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Leptin and its receptor in turbot *Scophthalmus maximus*: cloning, characterization and expression response to ratios of dietary carbohydrate–lipid

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Abstract In the present study, the full-length cDNA sequences of leptin (LEP) and its receptor (LEPR) from turbot *Scophthalmus maximus* were cloned. The cDNA of tLEP was 1126 bp in length encoding 157 amino acids. The amino acid sequence shared low identity with human LEP (18.8 %), but the three-dimensional structures of these two LEPs were strongly conserved. The deduced 1173-amino acid sequence of tLEPR was 28 % identical to human LEPR, and 82 % too range-spotted grouper LEPR, containing all functionally important domains conserved in vertebrate LEPR. Tissue distribution analysis showed that tLEP was abundantly expressed in brain, eyes and liver. The highest level of tLEPR mRNA was found in liver and kidney. After a 9-week feeding trial using diets with different ratios of carbohydrate–lipid (1:6, 1:2, 2:1 and 14:1), it was found that the increase in dietary carbohydrate-to-lipid ratios from 1:6 to 2:1 did not significantly influence tLEP and tLEPR expression in turbot liver ($P > 0.05$). The hepatic tLEP expression was significantly elevated in treatment with 14:1 dietary carbohydrate-to-lipid ratio ($P < 0.05$). The hepatic tLEPR mRNA level in group with 14:1 dietary carbohydrate-to-lipid ratio was significantly lower than that in 1:6 group ($P < 0.05$), but had no significant difference with the other two groups ($P > 0.05$). These results revealed the important relationship between dietary carbohydrate-to-lipid ratio and LEP expression in turbot.

Keywords Leptin · Leptin receptor · Turbot · Carbohydrate · Lipid

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Introduction

Leptin (LEP) is a hormone protein belonging to class-I helical alpha cytokine family. It was firstly discovered by Zhang et al. (1994) in adipose tissue of mouse. After that, LEP or thologues have been extensively explored in many vertebrate animals. Especially during recent years, many researches on LEP in fish species have done, such as common carp *Cyprinus carpio* (Huising et al. 2006), puffer fish *Takifugu rubripes* (Kurokawa et al. 2008), zebrafish *Danio rerio* (Gorissen et al. 2009), Atlantic salmon *Salmo salar* (Ronnestad et al. 2010) and yellow catfish

Pelteobagrus fulvidraco (Gong et al. 2013a, b). Different from mammals, two duplication copies of LEP (LEP-A and LEP-B) are identified in some fish species, including medaka, zebrafish and grouper (Gorissen et al. 2009; Wong et al. 2007; Zhang et al. 2013), and duplicated orthologues of LEP-A, named LEP-A1 and LEP-A2, are also identified in some tetraploid fish species, such as common carp and Atlantic salmon (Huising et al. 2006; Ronnestad et al. 2010), but only one kind of LEP can be found in others, such as puffer fish and yellow catfish (Gong et al. 2013a, b; Kurokawa et al. 2008). Although they share low amino acid (AA) identity with mammalian LEP, the three-dimensional structure of the predicted LEP protein in fish is well conserved (Ronnestad et al. 2010; Zhang et al. 2013).

The physiological actions of LEP are mediated by membrane-associated leptin receptor (LEPR) (Denver et al. 2011). In mammals, LEPR has at least six types of isoforms generated by alternate splicing of transcripts derived from a single LEPR gene. However, only the long isoform contains intracellular tyrosine residues necessary for signaling and physiological functions (Chua et al. 1997; Denver et al. 2011). In fish, the long-form LEPR was firstly identified in marine medaka *Oryzias melastigma* (Wong et al. 2007), and then it has been found in many other species (Ronnestad et al. 2010; Cao et al. 2011; Zhang et al. 2013; Gong et al. 2013a, b; Gong and Björnsson 2014). However, little information on LEPR isoforms is available in fish. It is reported that there are three kinds of isoforms in crucian carp *Carassius carassius* (Cao et al. 2011), five kinds of isoforms in Atlantic salmon (Ronnestad et al. 2010) and two kinds of isoforms in rainbow trout (Gong and Björnsson 2014).

The main function of LEP is to maintain the energy homeostasis through modulating food intake, lipid metabolism and glucose metabolism by the central nervous system or via a direct action on target peripheral tissue (Ahima and Flier 2000; Bjorbaek and Kahn 2004; Frühbeck and Salvador 2000; Reidy and Weber 2000). Effects of LEP on glucose metabolism have been proved, such as suppression of insulin gene expression and secretion in beta cells (Pallett et al. 1997), regulation of hepatic glycogenolysis and gluconeogenesis (Nemecz et al. 1999), and increasing glucose uptake and glycogenesis in muscle (Frühbeck and Salvador 2000). LEP has also been shown to promote lipid oxidation and decrease tissue triglycerides at liver and skeletal muscle (Muoio et al. 1997; Shimabukuro et al. 1997). It is conceivable that LEP is involved in switching hepatic substrate oxidation from carbohydrates to lipids and participates in the control of liver glycogen stores in obesity (Frühbeck and Salvador 2000). In fish, LEP is also reported to reduce food intake in goldfish (De Pedro et al. 2006), rainbow trout (Murashita et al. 2008; Gong et al. 2015) and grass carp (Li et al. 2010). Besides, LEP is demonstrated to stimulate lipolysis and fatty acid β-oxidation, inhibit adipogenesis in fat degenerated hepatocytes of grass carp (Lu et al. 2012), induce glucosensing in brain of rainbow trout (Aguilar et al. 2010) and decrease hepatic glycogen level in tilapia (Baltzegar et al. 2014). The gene expression of LEP is influenced by feeding and fasting in both mammals and some fish (De Vos et al. 1995; Kolaczynski et al. 1996a; Ronnestad et al. 2010; Won et al. 2012). Additionally, insulin and glucose state are also reported to affect LEP expression in human (Kolaczynski et al. 1996b; Wang et al. 1998). Although LEP function and signaling pathway have been researched deeply in mammals, little is known in fish, particularly in LEP response to dietary glucose and lipid level.

Turbot *Scophthalmus maximus* is a kind of carnivorous fish. It belongs to the order Pleuronectiformes. Although Kling et al. (2009) are succeeded in quantifying the plasma LEP level in turbot, the nucleotide sequence of turbot LEP has not been reported yet. The aim of this study is to clone the full-length cDNA sequence of LEP and LEPR of turbot and investigate the response of LEP and LEPR gene expression to ratios of dietary carbohydrate–lipid.

Materials and methods

All animal care and handling procedures in this study were approved by the Animal Care Committee of Ocean University of China.

Cloning of turbot LEP (tLEP) and LEPR (tLEPR)

Experimental turbots were obtained from a commercial farm in Qingdao, China. Juvenile turbots with 215–235 g of body weight were used to clone the genes and investigate their tissue distribution. Before sampling, fish were anesthetized with eugenol. Tissues

Table 1 Sequences of primers for cDNA cloning and real-time quantitative PCR (Q-PCR) of turbot leptin and its receptor genes

Primers	Sequences (5′–3′)
Primers for partial cDNA	
LEP F1	CCGGTGGAAGTCGTGRARATGAARWS
LEP R1	ACTGAGGAATCCCGTCAGCGANGADATNTC
LEPR F1	CGTSACGGTNTAYTGYGTGTTC
LEPR R1	GARAAATTDCCCTCRTCRCTGG
Primers for RACE PCR	
3′-LEP F2	AGTGACCGTCTTGGAGGGTTACAACAGC
3′-LEP F3	ATGGCGTCTCCCAGGTCAAGTCCG
5′-LEP R2	AAGGTGTCGGAGATCAGG
3′-LEPR F2	CAGCCCTGGTCCAAACCCCTCTCG
3′-LEPR F3	GCAGCTCCAGCGACGAGGGTAATTT
5′-LEPR R2	GTGTGGGGTTTCACATGATCTATGGGTG
5′-LEPR R3	GCAGCAGGTCATACATCCGGGTCTCC
UPM	Long—ACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
	Short—CTAATACGACTACTATAGGGC
NUP	AAGCAGTGGTATCAACGCAGAGT
Primers for real-time Q-PCR	
RT-LEP F	CTCTGGTCCTCCTGTTTTCTCTG
RT-LEP R	AGGCTAGAAGGGAACTGGAAATC
RT-LEPR F	CATGTGGAAGGATGTTCCCAACC
RT-LEPR R	CGCAGAGGTCAGCTTTGTCC
β-Actin F	TAGGTGATGAAGCCCAGAGC
β-Actin R	CTGGGTCATCTTCTCCCTGT

Mix bases: R-A/G; Y-C/T; M-A/C; K-G/T; S-G/C; W-A/T; H-A/T/C; B-G/T/C; V-G/A/C; D-G/A/T

for cloning and gene expression were collected and frozen immediately in liquid nitrogen and stored at −80 °C for RNA extraction.

Total RNA was isolated from turbot liver with RNAiso Plus (Takara, Japan) according to the manufacturer's protocol. The integrity of RNA was assessed by agarose gel electrophoresis. The purity and quantification of RNA were measured by NANODROP ND2000 spectrophotometer (Thermo Electrom Corporation, USA). One microgram of total RNA was used for reverse transcription with PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan). To obtain the partial cDNA sequence of tLEP and tLEPR, degenerated primers (Table 1) were designed based on the conserved cDNA sequence from other fish available in the GenBank database. The PCR reaction system consisted of 1 μl of cDNA, 12.5 μl 2× EsTaqMsterMix (CWbiotech, China), 9.5 μl of EDPC water and 1 μl of each primer (10 μM), carrying out under the following condition: 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 42 °C as annealing temperature (T_m) for 30 s and 72 °C for 1 min as extension time, ending with a 10-min final extension at 72 °C. The expected PCR products detected by 1.2 % agarose gel were purified using Quick Gel Extraction Kit (CWbiotech, China). After ligated to pEASY T1 vector and transformed into *Escherichia coli* DH5α competent cells (Trans Gen Biotech, China), the target products were sequenced by the BGI (Shanghai, China).

The full-length cDNA used to clone the 5′- and 3′-end of tLEP and tLEPR was transcribed, respectively, by 1 μg of total RNA with SMARTer RACE cDNA Amplification Kit (Clontech, USA). Specific outer and inner primers (Table 1) for race nest-PCR were designed based on the partial conserved sequence of tLEP and tLEPR. In the first PCR, full-length cDNA was amplified with the outer primer and UPM (Universal Primer A Mix, provided by the Clontech kit), of which the proper Tm is 65–66 °C. In the second PCR, the inner primer and NUP (Nested Universal Primer A, provided by the Clontech kit) with T_m at 61–62°C were used. Other PCR reaction mixture components and parameters were same with

descriptions above except that extension time was changed to 1.5 min. All the race PCR products were sequenced in the same way.

Molecular characterization and phylogenic analysis of tLEP and tLEPR

The open reading frame (ORF) and AA sequences of the full-length cDNA sequence of tLEP and tLEPR were predicted by DNAMAN software. DNA sequences and deduced protein sequences were analyzed with the BLAST program on the National Center for Biotechnology Information (NCBI) Web site (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Tertiary protein structures were estimated by the ProModII program at the SWISS-MODEL automated protein modeling server (http://swissmodel.expasy.org/). Putative signal peptide was predicted using Signal P 4.1 (http://www.cbs.dtu.dk/services/SignalP/). Putative transmembrane domain was predicted with the program TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Multiple sequence alignments of AAs were performed with BioEdit Sequence Alignment Editor. Protein phylogenetic tree based on the AA sequences was constructed using the neighbor-joining methods of the program MEGA 4.0.

Tissue distribution and expression of tLEP and tLEPR in turbot

Tissue distribution of tLEP and tLEPR mRNA was detected in brain, eyes, gill, heart, liver, spleen, kidney, stomach, intestine and muscle, by semiquantitative RT-PCR. Specific primers for tLEP (RT-LEP F/R), tLEPR (RT-LEPR F/R) and β-actin (reference gene, GenBank Accession No. AY008305.1) are shown in Table 1. The isolation of total RNA and synthesis of first-strand cDNA were carried out as described in 2.1. 58 °C-T_m and 30-s extension time were used in this PCR procedure.

Effects of ratios of dietary carbohydrate–lipid on tLEP and tLEPR gene expression in juvenile turbot after a 9-week feeding trial

Experimental diets and feeding

The basal diet was formulated to contain 50.22 % crude protein and 12.36 % crude lipid. Dextrin was used as the dietary carbohydrate source, and fish oil was used as the dietary lipid source. There were four experimental diets with different ratios of carbohydrate–lipid. They were as follows: 1:6, 1:2, 2:1 and 14:1. The four experimental diets were named as Diet-1 (control), Diet-2, Diet-3 and Diet-4, respectively (Table 2).

Juvenile turbots (body weight: 8.06 ± 0.08 g) were used for the feeding trial. Prior to the start of feeding trial, fish were fed a commercial diet (Great Seven Bio-Tech, Qingdao, China) for 2 weeks to acclimate to the experimental conditions. Following a 24-h fasting, fish in similar size were randomly assigned to four groups for the four diets with three replicates per group. Each tank (500 l) was stocked with 30 fish. The feeding trial was conducted in an indoor circulating seawater system for 9 weeks. Fish were hand-fed to apparent satiation twice daily at 07:00 and 18:00. During the rearing period, water temperature was control at 19 ± 1 °C, pH at 7.7 ± 0.1, salinity at 25.2 ± 1.0 ‰ and dissolved oxygen was ≥7.0 mg/l.

Sample collection and real-time quantitative RCR analysis

Before sampling, fish was fasting for 24 h. Three fishes per tank were randomly selected for liver collection. After total RNA extraction and first-strand cDNA synthesis, real-time quantitative PCR (Q-PCR) was performed on Mastercycler ep realplex system (Eppendorf, Germany) using SYBR Green I (CW biotech, China) according to the manufacturer's instructions. The total reaction volume was 25 μl, including 150 ng cDNA template, 12.5 μl 2× Ultra SYBR Mixture, 1 μl each gene-specific primer (10 μM, Table 1) and 7.5 μl DEPC water. The PCR cycling parameters were 95 °C hold for 10 min, then 40 cycles at 95 °C for 10 s, 58 °C for 10 s, 72 °C for 20 s. All real-time Q-PCR was performed in triplicate biological replicates.

Melt curve analysis conducted over a range from 58 to 95 °C verified that primers generated one single product and no primer-dimer artifacts. The specificity of single target amplification of tLEP and tLEPR was confirmed by separating the Q-PCR products in 1.2 % of agarose gel electrophoresis followed by cloning and sequencing of the purified fragments. β-Actin was amplified as reference gene, which we have shown not to change with

Table 2 Ingredients and proximate compositions of the experimental diets (%)

Ingredients	Diet-1	Diet-2	Diet-3	Diet-4
White fish meal	36.00	36.00	36.00	36.00
Casein	21.20	21.20	21.20	21.20
Gelatin	5.30	5.30	5.30	5.30
Sodium alginate	1.00	1.00	1.00	1.00
Dextrin	0.00	5.00	15.00	28.00
Microcrystalline cellulose	20.50	17.50	11.50	3.50
Soybean lecithin	2.00	2.00	2.00	2.00
Fish oil	11.00	9.00	5.00	0.00
Attractant[a]	0.60	0.60	0.60	0.60
Vitamin premix[b]	0.80	0.80	0.80	0.80
Mineral premix[c]	1.50	1.50	1.50	1.50
Ethoxyquine	0.10	0.10	0.10	0.10
Analyzed nutrients compositions (dry matter basis)				
Carbohydrate (%)	1.91	5.78	16.27	28.14
Crude protein (%)	50.22	49.47	50.43	50.08
Crude lipid (%)	12.36	11.78	7.35	2.39
Gross energy (kJ/g)	20.37	20.64	20.00	19.02

[a] Attractant composition: taurine: glycine: betaine = 1:3:3

[b] Vitamin premix (mg/kg diet): vitamin A, 32 mg; vitamin B1, 25 mg; vitamin B2, 45 mg; vitamin B6, 20 mg; vitamin B12, 10 mg; vitamin C, 2000 mg; vitamin D, 5 mg; vitamin E, 240 mg; vitamin K, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 200 mg; biotin, 60 mg; folic acid, 20 mg; inositol, 800 mg; microcrystalline cellulose, 4292.54 mg

[c] Mineral premix (mg/kg diet): $CuSO_4 \cdot 5H_2O$, 10 mg; Na_2SeO_3, 20 mg; $MnSO_4 \cdot H_2O$, 45 mg; $CoCl_2 \cdot 6H_2O$ (1 %), 50 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $Ca(IO_3)_2$, 60 mg; $FeSO_4 \cdot H_2O$, 80 mg; $MgSO_4 \cdot 7H_2O$, 1200 mg; zeolite powder, 18,485 mg

Table 3 Expression of reference gene β-actin in liver of turbot fed with different diets

Diets	Relative expression of β-actin
Diet-1	1.03 ± 0.31
Diet-2	1.00 ± 0.20
Diet-3	1.06 ± 0.11
Diet-4	1.05 ± 0.17

Values are means with standard errors and were analyzed by one-way ANOVA followed by Tukey's multiple range test. No expression changes of β-actin were observed in the liver of turbot feed on different diets

treatments (Table 3) (Yun et al. 2012). The relative expression of tLEP and tLEPR was calculated using "$2^{-\Delta\Delta C_t}$" method, and the value stood for *n*-fold difference relative to the calibrator (Livak and Schmittgen 2001).

Statistical analysis

Statistical analysis was performed using SPSS 17.0. A one-way analysis of variance (ANOVA) was used to compare the differences in relative tLEP and tLEPR gene expression among treatments. When overall differences were significant at <5 % level, Tukey's test was used to compare the mean values between individual treatments.

Results

Characterization of tLEP and tLEPR cDNA

The full-length cDNA sequence of tLEP (GenBank Accession No. KP197049) was 1126 bp, containing a 73-bp 5′-untranslated region (5′-UTR), a 672-bp 3′-untranslated region (3′-UTR) and a 471-bp open reading frame (ORF) coding for a 157-AA protein. The predicted peptide had signal sequence of 20 AAs, and two cysteine residues for a disulfide bond conserved in vertebrate LEPs (Fig. 1a).

Multiple sequence alignment was performed based on the AA sequences of the vertebrate LEPs (Fig. 2a). The mature tLEP showed very low identity to mammalian LEPs, 18.8 % identity to human. Additionally, the mature tLEP peptide showed 80.1 and 76.6 % identity to grouper LEP-A and yellow croaker LEP, respectively. Thus in the phylogenetic analysis of AA sequences, tLEP, grouper LEP-A and yellow croaker LEP formed an independent cluster, revealing that tLEP was more evolutionarily related to Acanthopterygii, but less related to Salmoniformes and Cypriniformes (Fig. 3a).

The estimated three-dimensional (3D) structure modeling of tLEP showed strong conservation of tertiary structure with human LEP. Both turbot and human LEP had the four-helix bundle topology (Fig. 4).

The tLEPR (GenBank Accession No. **KP197050**) was 4248 bp containing a 257-bp 5′-UTR, a 732-bp 3′-UTR and a 3411-bp ORF coding for an 1173-AA protein. The deduced tLEPR protein possessed a 27-AA signal peptide, an 808-AA extracellular segment, a 23-AA single transmembrane domain and a


A
1 ACACAAGCCAGCACCAGCCGAGGAATAGCAGTGTTATCTTTGAATTCTACCCATCTCCAGAAACACCACAACTATGGATTACACTCTGGT
M D Y T L V
Signal peptide
91 CCTCCTGTTTTCTCTGCTGCAAGTTTTAAGTGTGTGCACAGCCGCTCCTCTGCCGGTGGAAGTGGTGAAGATGAAATCCAAAGTGAAATG
L L F S L L Q V L S V C T A A P L P V E V V K M K S K V K W
181 GATGGCTGAACAGCTTATTATCAGGATGGAAGATTTCCAGTTCCCTTCTAGCCTGACACCTACTGATGACCTGGACGGAGCTTCCTCCAT
M A E Q L I I R M E D F Q F P S S L T P T D D L D G A S S I
271 AGTGACCGTCTTGGAGGGTTACAACAGCCTGATCTCCGACACCTTTGATGGCGTCTCCCAGGTCAAGTCCGAAATCTCTTCGTTGACGGG
V T V L E G Y N S L I S D T F D G V S Q V K S E I S S L T G
361 TTACGTCGATCAGTGGAGGCGGGGGCACTGCAGCGAGCAGCGGCCAAAGCCCTCGGTGCCGGGGCCGCTGCAAAATCTACTGAGTCGAAA
Y V D Q W R R G H C S E Q R P K P S V P G P L Q N L L S R K
451 GGAGTTCGTTCACACGGTGACCATCGAAGCCCTCATGAGGGTGAAAGAGCTGCTCAATCTTCTCCTGAAAAACCTGGATCATCTGGAGAG
E F V H T V T I E A L M R V K E L L N L LL K N L D H L E S
541 TTGCTAACAGACGGACATGAGGACCAATATTTATCAGCTGTTGGCCTGGTTTTTGGACAAGAGATCATCAGTGATGGGCAACTTTTCATT
C*
631 TCCTCCTGGCCATGTTCAACTTTTGCAATGTATTTTGAAGTAAGTTCATTCTTGAACGTGCACTTATTTATATACATATGTATTTATTTA
721 TCTACTTTATTAGAAAAAAATTGTATTTCGCAAAAAACATTGTCTTTTCATCAGCATGTCTGCAGCAAAGGGTGTTGTTGCCACAGGAAT
811 GTTTTTTATACAAATTAATCTACGGCACGTTTTGCACACAAAGACACTTCAGCTGCTCGTAGGTTTGTATCATGTGACAACTGTTGTGGA
901 AACAAAACTTGAAAATATGAATCTACTCATTAATTCAGTCCTGAATCATCAGTGTGAGAGGGCATCACGTTGAGCTGTGATGTCAGCGAT
991 GGAGGAAACATGCCAAGAAATTGTTTGTATGAGAACTAGGACAGGAAGGGTAAAGGGCAACTTTTATCTCAATGCAATTTCATCTAAATT
1081 GTTATTTATATCATAGATATTTAGAATATTTTTGTACAGTTTAAGCTTTATTACTTTGTATTTTTTATAACTTGAATTTTAAAAATTAAT
1171 AAATATTTTACAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
B
1 AACAGCGGCAGTGTGCGTGTGAGTGACAGAGGAATTCGTCATCGGGCTCCTCGTGTCGTTGGCCCCTGCTCTCGGCCGTGGACAGCCGAC
91 TCCGACAGAGACGCGCAGACCCTCAGTGGGGATTGGAACGGGCAGACTCTGACTTTGGGGGCAACACGGCAACAAAAAGAGGAAGCCCGG
181 ACACCGAAAGCAGAGGAGACCTCCGGCAGCGACTGAGATTCACACCAGCCAAAACCCAGCCAGGCCATGCTCACACTATGACCACTACAA
M T T T
271 TGTCTCGGTCTGTGATGCTGACAGTCCTGATGCATAATATCCTCCTGGTGTCCCATGGTGTTTGGTGTTCGGAGCCGGAGGACGGAGCGT
M S R S V M L T V L M H N I L L V S H G V W C S E P E D G A
Signal peptide
361 CTCTCCAGGCTGGTGCCCTGGTTCTCCCCTGGCAGGACGAGCTGTGCTGTGACTCACGGACAGCTCCCTTTAATGAGGCGGGCGGCGACA
S L Q A G A L V L P W Q D E L C C D S R T A P F N E A G G D
451 CGGACGCACCGGAGACAAACCGCTCTGAATCAAAGCTCCCACACTCTCCCCGCTGCCGCTTCAGGAGCTTGACAACCGGGACACGTCCTC
T D A P E T N R S E S K L P H S P R C R F R S L T T G T R P
541 GTGAGCTCTCTGGCCACAAGAGTTCATTCAACAATGGCACCTGTTTGGACATACTGTGCAGAATCGATGAAAACTGGGAAAAATTGACTT
R E L S G H K S S F N N G T C L D I L C R I D E N W E K L T
631 GCGATCTGACGTCTGGCGGCCGAACCTCAACGGCTCTGGACGCTGGTCTCATGGCGGTCAGCTTACGGCGTGTGTTGTCCCAGAAAGAGG
C D L T S G G R T S T A L D A G L M A V S L R R V L S Q K E
721 ATTCACTTGTGAACAGCGGGAATACTTCACCTGATAAACCGGTCGCCTGTGAAGCGAAGGATTCCTTTGTTTGCTCCATCGCTCTTGACA
D S L V N S G N T S P D K P V A C E A K D S F V C S I A L D
811 CCACGACAAGCTTTATCGCCGTGGTAACCGTCAGCGTCTCCGACGCCGAAGCTCTGCCGGTTCAGCTCAGAATTCCTGCCAGACCTGTGA
T TT S F I A V V T V S V S D A E A L P V Q L R I P A R P V
901 AACCGAGTCCTCCGGTCAACCTCTCTCACATTCAGACCACGGAGGCAGAACTGATCCTTCTCTGGGACGGCCCGACAGACTTTGACACCG
K P S P P V N L S H I Q T T E A E L I L L W D G P T D F D T
Fibronectin type 3 domain
991 GCCCGCTGAGATACGAGGTCCGATACTCCTTCAACACCACTCGTCCAGCCTGGCAGGTGATGTCTGCACCTGCAGGGCCCCGACTGCCTC
G P L R Y E V R Y S F N T T R P A W Q V M S A P A G P R L P
1081 TAGACCTGAAACCCAGGCTGAACTACACCATCCAGGTCCGCTGCTCCGGCCCGGGTGAACCTCCGCTGTGGAGCGACTGGAGTGAAAGTC
L D L K P R L N Y T I Q V R C S G P G E P P L W S D W S E S
WSXWS motif
1171 ACCACATCTACCTAGACACCGTGAGCTACATCCCCGAGAAGGTAGTGGCACGACCGGGGGAAAACGTCACGGTCTATTGTGTGTTCAACG
H H I Y L D T V S Y I P E K V V A R P G E N V T V Y C V F N

◀**Fig. 1** Turbot leptin and its receptor nucleotide sequences and predicted amino acid sequence. **a** Turbot leptin (Genbank Accession No. **KP197049**). Initiation codon (ATG) and termination codon (TAA) are shown in *shadow*. The signal peptide is shown using *bold letters*. The cysteine residues to form disulfide linkage are *circled*. **b** Turbot leptin receptor (Genbank Accession No. **KP197050**). Initiation codon (ATG) and termination codon (TGA) are shown in *shadow*. The signal peptide is shown using *bold letters*. *Underlined* amino acid sequences denote conserved domains (fibronectin type 3 domain and immunoglobulin-like C2-type domain). *Shaded areas* in the amino acid sequences represent leptin-binding domain. *Double underlined* amino acid sequences show the transmembrane domain. The conserved motifs of turbot leptin receptor (WSXWS repeated tryptophan/serine motifs, JAK2-binding motif boxes and STAT-binding motif box) are *boxed*

342-AA intracellular segment. The extracellular domain contained seven pairs of cysteine residues available for potential formation of an intramolecular disulfide bond (Fig. 1b).

All functionally important domains conserved among vertebrate LEPRs were found in tLEPR, including three fibronectin type 3 domains, an immunoglobulin C2-like domain, a pair of repeated tryptophan/serine motifs (WSXWS) at extracellular segment, two JAK2-binding motif boxes and a STAT-binding domain at intracellular segment (Fig. 1b). The leptin-binding domain (LBD) of tLEPR was estimated to be from the 394th to 603rd AA residue, which shared identities with that of human (29 %), zebrafish (35 %), grouper (86 %) and pufferfish (71 %), respectively (Fig. 2b).

The predicted full-length AA sequence of tLEPR showed 60 % and 82 % identities with puffer fish and grouper. In contrast, the identities of tLEPR with LEPR of human (28 %) and chicken (47 %) were lower than that of fish LEPR. Phylogenetic tree analysis revealed that the deduced tLEPR formed cluster with grouper, tilapia and puffer fish belonging to Acanthopterygii, but separated from mammals and other fish species (Fig. 3b).

Tissue distribution of tLEP and tLEPR

The tissue expression of tLEP and tLEPR was analyzed with semiquantitative RT-PCR, using β-actin as reference gene (Fig. 5). The expression of tLEP was relatively high in the eyes, brain and liver, but little in the muscle. The tLEPR showed a highest expression level in the liver and kidney. Other tissues with high expression of tLEPR were gill and stomach.

Effects of dietary carbohydrate-to-lipid ratios on tLEP and tLEPR gene expression

As liver considered as the important site of LEP and LEPR expression and energy metabolic center, we investigated hepatic tLEP and tLEPR expression response to ratios of dietary carbohydrate–lipid (C/L). The increasing in ratios of C/L from 1:6 to 2:1 did not result in significant differences in tLEP expression in liver ($P > 0.05$). However, the hepatic tLEP expression was significantly elevated in treatment with dietary C/L ratio in 14:1 ($P < 0.05$) (Fig. 6a). The expression of tLEPR was also not significantly affected by the increasing ratios from 1:6 to 2:1 ($P > 0.05$). The hepatic tLEPR mRNA level in the group with 14:1 ratio of dietary C/L was significantly lower than that in the group with ratio in 1:6 ($P < 0.05$). However, it did not significantly differ from those in the other two treatments ($P > 0.05$) (Fig. 6b).

Discussion

Structure and phylogeny of tLEP and tLEPR

There were two main types of LEP genes with low interspecies AA identity found in several species of fish (Gorissen et al. 2009; Kurokawa and Murashita 2009; Zhang et al. 2013). However, the present study only identified one full-length sequence of LEP from turbot, because the degenerate primers for cloning tLEP were based upon the available LEP-A type gene. Phylogenetic analysis showed that tLEP belonged to LEP-A type of Acanthopterygii. As members of Acanthopterygii, grouper and medaka had confirmed to have the B type of LEP gene (Kurokawa and Murashita 2009; Zhang et al. 2013). So that it is possible that turbot may have LEP-B type gene we have not found. Further study is still needed, which is important to explore the diverse function of LEP in fish.

Similar to other reported fish LEPs, the cDNA sequence of tLEP showed low identity with LEPs of mammals including human. However, the two characteristic cysteine residues were really conserved between tLEP and other LEPs identified (Figs. 1a,

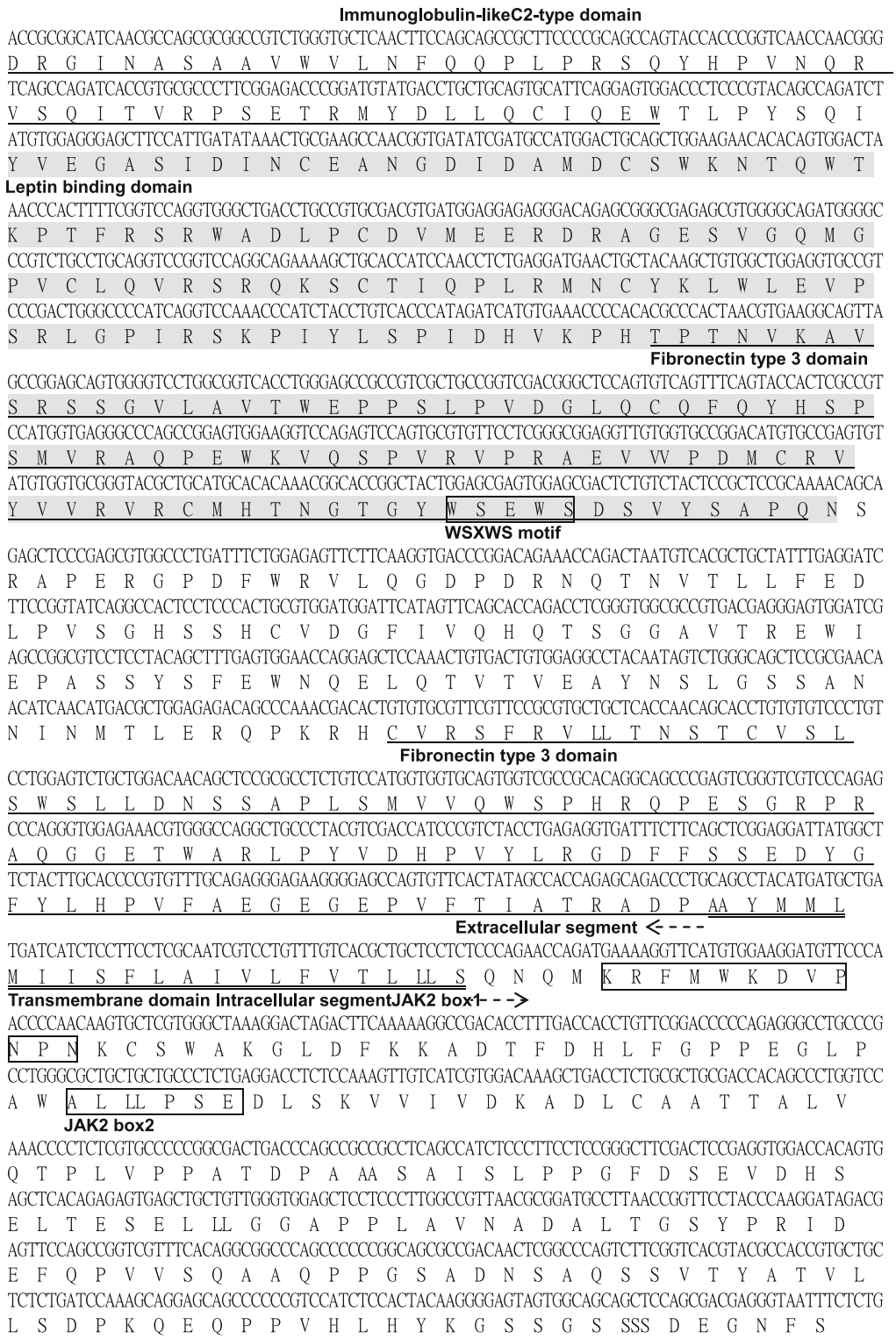

Fig. 1 continued

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3331  CCAACAACTCGGACATTTCAGGATCTTTCCCCGGCGGCCTGTGGGAGCTGGACAGCTGCCGGGGTGGGGAGACGGACGACCCGCGGCGCT
      A  N  N  S  D  I  S  G  S  F  P  G  G  L  W  E  L  D  S  C  R  G  G  E  T  D  D  P  R  R
3421  CCTGCTCCTACAACTCGGTGGAGGAGCTTTACGAAACGTCGGAGCAAGAGGACGACGGGGAGGCGAGGGACGAGAAGGACTTGTATTACC
      S  C  S  Y  N  S  V  E  E  L  Y  E  T  S  E  Q  E  D  D  G  E  A  R  D  E  K  D  L  Y  Y
3511  TAGGAATGGACTATCCGGCAGAGGATGAGGCGAGTGAGGACGAGGACGAGAGGCGAGAGGAGGCGGCGAAAATTGACCTGCGTAAAAACG
      L  G  M  D  Y  P  A  E  D  E  A  S  E  D  E  D  E  R  R  E  E  A  A  K  I  D  L  R  K  N
3601  TAGTTTTGACCAGAGACGACTGCTCCGTGGAGTCGCGCCCTCTGCTCTGCCCCGGGGACTCCAGCAGTCGCACAGGGCCGCTGTCGGCGT
      V  V  L  T  R  D  D  C  S  V  E  S  R  P  L  LC  P  G  D  S  SS  R  T  G  P  L  S  A
3691  CGACGCGGGGCTTTACCCCACTGTACCTGCCTCAGTTCAGAACTGCCCCGTGCACGAGGCAACTGCAGGACAGTGAACCCGAGCTGTGAC
      S  T  R  G  F  T  P  L  Y  L  P  Q  F  R  T  A  P  C  T  R  Q  L  Q  D  S  E  P  E  L  *
                              STAT box
3781  GGCCTACTATTTAAATGGAAATGTGCCCCATTAAAACCATTGTGTCGCAAACTGATTCAGCATTTGCCAAAGTGGATCTGCAGCTCCTCC
3871  TTCAGACACAGTGAAGTGCGGAGCTGGACACTTAGAAAAAACAACTTGACTGATGTACGATTGCTATTTGATGTTGGTCAACGCTGCTAT
3961  TGTGTTCCGTTGGAAAAAGACATCTCAAGGGGCACAAGCCTCGATCACAGGCATTTCTTTCTTTCTTTTCTTTATGGGGCTTTTGCTCTG
4051  ATTCTACTGTGCCACCCTGCTCCTCTGCTGTTGTGCAGTAGCGGTCATTGATGTAGGAGTATCGCAGAGGAAAATGAGCTCGCAGATCTT
4141  GTCCATGGGAGGTTAGGCTGGTGTAAAGTACAGTTAATCATTAAAGAGAGCAGCAGACAGCCTGTTCAAGCCTGTTCATTTGGCATCATA
4231  CATGATATGTAGGTAATGACATGTGTGCCTAGGTTCACTCTTCTGCTCTTTGTGTGGAAAAGGTGTTCATTTTCTGCCGAACTATTCCGG
4321  CTGCAACTTGACGGCAACGTGAAACGGGTTCAAAGAGAATGAGTAAAACGCACAGAACGAGAGAGAGAGAGAGAGAAAAAGCTCATAATC
4411  TGCACGGCCTTGTTTTTTTTCAAAGCACATTTGACTGTGAAGTGACAAAAAAGAAATATGATGAAACGTAATTATTTTTTTGGGCCGCTG
4501  CATTCATTGGCAACATCTGCACGGACTAACTCACCCACAAAGCCTAAGAATGTATTTTTGTACTTTGTGAAGTAACGTATTTGAGAATTT
4591  GTAACAGTATTTGAGAAATGTGATCTATTTTTTTTCCTGTGTTTGACCGTTGACATTGACACTTTCTTTGTGGTGTAAAGTTAAAGACAA
4681  AGTACTATATTGTATATTTCAAACAACAGTGGGACGTTCGGACGCCGTTGACAAGTTGTTAGTGCACATGAAGATCTTTTGTCTATTTTT
4771  GATGGTGAGATAAAATAAAAGTATTTATTGCAAAAAAAAAAAAAAAAAAAAAAAAAA
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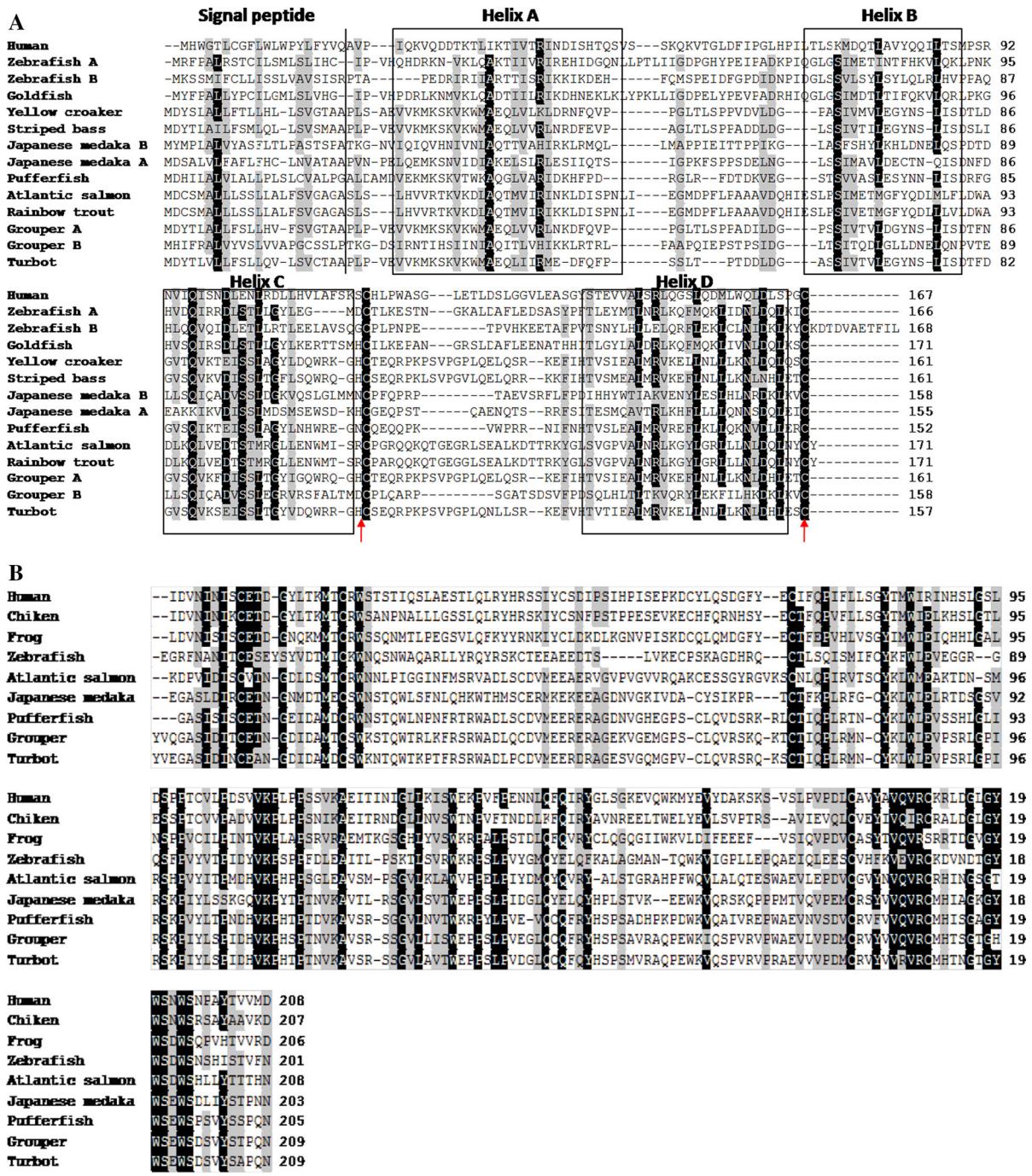
Fig. 1 continued

2a). Rock et al. (1996) had proved that the two cysteine residues could form a disulfide bond in LEP, which was prerequisite for the 3D configuration and bioactivity of human LEP. Therefore, the deduced 3D structure modeling of tLEP predicted strong conservation of tertiary structure with that of human and other vertebrates (Fig. 4) (Huising et al. 2006; Kurokawa et al. 2008; Murashita et al. 2008). Based on this, Kling et al. (2009) quantified plasma tLEP level using salmonid LEP radioimmunoassay. Crespi and Denver (2006) proved that LEP of frog with low identity of AA sequence and high conservation of tertiary structure to mammalian LEP could activate both mouse and frog LEPR in vitro. Meanwhile, they also found that both frog and human LEPs exhibited similar potencies on mouse and frog LEPRs in transfection assays, and both had similar anorectic potencies in juvenile frog (Crespi and Denver 2006). These results suggest that the conserved 3D structure is more important to keep the LEP–LEPR-binding affinity and LEP activity, and it could be constrained by the structure of the receptor-binding pocket (Crespi and Denver 2006). It could explain why mammalian LEP can have effect on fish in some studies, such as increasing gonadotropins release in pituitary cells from rainbow trout throughout gonad maturation (Weil et al. 2003) and decreasing food intake of goldfish (De Pedro et al. 2006) and striped bass (Won et al. 2012).

To date, six splice variants of LEPR have been identified in mammals, of which the long isoform is the only one with clearly demonstrated signaling capability (Zabeau et al. 2003). Nevertheless, duplicates are uncommon in fish. As was reported, five types, three types and two types of LEPR were cloned only in Atlantic salmon, crucian carp and rainbow trout, respectively (Cao et al. 2011; Ronnestad et al. 2010; Gong and Björnsson 2014). Except that, there is only long-form LEPR found in other fish species, such as pufferfish (Kurokawa et al. 2008), Japanese medaka (Kurokawa and Murashita 2009), yellow catfish (Gong et al. 2013a, b) and grouper (Zhang et al. 2013). In turbot, we also found only one copy of the LEPR gene. Phylogenetic analysis showed tLEPR formed cluster with fish long-form LEPR genes belonging to Acanthopterygii LEPR (Fig. 3b). Although the AA sequence of tLEPR shared low identity with that of mammals, tLEPR had all functionally important domains, such as fibronectin type 3 domains,



immunoglobulin C2-like domain, WSXWS motifs, JAK2 boxes and STAT box, coincident with other reported fish LEPRs (Fig. 1b). It is demonstrated that mammalian LEPR plays an important role in maintaining body weight and energy homeostasis via JAK/STAT pathway. The conserved functional domains

◀**Fig. 2** Comparison of amino acid sequences of leptins and the putative leptin-binding region of leptin receptors. **a** Leptins. The signal peptides are indicated by *vertical bar*. The four α-helices, inferred from human leptin, are *boxed*. Conserved cysteine residues are shown by up *arrows*. GenBank Accession Nos.: human (**AAH69452.1**); zebrafish A (**NP_001122048.1**); zebrafish B (**NP_001025357.2**); goldfish (**ACL68083.1**); yellow croaker (**AGR51148.1**); striped bass (**AFD34357.1**); Japanese medaka A(**NP_001098190.2**); Japanese medaka B (**NP_001153914.1**); pufferfish (**BAD94444.1**); Atlantic salmon (**ACZ02412.1**); rainbow trout (**CAR67819.1**); grouper A (**AFU55260.1**); grouper B (**AFU55261.1**). **b** Leptin receptor leptin-binding domains. GenBank Accession Nos.: human (**AAB09673.1**); chicken (**BAA94292.1**); frog (**ABD63000.2**); zebrafish (**NP_001106847.1**); Atlantic salmon (**BAI23197.1**); Japanese medaka(**BAH24203.2**); pufferfish (**BAG67079.1**); grouper (**AFU55262.1**). Deletions are indicated by *dashes* and *shaded areas* indicate residues shared by 75 % of all sequences

analysis indicates tLEPR possesses JAK and STAT-binding boxes and may be able to active JAK/STAT signaling pathway.

Tissue expression of tLEP and tLEPR

The tLEP was predominately expressed in brain, eyes and liver, tissues associated with lipid storage. Limit tLEP was detected in other tissues, especially in muscle with low fat content (approximately 1.25 %). Hypothalamus and hindbrain are the glucose and fatty acid sensing and food intake control area in fish (Polakof et al. 2007; Soengas 2014). In rainbow trout, intraperitoneal injection of LEP transiently reduced NPY expression and elevated POMCs expression in hypothalamus (Murashita et al. 2008), and intracerebroventricular injection of LEP could induce changes related to glucosensing in hypothalamus and hindbrain (Aguilar et al. 2010). The high expression of tLEP in brain indicates its potential important function in metabolic and appetitive regulation. Adipose tissue is the main production site of LEP in mammals, but most fish LEP hardly expressed in it. Most fish LEP mRNA is expressed in the liver, such as Japanese medaka (Kurokawa and Murashita 2009), Atlantic salmon (Ronnestad et al. 2010), grouper (Zhang et al. 2013) and yellow catfish (Gong et al. 2013a, b). In particular, the whole body composition assessment shows turbot has low lipid content, about 3–4 % on a wet weight. These results support the hypothesis that liver, rather than adipose tissue, is the energy metabolic center in fish, including turbot.

The relative ubiquitous expression of LEPR in turbot tissues made it plausible that tLEP was involved in physiological activities in various tissues similar to that in mammals. LEP could cooperate with stress hormones to influence energy metabolism, and the best characterized stress hormones are the catecholamines of the sympathetic nervous system and the terminal glucocorticoids of the hypothalamic–pituitary–adrenal axis (Copeland et al. 2011). Moreover, LEP signaling responds to hypoxia exposure in both mammals and fish. It has been reported that hypoxia triggers LEPR expression in gill, liver and heart of marine medaka (Wong et al. 2007). Further studies are required to explore the tLEP's role in these functions occurring in peripheral tissues.

Effects of dietary carbohydrate-to-lipid ratios

The present findings show some relevance between dietary carbohydrate and liver tLEP expression in turbot. According to our data unpublished, plasma glucose at 24 h after feeding in treatment with 14:1 ratio of dietary C/L increased about twofold than that in control group. The persistent hyperglycemia may result in the high expression of tLEP in liver. Similar effects of hyperglycemia on increase in LEP mRNA levels are also reported in skeletal muscle and adipose tissue of rats (Wang et al. 1998). LEP expression increases several days after insulin infusion in human and may be predictive of insulin resistance (Kolaczynski et al. 1996b). Consistently, insulin stimulates LEP expression in isolated adipocytes of mice (Rentsch and Chiesi 1996). In the present study, insulin level in treatment with 14:1 ratio of dietary C/L was the highest one (unpublished data). It implied the possible stimulation of insulin to tLEP expression in turbot liver. Unlike the tLEP expression, hepatic tLEPR expression decreased with the increasing dietary carbohydrate levels. It is speculated that the low expression of tLEPR could be a negative feedback mediation of organism to the excessive tLEP expression.

LEP has been shown to remarkably suppress hepatic glycogenolysis in perfused rat liver (Nemecz

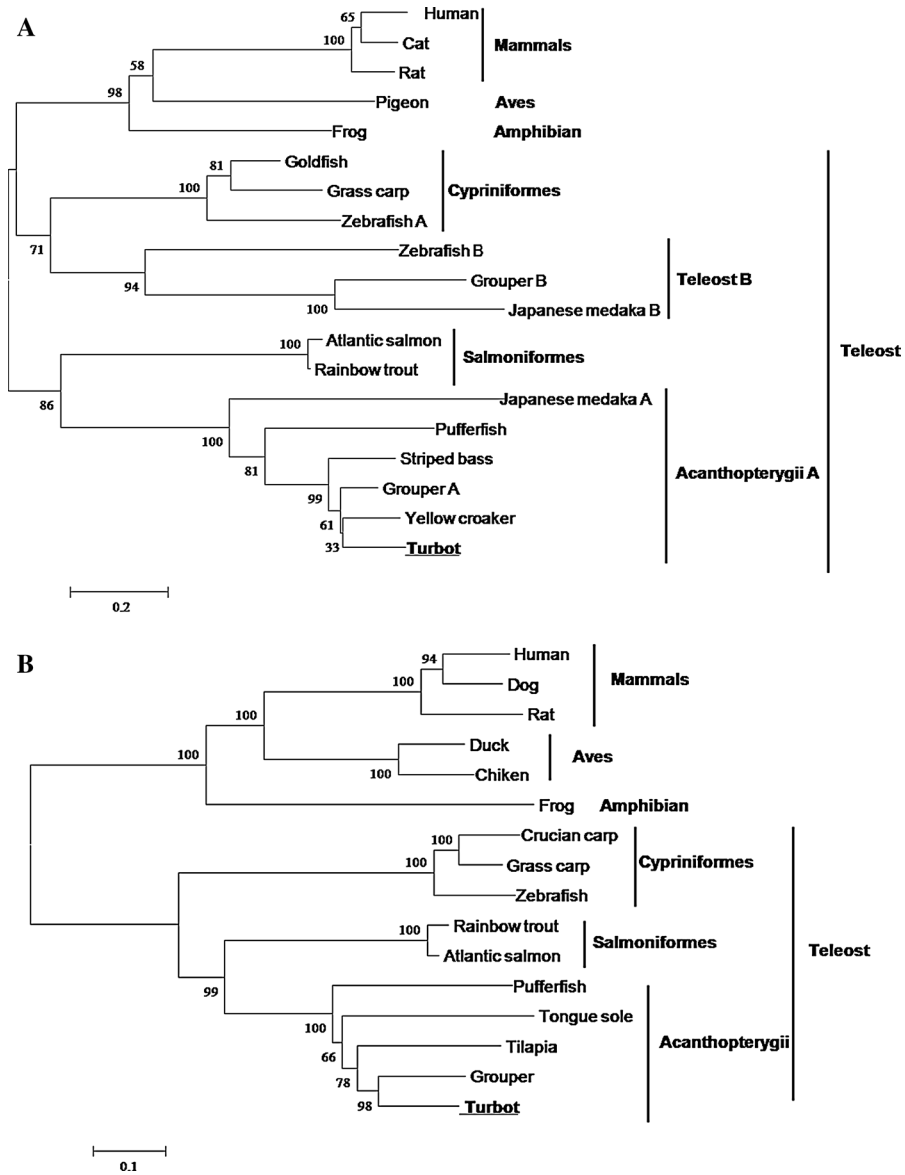


Fig. 3 Phylogenetic analysis of leptins and leptin receptors. *Numbers at nodes* indicate the bootstrap value (%), obtained for 1000 replicates. **a** Phylogenetic relationship of leptins. GenBank Accession Nos.: human (**AAH69452.1**); cat (**NP_001009850.1**); rat (**NP_037208.1**); pigeon (**CDL67225.1**); frog (**NP_001089183.1**); zebrafish A (**NP_001122048.1**); zebrafish B (**NP_001025357.2**); goldfish (**ACL68083.1**); grass carp (**ACI32423.1**); yellow croaker (**AGR51148.1**); striped bass (**AFD34357.1**); Japanese medaka A(**NP_001098190.2**); Japanese medaka B (**NP_001153914.1**); pufferfish (**BAD94444.1**); Atlantic salmon (**ACZ02412.1**); Rainbow trout (**CAR67819.1**); grouper A (**AFU55260.1**); grouper B (**AFU55261.1**). **b** Phylogenetic relationship of leptin receptors. GenBank Accession Nos.: human (**AAB09673.1**); dog (**NP_001019805.1**); rat (**NP_036728.1**); chicken (**BAA94292.1**); frog (**ABD63000.2**); zebrafish (**NP_001106847.1**); crucian carp (**ADZ75460.1**); grass carp (**AFU35431.1**); Atlantic salmon (**BAI23197.1**); pufferfish (**BAG67079.1**); tongue sole (**XP_008331553.1**); tilapia (**AGT28753.1**); grouper (**AFU55262.1**)

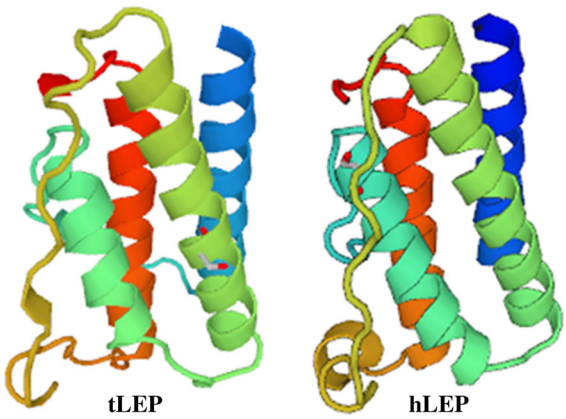


Fig. 4 Tertiary structure of turbot leptin (tLEP) and human leptin (hLEP). Tertiary protein structures were modeled using the ProMod II program at the SWISS-Model automated protein modeling server, based upon human leptin (1AX8.pdb) Protein Data Bank structure file

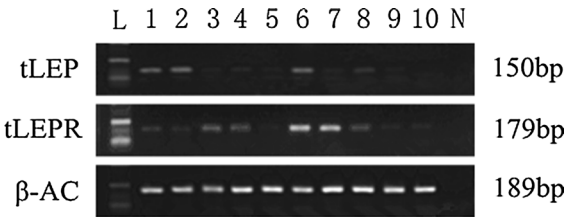


Fig. 5 Tissue distribution of turbot leptin (tLEP) and its receptor (tLEPR) expression. *L* ladder, *1* eyes, *2* brain, *3* gill, *4* stomach, *5* intestine, *6* liver, *7* kidney, *8* spleen, *9* pyloric caeca, *10* muscle, *N* negative control. Expression of housekeeping gene β-actin (β-AC) was observed to ensure the integrity of the cDNA template of each tissue sample

et al. 1999). Meanwhile, it increased gene expression of the gluconeogenesis enzyme phosphoenol pyruvate carboxykinase (PEPCK) at the end of 6-h infusion to rat (Rossetti et al. 1997), whereas intraperitoneal injection of tilapia recombinant LEP induced hepatic glycogenolysis in tilapia (Baltzegar et al. 2014). In present study, while the liver glycogen content in tLEP mRNA high-level group (Diet-4) was much more than other groups, the expression of hepatic PEPCK was much lower than the control group (Nie et al. 2013). These results seem to confirm the impeditive function on glycogenolysis of LEP in rat, but resist its positive effect on PEPCK expression reported in rodents and human. It is also conversed with the results investigated in tilapia. One possible reason could be the contrary low expression of tLEPR leading to "LEP resistance" in turbot. Under such circumstance, the inhibition effect of insulin could be more effective than the stimulation effect of tLEP on PEPCK gene expression.

In conclusion, the full-length cDNA sequences of LEP and LEPR were cloned from the liver of turbot. Meanwhile, phylogenetic analysis and tissue distribution of the two genes were characterized. Furthermore, the response of tLEP and tLEPR expression to ratios of dietary carbohydrate–lipid suggested some relevance between dietary carbohydrate and liver tLEP expression in turbot. However, as the lack of the protein of tLEP and tLEPR, the specific function and effect of tLEP on glucose metabolism and insulin regulation need further study.

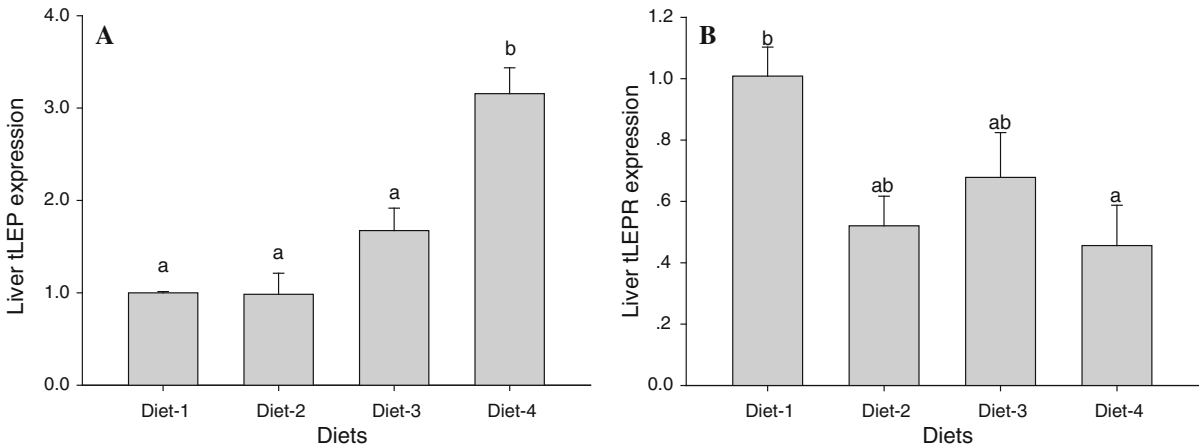


Fig. 6 Effects of dietary carbohydrate-to-lipid ratios on turbot leptin (tLEP) and its receptor (tLEPR) expression in liver. The mRNA levels were normalized with β-actin. *Each bar* represents the mean ± SEM of three samples. Significant differences among the diets are indicated by *different letters* ($P < 0.05$)

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