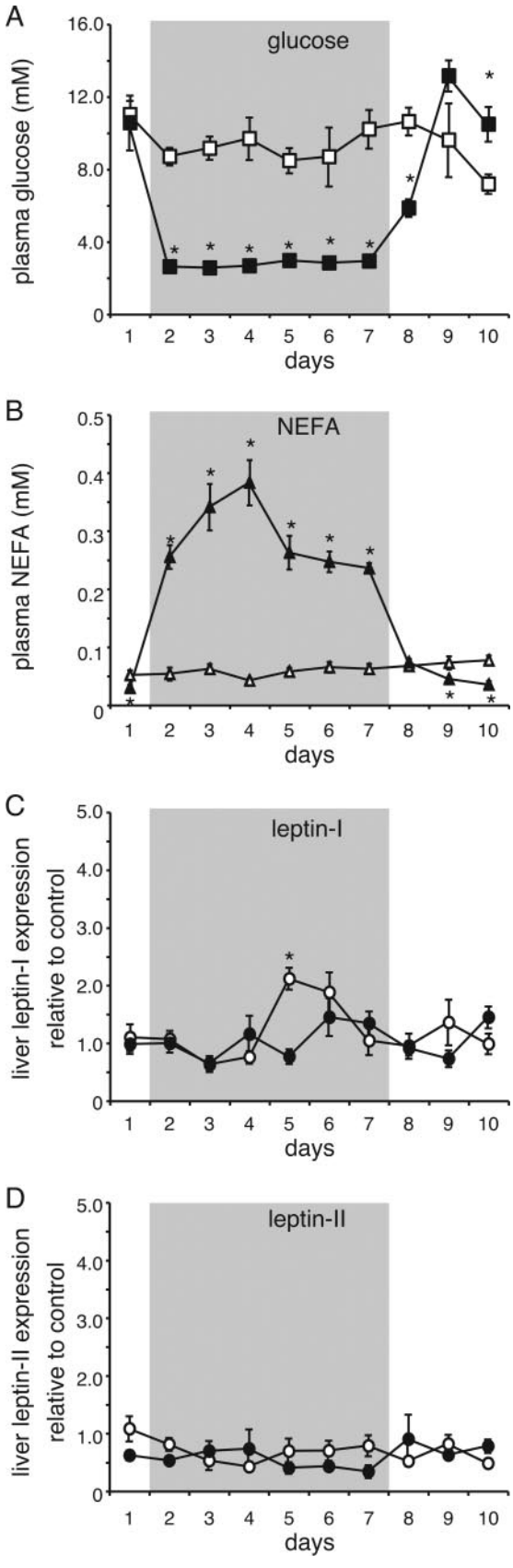

FIG. 7. The response to short-term fasting and re-feeding. Plasma glucose values in the experimental animals (*closed symbols*) are maintained at 2.5–3.0 mM (A), whereas plasma NEFA values are significantly elevated in the experimental animals, compared with controls (*open symbols*), throughout the fasting period (d 2–7, indicated by the *gray background*) (B). Liver expression of leptin-I (C) and leptin-II (D) does not respond to fasting or to subsequent refeeding. Leptin expression is standardized to expression of 40S ribosomal protein S11 and expressed relative to preexperimental controls. *Error bars* indicate the SE of four to five replicates; *asterisks* indicate significant differences with the corresponding control group; $P < 0.05$ was accepted as fiducial limit.

period (Fig. 9D). Also the average length of fish fed to satiation was significantly larger than that of controls after 6 wk (223 ± 13 *vs.* 192 ± 9 mm; not shown). Yet these profound differences in growth rate and body weight after 6 wk of feeding to satiation had no effect on the expression of leptin-I and leptin-II in the liver of carp (Fig. 9E).

Postprandial changes in leptin expression

Because we could not link the expression of either leptin gene with feeding to satiation or fasting for periods that span multiple meals, we next investigated the hepatic expression of leptin-I and leptin-II in response to a single meal. Feeding results in a characteristic postprandial peak in plasma glucose (Fig. 10A) that is accompanied by inhibited lipolysis as evidenced by a drop in circulating NEFA values (32). The postprandial peak in plasma glucose is followed by a marked postprandial rise in the hepatic expression of leptin-I and leptin-II at 3 h and 6 h after feeding, respectively (Fig. 10B). Interestingly, in the initial hours after feeding, before its postprandial peak, liver leptin-II expression slightly but significantly dropped below the prefeeding expression values.

Discussion

Carp, a contemporary representative of an early vertebrate lineage, expresses duplicate *ob* genes that encode two similar leptins that we designated leptin-I and leptin-II. Their high level of amino acid similarity suggests that both carp *ob* genes are the likely result of a recent gene duplication event, possibly the recent tetraploidization of the carp genome that occurred less than 16 million years ago (33). The carp leptins share a relatively low amino acid similarity of less than 25% with mammalian leptins and the amino acid identity to pufferfish leptins is only marginally higher. The observation that orthologous class-I helical cytokines of pufferfish and cyprinid fish species are only slightly more identical with each other than to their human or mouse orthologs is quite common; we observed this also for IL-11 and IL-12 (34, 35). This considerable sequence dissimilarity is explained by the large evolutionary distance between cyprinids and puffer fishes, which diverged approximately 300 million years ago, merely 150 million years after the fish-tetrapod split (36, 37).

Despite the relatively poor primary sequence identity between vertebrate leptins, we are confident in the assignment of orthology to carp and mammalian leptins based on several key observations. First, both coding exons of the carp *ob* genes differ merely one and three codons in length, respectively, from those of human and mouse *ob*. Importantly, their exon structure offers strong support to the orthology of carp and human leptins because vertebrate class-I helical cyto-

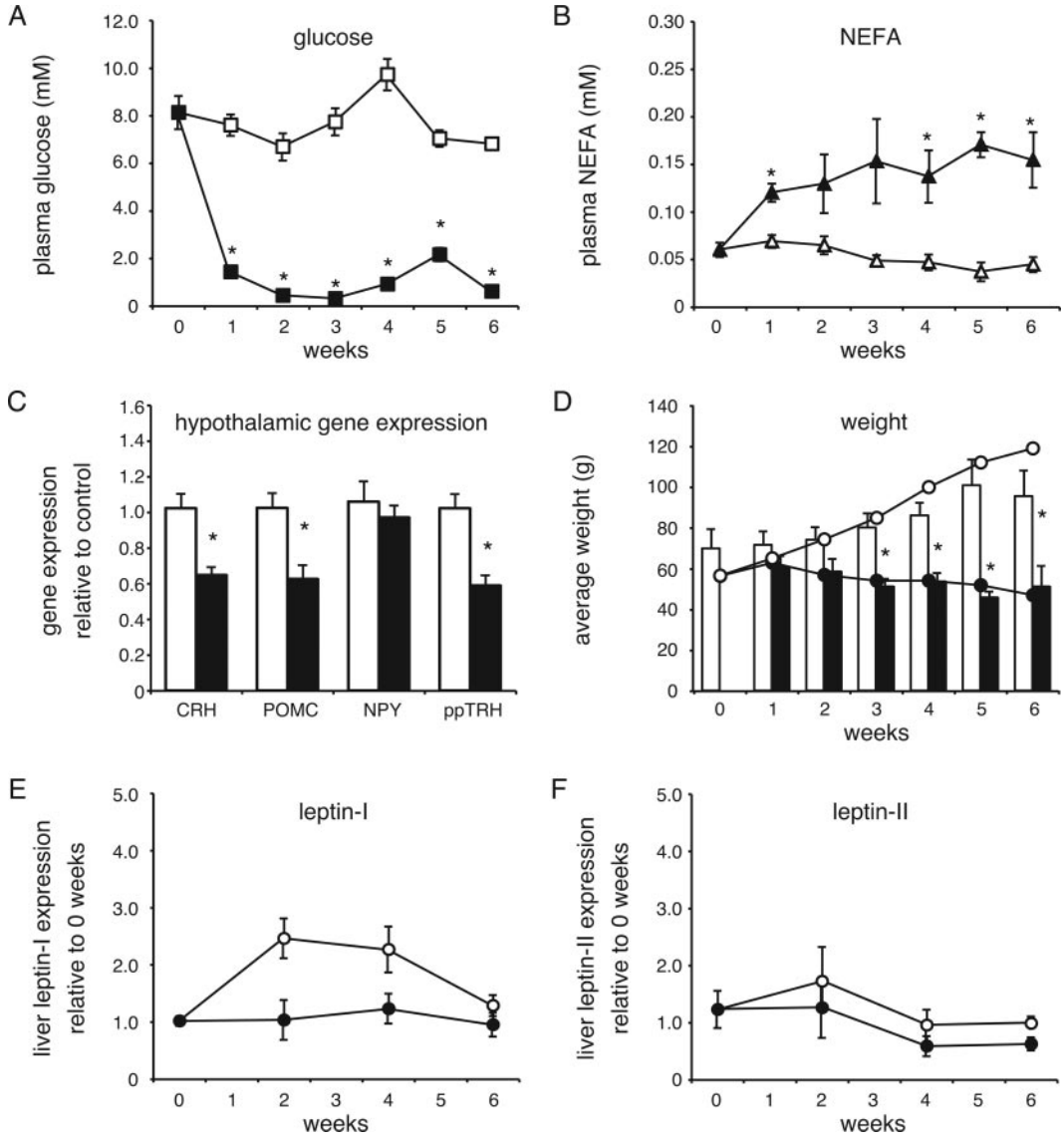

FIG. 8. The response to prolonged fasting. Plasma glucose values in the fasted animals (*closed symbols* or *bars*) are maintained less than 2 mM (A), whereas plasma NEFA values are significantly elevated in the fasted animals, compared with controls (*open symbols* or *bars*) throughout the fasting period (B). Despite the lack of changes in leptin expression throughout the experiment, hypothalamic expression of CRH, POMC, and ppTRH was significantly reduced at the end of the 6-wk fasting period, compared with controls on a normal feeding regimen, with NPY expression remaining unchanged (C). During the 6 wk of food deprivation, animals displayed a considerable reduction in weight, with the control animals on the normal feeding regimen weighing approximately twice as much at the end of the experiment (D). *Bars* (D) display the average individual weight of the eight fish sampled at each week. The *lines* indicate the average weight per animal, as derived from the total animal mass in each of the tanks, of the animals that were left after each sample point. Despite these changes in plasma parameters, hypothalamic gene expressions and body weight and growth rate, liver expression of leptin-I (E) and leptin-II (F) did not respond to prolonged fasting for a period of 6 wk. Hypothalamic gene expression is normalized to the expression of 40S ribosomal protein S11 and is expressed relative to fed controls. Leptin expression is standardized to expression of 40S ribosomal protein S11 and expressed relative to the expression in preexperimental controls. *Error bars* indicate the SE of seven to eight replicates, *asterisks* indicate significant differences with the corresponding control group, and $P < 0.05$ was accepted as fiducial limit.

kines are almost invariably encoded by at least three and in general five exons (38). The only other class-I helical cytokine gene encoded by two exons is ciliary neurotrophic factor (CNTF), but its exons differ clearly in size from those of leptin. Second, the spacing of the cysteines that constitute the single disulfide bridge of leptin, which are conserved in both carp leptins, is unique among class-I helical cytokines. Finally, the stable clustering in phylogenetic analyses, the predicted conformation of both carp leptins to the characteristic four-helix bundle topology of class-I helical cytokines, and the conservation of synteny between the zebrafish and human *ob* genes further support unambiguously the orthology of fish and mammalian leptins. Nevertheless, the poor overall sequence similarity between mammalian and fish leptins has likely contributed to the difficulties in retrieving *ob* orthologs in nonmammalian vertebrates. Importantly, this low (<25%) amino acid identity serves as a reminder that we assign the name leptin solely on the basis of the structural

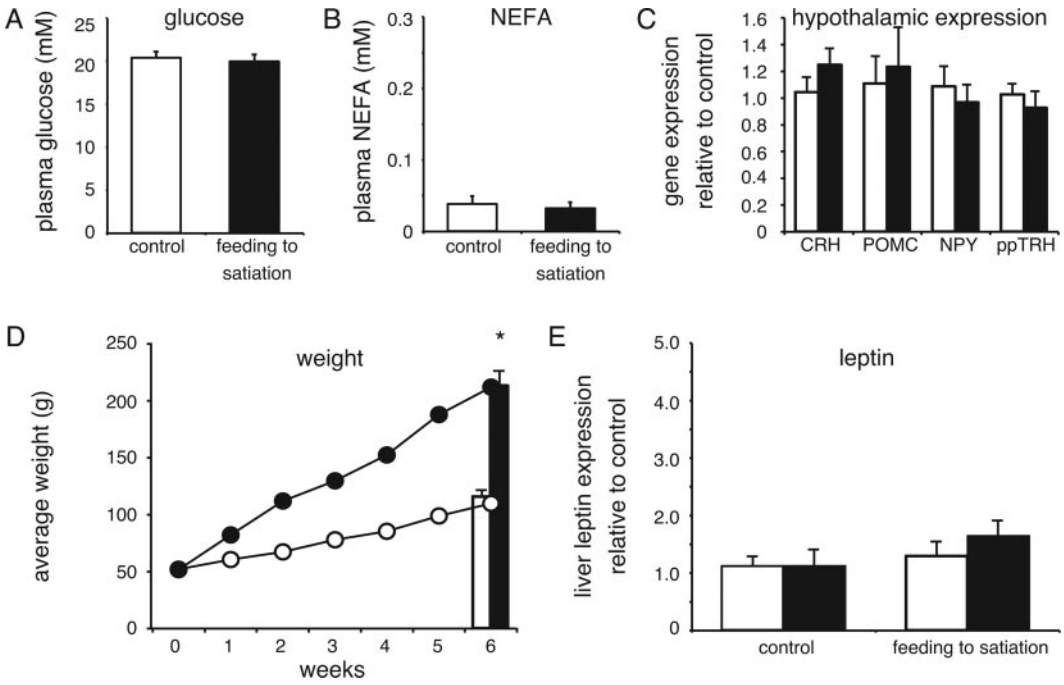

FIG. 9. The response to long-term feeding to satiation. After 6 wk of feeding to satiety, plasma glucose values were high (~20 mM) at 1 h after feeding in both groups and did not differ between controls (*open bar*) and animals fed to satiation (*closed bar*) (A). Plasma NEFA values determined at the same time were low (<0.05 mM) and very similar in both groups (B). No changes were observed in the hypothalamic gene expression of CRH, POMC, NPY, and ppTRH between control animals (*open bars*) and animals to satiation (*closed bars*) (C). Despite the lack of detectable changes in gene expression and plasma parameters, animals on the satiation feeding regimen (*closed symbols* and *bars*) did grow more than twice as fast as the control animals (*open symbols* and *bars*) on a 2% feeding regimen (D). *Bars* (D) display the average individual weight of the eight fish sampled at 6 wk. The *lines* indicate the average weight per animal, as derived from the total animal mass in each of the tanks determined weekly. Liver expression of leptin-I (*open bars*) and leptin-II (*closed bars*) remained unchanged in response to feeding to satiation for a period of 6 wk (E). Gene expression is normalized to the expression of 40S ribosomal protein S11 and is expressed relative to controls on the normal feeding regimen. *Error bars* indicate the SE of seven to eight replicates, *asterisks* indicate significant differences with the corresponding control group, and $P < 0.05$ was accepted as fiducial limit.

similarities described above. Orthologous proteins do not by default share analogous roles, and this may be particularly true for proteins that share so little of their primary amino acid sequences as the leptins of teleostean fishes and mammals.

Although several reports exist on nonmammalian leptin genes, our report on duplicate carp *ob* genes is the first that attempts to address the gene regulation of a nonmammalian leptin ortholog. A chicken leptin sequence that is nearly identical with mouse leptin has been reported several years ago (39, 40). However, the inability of several groups to repeat the amplification of the chicken *ob* gene (41, 42) and the absence of chicken leptin (as it was originally reported) in the recently published chicken genome question the validity of this chicken leptin sequence and would dismiss it as artifactual. Recently a leptin sequence from pufferfish (*T. rubripes*) was reported, together with leptin sequences of the spotted green pufferfish (*Tetraodon nigrovirides*) and medaka (*Oryzias latipes*) (24). In contrast to the leptin proteins of carp and zebrafish that match the size of their mammalian orthologs well, the pufferfish leptin sequence is 15 amino acids shorter than mammalian leptins. Moreover, pufferfish and human leptin share only 14–18% amino acid identity (Table 2), which is very low, even when compared with the modest amino acid identity shared by cyprinid and mammalian leptins.

In contrast to mammals, leptin is in carp only modestly expressed in visceral adipose tissue. The expression of leptin in adipose tissue of pufferfish was not addressed because adipose tissue could not be identified in this species (24). The modest leptin expression that is observed in the visceral adipose tissue of carp may be in agreement with the notion that the contribution of the visceral adipose compartment to circulating leptin levels in mammals is limited because plasma leptin is mainly secreted from sc adipose tissue (43, 44). Instead, the liver appears to be an important site for leptin expression in fish because it expresses both carp *ob* genes and is the major site for leptin-II expression as well as the exclusive site of leptin expression in pufferfish (24). Also, because the liver is the largest visceral organ in carp and many other species of fish, its sheer number of cells would ensure a significant leptin output, even though it is not the only organ that expresses leptin. Moreover, our findings in carp are in line with previous studies that suggest that the liver, rather than adipose tissue, is one of the main sites of immunoreactive leptin in fish (18). Both carp *ob* genes are expressed ubiquitously at other organs and tissues, most notably in the thymus. Although we have not investigated this extrahepatic expression in detail, several of these tissues and organs have precedent with regard to leptin expression in mammalian species (45–47).

The marked postprandial rise in the expression of both carp *ob* genes in the liver is similar to the postprandial rise in leptin mRNA that is observed in the hours after eating in

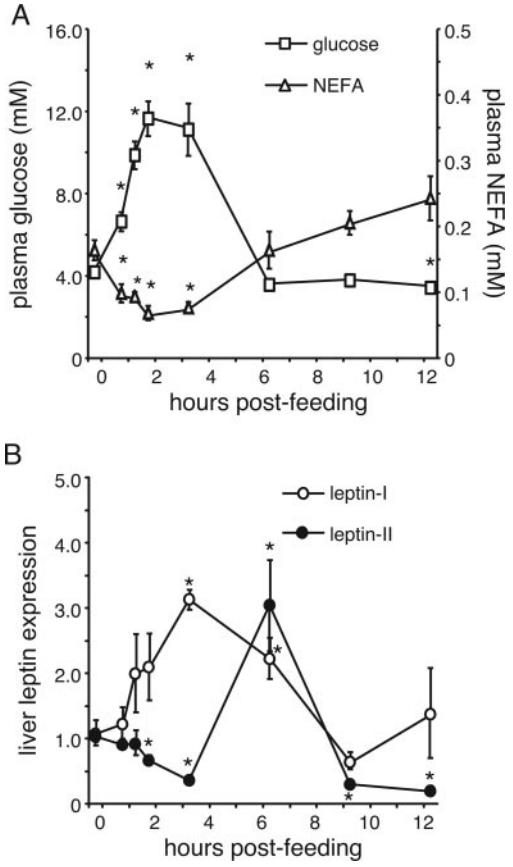

FIG. 10. During the course of a single day, fish from the same tank were sampled before and at various times after feeding. Feeding is followed by a postprandial rise in plasma glucose that is accompanied by a concomitant drop in plasma NEFA values (A). The liver expression of leptin-I and leptin-II displays a marked and significant postprandial peak in expression at 3 and 6 h after feeding, respectively (B). The peak in hepatic leptin expression follows the postprandial glucose peak. Directly after feeding (2.0% of their estimated body weight) leptin-I expression inclines toward its postprandial peak, whereas leptin-II expression is slightly reduced at that time. Leptin expression is standardized to expression of 40S ribosomal protein S11 and expressed relative to prefeeding controls. *Error bars* indicated the SE of four to five replicates, *asterisks* indicate significant differences with the prefeeding controls, and $P < 0.05$ was accepted as fiducial limit.

mice (6) and correlates leptin expression to short-term food intake in carp. Furthermore, the peak in postprandial hepatic leptin mRNA expression follows the postprandial increase in plasma glucose and concomitant drop in plasma NEFA. It is possible that the postprandial changes in leptin expression are under direct glucose control. Glucose administration by ip injection, which results in acutely increased plasma glucose levels, causes a rapid elevation of *ob* mRNA levels in lean wild-type mice (48). Furthermore, increased adipocyte glucose metabolism enhances the amount of leptin protein that is secreted from the cell (49). However, during 6 wk of fasting plasma glucose levels dropped to less than 1 mM for 3 consecutive weeks without apparent effects on the constitutive hepatic leptin expression and this suggest that basal leptin expression is maintained independently of plasma glucose levels. Alternatively, it is possible that the postprandial rise in *ob* expression is, in part or completely, brought about indirectly via the actions of other anorexigenic hormones such as insulin and cholecystokinin. There are some intriguing data available that suggest that Indian major carp (*Catla catla*) adipocytes from visceral fat tissue, liver, and kidney combine features of insulin target cells with the characteristics of pancreatic β-cells. These cells contain peroxisomal proliferator-activated receptor-γ and glucose transporter-4 and express and release bioactive insulin (20). When taken together with our observations, this suggests that in cyprinid fishes leptin and insulin are released from the same tissue and are thus poised to interact in a paracrine fashion.

The rapid and transient changes in *ob* gene expression that we observe in response to a single meal are in agreement with work of the group of Peter, who observed a rapid inhibitory effect on goldfish food intake after injection of recombinant murine leptin into the third ventricle of the brain (23). Nevertheless, caution with the interpretation of heterologous assays for the presence and actions of leptin protein is indicated and calls for the development of species-specific leptin assays.

Although changes in leptin expression are linked to acute food intake, we did not detect differences in the hepatic expression of either carp *ob* gene in response to longer-term changes in feeding regimen. Upon fasting, carp rapidly switch from carbohydrate to lipid metabolism, as indicated by the rapid and profound increase in plasma NEFA levels that are characteristic of starvation (50). Moreover, after 6 wk of fasting, the hypothalamic expression of the anorexigens POMC (precursor to α-MSH) and CRH are depressed, whereas the expression of the orexigen NPY remains at control levels. Moreover, the expression of ppTRH is reduced after long-term fasting as has previously been observed in fasted rats (51). Nevertheless, these obvious changes in metabolic status and hypothalamic gene expressions are not reflected by a decrease of leptin expression after 6 d or 6 wk of fasting. We predicted such regulation as rodents, in contrast to our current findings in carp, respond to fasting with a drop in leptin expression that is rapidly reverted on subsequent refeeding (6). Fasting rats for 6 d caused a considerable 36% reduction in total body weight of rats (50). By comparison, it required 5 wk of food deprivation for carp to lose a similar percentage of their starting body weight. These differences obviously relate directly to the overt differences in energy metabolism between endotherms and ectotherms, the latter enjoying much more flexibility in their metabolic regulation because they do not require to thermoregulate. This flexibility enables fish to cope with periods of starvation of up to several months, which many species encounter as a result of reproductive strategy or seasonal variation in food availability. Because the transition from ectothermia to endothermia was accompanied by the development of a continuous layer of adipose tissue, we speculate that this layer may have served a dual purpose; besides providing insulation to prevent heat loss the subcutaneous adipose tissue may at the same time have adopted the role of actively providing the rapid and continuous feedback to the central nervous system on the nutrient status of the body that enables the accurate matching of energy intake and expenditure required for endothermia.

In their seminal paper on the identification of leptin, Zhang *et al.* (1) addressed the question of the conservation of

the obese gene by a Southern blot experiment. Their successful hybridization of a murine *ob* probe to genomic DNA of chicken and eel led to the conclusion that leptin is "evolutionarily conserved" (1). Surely, this is a plausible view, given the key role leptin plays in the regulation of food intake and energy metabolism (certainly in mammals), physiological processes that are vital for survival and thus evolutionary fitness. Although the mere presence of leptin in bony fish can be regarded as testimony to its conservation, it is unlikely that an amino acid identity of less than 25% between leptins of different vertebrate species was what investigators had in mind when referring to leptin as evolutionarily conserved. We now know the actual extent of the amino acid differences between leptins of fish and mammals, and this low degree of amino acid similarity serves as a reminder that the physiological role of leptin in ectotherms may differ substantially from the role of leptin that we have come to associate with mammals. We propose that an increased understanding of the role of leptin in ectothermic vertebrates such as carp will provide us with a novel perspective that can assist our appreciation of the complex role that this key hormone plays in the regulation of energy metabolism in humans and mammalian model systems.

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