Title

Elongation of long-chain fatty acids in rabbitfish *Siganus canaliculatus*: Cloning, functional characterisation and tissue distribution of Elovl5- and Elovl4-like elongases

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Abstract

Elongases of very long-chain fatty acids (Elovl) catalyse the rate-limiting step of the elongation pathway that results in net 2C elongation of pre-existing fatty acyl chains. As the biosynthesis of long-chain polyunsaturated fatty acids (LC-PUFA) is particularly relevant in fish, Elovl involved in the pathway have been investigated in various of studies. Here we report the molecular cloning, functional characterisation and tissue distribution of two distinct *elovl*-like cDNAs isolated from the herbivorous marine teleost *Siganus canaliculatus*. Unlike the carnivorous marine fish previously investigated, we hypothesise that the rabbitfish has an enhanced LC-PUFA biosynthetic capability as previously anticipated in a former study on fatty acyl desaturases (Fad). The results of the present study showed that rabbitfish expresses at least two *elovl* cDNAs, which have high homology in sequence and function to Elovl5 and Elovl4 elongases that have been investigated previously in other fish species. Furthermore, the results confirm that the activities of the Elovl5 and Elovl4 enzymes enable rabbitfish to perform all the elongation reactions required for the biosynthesis of the physiologically essential C_{20-22} LC-PUFA including eicosapentaenoic (20:5n-3), arachidonic (20:4n-6) and docosahexaenoic (22:6n-3, DHA) acids, as well as the less common very long-chain fatty acids (>C_{24}). Rabbitfish is thus the first marine teleost in which genes encoding Fad and Elovl enzymes, with all the activities required for the production of DHA from C_{18} PUFA, have been characterised.

Keywords

Elovl4; Elovl5; fatty acid biosynthesis; *Siganus canaliculatus*.

Introduction

The molecular and biochemical mechanisms controlling the production of long-chain polyunsaturated fatty acids (LC-PUFA), including eicosapentaenoic (20:5n-3, EPA), docosahexaenoic (22:6n-3, DHA) and arachidonic (20:4n-6, ARA) acids, have been intensively investigated in fish. These studies have been driven by the role that fish play as unique dietary sources of these health-promoting compounds, particularly n-3 LC-PUFA, for human consumers (Bardon et al., 1996; Brouwer et al., 2006; Calder, 2006; Calder and Yaqoob, 2009; Eilander et al., 2007; Ruxton et al., 2007). In addition, a comprehensive understanding of the *de novo* biosynthetic capacity of farmed fish is required to determine which PUFA are the essential fatty acids that must be provided in the diet to ensure normal growth and development (Tocher et al., 2003). Elongases of very long-chain fatty acids (Elovl) are key microsomal enzymes involved in the biosynthesis of fatty acids (FA) with C_{18} or longer chain-lengths. Elovl catalyse the condensation reaction, which is the rate-limiting step in the two carbon elongation of pre-existing fatty acyl chains (Nugteren, 1965). The mammalian Elovl protein family consists of seven members (Elovl 1-7) and, generally, Elovl2, Elovl4 and Elovl5 are regarded as critical enzymes in the elongation of PUFA (Jakobsson et al., 2006).

The zebrafish (*Danio rerio*) Elovl5 was the first Elovl-like cDNA that was cloned and functionally characterised from a fish species (Agaba et al., 2004). Subsequently, further Elovl5-encoding cDNAs were investigated in other species including Atlantic salmon (*Salmo salar*), African catfish (*Clarius gariepinus*), tilapia (*Oreochromis niloticus*), turbot (*Psetta maxima*), gilthead sea bream (*Sparus aurata*), Atlantic cod (*Gadus morhua*), cobia (*Rachycentron canadum*), barramundi (Lates calcarifer) and Southern (*Thunnus maccoyii*) and Northern bluefin (*Thunnus thynnus*) tuna (Agaba et al., 2004, 2005; Hastings et al., 2005; Morais et al., 2009, 2011; Zheng et al., 2009;

Gregory et al., 2010; Mohd-Yusof et al., 2010). These studies confirmed that fish Elovl5, similar to mammalian homologues (Jakobsson et al., 2006), have the ability to preferentially elongate C_{18} (18:4n-3 and 18:3n-6) and C_{20} (20:5n-3 and 20:4n-6) PUFA, with only low activity towards C_{22} PUFA (22:5n-3 and 22:4n-6).

Studies on other Elovl enzymes involved in the LC-PUFA biosynthetic pathways have enabled a fuller understanding of the FA elongation pathways in fish. Thus, *elovl2* have been cloned and functionally characterised from Atlantic salmon (Morais et al., 2009) and zebrafish (Monroig et al., 2009). While activity towards C_{18} PUFA was very low, fish Elovl2 had the ability to elongate C_{20} PUFA, similar to Elovl5, but, in addition, also efficiently elongated the C_{22} substrates, 22:5n-3 and 22:4n-6 (Monroig et al., 2009; Morais et al., 2009). The ability of Elovl2 to elongate 22:5n-3 to 24:5n-3 has been regarded as critical for DHA biosynthesis, as two consecutive elongation steps from 20:5n-3 to 24:5n-3 are required prior to Δ6 desaturation and the peroxisomal chain-shortening steps (Sprecher, 2000). To date, no *elovl2* cDNA has been isolated from a marine fish species, and this had been hypothesised as a factor potentially contributing to their limited ability for DHA biosynthesis (Leaver et al., 2008; Morais et al., 2009).

Recent investigations, however, have suggested that fish Elovl4 exhibit functional similarities to Elovl2, and thus may partly compensate for the apparent absence of Elovl2 in marine species (Monroig et al., 2011a). Specifically, some fish Elovl4 have been demonstrated to effectively elongate C_{20} and C_{22} PUFA, in contrast to mammalian ELOVL4 that appear to operate only towards longer chain (C_{26}) PUFA (Agbaga et al., 2008). Thus, the ability of fish Elovl4 to elongate 22:5n-3 to 24:5n-3 demonstrates that these enzymes have the potential to participate in the production of DHA, similar to Elovl2. Furthermore, similar to mammalian orthologues, teleost Elovl4 have been shown to participate in the biosynthesis of very long-chain fatty acids (VLC-FA)

including saturated and polyunsaturated compounds with chain-lengths $>C_{24}$ (Monroig et al., 2010a, 2011a; Carmona-Antoñanzas et al., 2011). Whereas VLC-FA have key functions in mammalian tissues including skin (Cameron et al., 2007), retina (Aveldaño, 1987, 1988), brain (Robinson et al., 1990) and testis (Furland et al., 2003, 2007a,b), their presence and roles in fish have been barely explored (Poulos, 1995).

Historically, marine fish have been regarded as species with limited capability for *de novo* LC-PUFA biosynthesis in comparison to freshwater and salmonid fish (Tocher, 2010). This view has been supported by a wide variety of evidence including FA compositional analysis obtained from feeding trials, biochemical assays assessing the LC-PUFA biosynthetic ability of primary cell cultures and fish cell lines, and lately through functional characterisation of key enzymes (desaturases and elongases) genes involved in the LC-PUFA biosynthetic pathway (Tocher et al., 2003; Leaver et al., 2008). Compared to freshwater ecosystems, LC-PUFA are readily available in marine environments, and this difference in evolutionary pressure has been hypothesised to account for the apparent loss of some enzymatic activities of the LC-PUFA biosynthetic pathway in marine fish. However, recent studies on the marine teleost rabbitfish have suggested that the above assumption may be too simplistic, as other factors such as trophic level, i.e. the position of an organism in the food chain, might also determine the capacity of a certain species for de novo synthesis of LC-PUFA (Li et al., 2010).

The rabbitfish (*Siganus canaliculatus*), a herbivore consuming algae and seagrasses, occupies a lower trophic level compared to the carnivorous/piscivorous marine finfish upon which the general concept above was forged and, trophically, is more similar to herbivorous freshwater species (Woodland, 1990; Tacon et al., 2010). Here we report on the molecular cloning, functional characterisation and tissue distribution of two Elovl-encoding cDNAs isolated from the rabbitfish. This study aimed to expand our

knowledge of the LC-PUFA biosynthesis in rabbitfish, complementing previous studies of other enzymes involved in the pathway, fatty acyl desaturases (Fad) (Li et al., 2008, 2010). Our results on the rabbitfish elongases are discussed within the overall context of LC-PUFA biosynthesis in this species, and the potential impact this could have on the diversification of marine finfish aquaculture to species that have low dependence on dietary LC-PUFA.

Materials and Methods

2.1. Molecular cloning of rabbitfish elovl5 and elovl4 cDNAs

One μg of total RNA extracted from rabbitfish liver and eye (Trizol reagent, Invitrogen, USA) was reverse transcribed into cDNA using random hexamer primers (Cloned AMV First-Strand cDNA Synthesis Kit, Invitrogen, USA). For *elovl5*, the primers ELO5F (5'-GGTACTACTTCTCCAAGCTCAT-3') and ELO5R (5'-GTGATGTATCTCTTCCACC-3') were designed, based on alignment of several fish *elovl5* including those of Atlantic salmon (AY170327), rainbow trout (AY605100), zebrafish (AF532782) and tilapia (AY170326), and they were used to amplify a first fragment of the putative rabbitfish *elovl5* by polymerase chain reaction (PCR) using liver cDNA as template. For *elovl4*, the primers ELO4F (5'-CAGCCTGTCAACTACTCCAATGA-3') and ELO4R (5'-GTGAGGTATTTCTTCCACCA-3') were designed, based on conserved regions from the alignment of zebrafish (NM_199972) and cobia (HM026361) *elovl4* sequences, and they were used to amplify a first fragment of the rabbitfish putative *elovl4* using eye cDNA as template for PCR. For both *elovl* cDNAs, PCR consisted of an initial denaturation at 94°C for 5 min, followed by 32 cycles of denaturation at 94 °C 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 1 min, followed by a final

extension at 72 °C for 5 min. The PCR fragments were sequenced (CEQ-8800 Beckman Coulter Inc., Fullerton, USA), and gene-specific primers (Table 1) were designed to produce the full-length cDNA by 5' and 3' rapid amplification of cDNA ends (RACE) PCR (GeneRacer™ Kit, Invitrogen, USA).

2.2. Sequence and phylogenetic analysis of Elovl5 and *Elovl4*

The deduced amino acid (aa) sequences of the newly cloned rabbitfish elongases were aligned with their corresponding orthologues from human (ELOVL4, NM_022726; ELOVL5, NP_068586) and zebrafish (Elovl4a, NM_200796, Elovl4b, NM_199972; Elovl5, NP_956747) using ClustalW2. The aa sequence identities between deduced Elovl proteins from rabbitfish and other vertebrate homologues were compared by the EMBOSS Needle Pairwise Sequence Alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). A phylogenetic tree comparing the deduced aa sequences of rabbitfish Elovl5 and Elovl4 proteins, and Elovl proteins from human (ELOVL2, ELOVL4 and ELOVL5), zebrafish (Elovl2, Elovl4a, Elovl4b and Elovl5), Atlantic salmon (Elovl2, Elovl4, Elovl5a and Elovl5b), and cobia (Elovl4 and Elovl5), was constructed using the Neighbour Joining method (Saitou and Nei, 1987).

2.3. Functional characterisation in yeast

PCR fragments corresponding to the open reading frame (ORF) of *elovl5* and *elovl4* were amplified from liver and eye cDNA preparations, respectively, using the high fidelity *Pfu* Turbo DNA polymerase (Stratagene, Agilent Technologies, Cheshire, UK). A two-round PCR approach was followed with the first round performed with primer pairs based on the 5’ and 3’ untranslated regions (UTR) ScE5U5F/ScE5U3R (Elovl5) and ScE4U5F/ScE4U3R (Elovl4) (Table 1). PCR conditions consisted of an initial denaturing step at 95 ºC for 2 min, followed by 32 cycles of denaturation at 95 ºC for 30 s, annealing at 57 ºC for 30 s, extension at 72 ºC for 2 min, followed by a final

extension at 72 ºC for 5 min. First round PCR products were used as template for nested PCR with thermal conditions described above, and with primers ScE5VF/ScE5VR (*elovl5*) and ScE4VF/ScE4VR (*elovl4*) containing restriction enzyme sites (underlined in Table 1) for *Hin*dIII (forward) and *Xho*I (reverse). The DNA fragments were then digested with the corresponding restriction endonucleases (New England BioLabs, Herts, UK) and ligated into a similarly restricted pYES2 yeast expression vector (Invitrogen, Paisley, UK). The purified plasmids (GenElute™ Plasmid Miniprep Kit, Sigma) containing either the *elovl5* or *elovl4* ORF were then used to transform *S. cerevisiae* competent cells (S.c. EasyComp Transformation Kit, Invitrogen).

Transformation of the yeast *S. cerevisiae* (strain InvSc1) with the recombinant plasmids (pYES2-*elovl5* or pYES2-*elovl4*) was carried out using the S.c.EasyComp Transformation Kit (Invitrogen Ltd, Paisley, UK). Selection of yeast containing the pYES2 constructs was on *S. cerevisiae* minimal medium (SCMM)-uracil. For each *elovl*, one single yeast colony was cultured overnight in SCMM-uracil broth and diluted to OD600 of 0.4 in one single Erlenmeyer flasks for each potential substrate assayed. When cultures OD600 reached 1, the expression of the transgene was induced by the addition of galactose to 2% (wt/vol) and the FA substrate added (Hastings et al., 2001). For Elovl5, stearidonic acid (18:4n-3), γ-linolenic acid (18:3n-6), EPA (20:5n-3), ARA (20:4n-6), docosapentaenoic acid (DPA, 22:5n-3) or docosatetraenoic acid (DTA, 22:4n-6) were tested. For Elovl4, lignoceric acid (24:0), EPA, ARA, DPA or DTA were tested. DPA and DTA (> 98 – 99 % pure) were purchased from Cayman Chemical Co. (Ann Arbor, USA) and the remaining FA substrates (> 99 % pure) and chemicals used to prepare the *S. cerevisiae* minimal medium$^{-uracil}$ were from Sigma Chemical Co. Ltd. (Dorset, UK). Lignoceric acid was dissolved in α-cyclodextrin (Singh and Kishimoto, 1983) at 5 μM and added to the yeast cultures at a final concentration of 0.6 μM,

whereas PUFA substrates were added at final concentrations of 0.5 (C_{20}), 0.75 (C_{20}) and 1.0 (C_{22}) mM as uptake efficiency decreases with increasing chain length and degree of unsaturation (Zheng et al., 2009). Yeast transformed with pYES2 containing no insert were grown under the same conditions as a control. After 2 days incubation at 30 ^{o}C, yeast cultures were harvested, washed, and lipid extracted by homogenisation in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxy toluene (BHT) as antioxidant (Folch et al., 1957). Results were confirmed by repeating the assay with a different recombinant colony.

2.4. FAME analysis by GC-MS

Fatty acid methyl esters (FAME) from yeast total lipids were prepared, extracted and purified (Christie, 2003). Identification and quantification were carried out using a gas chromatograph (GC8000) coupled to an MD800 mass spectrometer (ThermoFisher Scientific, Hemel Hempstead, UK) using the methodology described by Monroig et al. (2010a). Elongation rates from PUFA substrates were calculated as the proportion of exogenously added substrate FA converted to elongated FA products, [individual product area/(all products areas + substrate area)] x 100. Because the newly produced FA from the exogenously added substrates may operate as substrates for further elongations, the accumulated conversion rates were also calculated by summing the individual conversion rate for each particular product and also those for longer products. Conversion rates from 24:0 by pYES2-*elovl4* yeast were not calculated as yeast endogenously contains several of the FA involved in the elongation pathway. Instead, contents of individual saturated FA $\geq$ C_{24} from *elovl4*-transformed yeast were calculated, and compared to control yeast as previously described (Monroig et al., 2010a).

2.5. Tissue distribution of rabbitfish elovl5 and elovl4 mRNA

The tissue distributions of *elovl5* and *elovl4* transcripts were examined by reverse transcription PCR (RT-PCR) using heart, liver, spleen, gill, muscle, eye, intestine and brain cDNA as templates. Tissue samples were obtained from fish (20-30 g) cultured in 250 L cylindrical tanks at ~25 °C, 32 ‰ salinity, natural photoperiod, and fed a full-nutrient diet based on fishmeal and fish oil. Fish were anaesthetised using MS-222 (Sigma), dissected and tissue samples frozen immediately in liquid nitrogen, and stored at -70 °C until RNA extraction. Total RNA (1 μg) from each tissue was reverse transcribed into cDNA (Cloned AMV First-Strand cDNA Synthesis Kit, Invitrogen). To confirm the absence of genomic DNA contamination, negative controls, consisting of reactions without reverse transcriptase, were also run. RT-PCR was carried out with an initial denaturing step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 40 s, and a final extension at 72 °C for 5 min. The expression of the housekeeping gene *β-actin* was used as internal control to check the efficiency of cDNA synthesis and cDNA integrity. The primer pairs used for RT-PCR are given in Table 1.

3. Results

3.1. Rabbitfish elongase elovl5 and elovl4 cDNA sequences and phylogenetics

The full-length rabbitfish elongase cDNAs (excluding the polyA tail) were 1254 bp (*elovl5*) and 1475 bp (*elovl4*), and their sequences were deposited in the GenBank database with the accession numbers GU597350 and JF320823, respectively. The *elovl5*-like cDNA consisted of an 876 bp ORF encoding a putative protein of 291 aa, while the *elovl4*-like cDNA contained a 909 bp ORF encoding a putative protein of 302 aa. The deduced aa sequences from the two rabbitfish elongases were 35% identical (aa)

to each other. Additionally, the rabbitfish Elovl5 was 74-81 % and 35-36 % identical to teleost Elovl5 and Elovl4 sequences, respectively, whereas the aa sequence of the rabbitfish putative Elovl4 was 71-93 % identical to other teleost (zebrafish, Atlantic salmon and cobia) Elovl4, and only 35-38 % identical to fish Elovl5 sequences.

The rabbitfish Elovl5 and Elovl4 deduced proteins contained the diagnostic histidine box (HXXHH) conserved in all members of the Elovl protein family (Fig. 1). They also possessed two lysine or arginine residues at the carboxyl terminus, KXRXX in Elovl5 and R(K)XKXX in Elovl4, which are putative ER retrieval signals. By sequence comparison with mouse ELOVL proteins, five putative transmembrane-spanning domains containing hydrophobic aa stretches were also predicted.

A phylogenetic tree was constructed on the basis of aa sequence comparisons of the rabbitfish Elovl5 and Elovl4 proteins, and other elongases from fish (zebrafish, Atlantic salmon and cobia) and human (Fig. 2). The phylogenetic analysis showed that the rabbitfish Elovl5 and Elovl4 clustered together with their corresponding human and teleost orthologues, and separately from the Elovl2 cluster.

3.2. Functional characterisation

The two putative Elovl elongases of rabbitfish were functionally characterised by determining the FA profiles of *S. cerevisiae* transformed with pYES2 containing either *elovl5* or *elovl4* ORF inserts and grown in the presence of potential FA substrates. The FA composition of wild yeast consists essentially of the main endogenous FA of *S. cerevisiae*, namely 16:0, 16:1 isomers (16:1n-9 and 16:1n-7), 18:0, 18:1n-9 and 18:1n-7 (Monroig et al., 2010b). Total lipid of yeast transformed with the pYES2 vector without elongase ORF inserts contained these FA together with whichever exogenous FA (if any) was added as substrate (data not shown). This was consistent with the well

established lack of PUFA elongase activity in *S. cerevisiae* (Hastings et al., 2001; Agaba et al., 2004).

The rabbitfish Elovl5 was functionally characterised by growing yeast in the presence of C_{18} (18:4n-3 and 18:3n-6), C_{20} (20:5n-3 and 20:4n-6) and C_{22} (22:5n-3 and 22:4n-6) PUFA substrates (Table 2). The results showed that the rabbitfish Elovl5 exhibited activity towards PUFA substrates with 18 to 22 carbons, with apparent preference for C_{18} and C_{20} over C_{22} FA substrates (Table 2). Thus, up to 67.7 % of the exogenously added 18:4n-3 was elongated to 20:4n-3, 22:4n-3 and 24:4n-3, with more modest conversion rates (55.6 %) observed for the n-6 FA, 18:3n-6 (Table 2). High elongation rates were also detected for C_{20} substrates such as 20:5n-3 (87.5 %) and 20:4n-6 (66.3 %), which were elongated to C_{22} and C_{24} products (Table 2). Elovl5 elongated C_{22} FA substrates, 22:5n-3 and 22:4n-6, to C_{24} PUFA to notably lower extents (3.9-10.6 %) (Table 2).

To test the ability of rabbitfish Elovl4 to biosynthesise saturated VLC-FA, transgenic yeast were grown in the presence of lignoceric acid (24:0). Yeast transformed with the empty vector (no elongase ORF insert) contained measurable levels of saturated VLC-FA, 24:0, 26:0, 28:0, 30:0 and 32:0 (Table 3). In contrast, yeast transformed with the *elovl4* ORF contained decreased amounts of 24:0 and 26:0, but increased amounts of 28:0, 30:0, 32:0, 34:0 and 36:0 (Table 3). The latter two FA were not detected in the control yeast.

The ability of the rabbitfish Elovl4 to biosynthesise very long-chain (>C_{24}) PUFA (VLC-PUFA) was also investigated. Thus, yeast transformed with the *elovl4* ORF were cultured in the presence of the C_{20} (20:5n−3 and 20:4n−6) and C_{22} (22:5n−3 and 22:4n−6) PUFA substrates, which were converted to VLC-PUFA up to C_{36} (Table 4).

Importantly, yeast expressing the rabbitfish Elovl4 could convert 20:5n−3 and 22:5n−3 to 24:5n−3.

3.3. Tissue expression of Elovl5 and Elovl4

Distribution of *elovl5* an *elovl4* transcripts was analysed by RT-PCR on cDNA samples from rabbitfish tissues (Fig. 3). Expression for *elovl5* was detected in liver, brain, intestine and eye and spleen. In contrast, expression of *elovl4* was only detected in eye and brain. The expression of the housekeeping gene *ß-actin* remained constant among analysed tissues (Fig. 3).

Discussion

Marine fish have been generally regarded as species with only very limited ability for LC-PUFA biosynthesis, resulting from evolutionary adaptation to environments with abundant availability of preformed LC-PUFA. In spite of its marine origin, the rabbitfish was recently demonstrated to have biosynthetic activities unique, not only among marine fish species, but among vertebrates in general (Li et al., 2010). Thus, rabbitfish possess a bifunctional Δ6/Δ5 desaturase, similar to that of zebrafish (Hastings et al., 2001), representing the first Fad with Δ5-desaturase activity among marine fish but, in addition, they also possess a further desaturase with predominantly Δ4 activity, a desaturation activity not reported previously in any vertebrate species. While these Fad enzymes enable rabbitfish to perform all the desaturations required to convert α-linolenic (18:3n-3) and linoleic (18:2n-6) acids into C_{20-22} LC-PUFA (Fig. 4), the present study now confirms that all the necessary elongase activities are also present, and thus rabbitfish is the first marine species where genes encoding Fad and Elovl enzymes, with all the activities required for the production of DHA from C_{18} PUFA, have been characterised.

Both Elovl5 and Elovl4 cDNAs isolated from rabbitfish possess all the main structural features common for Elovl protein family members, including the predicted transmembrane domains, the histidine box (HXXHH), and the canonical C-terminal ER retrieval signal (KXRXX for Elovl5 and RXKXX for Elovl4) (Jakobsson et al., 2006). The phylogenetic analysis confirmed that the newly isolated rabbitfish Elovl cDNAs encoded distinct Elovl5 and Elovl4 proteins whose deduced aa sequences showed high homology to their respective orthologues in other vertebrates. However, further evidence of the specific Elovl type of the cDNAs from rabbitfish was obtained through functional charaterisation in yeast. The rabbitfish Elovl5 demonstrated the ability to elongate C_{18} and C_{20} PUFA substrates, with lesser activity observed towards C_{22} PUFA. These results are consistent with previously reported specificities for mammal (Leonard et al., 2000) and teleost Elovl5 proteins (Agaba et al., 2004, 2005; Hastings et al., 2005; Morais et al., 2009, 2011; Zheng et al., 2009a; Gregory et al., 2010; Mohd-Yusof et al., 2010), clearly indicating that vertebrate Elovl generally have broad substrate specificity. Moreover, the rabbitfish Elovl5 has a preference for n-3 over n-6 PUFA substrates, in agreement with results obtained for most species studied previously, including both marine and freshwater fish (Agaba et al., 2005; Mohd-Yusof et al., 2010; Morais et al., 2011).

In addition to 18:4n-3 and 18:3n-6, assayed in the present study, other potential C_{18} PUFA substrates for Elovl5 could include 18:3n-3 and 18:2n-6 (Guillou et al., 2010). In contrast to the 'classical' pathway of "Δ6 desaturation → elongation → Δ5 desaturation", the Δ8 pathway for the biosynthesis of EPA and ARA is achieved through "elongation → Δ8 desaturation → Δ5 desaturation" (Fig. 4) (Monroig et al., 2011b). Although not determined in the present study, it is possible that the rabbitfish enzyme, like its homologue from the Southern bluefin tuna (Gregory et al., 2010), could

also elongate 18:3n-3 and 18:2n-6 to 20:3n-3 and 20:2n-6, respectively. This hypothesis is supported by the recent demonstration that the rabbitfish Δ6/Δ5 Fad was also able to effectively operate as a Δ8 desaturase, possibly limiting the production of "dead-end" metabolic products in rabbitfish (Monroig et al., 2011b).

In contrast to the Elovl5 enzyme, rabbitfish Elovl4 showed the ability to elongate a variety of FA substrates, generating products up to C_{36} in length. More specifically, the rabbitfish Elovl4 demonstrated a role in the biosynthesis of both saturated and polyunsaturated VLC-FA, similar to previous observations with zebrafish (isoform Elovl4b) (Monroig et al., 2010a), cobia (Monroig et al., 2011a) and Atlantic salmon (Carmona-Antoñanzas et al., 2011) Elovl4 proteins. In particular, yeast expressing the rabbitfish Elovl4 were capable of elongating saturated VLC-FA such as 24:0, 26:0 and 28:0 up to 36:0. Similarly, C_{20} and C_{22} PUFA substrates could be efficiently elongated to their corresponding n-3 or n-6 polyenoic products with C_{36} chain-lengths. Saturated and polyunsaturated VLC-FA have been detected in specific lipid classes in tissues such as brain, retina, and testes of terrestrial vertebrates (Poulos, 1995; McMahon et al., 2009; Agbaga et al., 2010), but the presence of VLC-FA in fish has only been reported in retinal lipids (Poulos, 1995). The tissue distribution of Elovl4 transcripts suggested that eye (possibly retina) and brain are also major metabolic sites for the biosynthesis of VLC-FA in fish as observed in other species (Monroig et al., 2010a, 2011a; Carmona-Antoñanzas et al., 2011). In contrast, Elovl5 mRNA showed a widespread distribution in rabbitfish tissues, consistent with the tissue distribution of Elovl5 in the majority of fish species investigated to date (Agaba et al., 2004, 2005; Hastings et al., 2005; Morais et al., 2009, 2011; Zheng et al., 2009; Gregory et al., 2010; Mohd-Yusof et al., 2010).

Beyond the role of Elovl4 in the biosynthesis of VLC-FA, it is also possible to speculate that the expression of Elovl4 in brain and eye is activated in the production of

DHA that accumulates in these neural tissues (Tocher and Sargent, 1990; Tocher et al., 1992; Tocher, 1993; Monroig et al., 2009). Supporting this hypothesis, functional characterisation of the rabbitfish Elovl4 confirmed that this enzyme can participate in the biosynthesis of DHA as previously shown for Elovl4 in other marine teleosts (Monroig et al., 2011a). Thus, unlike terrestrial vertebrate orthologues, Elovl4 from fish, including rabbitfish, have the ability to catalyse the conversion of 22:5n-3 to 24:5n-3, which is a step required in the biosynthesis DHA through the so-called "Sprecher pathway" (Sprecher, 2000). This pathway was initially demonstrated in rat but there is evidence that it may also operate in some fish species including rainbow trout (Buzzi et al., 1996, 1997), Atlantic salmon and zebrafish (Tocher et al., 2003).

In summary, rabbitfish express at least two Elovl cDNAs with high homology in sequence and function to Elovl5 and Elovl4 elongases previously investigated in other fish species. Moreover, our results confirmed that these enzymes enable rabbitfish to perform all the elongation reactions required for the biosynthesis of the physiologically essential C_{20-22} LC-PUFA, and also the less common VLC-FA. Rabbitfish is thus the first marine species where genes encoding Fad and Elovl enzymes, with all the activities required for the production of DHA from C_{18} PUFA, have been characterised.

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References

Agaba, M., Tocher, D.R., Dickson, C., 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. Mar. Biotechnol. 6, 251-261.

Agaba, M.K., Tocher, D.R., Dickson, C.A., Zheng, X., Dick, J.R., Teale, A.J., 2005. Cloning and functional characterisation of polyunsaturated fatty acid elongases from marine and freshwater teleost fish. Comp. Biochem. Physiol. 142B, 342–352.

Agbaga, M.P., Brush, R.S., Mandal, M.N.A., Henry, K., Elliott, M.H., Anderson, R.E., 2008. Role of Stargardt-3 macular dystrophy protein (ELOVL4) in the biosynthesis of very long chain fatty acids, Proc. Natl. Acad. Sci. USA. 105, 12843-12848.

Agbaga, M.P., Mandal, M.N.A., Anderson, R.E., 2010. Retinal very long chain polyunsaturated fatty acids: new insights from studies on ELOVL4 protein. J. Lipid Res. 51, 1624 – 1642.

Aveldaño, M.I., 1987. A novel group of very long chain polyenoic fatty acids in dipolyunsaturated phosphatidylcholines from vertebrate retina. J. Biol. Chem. 262, 1172–1179.

Aveldaño, M.I., 1988. Phospholipid species containing long and very long polyenoic fatty acids remain with rhodopsin after hexane extraction of photoreceptor membranes. Biochemistry 27, 1229-1239.

Bardon, S., Le, M.T., Alessandri, J.M., 1996. Metabolic conversion and growth effects of n-6 and n-3 polyunsaturated fatty acids in the T47D breast cancer cell line. Cancer Lett. 99, 51–58.

Brouwer, I.A., Geelen, A., Katan, M.B., 2006. n-3 Fatty acids, cardiac arrhythmia and fatal coronary heart disease. Prog. Lipid Res. 4, 357-367.

Buzzi, M., Henderson, R.J., Sargent, J.R., 1996. The desaturation and elongation of linolenic acid and eicosapentaenoic acid by hepatocytes and liver microsomes from rainbow trout (*Oncorhynchus mykiss*) fed diets containing fish oil or olive oil. Biochim. Biophys. Acta 1299, 235-244.

Buzzi, M., Henderson, R.J., Sargent, J.R., 1997. Biosynthesis of docosahexaenoic acid in trout hepatocytes proceeds via 24-carbon intermediates. Comp. Biochem. Physiol. 116B, 263–267.

Calder, P.C., 2006. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am. J. Clin. Nutr. 83, 1505S-1519S.

Calder, P.C., Yaqoob, P., 2009. Understanding omega-3 polyunsaturated fatty acids. Postgrad. Med. 121, 148-57.

Cameron, D.J., Tong, Z., Yang, Z., Kaminoh, J., Kamiyah, S., Chen, H., Zeng, J., Chen, Y., Lou, L., Zhang, K., 2007. Essential role of Elovl4 in very long chain fatty acid synthesis, skin permeability barrier function, and neonatal survival. Int. J. Biol. Sci. 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. Comp. Biochem. Physiol. 159B, 122-129.

Christie, W.W., 2003. Lipid Analysis, third ed. Oily Press, Bridgwater.

Eilander, A., Hundscheid, D.C., Osendarp, S.J., Trander, 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 Essent. Fatty Acids 76, 189-203.

Folch, J., Lees, N., Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509

Furland, N.E., Maldonado, E.N., Aveldaño, M.I., 2003. Very long chain PUFA in murine testicular triglycerides and cholesterol esters. Lipids 38, 73-80.

Furland, N.E., Oresti, G.M., Antollini, S.S., Venturino, A., Maldonado, E.N., Aveldaño, M.I., 2007a. Very long-chain polyunsaturated fatty acids are the major acyl groups of sphingomyelins and ceramides in the head of mammalian spermatozoa. J. Biol. Chem. 282, 18151-18161.

Furland, N.E., Maldonado, E.N., Ayuza-Aresti, P., Aveldaño, M.I., 2007b. Changes in lipids containing long- and very long-chain polyunsaturated fatty acids in cryptorchid rat testes. Biol. Reprod. 77, 181-188.

Gregory, M., See, V.H.L., Gibson, R.A., Shuller, K.A., 2010. Cloning and functional characterisation of a fatty acyl elongase from southern bluefin tuna (*Thunnus maccoyii*). Comp. Biochem. Physiol. 155B, 178-185.

Guillou, H., Zadravec, D., Martin, P.G.P., Jacobsson, A., 2010. The key roles of elongases and desaturases in mammalian fatty acid metabolism: Insights from transgenic mice. Prog. Lipid Res. 49, 186-199.

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. Proc. Natl. Acad. Sci. USA 98, 14304-14309.

Hastings, N., Agaba, M.K., Tocher, D.R., Zheng, X., Dickson, C.A., Dick, J.R., Teale, A.J., 2005. 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*). Mar. Biotechnol. 6, 463-474.

Jakobsson, A., Westerberg, R., Jacobsson, A., 2006. Fatty acid elongases in mammals: Their regulation and roles in metabolism. Prog. Lipid Res. 45, 237-249.

Leaver, M.J., Bautista, J.M., Björnsson, T., Jönsson, E. Krey, G., Tocher, D.R., Torstensen, B.E., 2008. Towards fish lipid nutrigenomics: current state and prospects for fin-fish aquaculture. Rev. Fisheries Sci. 16(S1), 71-92.

Leonard, A.E., Bobik, E.G., Dorado, J., Kroeger, P.E., Chuang, L.-T., Thurmond, J.M., Parker–Barnes, J.M., Das, T., Huang, Y.-S., Murkerji, P., 2000. Cloning of a human cDNA encoding a novel enzyme involved in the elongation of long-chain polyunsaturated fatty acids. Biochem. J. 350, 765–70

Li, Y., Hu, C., Zheng, Y., Xia, X., Xu, W., Wang, S., Chen, W., Sun, Z., Huang, J., 2008. The effects of dietary fatty acids on liver fatty acid composition and delta 6-desaturase expression differ with ambient salinities in *Siganus canaliculatus*. Comp. Biochem. Physiol. 151B, 183–190.

Li, Y., Monroig, Ó., Zhang, L., Wang, S., Zheng, X., Dick, J.R., You, C., Tocher, D.R., 2010. Vertebrate fatty acyl desaturase with Δ4 activity. *Proc. Natl. Acad. Sci. USA* 107, 16840-16845.

A. McMahon, W. Kedzierski, 2010. Polyunsaturated extremely long chain C28-C36 fatty acids and retinal physiology, Br. J. Ophthalmol. 94, 1127-1132.

Mohd-Yusof, N.Y., Monroig, Ó., 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 Physiol. Biochem. 3, 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. Biochim. Biophys. Acta 1791, 1093–1101.

Monroig, Ó., Rotllant, J., Cerdá-Reverter, J.M., Dick, J.R., Figueras, A., Tocher, D.R., 2010a. Expression and role of Elovl4 elongases in biosynthesis of very long-chain fatty acids during zebrafish *Danio rerio* early embryonic development. Biochim. Biophys. Acta 1801, 1145-1154.

Monroig, Ó., Zheng, X., Morais, S., Leaver, M.J., Taggart, J.B., Tocher, D.R., 2010b. Multiple genes for functional Δ6 fatty acyl desaturases (Fad) in Atlantic salmon (*Salmo salar* L.): Gene and cDNA characterization, functional expression, tissue distribution and nutritional regulation. Biochim. Biophys. Acta 180, 1072–1081.

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

Monroig, Ó., Li, Y., Tocher, D.R., 2011b. Delta-8 desaturation activity varies among fatty acyl desaturases of teleost fish: high activity in delta-6 desaturases of marine species. Comp. Biochem. Physiol. 159B, 206-213.

Morais, S., Monroig, Ó., Zheng, X., Leaver, M.J., Tocher, D.R., 2009. Highly unsaturated fatty acid synthesis in Atlantic salmon: characterization of Elovl5- and Elovl2-like elongases. Mar. Biotechnol. 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 Atlantic bluefin tuna (*Thunnus thynnus* L.). Aquaculture 313, 129-139.

Nugteren, D.H., 1965. The enzymatic chain elongation of fatty acids by rat-liver microsomes. Biochim. Biophys. Acta 106, 280-290.

Poulos, A., 1995. Very long chain fatty acids in higher animals – A review. Lipids 30, 1-14.

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. Biochem. J. 265, 763-767.

Ruxton, C.H.S., Reed, S.C., Simpson, M.J.A., Millington, K.J., 2007. The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. J. Hum. Nutr. Dietet. 20, 275-285.

Saitou, N., Nei, M., 1987. The neighbor-joining method. A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406-425.

Singh, I., Kishimoto, Y., 1983. Effect of cyclodextrins on the solubilization of lignoceric acid, ceramide, and cerebroside, and on the enzymatic reactions involving these compounds. J. Lipid Res. 24, 662-665.

Sprecher, H., 2000. Metabolism of highly unsaturated n-3 and n-6 fatty acids. Biochim. Biophys. Acta. 1486, 219-231.

Tacon, A.G.J., Metian, M., Turchini, G.M., DeSilva, S.S., 2010. Responsible aquaculture and trophic level implications to global fish supply. Rev. Fish. Sci. 18, 94–105.

Tocher, D.R., Sargent, J.R., 1990a. Incorporation into phospholipid classes and metabolism via desaturation and elongation of various ^{14}C-labelled (n-3) and (n-6) polyunsaturated fatty acids in trout astrocytes in primary culture. J. Neurochem. 54, 2118-2124.

Tocher, D.R., Mourente, G., Sargent, J.R., 1992. Metabolism of [1-^{14}C]docosahexaenoate (22:6n-3), [1-^{14}C]eicosapentaenoate (20:5n-3) and [1-^{14}C]linolenate (18:3n-3) in brain cells from juvenile turbot *Scophthalmus maximus*. Lipids 27, 494-499.

Tocher, D.R., 1993. Elongation predominates over desaturation in the metabolism of 18:3n-3 and 20:5n-3 in turbot (*Scophthalmus maximus*) brain astroglial cells in primary culture. Lipids 28, 267-272.

Tocher, D.R., Agaba, M., Hastings, N., Teale, A.J., 2003. Biochemical and molecular studies of the fatty acid desaturation pathway in fish. In: Browman, H.I., Skiftesvik, A.B. (Eds.), The Big Fish Bang – Proceedings of the 26th Annual Larval Fish Conference, pp. 211-227. Institute of Marine Nutrition, Bergen.

Tocher, D.R., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquac. Res. 41, 717–732.

Woodland, D.J., 1990. Revision of the fish family Siganidae with descriptions of two new species and comments on distribution and biology. Indo-Pacific Fishes 19, 1–136.

Zheng, X., Ding, Z., Xu, Y., Monroig, O., Morais, S., Tocher, D.R., 2009. Physiological roles of fatty acyl desaturase and elongase in marine fish: Characterisation of cDNAs of fatty acyl Δ6 desaturase and Elovl5 elongase of cobia (*Rachycentron canadum*). Aquaculture 290, 122-131.

Tables

Table 1. Sequences of the primer pairs used and accession numbers of the sequences used as references for primer design in the cloning of the rabbitfish elongase of very long-chain fatty acids (Elovl) ORF and for RT-PCR analysis of gene expression in rabbitfish tissues.

Aim	Transcript	Primer	Primer sequence	Accession No[1].
RACE PCR	*elovl5*	ScE5F1	5'-TCATGAACTGGATCCCCTGT-3'	GU597350.1
		ScE5F2	5'-GAGACCGTACCTTTGGTGGA-3'	
		ScE5R1	5'-GTTCATGACGAACCACCAGA-3'	
		ScE5R2	5'-GTGTCCATGAACTCGATAAGA-3'	
	elovl4	ScE4F1	5'-AACCAAGTCAGCTTCCTCCA-3'	JF320823.1
		ScE4F2	5'-TATGGTTACTACGGGCTGGC-3'	
		ScE4R1	5'-AGACTGTGTCCAGGAACTCCA-3'	
		ScE4R2	5'-GTAGGAGCTCTTTGGCGATG-3'	
ORF cloning	*elovl5*	ScE5U5F	5'-GGGGGACTTTATGGTGACAA-3'	GU597350.1
		ScE5U3R	5'-TGCGCTACATTGAGAACTGTG-3'	
		ScE5VF	5'-CCCAAGCTTAGGATGGAGGACTTCAATC-3'	
		ScE5VR	5'-CCGCTCGAGTCAATCCACCCTCAGCT-3'	
	elovl4	ScE4U5F	5'-TGTGGAAGCGCTGAGTAGAA-3'	JF320823.1
		ScE4U3R	5'-ACTTGCAGGGATGATGAAGC-3'	
		ScE4VF	5'-CCCAAGCTTAGGATGGAGGTTGTAACGC-3'	
		ScE4VR	5'-CCGCTCGAGTTACTCCCTCTTGGCTC-3'	
RT-PCR	*elovl5*	ScE5F2	5'-TTTGGTTTGGAGGCTACCAC-3'	GU597350.1
		ScE5R2	5'-TCCACCAAAGGTACGGTCTC-3'	
	elovl4	ScE4F2	5'-TCCACGTGCTCATGTATGGT-3'	JF320823.1
		ScE4R2	5'-CTTCCTCCTCCACTTTGCTG-3'	
	ß-actin	ScACTF	5'-CTTCCTTCCTCGGTATGGAGTC-3'	EU107278.1
		ScACTR	5'-AGGTGGAGCAATGATCTT GATC-3'	

[1] GenBank (http://www.ncbi.nlm.nih.gov/)

Table 2. Functional characterisation of rabbitfish Elovl5 elongase in *Saccharomyces cerevisiae*. Individual conversion rates were calculated according to the formula [individual product area/(all products areas + substrate area)] x 100. Accumulated conversions were computed by summing the individual conversion rate for each particular product and also those for longer products.

FA substrate	Product	% Individual conversion	% Accumulated conversion
18:4n-3	20:4n-3	34.8	67.6
	22:4n-3	31.7	32.8
	24:4n-3	1.1	1.1
18:3n-6	20:3n-6	36.9	55.6
	22:3n-6	12.4	18.7
	24:3n-6	6.3	6.3
20:5n-3	22:5n-3	80.8	87.5
	24:5n-3	6.7	6.7
20:4n-6	22:4n-6	62.6	66.3
	24:4n-6	3.7	3.7
22:5n-3	24:5n-3	10.6	10.6
22:4n-6	24:4n-6	3.9	3.9

Table 3. Functional characterisation of rabbitfish 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 empty pYES2 vector (Control) 7or rabbitfish *elovl4* ORF.

FA	Control	Elovl4
24:0*	11.0	8.3
26:0	74.8	45.6
28:0	9.2	30.3
30:0	4.1	12.1
32:0	0.9	2.7
34:0	0.0	0.9
36:0	0.0	0.2

* Lignoceric acid used as exogenously added substrate.

Table 4. Functional characterisation of the rabbitfish Elovl4 elongase: conversions of polyunsaturated fatty acid (FA) substrates. Individual conversion rates were calculated according to the formula [individual product area/(all products areas + substrate area)] x 100. Accumulated conversions were computed by summing the individual conversion rate for each particular product and also those for longer products.

FA substrate	Product	% Individual conversion	% Accumulated conversion
20:5n-3	22:5n-3	13.9	41.4
	24:5n-3	4.0	27.5
	26:5n-3	0.2	23.5
	28:5n-3	0.1	23.3
	30:5n-3	1.6	23.2
	32:5n-3	13.5	21.6
	34:5n-3	7.7	8.1
	36:5n-3	0.4	0.4
20:4n-6	22:4n-6	9.6	28.8
	24:4n-6	3.5	19.2
	26:4n-6	0.6	15.7
	28:4n-6	0.4	15.1
	30:4n-6	4.2	14.7
	32:4n-6	8.9	10.5
	34:4n-6	1.5	1.6
	36:4n-6	0.1	0.1
22:5n-3	24:5n-3	3.3	20.7
	26:5n-3	0.3	17.4
	28:5n-3	0.1	17.1
	30:5n-3	1.1	17
	32:5n-3	10.3	15.9
	34:5n-3	5.4	5.6
	36:5n-3	0.2	0.2
22:4n-6	24:4n-6	2.4	23.5
	26:4n-6	0.5	21.1
	28:4n-6	0.3	20.6
	30:4n-6	4.0	20.3
	32:4n-6	13.4	16.3
	34:4n-6	2.7	2.9
	36:4n-6	0.2	0.2

Figures

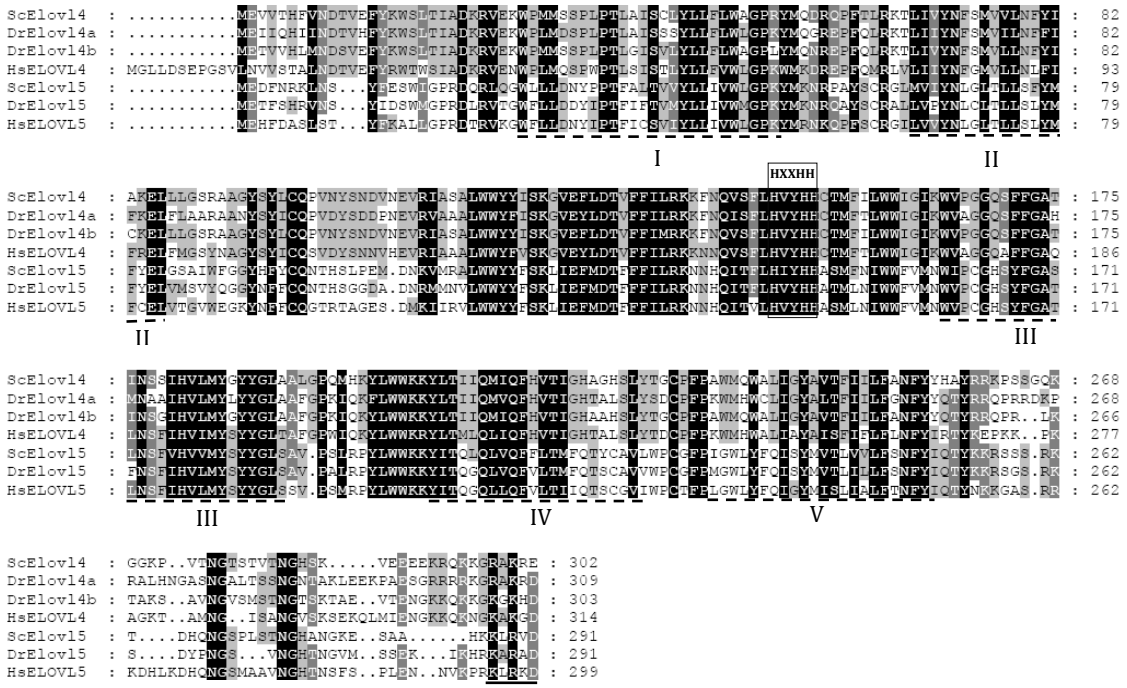

Fig. 1. Clustal W2 multiple alignment of the deduced amino acid (aa) sequences of the rabbitfish *Siganus canaliculatus* elongases Elovl4 and Elovl5 with their human and zebrafish orthologues. The aa sequences analysed were the *rabbitfish* (S. *canaliculatus*, Sc) Elovl4 (gb|ADZ73580|) and Elovl5 (gb|ADE34561|), human (*Homo sapiens*, Hs) ELOVL4 (gb|NP_073563.1|) and ELOVL5 (gb|NP_068586|), zebrafish (*Danio rerio*, Dr) Elovl4a (gb|NP_957090|), Elovl4b (gb|NP_956266|) and Elovl5 (gb|NP_956747|). AA numbers are shown on the right. Identical residues are shaded black and similar residues (based on the Gonnet matrix, using GeneDoc default parameters) are shaded grey. The conserved histidine box motif HXXHH is framed, five (I-V) putative membrane-spanning domains are dash-underlined, and the putative ER retrieval signal is solid underlined.

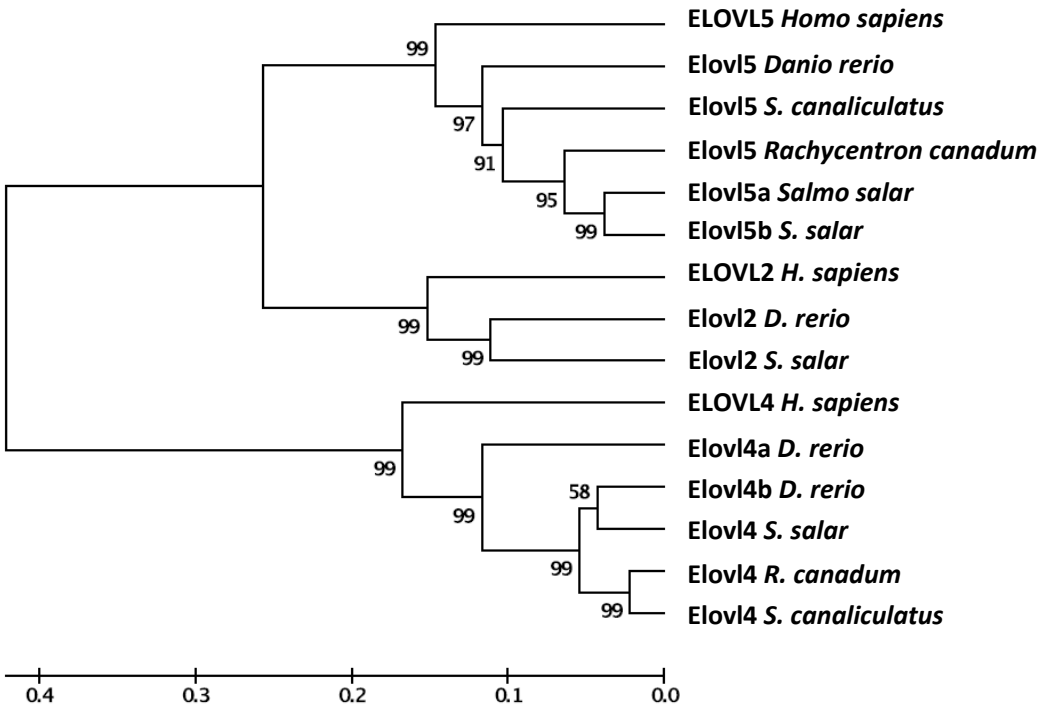

Fig. 2. Phylogenetic tree comparing the deduced amino acid (aa) sequences of the rabbitfish Elovl4 (gb|ADZ73580|) and Elovl5 (gb|ADE34561|) with the human ELOVL2 (gb|NP_060240|), ELOVL4 (gb|NP_022726|) and ELOVL5 (gb|NP_073563.1|), the zebrafish Elovl2 (gb|NP_001035452), Elovl4a (gb|NP_957090|), Elovl4b (gb|NP_956266|) and Elovl5 (gb|NP_956747|), the Atlantic Salmon Elovl2 (gb|FJ237532|), Elovl5a (gb|NP_001117039|) and Elovl5b (gb|FJ237531|), and the cobia Elovl4 (gb|HM026361|) and Elovl5 (gb|FJ440239|). The tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) with MEGA4. The horizontal branch length is proportional to aa substitution rate per site. The numbers represent the frequencies (%) with which the tree topology presented was replicated after 10000 iterations.

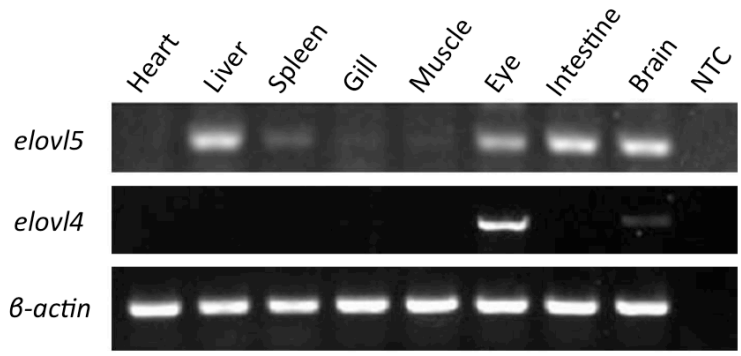

Fig. 3. Tissue distribution of *elovl4* and *elovl5* mRNA transcripts in *S. canaliculatus* examined by RT-PCR. The expression of the housekeeping gene *β-actin* was used as internal control. NTC, no template control.

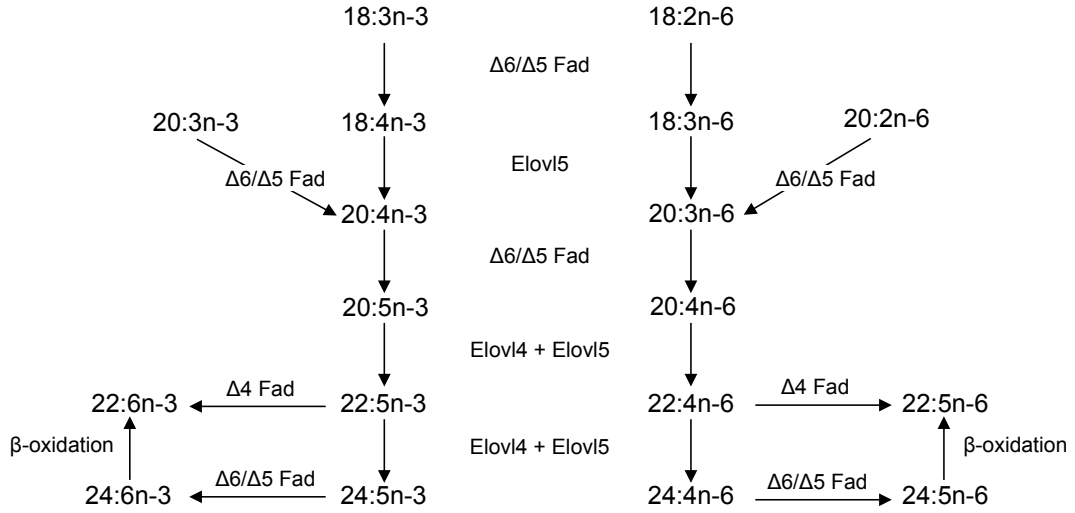

Fig 4. The biosynthesis pathway of long-chain polyunsaturated fatty acids ($\leq C_{24}$) from α-linolenic (18:3n-3) and linoleic (18:2n-6) acids in rabbitfish. Enzymatic activities shown in the scheme are predicted from heterologous expression in *S. cerevisiae* of the Δ6/Δ5 fatty acyl desaturase (Δ6/Δ5 Fad), the Δ4 Fad (Li et al., 2010) and the herein reported Elovl4- and Elovl5-like elongases.