Title

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

Authors

Óscar Monroig[1,a], Shuqi Wang[2,a], Liang Zhang[2], Cuihong You[2], Douglas R. Tocher[3], Yuanyou Li[2,b]

Addresses

[1]Instituto de Acuicultura Torre de la Sal (IATS-CSIC), 12595 Ribera de Cabanes, Castellón, Spain

[2]Guangdong Provincial Key Laboratory of Marine Biology, Shantou University, Shantou, Guangdong 515063, China

[3]Institute of Aquaculture, University of Stirling, Stirling, FK9 4LA, Scotland, UK

[a]Joint first authorship

[b]To whom correspondence should be addressed

Yuanyou Li

E-mail address: yyli@stu.edu.cn

Guangdong Provincial Key Laboratory of Marine Biology, Shantou University, Shantou, Guangdong 515063, China

Tel: (+86) 75482903157

Fax: (+86) 75482903473

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;

74 Gregory et al., 2010; Mohd-Yusof et al., 2010). These studies confirmed that fish
75 Elovl5, similar to mammalian homologues (Jakobsson et al., 2006), have the ability to
76 preferentially elongate C_{18} (18:4n-3 and 18:3n-6) and C_{20} (20:5n-3 and 20:4n-6) PUFA,
77 with only low activity towards C_{22} PUFA (22:5n-3 and 22:4n-6).
78 Studies on other Elovl enzymes involved in the LC-PUFA biosynthetic pathways have
79 enabled a fuller understanding of the FA elongation pathways in fish. Thus, *elovl2* have
80 been cloned and functionally characterised from Atlantic salmon (Morais et al., 2009)
81 and zebrafish (Monroig et al., 2009). While activity towards C_{18} PUFA was very low,
82 fish Elovl2 had the ability to elongate C_{20} PUFA, similar to Elovl5, but, in addition, also
83 efficiently elongated the C_{22} substrates, 22:5n-3 and 22:4n-6 (Monroig et al., 2009;
84 Morais et al., 2009). The ability of Elovl2 to elongate 22:5n-3 to 24:5n-3 has been
85 regarded as critical for DHA biosynthesis, as two consecutive elongation steps from
86 20:5n-3 to 24:5n-3 are required prior to Δ6 desaturation and the peroxisomal chain-
87 shortening steps (Sprecher, 2000). To date, no *elovl2* cDNA has been isolated from a
88 marine fish species, and this had been hypothesised as a factor potentially contributing
89 to their limited ability for DHA biosynthesis (Leaver et al., 2008; Morais et al., 2009).
90 Recent investigations, however, have suggested that fish Elovl4 exhibit functional
91 similarities to Elovl2, and thus may partly compensate for the apparent absence of
92 Elovl2 in marine species (Monroig et al., 2011a). Specifically, some fish Elovl4 have
93 been demonstrated to effectively elongate C_{20} and C_{22} PUFA, in contrast to mammalian
94 ELOVL4 that appear to operate only towards longer chain (C_{26}) PUFA (Agbaga et al.,
95 2008). Thus, the ability of fish Elovl4 to elongate 22:5n-3 to 24:5n-3 demonstrates that
96 these enzymes have the potential to participate in the production of DHA, similar to
97 Elovl2. Furthermore, similar to mammalian orthologues, teleost Elovl4 have been
98 shown to participate in the biosynthesis of very long-chain fatty acids (VLC-FA)

99 including saturated and polyunsaturated compounds with chain-lengths >C_{24} (Monroig
100 et al., 2010a, 2011a; Carmona-Antoñanzas et al., 2011). Whereas VLC-FA have key
101 functions in mammalian tissues including skin (Cameron et al., 2007), retina (Aveldaño,
102 1987, 1988), brain (Robinson et al., 1990) and testis (Furland et al., 2003, 2007a,b),
103 their presence and roles in fish have been barely explored (Poulos, 1995).
104 Historically, marine fish have been regarded as species with limited capability for *de*
105 *novo* LC-PUFA biosynthesis in comparison to freshwater and salmonid fish (Tocher,
106 2010). This view has been supported by a wide variety of evidence including FA
107 compositional analysis obtained from feeding trials, biochemical assays assessing the
108 LC-PUFA biosynthetic ability of primary cell cultures and fish cell lines, and lately
109 through functional characterisation of key enzymes (desaturases and elongases) genes
110 involved in the LC-PUFA biosynthetic pathway (Tocher et al., 2003; Leaver et al.,
111 2008). Compared to freshwater ecosystems, LC-PUFA are readily available in marine
112 environments, and this difference in evolutionary pressure has been hypothesised to
113 account for the apparent loss of some enzymatic activities of the LC-PUFA biosynthetic
114 pathway in marine fish. However, recent studies on the marine teleost rabbitfish have
115 suggested that the above assumption may be too simplistic, as other factors such as
116 trophic level, i.e. the position of an organism in the food chain, might also determine the
117 capacity of a certain species for de novo synthesis of LC-PUFA (Li et al., 2010).
118 The rabbitfish (*Siganus canaliculatus*), a herbivore consuming algae and seagrasses,
119 occupies a lower trophic level compared to the carnivorous/piscivorous marine finfish
120 upon which the general concept above was forged and, trophically, is more similar to
121 herbivorous freshwater species (Woodland, 1990; Tacon et al., 2010). Here we report on
122 the molecular cloning, functional characterisation and tissue distribution of two Elovl-
123 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

149 extension at 72 °C for 5 min. The PCR fragments were sequenced (CEQ-8800 Beckman
150 Coulter Inc., Fullerton, USA), and gene-specific primers (Table 1) were designed to
151 produce the full-length cDNA by 5' and 3' rapid amplification of cDNA ends (RACE)
152 PCR (GeneRacer™ Kit, Invitrogen, USA).
153 (TABLE 1)
154 *2.2. Sequence and phylogenetic analysis of Elovl5* and *Elovl4*
155 The deduced amino acid (aa) sequences of the newly cloned rabbitfish elongases were
156 aligned with their corresponding orthologues from human (ELOVL4, NM_022726;
157 ELOVL5, NP_068586) and zebrafish (Elovl4a, NM_200796, Elovl4b, NM_199972;
158 Elovl5, NP_956747) using ClustalW2. The aa sequence identities between deduced
159 Elovl proteins from rabbitfish and other vertebrate homologues were compared by the
160 EMBOSS Needle Pairwise Sequence Alignment tool
161 (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). A phylogenetic tree comparing the
162 deduced aa sequences of rabbitfish Elovl5 and Elovl4 proteins, and Elovl proteins from
163 human (ELOVL2, ELOVL4 and ELOVL5), zebrafish (Elovl2, Elovl4a, Elovl4b and
164 Elovl5), Atlantic salmon (Elovl2, Elovl4, Elovl5a and Elovl5b), and cobia (Elovl4 and
165 Elovl5), was constructed using the Neighbour Joining method (Saitou and Nei, 1987).
166 *2.3. Functional characterisation in yeast*
167 PCR fragments corresponding to the open reading frame (ORF) of *elovl5* and *elovl4*
168 were amplified from liver and eye cDNA preparations, respectively, using the high
169 fidelity *Pfu* Turbo DNA polymerase (Stratagene, Agilent Technologies, Cheshire, UK).
170 A two-round PCR approach was followed with the first round performed with primer
171 pairs based on the 5’ and 3’ untranslated regions (UTR) ScE5U5F/ScE5U3R (Elovl5)
172 and ScE4U5F/ScE4U3R (Elovl4) (Table 1). PCR conditions consisted of an initial
173 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,

199 1983) at 5 μM and added to the yeast cultures at a final concentration of 0.6 μM,
200 whereas PUFA substrates were added at final concentrations of 0.5 (C_{20}), 0.75 (C_{20}) and
201 1.0 (C_{22}) mM as uptake efficiency decreases with increasing chain length and degree of
202 unsaturation (Zheng et al., 2009). Yeast transformed with pYES2 containing no insert
203 were grown under the same conditions as a control. After 2 days incubation at 30 ^{o}C,
204 yeast cultures were harvested, washed, and lipid extracted by homogenisation in
205 chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxy toluene (BHT) as
206 antioxidant (Folch et al., 1957). Results were confirmed by repeating the assay with a
207 different recombinant colony.

208 *2.4. FAME analysis by GC-MS*

209 Fatty acid methyl esters (FAME) from yeast total lipids were prepared, extracted and
210 purified (Christie, 2003). Identification and quantification were carried out using a gas
211 chromatograph (GC8000) coupled to an MD800 mass spectrometer (ThermoFisher
212 Scientific, Hemel Hempstead, UK) using the methodology described by Monroig et al.
213 (2010a). Elongation rates from PUFA substrates were calculated as the proportion of
214 exogenously added substrate FA converted to elongated FA products, [individual
215 product area/(all products areas + substrate area)] x 100. Because the newly produced
216 FA from the exogenously added substrates may operate as substrates for further
217 elongations, the accumulated conversion rates were also calculated by summing the
218 individual conversion rate for each particular product and also those for longer products.
219 Conversion rates from 24:0 by pYES2-*elovl4* yeast were not calculated as yeast
220 endogenously contains several of the FA involved in the elongation pathway. Instead,
221 contents of individual saturated FA $\geq$ C_{24} from *elovl4*-transformed yeast were
222 calculated, and compared to control yeast as previously described (Monroig et al.,
223 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)

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

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

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

264 (FIGURE 1) (FIGURE 2)

265 *3.2. Functional characterisation*

266 The two putative Elovl elongases of rabbitfish were functionally characterised by
267 determining the FA profiles of *S. cerevisiae* transformed with pYES2 containing either
268 *elovl5* or *elovl4* ORF inserts and grown in the presence of potential FA substrates. The
269 FA composition of wild yeast consists essentially of the main endogenous FA of *S.*
270 *cerevisiae*, namely 16:0, 16:1 isomers (16:1n-9 and 16:1n-7), 18:0, 18:1n-9 and 18:1n-7
271 (Monroig et al., 2010b). Total lipid of yeast transformed with the pYES2 vector without
272 elongase ORF inserts contained these FA together with whichever exogenous FA (if
273 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).

(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).

299 Importantly, yeast expressing the rabbitfish Elovl4 could convert 20:5n−3 and 22:5n−3
300 to 24:5n−3.
301 (TABLE 3) (TABLE 4)
302 *3.3. Tissue expression of Elovl5 and Elovl4*
303 Distribution of *elovl5* an *elovl4* transcripts was analysed by RT-PCR on cDNA samples
304 from rabbitfish tissues (Fig. 3). Expression for *elovl5* was detected in liver, brain,
305 intestine and eye and spleen. In contrast, expression of *elovl4* was only detected in eye
306 and brain. The expression of the housekeeping gene *ß-actin* remained constant among
307 analysed tissues (Fig. 3).
308 (FIGURE 3)
309 **Discussion**
310 Marine fish have been generally regarded as species with only very limited ability for
311 LC-PUFA biosynthesis, resulting from evolutionary adaptation to environments with
312 abundant availability of preformed LC-PUFA. In spite of its marine origin, the
313 rabbitfish was recently demonstrated to have biosynthetic activities unique, not only
314 among marine fish species, but among vertebrates in general (Li et al., 2010). Thus,
315 rabbitfish possess a bifunctional Δ6/Δ5 desaturase, similar to that of zebrafish (Hastings
316 et al., 2001), representing the first Fad with Δ5-desaturase activity among marine fish
317 but, in addition, they also possess a further desaturase with predominantly Δ4 activity, a
318 desaturation activity not reported previously in any vertebrate species. While these Fad
319 enzymes enable rabbitfish to perform all the desaturations required to convert α-
320 linolenic (18:3n-3) and linoleic (18:2n-6) acids into C_{20-22} LC-PUFA (Fig. 4), the
321 present study now confirms that all the necessary elongase activities are also present,
322 and thus rabbitfish is the first marine species where genes encoding Fad and Elovl

323 enzymes, with all the activities required for the production of DHA from C_{18} PUFA,
324 have been characterised.
325 (FIGURE 4)
326 Both Elovl5 and Elovl4 cDNAs isolated from rabbitfish possess all the main
327 structural features common for Elovl protein family members, including the predicted
328 transmembrane domains, the histidine box (HXXHH), and the canonical C-terminal ER
329 retrieval signal (KXRXX for Elovl5 and RXKXX for Elovl4) (Jakobsson et al., 2006).
330 The phylogenetic analysis confirmed that the newly isolated rabbitfish Elovl cDNAs
331 encoded distinct Elovl5 and Elovl4 proteins whose deduced aa sequences showed high
332 homology to their respective orthologues in other vertebrates. However, further
333 evidence of the specific Elovl type of the cDNAs from rabbitfish was obtained through
334 functional charaterisation in yeast. The rabbitfish Elovl5 demonstrated the ability to
335 elongate C_{18} and C_{20} PUFA substrates, with lesser activity observed towards C_{22} PUFA.
336 These results are consistent with previously reported specificities for mammal (Leonard
337 et al., 2000) and teleost Elovl5 proteins (Agaba et al., 2004, 2005; Hastings et al., 2005;
338 Morais et al., 2009, 2011; Zheng et al., 2009a; Gregory et al., 2010; Mohd-Yusof et al.,
339 2010), clearly indicating that vertebrate Elovl generally have broad substrate specificity.
340 Moreover, the rabbitfish Elovl5 has a preference for n-3 over n-6 PUFA substrates, in
341 agreement with results obtained for most species studied previously, including both
342 marine and freshwater fish (Agaba et al., 2005; Mohd-Yusof et al., 2010; Morais et al.,
343 2011).
344 In addition to 18:4n-3 and 18:3n-6, assayed in the present study, other potential C_{18}
345 PUFA substrates for Elovl5 could include 18:3n-3 and 18:2n-6 (Guillou et al., 2010). In
346 contrast to the 'classical' pathway of "Δ6 desaturation → elongation → Δ5
347 desaturation", the Δ8 pathway for the biosynthesis of EPA and ARA is achieved

348 through “elongation → Δ8 desaturation → Δ5 desaturation” (Fig. 4) (Monroig et al.,
349 2011b). Although not determined in the present study, it is possible that the rabbitfish
350 enzyme, like its homologue from the Southern bluefin tuna (Gregory et al., 2010), could
351 also elongate 18:3n-3 and 18:2n-6 to 20:3n-3 and 20:2n-6, respectively. This hypothesis
352 is supported by the recent demonstration that the rabbitfish Δ6/Δ5 Fad was also able to
353 effectively operate as a Δ8 desaturase, possibly limiting the production of “dead-end”
354 metabolic products in rabbitfish (Monroig et al., 2011b).

355 In contrast to the Elovl5 enzyme, rabbitfish Elovl4 showed the ability to elongate a
356 variety of FA substrates, generating products up to C_{36} in length. More specifically, the
357 rabbitfish Elovl4 demonstrated a role in the biosynthesis of both saturated and
358 polyunsaturated VLC-FA, similar to previous observations with zebrafish (isoform
359 Elovl4b) (Monroig et al., 2010a), cobia (Monroig et al., 2011a) and Atlantic salmon
360 (Carmona-Antoñanzas et al., 2011) Elovl4 proteins. In particular, yeast expressing the
361 rabbitfish Elovl4 were capable of elongating saturated VLC-FA such as 24:0, 26:0 and
362 28:0 up to 36:0. Similarly, C_{20} and C_{22} PUFA substrates could be efficiently elongated
363 to their corresponding n-3 or n-6 polyenoic products with C_{36} chain-lengths. Saturated
364 and polyunsaturated VLC-FA have been detected in specific lipid classes in tissues such
365 as brain, retina, and testes of terrestrial vertebrates (Poulos, 1995; McMahon et al.,
366 2009; Agbaga et al., 2010), but the presence of VLC-FA in fish has only been reported
367 in retinal lipids (Poulos, 1995). The tissue distribution of Elovl4 transcripts suggested
368 that eye (possibly retina) and brain are also major metabolic sites for the biosynthesis of
369 VLC-FA in fish as observed in other species (Monroig et al., 2010a, 2011a; Carmona-
370 Antoñanzas et al., 2011). In contrast, Elovl5 mRNA showed a widespread distribution
371 in rabbitfish tissues, consistent with the tissue distribution of Elovl5 in the majority of

372 fish species investigated to date (Agaba et al., 2004, 2005; Hastings et al., 2005; Morais
373 et al., 2009, 2011; Zheng et al., 2009; Gregory et al., 2010; Mohd-Yusof et al., 2010).
374 Beyond the role of Elovl4 in the biosynthesis of VLC-FA, it is also possible to
375 speculate that the expression of Elovl4 in brain and eye is activated in the production of
376 DHA that accumulates in these neural tissues (Tocher and Sargent, 1990; Tocher et al.,
377 1992; Tocher, 1993; Monroig et al., 2009). Supporting this hypothesis, functional
378 characterisation of the rabbitfish Elovl4 confirmed that this enzyme can participate in
379 the biosynthesis of DHA as previously shown for Elovl4 in other marine teleosts
380 (Monroig et al., 2011a). Thus, unlike terrestrial vertebrate orthologues, Elovl4 from
381 fish, including rabbitfish, have the ability to catalyse the conversion of 22:5n-3 to
382 24:5n-3, which is a step required in the biosynthesis DHA through the so-called
383 "Sprecher pathway" (Sprecher, 2000). This pathway was initially demonstrated in rat
384 but there is evidence that it may also operate in some fish species including rainbow
385 trout (Buzzi et al., 1996, 1997), Atlantic salmon and zebrafish (Tocher et al., 2003).
386 In summary, rabbitfish express at least two Elovl cDNAs with high homology in
387 sequence and function to Elovl5 and Elovl4 elongases previously investigated in other
388 fish species. Moreover, our results confirmed that these enzymes enable rabbitfish to
389 perform all the elongation reactions required for the biosynthesis of the physiologically
390 essential C_{20-22} LC-PUFA, and also the less common VLC-FA. Rabbitfish is thus the
391 first marine species where genes encoding Fad and Elovl enzymes, with all the activities
392 required for the production of DHA from C_{18} PUFA, have been characterised.
393
394 **Acknowledgements**
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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/)

587 Table 2. Functional characterisation of rabbitfish Elovl5 elongase in *Saccharomyces*
588 *cerevisiae*. Individual conversion rates were calculated according to the formula
589 [individual product area/(all products areas + substrate area)] x 100. Accumulated
590 conversions were computed by summing the individual conversion rate for each
591 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

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599 Table 3. Functional characterisation of rabbitfish Elovl4 elongase: Role in
600 biosynthesis of very long-chain saturated fatty acids (FA). Results are expressed as
601 an area percentage of total saturated FA C ≥ 24 found in yeast transformed with
602 either empty pYES2 vector (Control) 7or rabbitfish *elovl4* ORF.

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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

604 * Lignoceric acid used as exogenously added substrate.
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618 Table 4. Functional characterisation of the rabbitfish Elovl4 elongase: conversions
619 of polyunsaturated fatty acid (FA) substrates. Individual conversion rates were
620 calculated according to the formula [individual product area/(all products areas +
621 substrate area)] x 100. Accumulated conversions were computed by summing the
622 individual conversion rate for each particular product and also those for longer
624 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

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626 **Figure captions**

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

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640 Fig. 2. Phylogenetic tree comparing the deduced amino acid (aa) sequences of *S.*
641 *canaliculatus* (Sc) Elovl4 and Elovl5 with Elovl4, Elovl5 and Elovl2 proteins from
642 human (*Homo sapiens*, Hs), zebrafish (*Danio rerio*, Dr), Atlantic Salmon (*Salmo salar*,
643 Ss) and cobia (*Rachycentron canadum*, Rc). The tree was constructed using the
644 neighbor-joining method (Saitou and Nei, 1987) with MEGA4. The horizontal branch
645 length is proportional to aa substitution rate per site. The numbers represent the
646 frequencies (%) with which the tree topology presented was replicated after 10000
647 iterations.

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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 (≤ 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.

Figures

Figure 1

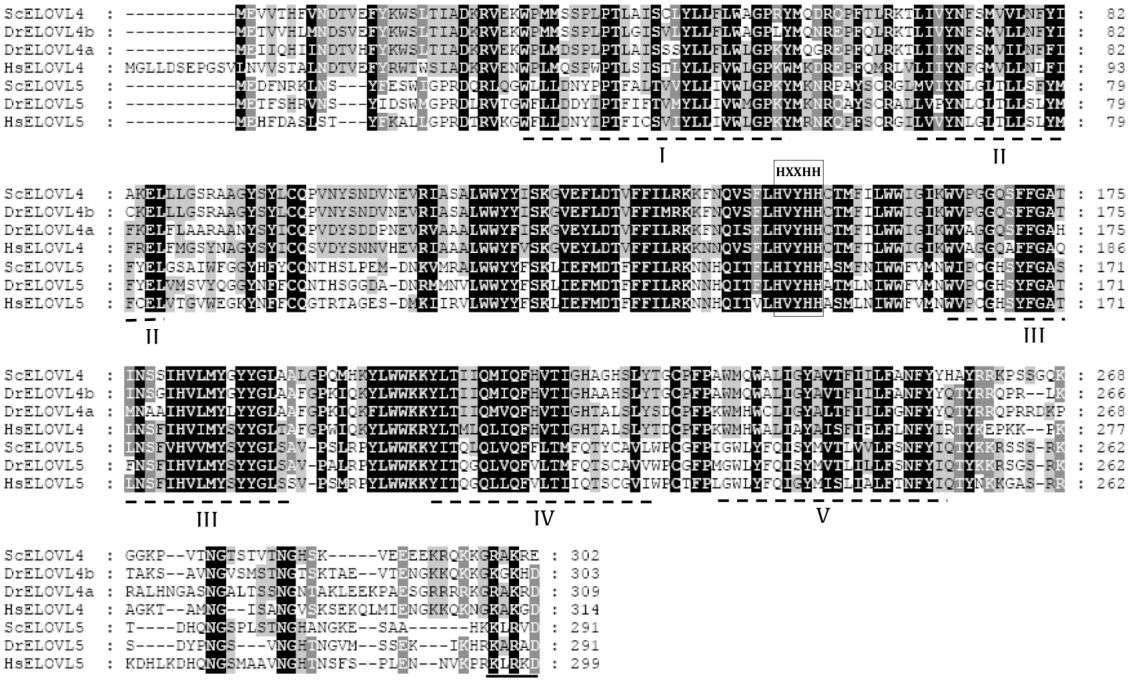
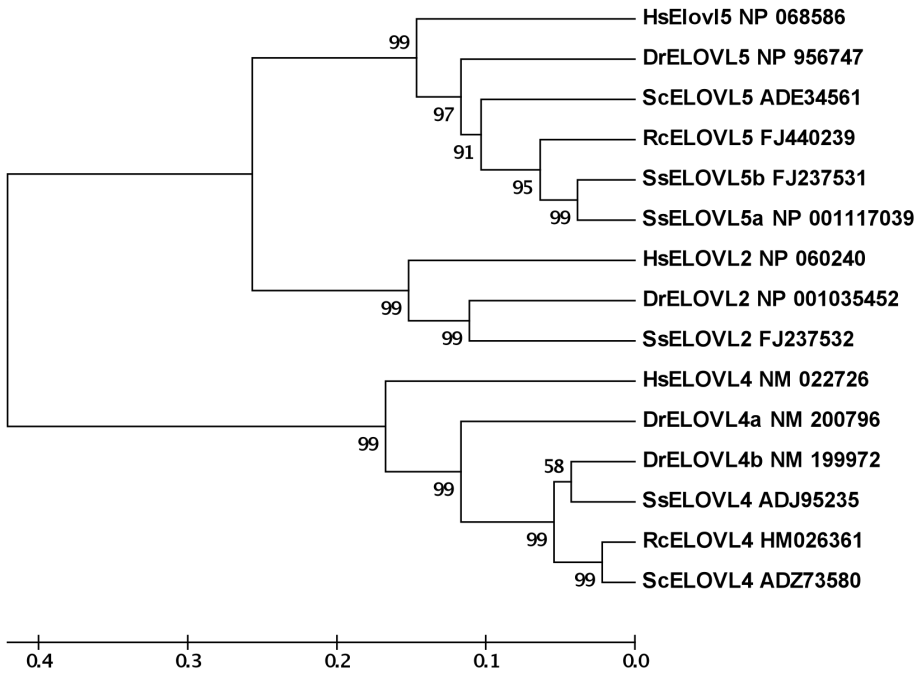


Figure 2



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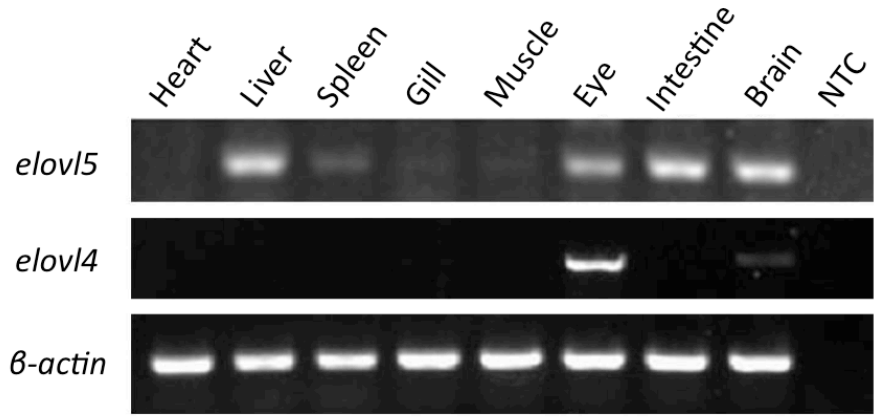
670 Figure 3



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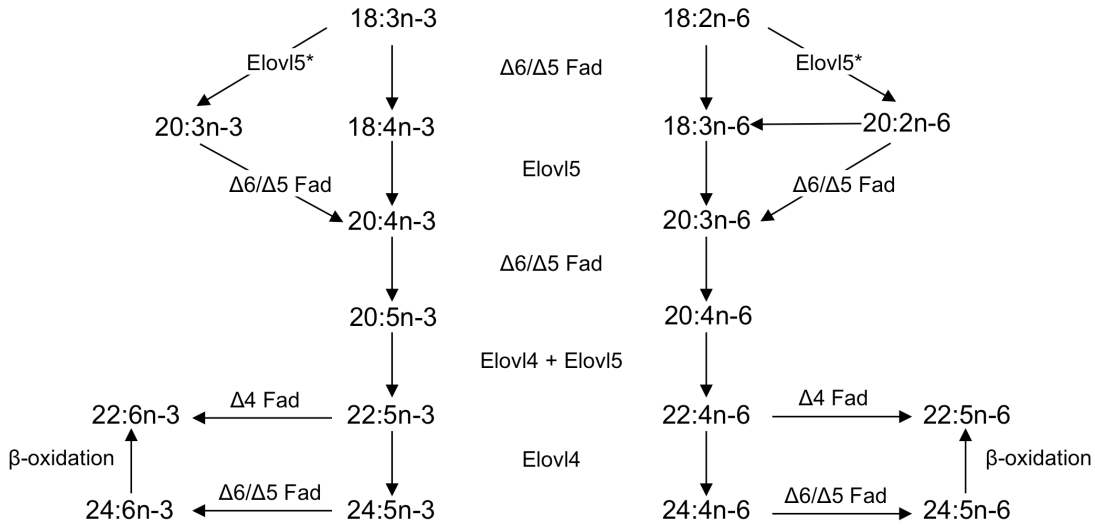
672 Figure 4


18:3n-3
Elovl5*
20:3n-3
18:4n-3
Δ6/Δ5 Fad
20:4n-3
20:5n-3
22:6n-3
Δ4 Fad
22:5n-3
β-oxidation
24:6n-3
Δ6/Δ5 Fad
24:5n-3
Δ6/Δ5 Fad
Elovl5
Δ6/Δ5 Fad
Elovl4 + Elovl5
Elovl4
18:2n-6
Elovl5*
18:3n-6
20:2n-6
Δ6/Δ5 Fad
20:3n-6
20:4n-6
22:4n-6
Δ4 Fad
22:5n-6
β-oxidation
24:4n-6
Δ6/Δ5 Fad
24:5n-6

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