Molecular cloning, functional characterization and nutritional regulation of two *elovl4b* elongases from rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Elongation of very long-chain fatty acids 4 (Elovl4) is a critical enzyme in the biosynthesis of long-chain and very long-chain polyunsaturated fatty acids (LC- and VLC-PUFAs) in animals. So far, very little is known about the molecular function of Elovl4 in biosynthetic process, especially in fish. In this study, two *elovl4* paralogs (*elovl4b1* and *elovl4b2*) were successfully cloned from rainbow trout (*Oncorhynchus mykiss*). The full-length cDNAs of *elovl4b1* and *elovl4b2* were shown to comprise 1896bp and 2227bp, respectively, and both contained 921 bp open reading frames (ORFs), encoded 306 amino acids in high homology. Heterologous expression in yeast showed that rainbow trout Elovl4b1 and Elovl4b2 proteins are able to effectively convert C18-22 polyunsaturated fatty acids (PUFAs) to the polyenoic products up to C32 or C36. Especially, compared with Elovl4b2, Elovl4b1 had much higher efficiency for converting 20:5n-3 and 22:5n-3 to 24:5n-3. Tissue distribution analysis revealed that rainbow trout *elovl4b* were widely transcribed in various tissues with the highest level in eye, brain and gill. Feeding experiment was conducted with three groups of rainbow trout fed diets with either fish oil (FO) or 100% of the FO replaced by soybean oil (SO) and linseeds oil (LO). Compared to the FO-fed group, *elovl4b* was significantly up-regulated in the liver of the SO-fed group and in the entire intestine of the LO-fed group, which suggested that *elovl4b* expression is regulated by dietary lipid sources. These results may contribute to better understand the LC-PUFAs and VLC-PUFAs biosynthetic pathway and the regulation mechanism in rainbow trout.

Key words: Elovl4b, Long-chain polyunsaturated fatty acids, heterologous expression, lipid sources, *Oncorhynchus mykiss*

1. Introduction

Some long-chain (C₂₀₋₂₄) polyunsaturated fatty acids (LC-PUFAs) have beneficial roles in human health and fish represent a main source of LC-PUFAs for humans (Simopoulos, 2000). Arguably, diet represents the most important factor determining the LC-PUFAs profile in fish but endogenous production (i.e. biosynthesis) can also have a major influence. The extent to which fish can biosynthesize LC-PUFAs varies among species and is associated with their capacity for fatty acyl desaturation and elongation (Monroig, *et al.*, 2018; Tocher, 2003).

Desaturase enzymes conduct dehydrogenation reactions and introduce a stereospecific double bond between the defined carbons of fatty acyl chains (Zhang, *et al.*, 2016). Elongation of very long-chain fatty acids (Elovl) proteins catalyze the rate-limiting condensation step in the elongation pathway (Jakobsson, *et al.*, 2006; Nugteren, 1965). Elovl's are composed of a family of at least seven members with Elovl2, Elovl4 and Elovl5 all known to be involved in LC-PUFAs biosynthesis in fish (Castro, *et al.*, 2016).

Elovl4 has been investigated in a variety of fish species, such as zebrafish, Atlantic salmon, cobia, rabbitfish, Nibe croaker, black seabream, orange-spotted grouper, large yellow croaker, and African catfish (Monroig, *et al.*, 2010; Carmona-Antoñanzas, *et al.*, 2011; Monroig, *et al.*, 2011b; Monroig, *et al.*, 2012; Kabeya, *et al.*, 2015; Jin, *et al.*, 2017; Li, *et al.*, 2017a; Li, *et al.*, 2017b; Oboh, *et al.*, 2017). Fish Elovl4 enzymes have, in contrast to mammalian Elovl4 (Agbaga, *et al.*, 2008), the potential to contribute to the biosynthesis of docosahexaenoic acid (22:6n-3, DHA) by elongating 20:5n-3 and 22:5n-3 to 24:5n-3 (Castro, *et al.*, 2016). Two distinct isoforms of Elovl4 are present in teleost genomes (Castro, *et al.*, 2016), which are typically termed as Elovl4a and Elovl4b following names of the zebrafish *Danio rerio* orthologs (Monroig, *et al.*, 2010). Functional differences between two isoforms were confirmed in zebrafish, in which only Elovl4b could elongate PUFA substrates to corresponding products up to C36. More recent investigations in black seabream *Acanthopagrus schlegelii* (Jin, *et al.*, 2017) and African catfish *Clarias gariepinus* (Oboh, *et al.*, 2017) revealed that both Elovl4a and Elovl4b can effectively elongate PUFA substrates.

Rainbow trout, *Oncorhynchus mykiss*, is a commercially important species that is farmed in many countries worldwide. Previous studies reported on LC-PUFA biosynthesizing genes include a fatty acyl desaturases 2 (*fads2*) and elongases *elovl5* and *elovl2* from rainbow trout (Astrid, *et al.*, 2004; Monroig, *et al.*, 2011a; Zheng, *et al.*, 2004). However, as far as we know, *elovl4* elongases have not yet been cloned from this freshwater carnivorous teleost. Thus, this study aimed at the molecular cloning, functional characterization, and tissue distribution of two *elovl4b* elongases from rainbow trout. Moreover, we investigated the nutritional regulation of the rainbow trout *elovl4b* when fish were fed diets containing different lipid sources.

2. Materials and Methods

2.1. Molecular Cloning

Total RNA was extracted from rainbow trout (85 g) liver using TRizol reagent (Takara, Japan). RNA quality and quantity were assessed by a 1.0% denaturing agarose gel and NanoDrop ND-1000 (Wilmington, DE, USA).

First strand cDNA was synthesized using Prime Script™ RT reagent Kit (Takara, Japan) following the manufacturer's instructions. Degenerate primers (Table 1) were designed based on highly conserved regions from the *elovl4* sequences of other fish, including zebrafish (NM_199972.1), cobia (HM026361.1), Atlantic salmon (NM_001195552.1) and northern pike (XM_010882381.2) in Genbank. Tks Gflex™ DNA Polymerase (Takara, Japan) was used for PCR amplification of first fragment. The PCR products were separated by electrophoresis on a 1.0% agarose gel, purified and ligated into pEASY-T1 vector (Transgen Biotech) for further sequencing (Sangon Biotech Co., Ltd, Shanghai, China). Nucleotide sequence was blasted on GenBank to confirm similarity with other *elovl4* sequences.

5'-and 3'-rapid amplification of cDNA ends (RACE) PCR template were obtained according to the manufacturer's instructions (SMARTer™ RACE cDNA Amplification Kit, Clontech, USA). The full-length cDNA sequences were obtained by 5'- and 3'-RACE according to the same first fragment template using four pairs of gene-specific primers. For 3' and 5' RACE of *elovl4b1*,

gene-specific primers, Elov14b-F1 and Elov14b-R2, and Universal Primer Mix (provided in the kit) were used in first round PCR. Subsequently, nested PCR was performed with Elov14b-F2 and Elov14b-R1 and a nested universal primer (provided in the kit) to obtain specific PCR product. For 3' and 5' RACE of *elov14b2*, Elov14b-F3 and Elov14b-R4, and Universal Primer Mix were used in first round PCR and Elov14b-F4, Elov14b-R3 and the nested universal primer were used in the second round PCR. The PCR products were purified, cloned into pEASY-T1 vector and sequenced as described above. The full length of the rainbow trout *elov14b* cDNAs were obtained by aligning the first PCR product with the corresponding RACE PCR products using DNAMAN software (Version 6.0, Lynnon BioSoft. Inc., USA).

2.2. Sequence and phylogenetic analysis

The BLAST sequence analysis service of National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) was used for sequence alignment and analysis. The deduced Elov14b1 and Elov14b2 protein sequences were aligned with those of Atlantic salmon Elov14b (*Salmo salar*, NP_001182481.1), northern pike Elov14 (*Esox lucius*, XP_010880683.1), zebrafish Elov14a and Elov14b (*Danio rerio*, NP_957090 and NP_956266). Multiple sequence alignment was performed with Mega 7.0 and a phylogenetic tree of Elov14 from rainbow trout and other vertebrates including fish, birds and mammals was constructed using the neighbor-joining method (Nei and Saitou, 1987). Confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping through 1,000 iterations.

2.3. Functional characterization of the rainbow trout Elov14b elongases in yeast

DNA fragment corresponding to the open reading frames (ORFs) of the rainbow trout Elov14b elongases were amplified with primers containing restriction sites for *Hind*III and *Xho*I (Table 1) using PrimeSTAR[®] HS DNA Polymerase (Takara, Japan). The DNA fragment was digested with corresponding restriction endonucleases (Takara, Japan) and then ligated into the similarly restricted pYES2 empty vector (Invitrogen, UK) to obtain the plasmid constructs

pYES2-Elov14b1 and pYES2-Elov14b2. The constructs were transformed into the *S. cerevisiae* competent strain INVSc1 (Invitrogen, Life Technologies™, USA) to produce transgenic yeast strains expressing the recombinant proteins. Selection of yeast with recombinant plasmids and yeast culture were conducted as described in detail previously (Jin, *et al.*, 2017). Recombinant yeast expressing Elov14b1 and Elov14b2 were grown in *S. cerevisiae* minimal medium^{-uracil} supplemented with the following PUFA substrates: stearidonic acid (18:4n-3), γ -linolenic acid (18:3n-6), eicosapentaenoic acid (20:5n-3), arachidonic acid (20:4n-6), docosapentaenoic acid (22:5n-3), DHA, and docosatetraenoic acid (22:4n-6) at the final concentrations of 0.5 mM (C18), 0.75 mM (C20) and 1.0 mM (C22) (Lopes-Marques, *et al.*, 2017). Yeast cells were harvested for analysis after 40 h of fatty acid supplementation at 30 °C. The cells were collected by centrifugation at 1500 g for 5 min, washed twice with 5 mL ice-cold HBSS, and freeze dried for 24 h for further analyses. Yeast transformed with empty pYES2 grown in the presence of all PUFA substrates were used as control.

2.4. Fatty acid analysis

Fatty acid analysis of transgenic yeast expressing the rainbow trout Elov14b1 and Elov14b2 were performed as described previously (Li, *et al.*, 2017a). Briefly, total lipid was extracted from yeast samples by homogenization in chloroform/methanol (2:1, v/v) containing 0.01% BHT as antioxidant, according to the method of Folch *et al.* (Folch, *et al.*, 1957). Fatty acid determinations were carried out through acid-catalysed transesterification according to the methodology described by Christie (Christie, 1982). Fatty acid methyl esters (FAME) were extracted with n-hexane/diethyl ether (1: 1, v/v) and purified by thin-layer chromatography (Silica gel G 60; Merck, Darmstadt, Germany) using a mixture of n-hexane/diethyl ether/acetic acid (85:15:1.5, v/v/v) as solvent system. FAMES were identified and quantified after splitless injection and run with temperature programming (50 – 300 °C) in an Agilent 6850 Gas Chromatograph system equipped with a Sapiens- 5MS (30 m $\times$ 0.25 μ m $\times$ 0.25 μ m) capillary column (Teknokroma, Barcelona, Spain) and coupled to a 5975 series MSD (Agilent

Technologies, Santa Clara, CA, USA) (Li, *et al.*, 2017b). Helium (1.5 mL/min.) was used as carrier gas. The elongation of exogenously added PUFA substrates (18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3, 22:4n-6 and 22:6n-3) was calculated by the step-wise proportion of substrate PUFAs converted to elongated product as [areas of first product and longer chain products/ (areas of all products with longer chain than substrate + substrate area)] $\times$ 100 (Li, *et al.*, 2017a).

2.5. Tissue distribution and nutritional regulation

Due to the highly homologous nucleotide sequences between *elovl4b1* and *elovl4b2*, one pair of primers (Table 1) were designed (Primer Premier 5.0) for gene expression analyses by quantitative PCR (qPCR) of both *elovl4b* genes from rainbow trout. In a first experiment, tissue distribution of *elovl4b* expression was determined in samples from muscle, liver, intestine, eye, spleen, kidney, brain, gill, heart and adipose tissue collected from a single individual (85 g). A second experiment investigating the effects of diet on *elovl4b* expression was performed with disease-free and equal size (initial mean weight 10.03 ± 0.02 g) rainbow trout from a commercial farm located in Weifang, Shandong, China. Before the initial feeding experiment, all rainbow trout were reared in the water circulation system and fed the control diet twice daily (05:00 and 17:00) for 2 weeks to acclimatize to experimental conditions and feed. Following this, fish were randomly divided into nine tanks (40 cm $\times$ 50 cm $\times$ 35 cm, 30 fish/tank) supplied with continuous flow-through freshwater supply and aeration, with water temperature maintained at 18 ± 2 °C and dissolved oxygen at 7-8 mg/L. Three isoproteic (41 % crude protein) and isolipidic (12 % crude lipid) diets were formulated to contain either fish oil (FO) or 100% of the FO replaced by soybean oil (SO) or linseeds oil (LO), according to the methodology described by Li (Li, 2017). Briefly, all ingredients were grounded into fine powder through a 180 μ m mesh and thoroughly blended with fish oil (or soybean oil, linseed oil) and lecithin, then water was added to product stiff dough. The dough was then pelleted with a granulator, dried for about 10 h in a ventilated oven at 55 °C, and stored at -20 °C until used.

Each diet was randomly allocated to triplicate groups of fish. The fish were slowly hand-fed twice daily at 05:00 and 17:00 until apparent satiation on the basis of visual observation for 10 weeks. At the end of the 10 weeks feeding trial, fish were fasted for 24 h. Liver and the entire intestine tissue samples from three fish per tank were collected and immediately frozen in liquid nitrogen.

2.6. Quantitative PCR (QPCR) analysis

Total RNA extracts from the tissue distribution and nutritional regulation experiments were reverse transcribed into cDNA as described above. The amplification was performed in a quantitative thermal cycler (Mastercycler EP Realplex, Eppendorf, Germany). Appropriate amount of cDNA, 1 μ L of each primer, 12.5 μ L 2 $\times$ qPCR SuperMix and water were added in a 25 μ L reaction mixture. QPCR were carried out with an initial denaturing step at 94 $^{\circ}$ C for 5 min, followed by 30 cycles of denaturation at 94 $^{\circ}$ C for 10 s, annealing at 57 $^{\circ}$ C for 30 s, extension at 72 $^{\circ}$ C for 30 s, and a final extension at 72 $^{\circ}$ C for 5 min. At the end of PCR reaction, melting curve analyses were systematically conducted to confirm that a single PCR product was present in each reaction. The expression of the rainbow trout *elovl4b* were normalized for the geometric mean of the expression levels of two housekeeping genes, β -actin and elongation factor 1 α , in relative units (Gregory, *et al.*, 2016). The expression levels of the rainbow trout *elovl4b* were calculated following the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

2.7. Statistical analysis

The Software SPSS 19.0 (SPSS Incorporation) was used for all statistical evaluations. The tissues distribution data were checked for homogeneity of variances using a Levene's test and then analyzed by one-way analysis of variance (ANOVA) followed by a Tukey's test ($P < 0.05$). For nutritional regulation data, the vegetable oil (VO) treatments including soybean-fed group and linseed-fed group were compared with the control (FO-fed group) by two-tailed Student's t test ($P < 0.05$).

3. Results

3.1. Sequence and phylogenetics of the rainbow trout *elovl4b* cDNAs

The full-length cDNA of the rainbow trout *elovl4b1* encompasses 1896 bp including a 317 bp 5'-untranslated region (UTR), a 921bp ORF encoding a putative protein of 306 amino acid (aa), and a 658 bp 3'UTR. Moreover, the full-length cDNA of the rainbow trout *elovl4b2* has 2227bp, and contained a 5' UTR OF 297bp, an ORF of 921bp encoding a putative protein of 306aa, and a 3' UTR of 989bp. Protein sequences analysis showed that the rainbow trout Elov4b1 and Elov4b2 putative proteins are 97.0% identical, with 12aa difference. Both proteins shared high identity with Elov4 from other fish including Atlantic salmon Elov4b (both presenting 98.0% identity), northern pike Elov4 (both presenting 95.10% identity) and zebrafish Elov4b (85.95% and 86.27% identity, respectively), but presented lower identity scores when compared to zebrafish Elov4a (75.1% and 74.7% identity, respectively).

The two Elov4 deduced proteins possess the conserved diagnostic histidine box HXXHH motif, five putative membrane-spanning domains, and single lysine and arginine residues (RXKXX) at the carboxyl terminus (Fig. 1). Phylogenetic tree analysis showed that rainbow trout both Elov4b elongases clustered with several other Elov4-like sequences from fish, bird and mammals, and separately with Elov2 and Elov5 from these vertebrate lineages. Furthermore, rainbow trout Elov4b1 and Elov4b2 clustered together with zebrafish Elov4b, Atlantic salmon Elov4b, but more distantly from zebrafish Elov4a (Fig. 2).

3.2. Functional characterization

Fatty acid composition of the yeast transformed with pYES2 vector alone shows four main fatty acids, namely 16:0, 16:1n-7; 18:0 and 18:1n-9, together with whichever exogenous PUFA added (data not shown). Rainbow trout Elov4b1 and Elov4b2 proteins could elongate PUFA substrates to the corresponding elongated polyenoic products up to C32 or C36 (Table 2), Approximately, rainbow trout Elov4b2 converted 1.3% and 2.4% of 20:5n-3 and 20:4n-6 to

22:5n-3 and 22:4n-6, respectively. In contrast, rainbow trout Elovl4b1 showed higher efficiency in converting 20:5n-3 and 20:4n-6 (12.6% and 14.2%, respectively), and 22:5n-3 and 22:4n-6 (22.0% and 10.3%, respectively) (Table 2).

3.3. Tissue distribution of *elovl4b*

Rainbow trout *elovl4b* gene was expressed in all tissues examined including muscle, liver, intestine, eye, spleen, kidney, brain, gill, heart and adipose tissues and showed predominant expression in the eye, followed by the brain and gill ($P < 0.05$) (Fig. 3).

3.4. Nutritional regulation of *elovl4b* expression

The expression of *elovl4b* gene, in response to dietary lipid sources, were detected in the liver and the entire intestine of rainbow trout that had been fed diets containing either FO or 100% of the FO replaced by SO and LO. Compared to the FO-fed fish, significant increase of *elovl4b* expression was detected in liver of SO-fed group and no significance was found in LO-fed group ($P < 0.05$) and (Fig.4-A). In the entire intestine, the expression of *elovl4b* gene was up-regulated in LO-fed group and no significant difference was detected in SO-fed group ($P < 0.05$) and (Fig.4-B).

4. Discussion

The two *elovl4b* genes in rainbow trout have been cloned in the present study, which is different from that in mammals with only one Elovl4-encoding gene (Zhang, *et al.*, 2003). This confirmed that the whole-genome duplication event in salmonids occurred 25 to 100 Mya ago (Berthelot, *et al.*, 2014). The putative rainbow trout Elovl4b1 and Elovl4b2 proteins possessed all the features of the elongase family including conserved histidine box (HXXHH), transmembrane domains, and the C-terminal endoplasmic reticulum (ER) retrieval signal (RXKXX), indicating the role in LC-PUFAs biosynthesis (Cook and McMaster, 2002). Phylogenetic analysis revealed that rainbow trout Elovl4b1 and Elovl4b2 proteins are more

similar to other Elovl4 orthologues from mammals, birds and other fish, in comparison to other Elovl proteins in fish species including Elovl5 and Elovl2. This suggested that Elovl4 proteins were highly conserved during evolution (Lagali, *et al.*, 2003). Furthermore, rainbow trout Elovl4b1, Elovl4b2 clustered together with zebrafish Elovl4b, Atlantic salmon Elovl4b, but more distantly from zebrafish Elovl4a. This indicate the members of the two clusters may have functional differences (Monroig, *et al.*, 2011b).

Functional characterization assays of rainbow trout Elovl4b1 and Elovl4b2 revealed the similarities with the previously characterized zebrafish Elovl4b, as they were all able to elongate PUFAs to corresponding polyenoic products up to C32 or C36 (Monroig, *et al.*, 2010). The functional differences between Elovl4b1 and Elovl4b2 were reflected mainly in their high or low efficiency to convert 20:5n-3 and 22:5n-3 to 24:5n-3, a potential substrate for DHA biosynthesis (Sprecher, 2000). Previous study has demonstrated that substitution of eight aa residues of rat $\Delta 6$ desaturase (D6d) with the corresponding residues of $\Delta 5$ desaturase (D5d) switched the substrate specificity (Watanabe, *et al.*, 2015). Also, the differences of three-residue substrate recognition motif in cysteine sulfinatase decarboxylase (SCD) of rainbow trout and Japanese flounder were confirmed to be responsible for high and low enzyme catalytic activity, respectively (Wang, *et al.*, 2016). Thus, the difference of 12 aa between Elovl4b1 and Elovl4b2 in rainbow trout might be the reason of the functional variations.

With the increasing use of VO in aquafeeds, the contents of LC-PUFAs in farmed fish decreased to a large extent, which may severely impact its quality and value for human consumers (Bell, *et al.*, 2002; Henriques, *et al.*, 2014; Sprague, *et al.*, 2016). Nutritional regulation of genes encoding Elovl and desaturases have been proposed as a potential strategy to improve the endogenous production of LC-PUFAs and VLC-PUFAs and enhance the effective utilization of VO in aquafeeds (Agaba, *et al.*, 2004; Lewis, *et al.*, 2013; Tocher, 2015; Tocher, *et al.*, 2010; Vagner and Santigosa, 2011). In our present study, the expression of rainbow trout *elovl4b* gene were significantly up-regulated by dietary VO. Similar results were also observed

in other teleost fishes such as orange-spotted grouper in which *elovl4* mRNA expression was up-regulated in response to low dietary LC-PUFA (Li, *et al.*, 2017a) and large yellow croaker in which n-3 LC-PUFA suppressed transcription of the *elovl4b* (Li, *et al.*, 2017b). It is largely accepted that high levels of dietary LC-PUFA could suppressed the expression of the elongase and desaturase genes (Tocher, *et al.*, 2003), which reflect the existence of negative feedback regulation in the LC-PUFA synthetic pathway (Lin, *et al.*, 2018). Meanwhile, over-expression or compensatory regulation in liver may be linked to fatty acid turnover and metabolism and, in these particular results, to the presence of 18:2n-6, presumably abundant after the fish oil substitution. In the entire intestine, the regulation could be associated to absorption and, in this case, to the 18:3n-3 abundance in linseed oil. Unfortunately we do not know the affinity-activity of Elov14b for these two particular substrates. However, it is interesting to note, that the two substituted diets provide an excess of 18 C unsaturated fatty acids for which Elov14b seem to have lower activity as shown by the functional analysis. It is tempting to hypothesize around the effects in terms of up-regulation in scenarios of over-abundance of substrates not especially conducive to the activity of the enzyme, and about their n-3/n-6/organ association, but this topic was not the objective of this work and deserves to be dealt with more detail in further research.

In conclusion, the present study demonstrated rainbow trout possess two *elovl4b* genes. Heterologous expression in yeast demonstrated that rainbow trout Elov14b1 and Elov14b2 differ in the ability to elongate PUFAs substrates. The expression of rainbow trout *elovl4b* gene were regulated by dietary lipid sources.

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7. Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Fig. 1. Comparison of the deduced amino acid (aa) sequences of Elovl4 from rainbow trout, other fish, mouse and human. The aa sequences were aligned using ClustalX, and identity/similarity shading was based on a 75% identity threshold. Identical residues are shaded black and similar residues are shaded grey. Indicated are the conserved HXXHH histidine box motif, five (I–V) putative membrane-spanning domains and the endoplasmic reticulum (ER) retrieval signal.

Fig. 2. Phylogenetic tree comparing the rainbow trout Elovl4b1, Elovl4b2 proteins with elongase proteins from other species. The tree was constructed using the neighbor-joining method using MEGA7. The numbers represent the frequencies (%) with which the tree topology presented was replicated after 1000 iterations.

Fig. 3. Tissue expression of *elovl4b* in rainbow trout. Values (means $\pm$ standard error of the mean, SEM) in bars that have the same letters are not significantly different ($p > 0.05$; Tukey's test) among tissues ($n = 3$).

Fig. 4. Nutritional regulation of *elovl4b* in rainbow trout fed diets containing fish oil, soybean oil (SO) or linseed oil (LO), in the liver (A) and intestine (B) tissues. Results are expressed as means $\pm$ standard error of the mean ($n = 3$). Significant differences between the FO and the other groups: * $p < 0.05$.

Table 1. Primers used for Cloning, function characterization and QPCR

Purpose	Primers name	Nucleotide sequence (5' - 3')
First fragment cDNA cloning	FFRA-F	GGAGCCTTACYATAGCAGAC
	FFRA-R	GATGCAGTGGGCGYYTGATTG
RACE PCR	Elov14b-R1	CCACAGGAAGAGCAAGTACAGGCAG
	Elov14b-R2	AAGCGGGGAAAGGGCAGCCAGTGTAG
	Elov14b-F1	GGACAGTCGTTCTTCGGAGCAGGCAT
	Elov14b-F2	AGTCAGGATAGCGTCTGCTCTGTGGT
	Elov14b-R3	CCACAGGAAGAGCAAGTACAGGCAG
	Elov14b-R4	AAGCGGGGAAAGGGCAGCCAGTGTAG
	Elov14b-F3	CAGGCTACTCCCTCTACACTGGCTGCC
	Elov14b-F4	GCCGCTTTCGGACCCAAGATCCAGAAAT
Function characterization	Elov14b1-orf-F	CCCAAGCTTATGGAAGCTGTCACCCACT
	Elov14b1-orf-R	CCGCTCGAGTTACTCCCTCTTCACTCTTC
	Elov14b2-orf-F	CCAAGCTTATGGAAGCTCTCACCCACTT
	Elov14b2-orf-R	CCGCTCGAGTTACTCCCTTTTCGCTCTTC
QPCR	<i>elov14b</i> -F	AGGATAGCGTCTGCTCTGTG
	<i>elov14b</i> -R	GGAATTTCTGGATCTTGGGT
	β -actin-F	TCCTTCCTCGGTATGGAGTCT
	β -actin-R	TTACGGATGTCCACGTCACAC
	<i>ef1α</i> -F	GGATGGAAGGTCGAACGTAA
	<i>ef1α</i> -R	TTGTAGACATCCTGGAGGGG

Table 2. Functional characterization of the rainbow trout Elovl4b1 and Elovl4b2 proteins

FA substrates	Products	Conversion(%)		Activity
		Elovl4b1	Elovl4b2	
18:4n-3	20:4n-3	1.9	0.6	C18→C36
	22:4n-3	6	3.3	C20→C36
	24:4n-3	25.8	N.D.	C22→C36
	26:4n-3	71.4	N.D.	C24→C36
	28:4n-3	100	N.D.	C26→C36
	30:4n-3	100	N.D.	C28→C36
	32:4n-3	63.4	N.D.	C30→C36
	34:4n-3	N.D.	N.D.	C32→C36
	36:4n-3	N.D.	N.D.	C34→C36
18:3n-6	20:3n-6	4.2	1.0	C18→C36
	22:3n-6	17.3	5.7	C20→C36
	24:3n-6	37	N.D.	C22→C36
	26:3n-6	80.1	N.D.	C24→C36
	28:3n-6	90.4	N.D.	C26→C36
	30:3n-6	84.9	N.D.	C28→C36
	32:3n-6	18.0	N.D.	C30→C36
	34:3n-6	N.D.	N.D.	C32→C36
	36:3n-6	N.D.	N.D.	C34→C36
20:5n-3	22:5n-3	12.6	1.3	C20→C36
	24:5n-3	40.5	10.4	C22→C36
	26:5n-3	68.4	35.1	C24→C36
	28:5n-3	94.2	N.D.	C26→C36
	30:5n-3	91.9	N.D.	C28→C36
	32:5n-3	27.5	N.D.	C30→C36
	34:5n-3	N.D.	N.D.	C32→C36
	36:5n-3	N.D.	N.D.	C34→C36
20:4n-6	22:4n-6	14.2	2.4	C20→C36
	24:4n-6	43.5	16.3	C22→C36
	26:4n-6	73.5	48.2	C24→C36
	28:4n-6	89.9	42.9	C26→C36
	30:4n-6	94.9	N.D.	C28→C36
	32:4n-6	53.8	N.D.	C30→C36
	34:4n-6	4.2	N.D.	C32→C36
	36:4n-6	N.D.	N.D.	C34→C36
22:5n-3	24:5n-3	22.0	0.8	C22→C36
	26:5n-3	63.1	47.4	C24→C36
	28:5n-3	92.0	88.9	C26→C36
	30:5n-3	87.2	80.5	C28→C36
	32:5n-3	21.0	25.1	C30→C36
	34:5n-3	2.5	N.D.	C32→C36
	36:5n-3	N.D.	N.D.	C34→C36
22:4n-6	24:4n-6	10.3	0.32	C22→C36
	26:4n-6	68.5	70.86	C24→C36
	28:4n-6	87.5	90.54	C26→C36
	30:4n-6	94.3	82.83	C28→C36
	32:4n-6	48.5	49.32	C30→C36
	34:4n-6	7.7	N.D.	C32→C36
	36:4n-6	41.8	N.D.	C34→C36
22:6n-3	24:6n-3	1.0	N.D.	C22→C36
	26:6n-3	25.8	N.D.	C24→C36
	28:6n-3	N.D.	N.D.	C26→C36
	30:6n-3	N.D.	N.D.	C28→C36
	32:6n-3	N.D.	N.D.	C30→C36
	34:6n-3	N.D.	N.D.	C32→C36
	36:6n-3	N.D.	N.D.	C34→C36

N.D., not detected