Aquaculture Research

Aquaculture Research, 2017, **48**, 537–552 doi: 10.1111/are.12902

Molecular cloning and functional characterization of a putative *Elovl4* gene and its expression in response to dietary fatty acid profiles in orange-spotted grouper *Epinephelus coioides*

Songlin Li[1,2], Óscar Monroig[3], Juan Carlos Navarro[4], Yuhui Yuan[1,2], Wei Xu[1,2], Kangsen Mai[1,2], Douglas R Tocher[3] & Qinghui Ai[1,2]

[1]Key Laboratory of Aquaculture Nutrition and Feed, Ministry of Agriculture, Ocean University of China, Qingdao, China

[2]Key Laboratory of Mariculture, Ministry Education of China, Ocean University of China, Qingdao, China

[3]Institute of Aquaculture, University of Stirling, Stirling, UK

[4]Instituto de Acuicultura Torre de la Sal (CSIC), Castellón, Spain

Correspondence: Q Ai, Key Laboratory of Aquaculture Nutrition and Feed, Ministry of Agriculture, Ocean University of China, Qingdao 266003, China. E-mail: qhai@ouc.edu.cn

Abstract

Elongase of very long-chain fatty acids (Elovl) 4 probably plays a crucial role in marine fish species, where lack of Elovl2 has been considered as one possible reason for their low long-chain polyunsaturated fatty acids' (LC-PUFAs) biosynthetic capability. Elongase of very long-chain fatty acids 4 is the most recent member of the Elovl family that has been investigated in fish. Here, we report the molecular cloning and functional characterization of putative *elovl4* cDNA isolated from marine teleost, *Epinephelus coioides*, and its expression in response to dietary n-3 LC-PUFA and docosahexaenoic acid (DHA) to eicosapentaenoic acid (EPA) ratio. The *elovl4* cDNA of grouper was 2341 bp including 301 bp of 5′-untranslated region (UTR), 918 bp of the coding region that encodes 305 amino acids (AA) and 1122 bp of 3′UTR. Heterologous expression in yeast demonstrated that grouper Elovl4 could elongate saturated fatty acids (FA), especially 24:0 and 26:0, up to 36:0. Also, grouper Elovl4 effectively converted C20 and C22 polyunsaturated FAs to elongated polyenoic products up to C36. Tissue distribution analysis revealed that Elovl4 were widely transcribed in various tissues with the highest level in eye, brain and testis as described in other teleosts. The transcript level of *elovl4* was significantly affected by dietary n-3 LC-PUFA and high LC-PUFA level repressess its expression. However, the ratio of DHA to EPA had no significant influence on its expression. These results may contribute to better understanding the LC-PUFA biosynthetic pathway in this fish species.

Keywords: Elovl4, cloning, functional characterization, nutrition regulation, orange-spotted grouper

Introduction

Long-chain polyunsaturated fatty acids (LC-PUFAs) (C ≥ 20) are beneficial for human health, promoting the development of neuronal tissues and protect against cardiovascular, immune and inflammatory condition (Salem, Litman, Kim & Gawrisch 2001; Ruxton, Reed, Simpson & Millington 2004; Calon & Cole 2007; Eilander, Hundscheid, Osendarp, Transler & Zock 2007). Fish species, especially marine species, are the main source of LC-PUFAs for humans. However, with increasing use of vegetable oils in aqua feed, the contents of docosapentaenoic acid (DHA) and eicosapentaenoic acid (EPA) in farmed fish decreased significantly due to the lack of LC-PUFA in vegetable oils (Lin, Liu, He, Zheng & Tian 2007; Peng, Xu, Mai, Zhou, Zhang, Liufu, Zhang & Ai 2014), which may severely impact its quality and value for human consumers. Therefore, the molecular mechanisms of the enzymes involved in

the biosynthesis of LC-PUFAs in teleost are urgently better understood (Tocher 2003).

The biosynthesis of LC-PUFAs in vertebrates is catalysed by fatty acyl desaturase (Fads) and elongation of very long-chain FAs (Elovl) enzymes, which could convert the dietary essential α-linolenic acid (18:3n-3) and linoleic acid (18:2n-6) to LC-PUFAs, including the physiologically important DHA, EPA and arachidonic acid (ARA), through consecutive desaturation and elongation reactions (Sprecher 2000; Nakamura, Cho, Xu, Tang & Clarke 2001). Generally speaking, most marine fish, unlike freshwater species, have low LC-PUFA biosynthetic capacity due to a lack of or low specific enzyme activities involved in the pathway. Up to now, no *Δ5 FAD* cDNA has been isolated from any marine fish species other than a bifunctional Δ6/Δ5 FAD found in rabbitfish (Li, Monroig, Zhang, Wang, Zheng, Dick, You & Tocher 2010). Additionally, marine fish species also appear to lack Elovl2 which could elongate C20 and C22 LC-PUFA and was regarded as an essential enzyme in DHA biosynthesis (Monroig, Rotllant, Sánchez, Cerdá-Reverter & Tocher 2009; Morais, Monroig, Zheng, Leaver & Tocher 2009). Elovl4 is the most recent member of Elovl family that has been investigated in fish (Monroig, Rotllant, Cerdá-Reverter, Dick, Figueras & Tocher 2010), although it has been proved to play a crucial rule in the biosynthesis of both saturated and polyunsaturated very long-chain FAs (VLC-FAs) ($C \geq 24$) in mice (Cameron, Tong, Yang, Kaminoh, Kamiyah, Chen, Zeng, Chen, Luo & Zhang 2007). The *Elovl4* cDNAs have been isolated and characterized in zebrafish (Monroig *et al.* 2010), Atlantic salmon (Carmona-Antoñanzas, Monroig, Dick, Davie & Tocher 2011), cobia (Monroig, Webb, Ibarra-Castro, Holt & Tocher 2011) and rabbitfish (Monroig, Wang, Zhang, You, Tocher & Li 2012). Zebrafish possesses two Elovl4 enzymes, Elovl4a and Elovl4b. Both zebrafish Elovl4 proteins efficiently elongated saturated FAs up to C36. However, only Elovl4b could elongate PUFA substrates to corresponding elongated polyenoic products up to C36, with C20 PUFA appearing as preferred substrates (Monroig *et al.* 2010). As for marine fish species, the function of Elovl4 in rabbitfish and cobia were similar to Elovl4b. The ability of Elovl4 to effectively elongate C22 PUFA to C24 PUFA indicates that these enzymes have the potential to participate in the production of DHA, similar to Elovl2.

During the past decades, a number of studies have been focused on the regulation of those enzymes involved in LC-PUFA biosynthetic pathway. A few of them have been proved to be regulated by spatial temporal (Ishak, Tan, Khong, Jaya-Ram, Enyu, Kuah & Shu-Chien 2008; Monroig *et al.* 2010; Tan, Chung & Shu-Chien 2010), environmental factors (Zheng, Torstensen, Tocher, Dick, Henderson & Bell 2005) as well as nutrients (Zheng *et al.* 2005; Ling, Kuah, Sifzizul, Muhammad, Kolkovski & Shu-Chien 2006; Morais, Mourente, Ortega, Tocher & Tocher 2011). Studies on the regulation of those enzymes have been mainly focused on Fads2, Elovl5 and Elovl2. However, to our knowledge, little information was available on the regulation of *elovl4*. In this study, the expression of *elovl4* in response to dietary FA was investigated.

Orange-spotted grouper, *Epinephelus coioides*, is a popular fish cultured in Southeast Asia and good candidates for intensive aquaculture for their fast growth, efficient feed conversion and high market value (Millamena 2002). However, only few studies have been conducted to investigate the regulation of enzymes involved in LC-PUFA biosynthetic pathway of grouper. Only Li, Mai, Xu, Yuan, Zhang and Ai (2014) reported the expression of *Δ6 FAD* in response to dietary n-3 LC-PUFA. Therefore, it is crucial to understand the molecular mechanisms underlying the biosynthesis of PUFA in grouper, which could provide the basis for the successful substitution of fish oil and maintaining high levels of n-3 LC-PUFA in the flesh. Elovl4 plays a crucial role in the early development of vertebrates (Monroig *et al.* 2010). In the present study, grouper larvae were chosen to investigate the effect of dietary FA on expression of *elovl4*. The aim of the present study was conducted to clone the *elovl4* cDNA, and investigate its characterization, tissue distribution and mRNA expression in response to dietary FA.

Materials and methods

Experimental fish

The grouper were bought from a local fish-rearing farm in Yandun, Hainan, China. The body mass of grouper used for cloning, rapid amplification of cDNA ends (RACE) and tissue distribution were 150.62 ± 2.35 g. The initial body weight of

grouper larvae used for nutritional regulation study was 70 ± 2 mg (29 days after hatch, DAH).

Cloning and sequencing of grouper Elovl4 cDNA

Total RNA was isolated from grouper liver using Trizol Reagent (Takara, Tokyo, Japan) followed by quality measurement on a 1.2% denaturing agarose gel and yield determination on NanoDrop® ND-1000 (Wilmington, DE, USA). The RNA was treated with RNA-Free DNase (Takara) to remove DNA contaminant and reversely transcribed to cDNA by PrimeScript™ RT reagent Kit (Takara) according to the instructions provided by the manufacturer.

First strand cDNA was synthesized using PrimeScript™ RT reagent Kit (Takara) following the instructions. To obtain the first fragment of *elovl4* cDNA of grouper, degenerate polymerase chain reaction (PCR) primers were designed based on highly conserved regions from the *elovl4* sequences of other fish (cobia, zebrafish and Atlantic salmon) in Genbank and were synthesized by Biosune Biotech (Shanghai, China). Two degenerate primers (Elovl4-F and Elovl4-R, Table 1) were designed to clone a fragment within the coding region by PCR. The PCR programme was carried out in Eppendorf Mastercycler Gradient (Eppendorf, Hamburg) and the PCR conditions were: 2 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 54°C, 40 s at 72°C; another 10 min at 72°C. The amplification products were separated by electrophoresis on a 1.5% agarose gel for length difference, and then the target band was ligated into the pEASY-T1 vector (TransGen Biotech, Beijing, China). The PCR fragment was sequenced in Biosune Biotech (Shanghai, China) and the nucleotide sequence was blasted on GenBank to confirm its high similarity with other Elovl4 proteins.

The full-length cDNA sequence of *elovl4* was obtained by 5′- and 3′-RACE using the SMARTer™ RACE cDNA Amplification Kit (Clontech, CA, USA). The 3′- and 5′-ends cDNA templates were synthesized according to the user's manual. Four gene-specific primers, Elovl4-F1, Elovl4-F2, Elovl4-R1 and Elvol4-R2 were designed for the amplification of RACE cDNA fragments based on the obtained Elovl4 cDNA fragment (Table 1). For 3′ and 5′ RACE, gene-specific primers, Elovl4-F2 and Elovl4-R1, and Universal Primer A Mix (provided in the kit) was used in first round PCR. Then, nested PCR was performed with the other gene-specific primer, Elovl4-F1 and Elovl4-R2, for 3′ and 5′ RACE PCRs, respectively, and a nested universal primer (provided in the kit) to obtain specific PCR product. The PCR products were purified, cloned into and sequenced as described above.

Sequence and phylogenetic analysis of Elovl4

Sequence alignment and analysis were conducted using the BLAST sequence analysis service of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Multiple alignments of *elovl4* were performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.

Table 1 Sequences of the PCR primers used in this work

Primer	Sequences (5′-3′)	Purpose
Elovl4-F	AGACAAGMGKGTGGAGAAATG	RT primer
Elovl4-R	AGGATGATGAARGTGACRGCG	RT primer
Elovl4-F1	CTTCCTGAGTGTATAGGGCTGGCGGTC	5′RACE primer
Elovl4-F2	GCATCTGAGGTCCCAGAGCTGCCAG	5′RACE primer
Elovl4-R1	GCAGGACCGCCAGCCCTATACACTCAG	3′RACE primer
Elovl4-R2	GGCTCTGATTGGCTACGCCGTCACCTT	3′RACE primer
gE4-HindIII-F	CCCAAGCTTATGGAGGTTGTAACACATCT	Functional characterization
gE4-EcorI-R1	CCGCTCGAGTTACTCCCTTTTCGCTCGTC	Functional characterization
UPM	Long: CTAATACGACTCACTATAG GGCAAGCAGTGGTATCAACGC AGAGT	RACE method
	Short: CTAATACGACTCACTATAGGGC	RACE method
NUP	AAGCAGTGGTATCAACGCAGAGT	RACE method
Elovl4-qF	CTTTCATCATCCTCTTCGCC	RT-qPCR
Elovl4-qR	TTACTCCCTTTTCGCTCGTC	RT-qPCR
βactin-F	TACGAGCTGCCTGACGGACA	RT-qPCR
βactin-R	GGCTGTGATCTCCTTCTGCA	RT-qPCR

uk/clustalw/). The deduced amino acid sequence of the newly cloned grouper *elovl4* cDNAs was aligned with their corresponding orthologues from different species including human (*Homo sapiens*, NP_073563), mouse (*Mus musculus*, NP_683743), rat (*Rattus norvegicus*, NP_001178725), zebrafish (*Danio rerio*, NP_957090 and NP_956266), cobia (*Rachycentron canadum*, ADG59898), Atlantic salmon (*Salmo salar*, NP_001182481). Multiple sequence alignment was performed with Mega 4.0. A phylogenetic tree was constructed on the basis of amino acid sequence between the grouper Elovl cDNAs, vertebrate Elovl4, Elovl2 and Elovl5 proteins and using the neighbour-joining method (Saitou & Nei 1987). Confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping through 1000 iterations.

Functional characterization of the grouper Elovl4 in yeast

The grouper Elovl4 function was determined by expressing its open reading frame (ORF) in *Saccharomyces cerevisiae* yeast cells as previously described (Monroig *et al.* 2010; Carmona-Antoñanzas *et al.* 2011). Briefly, the ORF of the grouper *elovl4* forms was amplified with primers containing restriction sites (Hind III and Ecor I) (Table 1) for further cloning into the yeast expression vector pYES2 (Invitrogen, Paisley, UK). The purified plasmids containing the putative *elovl4* ORFs were then transformed into *S. cerevisiae* competent cells InvSc1 (Invitrogen) as previously described (Hastings, Agaba, Tocher, Leaver, Dick, Sargent & Teale 2001; Agaba, Tocher, Zheng, Dickson, Dick & Teale 2005). One single recombinant yeast colony was grown in *S. cerevisiae* minimal medium-uracil broth to produce a bulk culture required to run the functional assay as follows. In order to assess the role of the grouper Elovl4 in the biosynthesis of very long-chain (C > 24) PUFA, individual flasks of transgenic yeast were supplemented with one of the following FA substrates: stearidonic acid (18:4n-3), γ-linolenic acid (18:3n-6), EPA (20:5n-3), ARA (20:4n-6), DHA (22:5n-3), docosatetraenoic acid (22:4n-6) or DHA (22:6n-3). In order to test the ability of the grouper Elovl4 to elongate saturated VLC-FA, yeast transformed with pYES2 containing the putative *elovl4* ORF or no insert (pYES2-empty) (control) were incubated in triplicate in the absence of exogenously added substrates. The VLC-FA profiles from yeast transformed with pYES2-*elovl4* and pYES2-empty were then compared. Docosapentaenoic and docosatetraenoic acids (>98–99% pure) were purchased from Cayman Chemical (Ann Arbor, MI, USA) and the remaining FA substrates (>99% pure) and chemicals used to prepare the *S. cerevisiae* minimal medium-uracil were from Sigma Chemical (Dorset, UK). After 2 days, yeast were harvested and washed for further analyses. Yeast transformed with pYES2 containing no insert were cultured under the same conditions as a control treatment.

Fatty acid analysis of yeast

Total lipids were extracted from yeast samples and used to prepare methyl esters (FAME) as described in detail previously by Monroig, Tocher, Hontoria and Navarro (2013). The FAME were identified and quantified after splitless injection and run in temperature programming, in an Agilent 6850 Gas Chromatograph system, equipped with a Sapiens-5MS (30 m × 0.25 μm × 0.25 μm) capillary column (Teknokroma, Sant Cugat del Vallés, Barcelona, Spain) coupled to a 5975 series MSD (Agilent Technologies, Santa Clara, CA, USA). The elongation of endogenous substrates was assessed by comparison of the areas of the FAs of control yeast with those of Elovl4 transformed yeast. The elongation of exogenously added PUFA substrates (18:4n-3, 18:3n-6, 18:4n-3, 20:5n-3, 20:4n-6, 22:5n-3 and 22:6n-3) was calculated by the stepwise proportion of substrate FA converted to elongated product as [areas of first product and longer chain products/(areas of all products with longer chain than substrate + substrate area)] × 100.

n-3 LC-PUFA levels and DHA/EPA study

For n-3 LC-PUFA level study, grouper larvae were obtained from tissue samples collected for a previous publication (Li *et al.* 2014). Briefly, Triplicate groups of grouper larvae (29DAH) were fed to apparent satiation six times daily for 4 weeks with five isoproteic (58% crude protein) and isolipidic (16% crude lipid) diets containing graded levels of n-3 LC-PUFA (0.52%, 0.94%, 1.57%, 1.97% and 2.43%) (Tables 2 and 3).

For DHA/EPA study, A total of 2100 larvae (29DAH, 70 ± 2 mg) were distributed into 15 white plastic tanks (water volume 100 L) at a stocking density of 140 individuals per tank. Triplicate groups of grouper larvae were fed to apparent satiation six times daily for 4 weeks with five

isoproteic (58% crude protein) and isolipidic (16% crude lipid) diets containing graded levels of DHA/EPA (0.82, 1.28, 1.67, 2.00 and 2.33) (Tables 4 and 5) and the total amount of n-3 LC-PUFA was approximately fixed at 2.0% of the dry weight. Five fish in each cage were pooled into 1.5 mL tube (RNase-Free; Axygen, Tewksbury, MA, USA), frozen in liquid nitrogen and then stored at −80°C for later analysis of Elovl4 expression.

Real-time quantitative PCR (RT-qPCR) analysis

The mRNA expression pattern of putative *elovl4* in various tissues (eye, brain, testis, heart, liver, kidney, stomach, intestine and muscle) and samples from the larval rearing experiments were measured by RT-qPCR. β-actin (GenBank ID: AY510710) was selected as reference gene, and the stability of β-actin was verified and confirmed. Gene-specific primers for RT-qPCR of Elovl4 and β-actin (Table 1) were designed by Primer Primier 5.0 based on the cloned nucleotide sequences.

The RT-qPCR was carried out in a quantitative thermal cycler (Mastercyclerepreplex, Eppendorf, Germany). The amplification was performed in a total volume of 25 μL containing 2 × SYBR® Premix Ex Taq™ (Perfect Real Time) (Takara), 0.5 μL of each primer (10 μmol L^{-1}), 1 μL of cDNA mix. The programme was as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 57°C for 10 s and 72°C for 20 s. At the end of each PCR reaction, melting curve analysis of amplification products was carried out to confirm that a single PCR product was present in these reactions. Standard curves were made with five different dilutions (in triplicate) of the cDNA samples and amplification efficiency was analysed according to the following equation $E = 10^{(-1/\text{Slope})} - 1$. The primer amplication efficiency was 0.9970 for *elovl4*, 1.008 for β-*actin*. The abosolute ΔCT value of the slope is 0.01, which indicated that ΔΔCT calculation for the relative quantification of target gene could be used. The expression levels of the target genes were calculated followed the $2^{-\Delta\Delta t}$ method described by Livak and Schmittgen (2001).

Statistical analysis

The results were given as means ± SEM (standard error of the mean). Data from each treatment were subjected to one-way ANOVA and correlation analysis where appropriate using SPSS 19.0 (SPSS Incor-

Table 2 Formulation and proximate analysis of the experimental diets with graded levels of n-3 LC-PUFA (% dry weight)

	Dietary n-3 LC-PUFA contents (% dry weight)				
Ingredients (%)	**0.52**	**0.94**	**1.57**	**1.97**	**2.43**
Casein*	13	13	13	13	13
Defatted white fish meal*	35	35	35	35	35
Defatted krill meal*	10	10	10	10	10
Squid meal*	5	5	5	5	5
Hydrolysed fish meal*	8	8	8	8	8
LT-Yeast*	2	2	2	2	2
Alginate sodium	2	2	2	2	2
α-starch	5	5	5	5	5
Vitamin premix†	1.5	1.5	1.5	1.5	1.5
Mineral premix‡	1.5	1.5	1.5	1.5	1.5
Attractant§	1.5	1.5	1.5	1.5	1.5
Ethoxyquin	0.1	0.1	0.1	0.1	0.1
Choline chloride	0.2	0.2	0.2	0.2	0.2
DHA-enriched oil¶	0.33	1.45	2.56	3.66	4.8
EPA-enriched oil**	0.02	0.66	1.31	1.95	2.58
Palmitin††	9.75	7.99	6.23	4.49	2.72
ARA-enriched oil‡‡	1	1	1	1	1
Soy lecithin	4	4	4	4	4
Mold inhibitor§§	0.1	0.1	0.1	0.1	0.1
Proximate analysis (n = 3)					
Crude protein (%)	57.98	57.21	57.15	57.20	57.71
Crude lipid (%)	15.09	15.24	15.11	15.46	15.75
Ash (%)	16.08	16.11	15.29	15.30	15.19

*Casein: crude protein 87.91% dry matter, crude lipid 1.69% dry matter; Defatted fish meal: crude protein 73.36% dry matter, crude lipid 1.52% dry matter; Defatted Krill meal: crude protein 71.80% dry matter, crude lipid 2.93% dry matter; Squid meal: crude protein 61.72% dry matter, crude lipid 3.16% dry matter; Hydrolysed fish meal: crude protein 77.10% dry matter, crude lipid 4.60% dry matter.

†Vitamin premix (IU or g kg^{-1} vitamin premix): retinal palmitate, 3 000 000 IU; cholecalciferol, 1 200 000 IU; DL-α-tocopherol acetate, 40.0 g; menadione, 8.0 g; thiamin-HCl, 5.0 g; riboflavin, 5.0 g; D-calcium pantothenate, 16.0 g; pyridoxine-HCl, 4.0 g; meso-inositol, 200.0 g; D-biotin, 8.0 g; folic acid, 1.5 g; para-aminobenzoic acid, 5.0 g; niacin, 20.0 g; cyanocobalamin, 0.01 g; ascorbyl polyphosphate (contained 25% ascorbic acid), 100.0 g.

‡Mineral premix (g kg^{-1}): $Ca(H_2PO_4)_2{\cdot}H_2O$, 675.0; $CoSO_4{\cdot}4H_2O$, 0.15; $CuSO_4{\cdot}5H_2O$, 5.0; $FeSO_4{\cdot}7H_2O$, 50.0; KCl, 50.0; KI, 0.1; $MgSO_4{\cdot}2H_2O$, 101.7; $MnSO_4{\cdot}4H_2O$, 18.0; NaCl, 80.0; $Na_2SeO_3{\cdot}H_2O$, 0.05; $ZnSO_4{\cdot}7H_2O$, 20.0.

§Attractant (g 100 g^{-1}): betaine, 50; glycine, 15; alanine, 10; argine, 10; taurine, 10; inosine-5′-monophosphoric acid, 5.

¶DHA-enriched oil: DHA content, 40.64% of TFA; in the form of DHA-methylester; JIANGSU TIANKAI Biotechnology, China.

**EPA-enriched oil: EPA content, 46.41% of TFA; DHA content, 23.66% of TFA; both in the form of triglyceride; HEBEI HAIYUAN Health biological Science and Technology, China.

††Palmitin: Palmitic acid content, 98.7% of TFA, in the form of methylester; Shanghai Zhixin Chemical, China.

‡‡ARA-enriched oil: ARA content, 53.69% of TFA, in the form ofof ARA-methylester; JIANGSU TIANKAI Biotechnology, China.

§§Mold inhibitor: contained 50% calcium propionic acid and 50% fumaric acid.

Table 3 Fatty acid composition of the experimental diets with graded levels of n-3 LC-PUFA (% total fatty acids)

	Dietary n-3 LC-PUFA contents (% dry weight)				
Fatty acid	**0.52**	**0.94**	**1.57**	**1.97**	**2.43**
14:0	1.17	1.51	2.08	2.35	2.72
16:0	69.64	59.20	47.90	44.71	34.05
18:0	1.50	1.81	1.98	1.91	2.10
20:0	0.73	0.85	1.05	1.09	1.21
∑SFA	73.04	63.37	53.01	50.06	40.08
18:1	6.23	6.47	6.87	6.63	6.96
∑MUFA	6.23	6.47	6.87	6.63	6.96
18:2n-6	8.36	8.95	10.76	9.00	9.25
18:3n-6	0.38	0.27	0.45	0.34	0.40
20:4n-6	2.81	3.20	3.48	3.41	3.77
∑n-6PUFA	11.55	12.42	14.69	12.75	13.42
18:3n-3	1.01	1.08	1.41	1.02	1.06
18:4n-3	0.29	0.32	0.44	0.37	0.43
20:5n-3	1.48	3.11	5.34	6.71	8.56
22:6n-3	2.92	6.09	11.04	13.78	18.22
∑n-3PUFA	5.70	10.59	18.23	21.88	28.27
n-3/n-6PUFA	0.48	0.85	1.25	1.73	2.14
n-3LC-PUFA	4.40	9.20	16.38	20.49	26.78
DHA/EPA*	1.98	1.96	2.07	2.05	2.13
EPA/ARA†	0.53	0.97	1.53	1.97	2.27

*DHA/EPA: 22:6n-3/20:5n-3.

†EPA/ARA: 20:5n-3/20:4n-6.

SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; n-6 PUFA, n-6 poly-unsaturated fatty acids; n-3 PUFA, n-3 poly-unsaturated fatty acids; n-3 LC-PUFA, n-3 long-chain polyunsaturated fatty acids.

poration, Chicago, IL, USA) for Windows. Tukey's multiple range test was chosen as a multiple comparison test and the significance level of 5% was used. For the Elovl4 functional characterization, the saturated VLC-FA profiles from yeast expressing the elovl4 were compared to those of the control yeast transformed with the empty pYES2 vector by a Student's *t*-test ($P < 0.05$).

Result

Sequence analyses of grouper Elovl4

Degenerate primers were used to amplify the PCR product of expected size (684 bp) from grouper liver and the deduced amino acid sequence from the 684 bp product was homologous to other known *elovl4*. Then, two end fragments were amplified by 3′-RACE and 5′-RACE PCR based on the RACE technology. The complete cDNA sequence of *elovl4* was obtained by assembling the three fragments (1st fragement and RACE prod-

Table 4 Formulation and proximate analysis of the experimental diets with graded levels of DHA/EPA (% dry weight)

	DHA/EPA ratio				
Ingredients	**0.82**	**1.28**	**1.67**	**2.01**	**2.33**
White fish meal*	49.00	49.00	49.00	49.00	49.00
Krill meal*	15.00	15.00	15.00	15.00	15.00
Squid meal*	4.00	4.00	4.00	4.00	4.00
Hydrolysed fish meal*	8.00	8.00	8.00	8.00	8.00
LT-Yeast	2.00	2.00	2.00	2.00	2.00
α-starch	4.50	4.50	4.50	4.50	4.50
Alginate sodium	2.00	2.00	2.00	2.00	2.00
Vitamin premix†	1.50	1.50	1.50	1.50	1.50
Mineral premix‡	1.50	1.50	1.50	1.50	1.50
Attractant§	1.50	1.50	1.50	1.50	1.50
Antioxidant	0.10	0.10	0.10	0.10	0.10
Choline chloride	0.20	0.20	0.20	0.20	0.20
DHA-enriched oil¶	0.55	1.92	2.82	3.45	3.89
EPA-enriched oil**	2.35	1.51	0.98	0.60	0.33
Palmitin††	2.70	2.17	1.80	1.55	1.38
ARA-enrich oil‡‡	1.00	1.00	1.00	1.00	1.00
Soy lecithin	4.00	4.00	4.00	4.00	4.00
Mold inhibitor§§	0.10	0.10	0.10	0.10	0.10
Proximate analysis (n = 3)					
Crude protein (%)	56.11	55.77	55.56	55.32	55.94
Crude lipid (%)	17.36	17.56	17.10	18.37	17.80
Ash (%)	16.86	16.71	17.42	16.82	17.13
DHA/EPA	0.82	1.28	1.67	2.01	2.33
n-3HUFA	2.02	2.01	2.02	2.02	2.02

*White fish meal: crude protein 71.18% dry matter, crude lipid 5.32% dry matter; Krill meal: crude protein 63.76% dry matter, crude lipid 12.95% dry matter; Squid meal: crude protein 61.72% dry matter, crude lipid 3.16% dry matter; Hydrolysed fish meal: crude protein 77.10% dry matter, crude lipid 4.60% dry matter.

†Vitamin premix (IU or g kg^{-1} vitamin premix): retinal palmitate, 3 000 000 IU; cholecalciferol, 1 200 000 IU; DL-α-tocopherol acetate, 40.0 g; menadione, 8.0 g; thiamin-HCl, 5.0 g; riboflavin, 5.0 g; D-calcium pantothenate, 16.0 g; pyridoxine-HCl, 4.0 g; meso-inositol, 200.0 g; D-biotin, 8.0 g; folic acid, 1.5 g; para-aminobenzoic acid, 5.0 g; niacin, 20.0 g; cyanocobalamin, 0.01 g; ascorbyl polyphosphate (contained 25% ascorbic acid), 100.0 g.

‡Mineral premix (g kg^{-1}): $Ca(H_2PO_4)_2{\cdot}H_2O$, 675.0; $CoSO_4{\cdot}4H_2O$, 0.15; $CuSO_4{\cdot}5H_2O$, 5.0; $FeSO_4{\cdot}7H_2O$, 50.0; KCl, 50.0; KI, 0.1; $MgSO_4.2H_2O$, 101.7; $MnSO_4{\cdot}4H_2O$, 18.0; NaCl, 80.0; $Na_2SeO_3{\cdot}H_2O$, 0.05; $ZnSO_4{\cdot}7H_2O$, 20.0.

§Attractant (g 100 g^{-1}): betaine, 50; glycine, 15; alanine, 10; argine, 10; taurine, 10; inosine-5′-monophosphoric acid, 5.

¶DHA-enriched oil: DHA content, 40.64% of TFA; in the form of DHA-methylester; JIANGSU TIANKAI Biotechnology, China.

**EPA-enriched oil: EPA content, 46.41% of TFA; DHA content, 23.66% of TFA; both in the form of triglyceride; HEBEI HAIYUAN Health biological Science and Technology, China.

††Palmitin: Palmitic acid content, 98.7% of TFA, in the form of methylester; Shanghai Zhixin Chemical, China.

‡‡ARA-enriched oil: ARA content, 53.69% of TFA, in the form of of ARA-methylester; JIANGSU TIANKAI Biotechnology, China.

§§Mold inhibitor: contained 50% calcium propionic acid and 50% fumaric acid.

Table 5 Fatty acid composition of the experimental diets with graded levels of DHA/EPA (% total fatty acids)

	Dietary DHA/EPA				
Fatty acid	**0.82**	**1.28**	**1.67**	**2.01**	**2.33**
14:0	3.08	3.53	3.93	4.09	4.13
16:0	28.91	29.06	27.34	27.02	26.07
18:0	2.06	2.08	2.08	2.06	2.01
20:0	2.95	2.72	2.63	2.64	2.72
∑SFA	36.99	37.39	35.98	35.82	34.93
16:1	3.74	3.89	3.81	3.96	3.69
18:1	12.32	12.30	12.16	12.18	11.75
∑MUFA	16.06	16.19	15.97	16.14	15.43
18:2n-6	8.84	8.82	9.01	8.93	8.62
20:4n-6	3.81	3.17	3.62	3.45	3.39
∑n-6PUFA	12.66	11.99	12.63	12.38	12.01
18:3n-3	1.24	1.14	1.13	1.10	1.11
20:5n-3	12.82	10.14	8.59	7.60	6.66
22:6n-3	10.52	12.96	14.35	15.26	15.50
∑n-3PUFA	24.57	24.24	24.07	23.96	23.28
n-3/n-6PUFA	1.94	2.02	1.91	1.93	1.94
n-3LC-UFA	23.33	23.10	22.94	22.86	22.17
ARA/EPA*	0.30	0.31	0.42	0.45	0.51
DHA/EPA†	0.82	1.28	1.67	2.01	2.33

*ARA/EPA: 20:4n-6/20:5n-3.

†DHA/EPA: 22:6n-3/20:5n-3.

SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; n-6 PUFA, n-6 polyunsaturated fatty acids; n-3 PUFA, n-3 polyunsaturated fatty acids; n-3 LC-UFA, n-3 long-chain polyunsaturated fatty acids.

ucts). The full-length sequence of *elovl4* mRNA and the deduced amino acids (AA) are shown in Fig. 1. The sequences corresponding to grouper elongase cDNAs (excluding the polyA tail) were 2341 bp. A 301 bp of 5′-untranslated region (UTR), 918 bp of the coding region that encodes a 305 AA protein (Genbank ID: KF533722) and 1122 bp of 3′UTR were included in the *elovl4* cDNA sequence. The calculated molecular mass of the protein was estimated as 35.437 KDa by using Compute pI/Mw (http://web.expasy.org/compute_pi/).

Multiple sequences alignment and phylogenetic analysis

The analysis of the deduced AA sequence of grouper *elovl4* by BLAST indicated that *elovl4* in orange-spotted grouper shares sequence identity with *elovl4* of other teleosts, such as cobia (*R. canadum*, 94%), white-spotted rabbitfish (*Siganus canaliculatus*, 95%), Atlantic salmon (*S. salar*, 86%), zebrafish (*D. rerio*, 85%), more than 60% identity with *elovl4* of human beings (*H. sapiens*, 65%), mouse (*M. musculus*, 65%) and cattle (*B. Taurus*, 65%). However, the deduced AA of grouper *elovl4* was 40% identical with grouper *elovl5* 36–40% identical to teleost *elovl5* sequences respectively.

The grouper *elovl4*-deduced proteins contained the diagnostic histidine box HXXHH motif conserved in all elongases and five membrane-spanning domines. It also possessed a single lysine and arginine residue at the carboxyl terminus, RXKXX in *elovl4* (Fig. 2). The phylogenetic tree was constructed on basis of AA sequence comparisons of grouper *elovl4* and other elongase from fish (zebrafish, Atlantic salmon and rabbitfish) and human (Fig. 3). The phylogenetic analysis showed that the grouper *elovl4* clustered together with their corresponding teleost orthologues, and separately from *elovl2* and *elovl5* cluster.

Functional characterization of grouper putative Elvol4

The putative Elovl4 of grouper was functionally characterized by determining the FA profiles of transformed *S. cerevisiae* with either empty pYES2 vector (control) or the vector containing *elovl4* ORF inserts and grown in the presence of potential FA substrates. To test the ability of grouper to elongate saturated VLC-FA, transgenic yeast was grown incubated with lignoceric acid (24:0). Yeast transformed with the empty vector contained measurable amounts of saturated VLC-FA, 24:0, 26:0, 28:0, 30:0 and 32:0 (Table 6). However, the *elovl4*-transformed yeast showed decreased amounts of 24:0 and 26:0, but increased amounts of 28:0, 30:0, 32:0 and 34:0 (Table 6). These results confirmed that grouper Elovl4 is involved in the biosynthesis of saturated VLC-FA and at least 24:0, 26:0 and 28:0 may be the good substrates for grouper Elovl4. The role of grouper Elovl4 in the biosynthesis of VLC-PUFA was also investigated and transgenic yeast transformed with *elovl4* ORF were incubated with C18 (18:4n-3 and 18:3n-6), C20 (20:5n-3 and 20:4n-6) and C22 (22:5n-3, 22:4n-6 and 22:6n-3) PUFA substrates (Table 7; Fig. 4). Fatty acid composition of the yeast transformed with only pYES2 shows four main FA, namely 16:0, 16:1n-7; 18:0 and 18:1n-9, together with whichever exogenous FA added. However, GC–MS analyses confirmed that grouper Elovl4 could elongate PUFA to the corresponding elongated polyenoic products up to C36 (Table 7; Fig. 4). Elovl4 showed higher activity towards C20

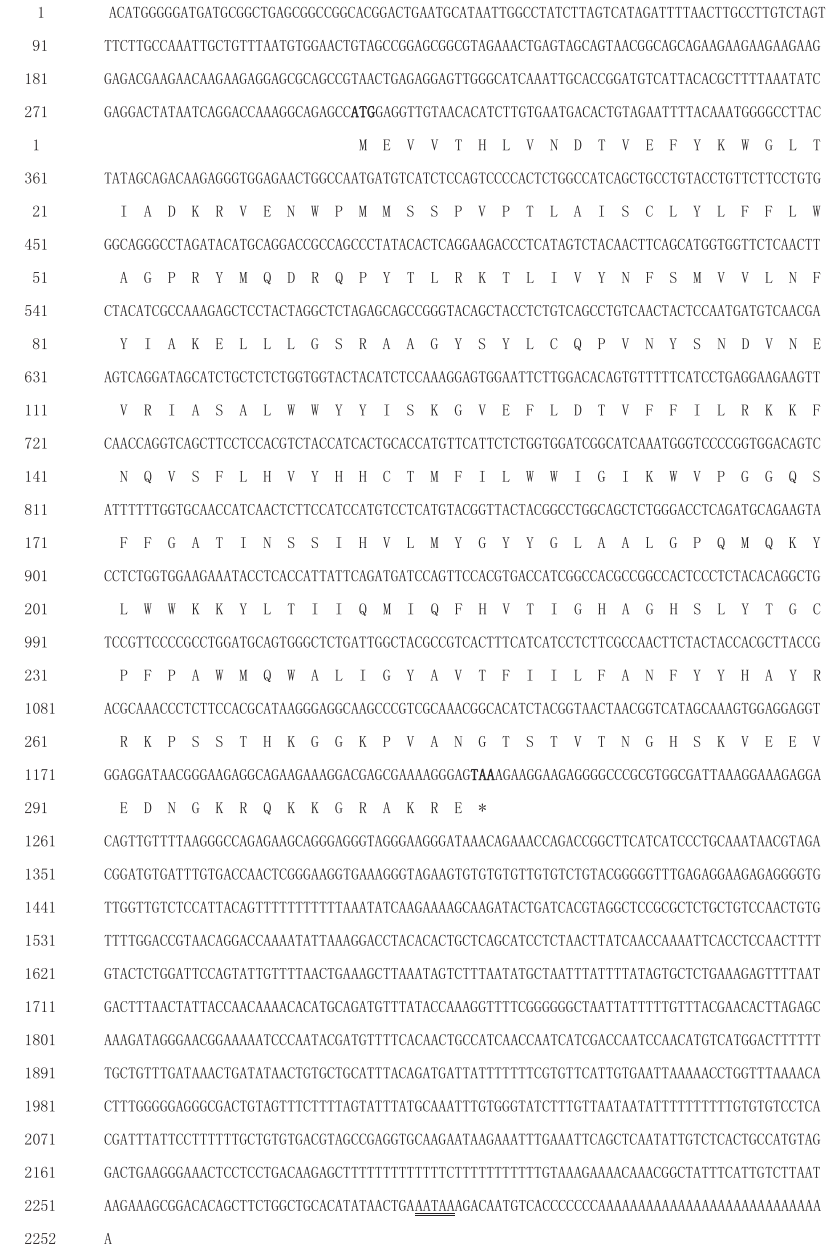

Figure 1 Nucleotide and deduced amino acid sequences of Elovl4 gene. Uppercase letters indicate the translated region and lowercase letters indicate the untranslated region. The start codon (ATG) and the stop codon (TAG) are in bold. Double-underlined letters indicate the polyadenylation signal (AATAA).

(20:5n-3, 29.9% and 20:4n-6, 33.1%) and C22 (22:5n-3, 43.8% and 22:4n-6, 51.0%) and low activity towards C18 (18:4n-3, 5.6% and 18:3n-6, 9.6%) (Table 7). It is noteworthy that grouper Elovl4 was able to convert both 20:5n-3 and 22:5n-3 to 24:5n-3, the substrate for DHA biosynthesis via the Sprecher shunt pathway (Sprecher 2000). However, grouper Elovl4 showed relative low activity towards DHA, consistent with the result in Suh and Clandinin (2005).

Tissue expression of the putative elovl4

The expression level of *elovl4* varied among different tissues. The transcription of *elovl4* was detected in eye, brain, testis, heart, liver, kidney, stomach,

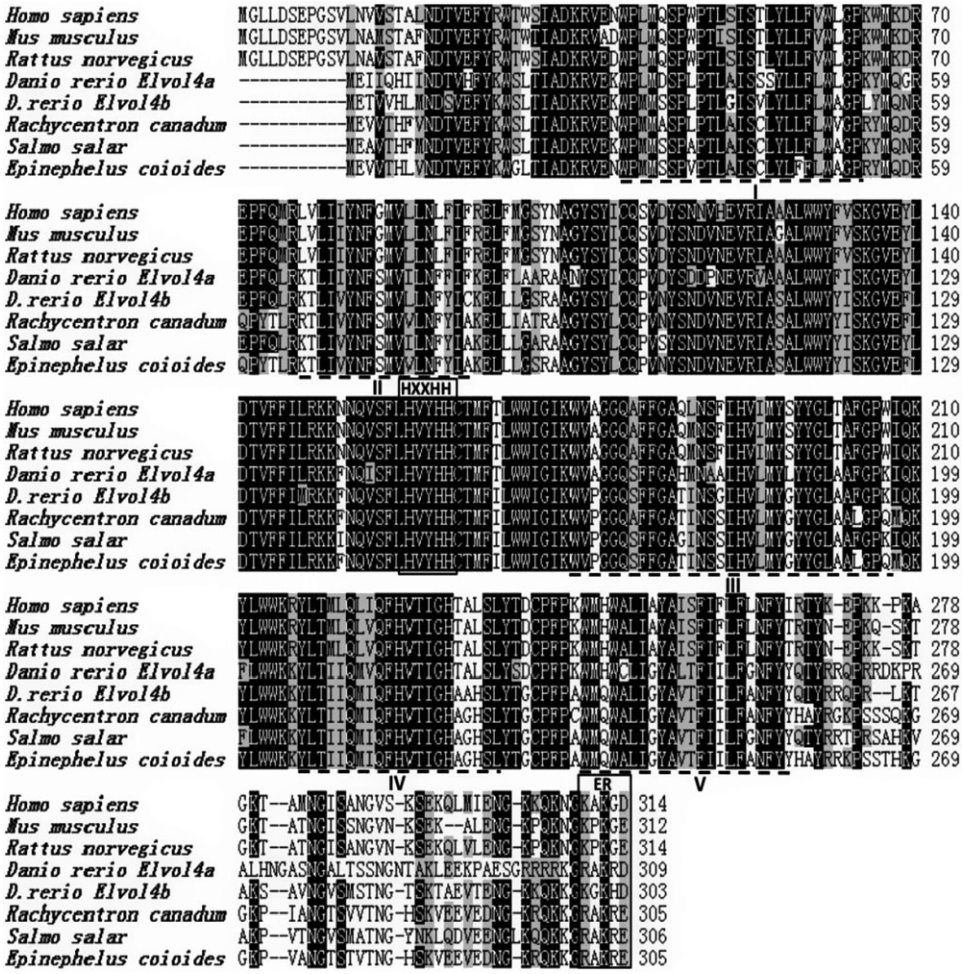

Figure 2 Comparison of the deduced amino acid sequences of Elovl4 from orange-spotted grouper, other fish, mouse and human. The AA sequences were aligned using ClustalX, and identity/similarity shading was based on a 75% identity threshold. Identical residues are shaded black and similar residues are shaded grey. Indicated are the conserved HXXHH histidine box motif, five (I–V) putative membrane-spanning domains and the ER retrieval signal predicted by Zhang, Yang, Karan, Hashimoto, Baehr, Yang and Zhang (2003).

intestine and muscle. Relatively high expression of *elovl4* were observed in eye, brain and testis, then in liver and kidney, and weekly in heart, muscle, stomach and intestine. The highest transcriptional level of *elovl4* was detected in eye and brain, more than 30 folds than the corresponding value in muscle, stomach and intestine (Fig. 5).

Effect of n-3LC-PUFA levels and DHA/EPA on the expression of the putative elovl4

Relative mRNA expression of *elovl4* of grouper larvae was significantly affected by dietary n-3 LC-PUFA ($P < 0.05$). The relative expression of *elovl4* in the first two treatments was significantly higher than 2.43% n-3 LC-PUFA group. The *elovl4* transcript levels were up-regulated by 0.41-fold, 0.79-fold, 0.31-fold and 0.26-fold in the level of 0.52%, 0.94%, 1.57% and 1.97% treatments compared with the treatment of 2.43% n-3 LC-PUFA respectively (Fig. 6a). The mRNA level of *elovl4* also showed a significantly negative linear relationship relative to dietary n-3 LC-PUFA with an $R = 0.7467$ ($Y = -0.1351X + 1.352$). However, no significant differences were observed in the expression of Elovl4 in larvae fed diets with graded levels of DHA/EPA (Fig. 6b).

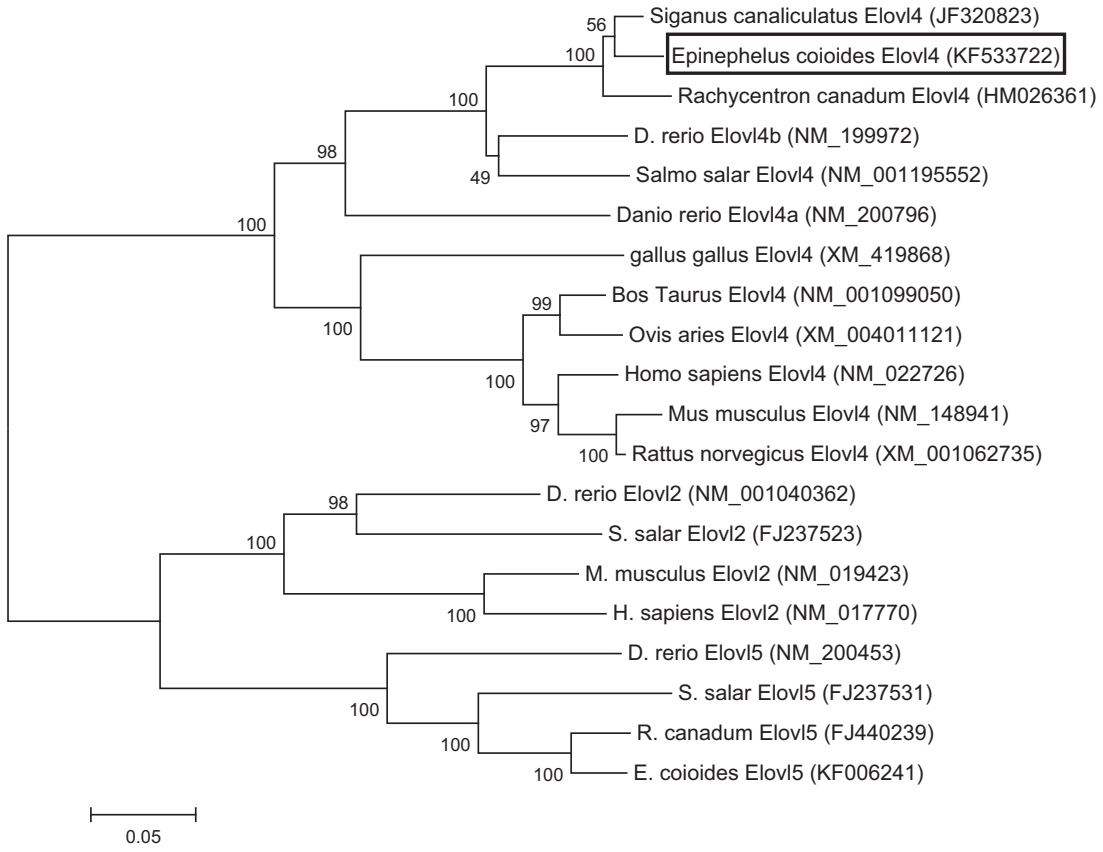

Figure 3 Phylogenetic tree comparing the grouper Elovl4 with elongase proteins from other organisms. The tree was constructed using the Neighbour-Joining method (Saitou & Nei 1987) using MEGA4. The horizontal branch length is proportional to amino acid substitution rate per site. The numbers represent the frequencies (%) with which the tree topology presented was replicated after 1000 iterations.

Discussion

Elovl are crucial enzymes for the condensation of activated FAs with malonyl-CoA in the long-chain FA elongation pathway (Nugteren 1965). Fish Elovl cDNAs including *elovl5* and *elovl2* have been cloned and functionally characterized from several species including freshwater species, the salmonids and marine species (Agaba, Tocher, Dickson, Dick & Teale 2004; Hastings, Agaba, Tocher, Zheng, Dickson, Dick & Teale 2004; Meyer, Kirsch, Domergue, Abbadi, Sperling, Bauer, Cirpus, Zank, Moreau & Heinz 2004; Agaba *et al.* 2005; Morais *et al.* 2009; Zheng, Ding, Xu, Monroig, Morais & Tocher 2009; Gregory, See, Gibson & Schuller 2010; Mohd-Yusof, Monroig, Mohd-Adnan, Wan & Tocher 2010; Monroig *et al.* 2012). In recent years, Elovl4 has been discovered in zebrafish (Monroig *et al.* 2010), Atlantic salmon (Carmona-Antoñanzas *et al.* 2011), cobia (Monroig *et al.* 2011) and rabbitfish (Monroig *et al.* 2012). Through the functional characterization in heterologous expression in *S. cerevisiae*, the ability of Elovl4 to effectively elongate C22 PUFA made it possible to form DHA from EPA.

In this study, a full-length cDNA of a putative *elovl4* was first cloned in orange-spotted grouper. The deduced 305 AA showed high identity with *elovl4* of other teleosts, particular cobia (*R. canadum*, 94%), white-spotted rabbitfish (*S. canaliculatus*, 95%), Atlantic salmon (*S. salar*, 86%), zebrafish (*D. rerio*, 85%). The putative grouper *elovl4*-deduced proteins possessed conserved region, the diagnostic histidine box HXXHH motif, conserved in all elongases and also characteristic of desaturase and hydrolase enzymes con-

Table 6 Functional characterisation of the grouper Elovl4 elongase: Role in biosynthesis of very long-chain saturated fatty acids (FA). Results are expressed as an area percentage of total saturated FA C ≥ 24 found in yeast transformed with either the empty pYES2 vector (Control) or the grouper *elovl4* ORF

FA	Control	Elovl4
24:0	9.1 ± 0.4	6.9 ± 1.2
26:0	81.5 ± 3.1	69.5 ± 2.5*
28:0	7.5 ± 2.8	20.9 ± 0.5*
30:0	1.5 ± 0.1	2.4 ± 0.8
32:0	0.3 ± 0.2	0.4 ± 0.2
34:0	nd	0.0 ± 0.1

Results are means ± standard deviations ($N = 3$). Asterisks ('*') indicate means are statistically different between treatments (Student's *t*-test, $P < 0.05$).

taining a di-iron-oxo cluster (Fe-O-Fe), which was involved in the coordination of electron reception during reactions occurring during FA elongation (Jakobsson, Westerberg & Jacobsson 2006). The putative grouper *elovl4* possessed an arginine residue and one lysine residue (RXKXX) in position-5 and -3 from the C-terminus of the protein, respectively, which was crucial for endoplasmatic reticulum retrieval signal function (Jackson, Nilsson & Peterson 1990). The RXKXX pattern, indicating its role in LC-PUFA synthesis (Cook & McMaster 2004), is common to other teleost *elovl4* including cobia, white-spotted rabbitfish, Atlantic salmon and zebrafish *elovl4a* (Monroig *et al.* 2010, 2011, 2012; Carmona-Antoñanzas *et al.* 2011).

The phylogenetic analysis revealed that the grouper *elovl4* cDNA encodes a protein more similar to the other Elovl4 orthologues from teleosts and mammals, than other Elovl family in fish species, the Elovl2 and Elovl5. Zebrafish have two Elovl4, Elovl4a and Elovl4b, and they showed marked differences in their substrate specificity (Monroig *et al.* 2011). Elovl4a could only efficiently elongate saturated VLC-FA up to C36, whereas Elovl4b could elongate PUFA substrates to corresponding elongated polyenoic products (Monroig *et al.* 2010). The teleost Elovl4 proteins were separated to two groups, with zebrafish Elovl4a separated with Zebrafish Elovl4b and other teleost Elovl4. Previous studies have found that the function of Elovl4 in cobia, white-spotted rabbitfish and Atlantic salmon was similar to Elovl4b in zebrafish (Carmona-Antoñanzas *et al.* 2011; Monroig *et al.* 2011, 2012). Compared with Elovl4a, the phylogenetic analysis indicate that the

Table 7 Functional characterisation of the grouper Elovl4 elongase: conversions of polyunsaturated fatty acid (FA) substrates. Conversions were calculated for each stepwise elongation according to the formula [areas of first product and longer chain products/(areas of all products with longer chain than substrate + substrate area)] × 100. The substrate FA varies as indicated in each step-wise elongation

FA substrate	FA Product	% Conversion	Elongation
18:4n-3	20:4n-3	5.6	C18→34
	22:4n-3	40.0	C20→34
	24:4n-3	80.8	C22→34
	26:4n-3	96.1	C24→34
	28:4n-3	97.8	C26→34
	30:4n-3	96.2	C28→34
	32:4n-3	80.6	C30→34
	34:4n-3	6.4	C32→34
18:3n-6	20:3n-6	9.6	C18→34
	22:3n-6	52.4	C20→34
	24:3n-6	79.6	C22→34
	26:3n-6	95.0	C24→34
	28:3n-6	97.0	C26→34
	30:3n-6	94.4	C28→34
	32:3n-6	32.9	C30→34
	34:3n-6	2.4	C32→34
20:5n-3	22:5n-3	29.9	C20→36
	24:5n-3	72.0	C22→36
	26:5n-3	86.4	C24→36
	28:5n-3	97.3	C26→36
	30:5n-3	99.2	C28→36
	32:5n-3	89.5	C30→36
	34:5n-3	25.5	C32→36
	36:5n-3	0.9	C34→36
20:4n-6	22:4n-6	33.1	C20→36
	24:4n-6	66.2	C22→36
	26:4n-6	80.9	C24→36
	28:4n-6	92.3	C26→36
	30:4n-6	95.0	C28→36
	32:4n-6	53.5	C30→36
	34:4n-6	5.4	C32→36
	36:4n-6	1.4	C34→36
22:5n-3	24:5n-3	43.8	C22→36
	26:5n-3	81.8	C24→36
	28:5n-3	96.1	C26→36
	30:5n-3	99.0	C28→36
	32:5n-3	85.4	C30→36
	34:5n-3	16.4	C32→36
	36:5n-3	0.7	C34→36
22:4n-6	24:4n-6	51.0	C22→36
	26:4n-6	86.0	C24→36
	28:4n-6	95.6	C26→36
	30:4n-6	97.5	C28→36
	32:4n-6	61.0	C30→36
	34:4n-6	7.7	C32→36
	36:4n-6	1.2	C34→36
22:6n-3	24:6n-3	8.8	C22→34
	26:6n-3	100.0	C24→34
	28:6n-3	100.0	C26→34
	30:6n-3	75.4	C28→34
	32:6n-3	89.1	C30→34
	34:6n-3	10.8	C32→34

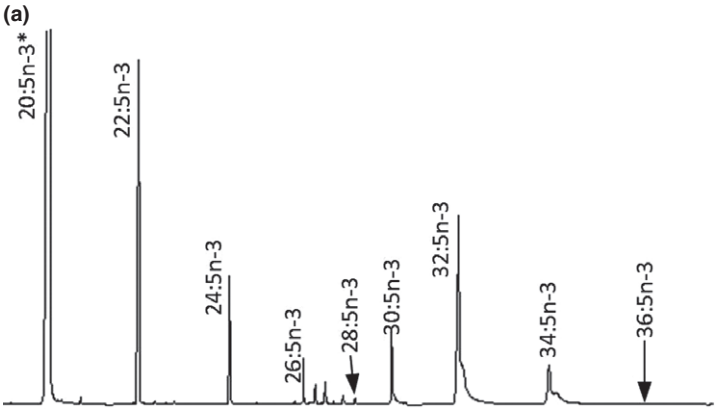

Figure 4 Role of grouper Elovl4 in the biosynthesis of very long-chain fatty acids (VLC-PUFA). Yeast transformed with pYES2 vector containing the *elovl4* ORF was grown in the presence of PUFA substrates 20:5n-3 (A) and 22:5n-3 (B), and fatty acid composition was determined. Substrates ('*') and their corresponding elongated products are indicated accordingly. Vertical axis, MS response; horizontal axis, retention time.

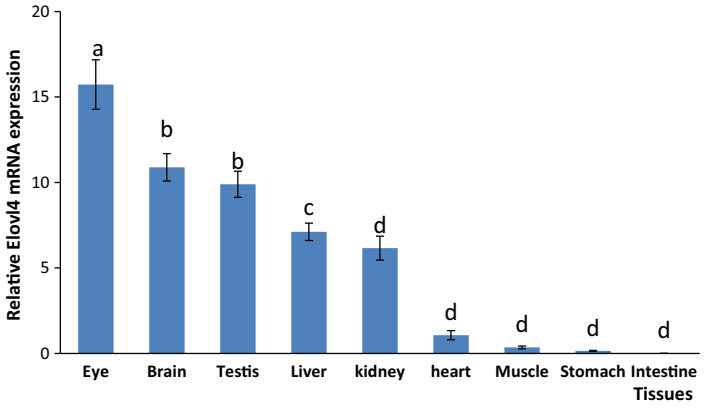

Figure 5 Tissue expression of Elovl4 in orange-spotted grouper. Results are expressed as mean± standard error ($n = 3$). Different letters above the bars denote significant ($P < 0.05$) differences among tissues.

grouper Elovl4 has close kinship to Elovl4b, which may indicate that the function of grouper Elovl4 was similar to Elovl4b.

The functional characterization of grouper Elovl4 was also investigated in this study and it was convinced that the function of grouper Elovl4 has great similarities to zebrafish Elovl4b. Briefly, grouper Elovl4 was efficient in the biosynthesis of saturated VLC-FA, with 24:0, 26:0 and 28:0 as preferred substrates. Meanwhile, grouper Elovl4 also showed high efficiency in the elongation of C20 (20:5n-3 and 20:4n-6) and C22 (22:5n-3 and 22:4n-6) PUFA to corresponding elongated polyenoic products with C36 chain-lengths. It has been reported that VLC-FAs were abundant in retina (Aveldaño 1987) and brain (Robinson, Johnson & Poulos 1990) and testis (Zadravec, Tvrdik, Guillou, Haslam, Kobayashi, Napier, Capecchi & Jacobsson 2011), which was consistent with the tissue distribution analysis of *elovl4*

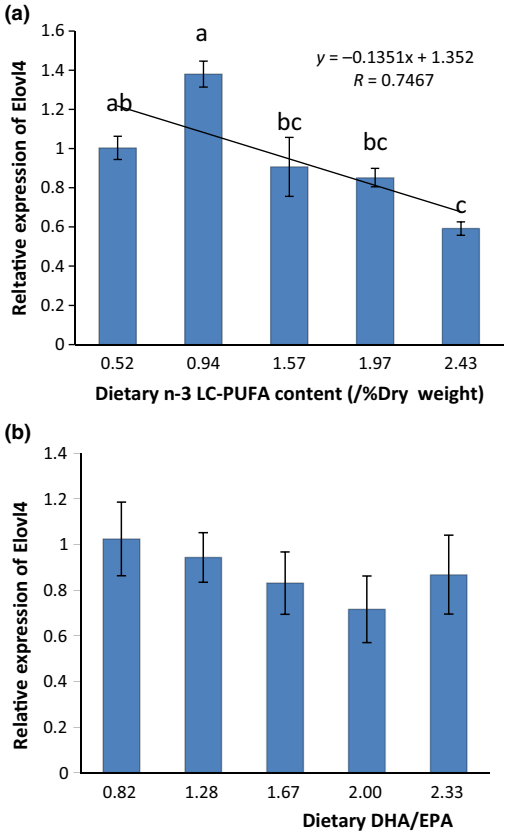

Figure 6 Relative mRNA expression of elovl4 in visceral mass of grouper fed diets with graded levels of n-3 LC-PUFA (a) and DHA/EPA (b). Results are expressed as means ± standard error (n = 3). Different letters above the bars denote significant ($P < 0.05$) differences among dietary groups. A line with R value was shown across the five bars if significant linear relationship was detected ($P < 0.05$).

mRNA transcripts. This may indicate that brain, eye and testis were the prominent metabolic sites for the biosynthesis of VLC-FA. Unlike freshwater species and the salmonids, marine fish species also appear to lack Elovl2, regarded as an essential enzyme in DHA biosynthesis (Monroig *et al.* 2009; Morais *et al.* 2009). However, along with the discovery of Elovl4, marine fish species could also synthesize DHA from EPA through sprecher pathway (Monroig *et al.* 2011). It was found in the present study that grouper Elovl4 have higher activity towards C20 (20:5n-3 and 20:4n-6) and C22 (22:5n-3 and 22:4n-6). In particular, grouper Elovl4 was able to effectively convert both 20:5n-3 and 22:5n-3 to 24:5n-3, the substrate for DHA biosynthesis via the Sprecher shunt pathway (Sprecher 2000). This may indicate the potential role of grouper Elovl4 in the biosynthesis of DHA and Elovl4 could compensate for the lacking of Elovl2 in marine fish species.

The content of DHA and EPA, potentially compromising their nutritional benefit to the human consumer, was reduced with the increasing use of vegetable oils, which are devoid of LC-PUFA (Izquierdo, Obach, Arantzamendi, Montero, Robaina & Rosenlund 2003; Torstensen, Frøyland, Ørnsrud & Lie 2004). The research on regulational mechanism of the enzymes involved in LC-PUFA biosynthesic pathway may make effective use of vegetable oil in aquafeeds (Tocher 2010). However, studies of those enzymes of teleosts involved in LC-PUFA biosynthetic pathway remain in transcriptional level, and mainly focused on Fads2, Elovl5 and Elovl2. As for Elovl4, Monroig *et al.* (2010) have studied spatial-temporal expression of zebrafish *elovl4* genes. However, to the best of our knowledge, little information was available on the nutritional regulation of *elovl4*. In the present study, the mRNA expression of *elovl4* was down-regulated by dietary n-3 LC-PUFA. Meanwhile, similar results have been found in the study of *elovl5* expression (Zheng *et al.* 2005; Ling *et al.* 2006; Morais *et al.* 2011). This may indicate that the nutritional regulation of *elovl4* may have some characteristics similar to *elovl5*. The same changing trend of *elovl4* and *elovl5* expression in response to dietary n-3 LC-PUFA may confirm the hypothesis to some extent. Unlike the response to dietary n-3 LC-PUFA, the expression of *elovl4* was not significantly affected by the level of DHA/EPA in this study. In the study of salmon, it was found that both DHA and EPA have similar effects on regulating the expression of LXR and SREBP-1, potential regulator of FA desaturase and elongation ((Minghetti, Leaver & Tocher 2011). Although Elovl4 has the ability to elongate EPA to form DHA, the similar effect on the transcription factor (LXR and SREBP-1) may account for the response of *elovl4* to dietary DHA/EPA and further study should be conducted to investigate the specific regulation mechanism.

In conclusion, the cDNA of *elovl4* was first cloned from orange-spotted grouper. The *elovl4* possessed all the features of Elovl proteins and is phylogenetically close to other *elovl4* orthologues of teleosts. Heterologous expression in yeast demonstrated that grouper Elovl4 could elongate saturated FAs, especially 24:0 and 26:0, up to

36:0. Also, grouper Elovl4 effectively converted C20 and C22 polyunsaturated FAs to elongated polyenoic products up to C36. High LC-PUFA level significantly decreased the expression of *elovl4*, whereas the level of DHA/EPA have no significant influence on it.

Acknowledgments

This study was financially supported by the National Science-technology Support Plan Projects (2011BAD13B01) and National Natural Science Foundation of China (31172425). We thank B. Han, H.B. Hu for their help in diet production and gratitude also is given to K. Liu, C. C. Su and X.L. Liu for their help during the sampling. We also thank X.J. Dong, R.T. Zuo for their help in gene clone and expression analysis.

Conflict of interest: No conflict of interest was existed.

References

Agaba M., Tocher D.R., Dickson C.A., Dick J.R. & Teale A.J. (2004) Zebrafish cDNA encoding multifunctional fatty acid elongase involved in production of eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids. *Marine Biotechnology* **6**, 251–261.

Agaba M.K., Tocher D.R., Zheng X., Dickson C.A., Dick J.R. & Teale A.J. (2005) Cloning and functional characterisation of polyunsaturated fatty acid elongases of marine and freshwater teleost fish. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **142**, 342–352.

Aveldaño M.I. (1987) A novel group of very long chain polyenoic fatty acids in dipolyunsaturated phosphatidylcholines from vertebrate retina. *Journal of Biological Chemistry* **262**, 1172–1179.

Calon F. & Cole G. (2007) Neuroprotective action of omega-3 polyunsaturated fatty acids against neurodegenerative diseases: evidence from animal studies. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **77**, 287–293.

Cameron D.J., Tong Z., Yang Z., Kaminoh J., Kamiyah S., Chen H., Zeng J., Chen Y., Luo L. & Zhang K. (2007) Essential role of Elovl4 in very long chain fatty acid synthesis, skin permeability barrier function, and neonatal survival. *International Journal of Biological Sciences* **3**, 111–119.

Carmona-Antoñanzas G., Monroig Ó., Dick J.R., Davie A. & Tocher D.R. (2011) Biosynthesis of very long-chain fatty acids (C > 24) in Atlantic salmon: cloning, functional characterisation, and tissue distribution of an Elovl4 elongase. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **159**, 122–129.

Cook H.W. & McMaster R.C.R. (2004) Fatty acid desaturation and chain elongation in eukaryotes. In: *Biochemistry of Lipids, Lipoproteins and Membranes* (ed. by D.E. Vance & J.E. Vance). Elsevier, Amsterdam, the Netherlands.

Eilander A., Hundscheid D.C., Osendarp S.J., Transler C. & Zock P.L. (2007) Effects of n-3 long chain polyunsaturated fatty acid supplementation on visual and cognitive development throughout childhood: a review of human studies. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **76**, 189–203.

Gregory M.K., See V.H., Gibson R.A. & Schuller K.A. (2010) Cloning and functional characterisation of a fatty acyl elongase from southern bluefin tuna (*Thunnus maccoyii*). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **155**, 178–185.

Hastings N., Agaba M., Tocher D.R., Leaver M.J., Dick J.R., Sargent J.R. & Teale A.J. (2001) A vertebrate fatty acid desaturase with Δ5 and Δ6 activities. *Proceedings of the National Academy of Sciences of the USA* **98**, 14304–14309.

Hastings N., Agaba M.K., Tocher D.R., Zheng X., Dickson C.A., Dick J.R. & Teale A.J. (2004) Molecular cloning and functional characterization of fatty acyl desaturase and elongase cDNAs involved in the production of eicosapentaenoic and docosahexaenoic acids from α-linolenic acid in Atlantic salmon (*Salmo salar*). *Marine Biotechnology* **6**, 463–474.

Ishak S.D., Tan S.H., Khong H.K., Jaya-Ram A., Enyu Y.L., Kuah M.K. & Shu-Chien A.C. (2008) Upregulated mRNA expression of desaturase and elongase, two enzymes involved in highly unsaturated fatty acids biosynthesis pathways during follicle maturation in zebrafish. *Reproductive Biology and Endocrinology* **6**, 56–65.

Izquierdo M.S., Obach A., Arantzamendi L., Montero D., Robaina L. & Rosenlund G. (2003) Dietary lipid sources for seabream and seabass: growth performance, tissue composition and flesh quality. *Aquaculture Nutrition* **9**, 397–407.

Jackson M.R., Nilsson T. & Peterson P.A. (1990) Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *The EMBO Journal* **9**, 3153–3162.

Jakobsson A., Westerberg R. & Jacobsson A. (2006) Fatty acid elongases in mammals: their regulation and roles in metabolism. *Progress in lipid research* **45**, 237–249.

Li Y.Y., Monroig Ó., Zhang L., Wang S.Q., Zheng X., Dick J.R., You C.H. & Tocher D.R. (2010) Vertebrate fatty acyl desaturase with Δ4 activity. *Proceedings of the National Academy of Sciences* **107**, 16840–16845.

Li S., Mai K., Xu W., Yuan Y., Zhang Y. & Ai Q. (2014) Characterization, mRNA expression and regulation of Δ6 fatty acyl desaturase (FADS2) by dietary n-3 long chain polyunsaturated fatty acid (LC-PUFA) levels in

grouper larvae (*Epinephelus coioides*). *Aquaculture* **434**, 212–219.

Lin H.Z., Liu Y.J., He J.G., Zheng W.H. & Tian L.X. (2007) Alternative vegetable lipid sources in diets for grouper, *Epinephelus coioides* (Hamilton): effects on growth, and muscle and liver fatty acid composition. *Aquaculture Research* **38**, 1605–1611.

Ling S., Kuah M.K., Sifzizul T., Muhammad T.S.T., Kolkovski S. & Shu-Chien A.C. (2006) Effect of dietary LC-PUFA on reproductive performance, tissue fatty acid profile and desaturase and elongase mRNAs in femaleswordtail *Xiphophorus helleri*. *Aquaculture* **261**, 204–214.

Livak K.J. & Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408.

Meyer A., Kirsch H., Domergue F., Abbadi A., Sperling P., Bauer J., Cirpus P., Zank T.K., Moreau H. & Heinz E. (2004) Novel fatty acid elongases and their use for the reconstitution of docosahexaenoic acid biosynthesis. *Journal of Lipid Research* **45**, 1899–1909.

Millamena O.M. (2002) Replacement of fish meal by animal by-product meals in a practical diet for grow-out culture of grouper *Epinephelus coioides*. *Aquaculture* **204**, 75–84.

Minghetti M., Leaver M.J. & Tocher D.R. (2011) Transcriptional control mechanisms of genes of lipid and fatty acid metabolism in the Atlantic salmon (*Salmo salar* L.) established cell line, SHK-1. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **1811**, 194–202.

Mohd-Yusof N.Y., Monroig O., Mohd-Adnan A., Wan K.L. & Tocher D.R. (2010) Investigation of highly unsaturated fatty acid metabolism in the Asian sea bass, *Lates calcarifer*. *Fish physiology and biochemistry* **36**, 827–843.

Monroig Ó., Rotllant J., Sánchez E., Cerdá-Reverter J.M. & Tocher D.R. (2009) Expression of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis genes during zebrafish *Danio rerio* early embryogenesis. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **1791**, 1093–1101.

Monroig Ó., Rotllant J., Cerdá-Reverter J.M., Dick J.R., Figueras A. & Tocher D.R. (2010) Expression and role of Elovl4 elongases in biosynthesis of very long-chain fatty acids during zebrafish (Danio rerio) early embryonic development. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **1801**, 1145–1154.

Monroig Ó., Webb K., Ibarra-Castro L., Holt G.J. & Tocher D.R. (2011) Biosynthesis of long-chain polyunsaturated fatty acids in marine fish: characterization of an Elovl4-like elongase from cobia (*Rachycentron canadum*) and activation of the pathway during early life stages. *Aquaculture* **312**, 145–153.

Monroig Ó., Wang S., Zhang L., You C., Tocher D.R. & Li Y.Y. (2012) Elongation of long-chain fatty acids in rabbitfish Siganus canaliculatus: cloning, functional characterisation and tissue distribution of Elovl5-and Elovl4-like elongases. *Aquaculture* **350**, 63–70.

Monroig Ó., Tocher D.R., Hontoria F. & Navarro J.C. (2013) Functional characterisation of a Fads2 fatty acyl desaturase with Δ6/Δ8 activity and an Elovl5 with C16, C18 and C20 elongase activity in the anadromous teleost meagre (*Argyrosomus regius*). *Aquaculture* **412**, 14–22.

Morais S., Monroig O., Zheng X., Leaver M.J. & Tocher D.R. (2009) Highly unsaturated fatty acid synthesis in Atlantic salmon: characterization of ELOVL5-and ELOVL2-like elongases. *Marine Biotechnology* **11**, 627–639.

Morais S., Mourente G., Ortega A., Tocher J.A. & Tocher D.R. (2011) Expression of fatty acyl desaturase and elongase genes, and evolution of DHA: EPA ratio during development of unfed larvae of Atlantic bluefin tuna (*Thunnus thynnus* L.). *Aquaculture* **313**, 129–139.

Nakamura M.T., Cho H.P., Xu J., Tang Z. & Clarke S.D. (2001) Metabolism and functions of highly unsaturated fatty acids: an update. *Lipids* **36**, 961–964.

Nugteren D. (1965) The enzymic chain elongation of fatty acids by rat-liver microsomes. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism* **106**, 280–290.

Peng M., Xu W., Mai K., Zhou H., Zhang Y., Liufu Z., Zhang K. & Ai Q. (2014) Growth performance, lipid deposition and hepatic lipid metabolism related gene expression in juvenile turbot (*Scophthalmus maximus* L.) fed diets with various fish oil substitution levels by soybean oil. *Aquaculture* **433**, 442–449.

Robinson B.S., Johnson D.W. & Poulos A. (1990) Unique molecular species of phosphatidylcholine containing very-long-chain (C24–C38) polyenoic fatty acids in rat brain. *Biochemical Journal* **265**, 763–767.

Ruxton C., Reed S.C., Simpson M. & Millington K. (2004) The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. *Journal of Human Nutrition and Dietetics* **17**, 449–459.

Saitou N. & Nei M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution* **4**, 406–425.

Salem N. Jr, Litman B., Kim H.Y. & Gawrisch K. (2001) Mechanisms of action of docosahexaenoic acid in the nervous system. *Lipids* **36**, 945–959.

Sprecher H. (2000) Metabolism of highly unsaturated n-3 and n-6 fatty acids. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **1486**, 219–231.

Suh M. & Clandinin M.T. (2005) 20:5n-3 but not 22:6n-3 is a preferred substrate for synthesis of n-3 very-long-chain fatty acids (C24–C36) in retina. *Current Eye Research* **30**, 959–968.

Tan S.H., Chung H.H. & Shu-Chien A.C. (2010) Distinct developmental expression of two elongase family mem-

bers in zebrafish. *Biochemical and biophysical research communications* **393**, 397–403.

Tocher D.R. (2003) Metabolism and functions of lipids and fatty acids in teleost fish. *Reviews in Fisheries Science* **11**, 107–184.

Tocher D.R. (2010) Fatty acid requirements in ontogeny of marine and freshwater fish. *Aquaculture Research* **41**, 717–732.

Torstensen B.E., Frøyland L., Ørnsrud R. & Lie Ø. (2004) Tailoring of a cardioprotective fillet fatty acid composition of Atlantic salmon (Salmo salar) fed vegetable oils. *Food Chemistry* **87**, 567–580.

Zadravec D., Tvrdik P., Guillou H., Haslam R., Kobayashi T., Napier J.A., Capecchi M.R. & Jacobsson A. (2011) Elovl2 controls the level of n-6 28:5 and 30:5 fatty acids in testis, a prerequisite for male fertility and sperm maturation in mice. *Journal of Lipid Research* **52**, 245–255.

Zhang X.M., Yang Z., Karan G., Hashimoto T., Baehr W., Yang X.J. & Zhang K. (2003) Elovl4 mRNA distribution in the developing mouse retina and phylogenetic conservation of Elovl4 genes. *Molecular Vision* **9**, 301–307.

Zheng X., Torstensen B.E., Tocher D.R., Dick J.R., Henderson R.J. & Bell J.G. (2005) Environmental and dietary influences on highly unsaturated fatty acid biosynthesis and expression of fatty acyl desaturase and elongase genes in liver of Atlantic salmon (*Salmo salar*). *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **1734**, 13–24.

Zheng X., Ding Z., Xu Y., Monroig O., Morais S. & Tocher D.R. (2009) Physiological roles of fatty acyl desaturases and elongases in marine fish: characterisation of cDNAs of fatty acyl Δ6 desaturase and elovl5 elongase of cobia (*Rachycentron canadum*). *Aquaculture* **290**, 122–131.

