1 **Title**

2 Biosynthesis of long-chain polyunsaturated fatty acids in marine fish: Characterization

3 of an Elovl4-like elongase from cobia *Rachycentron canadum* and activation of the

4 pathway during early life stages

5

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25   **Summary**

26   Marine fish, unlike freshwater species, have been generally considered to have a

27   limited ability to biosynthesize long-chain polyunsaturated fatty acids (LC-PUFA) from

28   C_{18} precursors due to apparent limited enzymatic activities involved in the pathway.

29   Although LC-PUFA play important physiological roles throughout the entire life cycle,

30   requirements for early life stages are especially high and provision of preformed LC-

31   PUFA in egg lipids appears critical to support the formation of developing tissues where

32   these compounds accumulate. No studies, however, have been conducted to explore the

33   capability of marine fish embryos (here referring to life stages from zygote to the

34   oesophagus opening) for *de novo* synthesis of the LC-PUFA required for normal growth

35   and development. The present study aimed to investigate the activation of the LC-PUFA

36   biosynthetic pathway during embryogenesis of the marine teleost cobia (*Rachycentron*

37   *canadum*). First, a fatty acyl elongase with sequence similarity to mammalian elongase

38   of very long-chain fatty acids 4 (Elovl4) was isolated, and its biochemical function

39   characterized showing that it catalyzed the production of very long-chain fatty acids

40   (VLC-FA) including both saturated and polyunsaturated fatty acids with chain lenghts ≥

41   24 carbons. Notably, cobia Elovl4 was able to elongate 22:5n-3 to 24:5n-3 and thus

42   could play a key role in the biosynthesis of docosahexaenoic acid (22:6n-3), a critical

43   fatty acid in neural tissues. Subsequently, the fatty acid dynamics of embryos at

44   different developmental stages and the temporal expression patterns of target genes

45   including *elovl4*, and the formerly characterized *elovl5* elongase and Δ6 fatty acyl

46   desaturase, were analyzed in order to elucidate the overall activation of the LC-PUFA

47   biosynthetic pathway in cobia embryos. Our results indicated that expression of the LC-

48   PUFA biosynthetic pathway in cobia embryos is initiated at 12-18 hours post-

49   fertilization.

1. Introduction

Fish are the primary source in the human diet of n-3 long-chain polyunsaturated fatty acids (LC-PUFA), which have been demonstrated to promote cardiovascular health and immune function, and protect against neurological and inflammatory conditions (Calder, 2007; Calon and Cole, 2007; Torrejon et al., 2007). With aquaculture increasing its contribution to the overall supply of fish in the human food basket (FAO, 2009), considerable efforts have been made to elucidate the biochemical and molecular mechanisms controlling the biosynthesis of LC-PUFA in fish (Tocher, 2003). These investigations have allowed us to determine that the capacity of fish to biosynthesize LC-PUFA varies with species, and ultimately depends on the enzymatic complement required in the metabolic process.

The accepted biosynthetic pathway in fish consists of consecutive enzymatic reactions that convert C_{18} PUFA 18:3n-3 (α-linolenic acid) and 18:2n-6 (linoleic acid) to LC-PUFA (Fig. 1). Two types of enzymes are responsible for these conversions (Sargent et al., 2002). Fatty acyl desaturases (Fad) introduce double bonds into fatty acyl chains, and elongases of very long-chain fatty acids (Elovl) are responsible for a condensation reaction resulting in a 2-carbon elongation of the pre-existing chain (Jakobsson et al., 2006). Thus for synthesis of arachidonic acid (20:4n-6, ARA), 18:2n-6 is desaturated by Δ6 Fad to 18:3n-6, which is elongated to 20:3n-6 and then desaturated by Δ5 Fad to ARA. Synthesis of eicosapentaenoic acid (20:5n-3, EPA) from 18:3n-3 requires the same enzymes and pathway as for ARA, but synthesis of docosahexaenoic acid (22:6n-3, DHA) reportedly requires two further elongation steps, a further Δ6 desaturation and a peroxisomal chain shortening step (Sprecher, 2000). An alternative more direct way for DHA biosynthesis has been recently described for the

3

74 first time in vertebrates in the herbivorous marine fish, *Siganus canaliculatus*, which

75 expresses a $\Delta 4$ Fad capable of desaturating 22:5n-3 to DHA (Li et al., 2010) (Fig. 1).

76   Most marine fish species have low LC-PUFA biosynthetic capacity due in part to the

77 apparent lack of specific enzymatic activities required in the pathway. Other than a

78 bifunctional $\Delta 6/\Delta 5$ Fad found in *S. canaliculatus* (Li et al., 2010), no $\Delta 5$ Fad has been

79 reported in marine species, and all *fad* genes have been characterized as monofunctional

80 $\Delta 6$ Fads (Zheng et al., 2004, 2009; Tocher et al., 2006; González-Rovira et al., 2009;

81 Mohd-Yusof et al., 2010). Additionally, marine teleosts appear to lack Elovl2, an

82 enzyme that elongates C_{20} and C_{22} LC-PUFA including 22:5n-3 to 24:5n-3 (Fig. 1) and

83 is thus regarded as an essential enzyme for DHA biosynthesis (Monroig et al., 2009;

84 Morais et al., 2009). Elovl2 functions differ from those of Elovl5, an elongase isolated

85 from a number of marine fish species (Agaba et al., 2005; Zheng et al., 2009; Gregory

86 et al., 2010; Mohd-Yusof et al., 2010), but which has virtually no activity towards C_{22}

87 LC-PUFA. However, marine fish species may have other Elovl enzymes whose

88 functions may partially compensate for the above mentioned incapacity to perform the

89 last elongation steps in the biosynthetic pathway of LC-PUFA.

90   Elovl4 is the most recent member of the Elovl family to be investigated in fish

91 (Monroig et al., 2010). Zebrafish possesses two Elovl4 enzymes that are responsible for

92 the biosynthesis of very long-chain fatty acids (VLC-FAs), including saturated and

93 polyunsaturated FA with chain-lengths $\geq C_{24}$. Studies in mammals have shown that

94 VLC-FA play pivotal functions in phototransduction, fertility and skin permeability

95 (Cameron et al., 2007; Agbaga et al., 2010; Zadravec, 2010), although they have been

96 barely investigated in fish (Alvedaño, 1987). Particularly interesting though, some fish

97 Elovl4 have, in contrast to mammalian Elovl4, the ability to facilitate the synthesis of

98 DHA by possessing Elovl2-like activity. Thus, whereas one zebrafish isoform, Elovl4a,

99   did not show relevant activity towards 22:5n-3, the other isoform, Elovl4b,

100   demonstrated the ability to elongate 22:5n-3 to 24:5n-3 when expressed in yeast

101   (Monroig et al., 2010). These results prompt the question whether marine species have

102   Elovl4 enzymes whose functions resemble those of zebrafish Elovl4a, or contrarily,

103   those of Elovl4b, the latter having important consequences for the production of

104   physiologically essential LC-PUFA including DHA, to compensate for the apparent

105   absence of Elovl2 in marine fish genomes.

106   The insufficiency in LC-PUFA biosynthesis in marine fish may be particularly

107   critical in early developmental stages, where physiological requirements for LC-PUFA,

108   especially DHA, are high due to the rapid formation and development of neural tissues

109   (Bell et al., 1995; Navarro et al., 1997; Benítez-Santana et al., 2007). Whereas dietary

110   LC-PUFA enhancement of broodstock has been shown to improve offspring viability

111   (Rodríguez et al., 1998; Mazorra et al., 2003), it is important to elucidate if early life-

112   stages of marine fish are capable of endogenous biosynthesis to supplement preformed

113   LC-PUFA deposited in the egg. The present study investigated the activation of the LC-

114   PUFA biosynthetic pathway during embryogenesis of the marine teleost, cobia

115   (*Rachycentron canadum*). Cobia is a rapidly emerging aquaculture species with

116   impressive growth performance, excellent flesh quality and many other favourable

117   production-related characteristics (Holt et al., 2007), and some pre-existing knowledge

118   of larval lipid nutrition and LC-PUFA synthesis (Faulk and Holt, 2003; Zheng et al.,

119   2009). Thus, an *elovl4*-like cDNA was isolated from cobia, and its function determined

120   in the yeast expression system, confirming its involvement in the biosynthesis of LC-

121   PUFA. The expression patterns of genes shown to participate in the LC-PUFA synthesis

122   pathway of cobia, including *elovl4* and the formerly characterized *elovl5* and Δ6*fad*

123 (Zheng et al., 2009), were then determined along with the fatty acid dynamics in
124 embryos collected at different stages of development.

125

126 **2. Materials and methods**

127 *2.1. Fish maintenance*

128   Fertilized eggs of cobia were obtained via photo-thermal conditioning of broodstock

129 maintained in a 42 m^3 recirculating aquaculture system (Holt et al., 2007) at the

130 Fisheries and Mariculture Laboratory of the University of Texas at Austin Marine

131 Science Institute in Port Aransas, Texas. The eggs were transferred to a 500 l tank and

132 incubated at 26 – 30 °C and salinity from 25.0 –33.0 ‰. Samples were collected at the

133 indicated sample points (see sections 2.6 and 2.7), rinsed with distilled water and

134 immediately frozen at -80 ºC until further analyses. Additionally, tissue samples were

135 collected from juvenile cobia (~250 g) reared in 8000 l tanks at the facilities.

136

137 *2.2. Cobia elovl4 cloning*

138   Total RNA was extracted from brain using TRIzol ® reagent (Gibco BRL, Grand

139 Island, NY, USA). First strand cDNA was synthesized using a Verso™ cDNA kit

140 (ABgene, Rockford, IL, USA) primed with random hexamers. The sequence of the

141 zebrafish *elovl4* (gb|NM_199972|) and the medaka EST (gb|DK113639.1|) were

142 aligned in order to design primers UNIE4F (5'- GTCTACAACTTCAGCATGGTG-3')

143 and UNIE4R (5'- GGAACTGGATCATCTGAATAAT-3') that were used for

144 polymerase chain reaction (PCR) using GoTaq$^®$ Colorless Master Mix (Promega,

145 Southampton, UK) on brain cDNA as template. The PCR included an initial denaturing

146 step at 95 ºC for 2 min, followed by 33 cycles of denaturation at 95 ºC for 30 s,

147 annealing at 55 ºC for 30 s, extension at 72 ºC for 40 s, followed by a final extension at

148 72 ºC for 5 min. The PCR fragment was sequenced (CEQ-8800 Beckman Coulter Inc.,

149 Fullerton, USA) and specific primers were designed to produce the full-length cDNA by

150 5' and 3' rapid amplification of cDNA ends (RACE) PCR (FirstChoice® RLM-RACE kit,

151 Ambion, Applied Biosystems, Warrington, UK) to produce full-length cDNA.

152

153 *2.3. Sequence and phylogenetic analyses*

154   The deduced amino acid (AA) sequence of the newly cloned cobia *elovl4* cDNA was

155 aligned with human ELOVL4 (NM_022726) and other fish orthologues including

156 zebrafish Elovl4a (gb|NM_200796|) and Elovl4b (gb|NM_199972|), pufferfish *Takifugu*

157 *rubripes* Elovl4 (derived from EST emb|ENSTRUT00000011027|) and *Tetraodon*

158 *nigroviridis* Elovl4 (emb|CAG01780|) using ClustalW2. AA sequence identity between

159 Elovl4-like proteins was compared by the EMBOSS Pairwise Alignment Algorithms

160 tool (http://www.ebi.ac.uk/Tools/emboss/align/). Phylogenetic analysis of the AA

161 sequences of Elovl4 from cobia and other vertebrates including fish, birds and mammals

162 was performed by constructing a tree using the Neighbor Joining method (Saitou and

163 Nei, 1987), with confidence in the resulting tree branch topology measured by

164 bootstrapping through 1000 iterations. The AA sequences of Elovl2 and Elovl5, both

165 proteins previously reported in teleosts, were also included in the phylogenetic analysis.

166

167 *2.4. Functional characterization of cobia Elovl4 by heterologous expression in*

168 *Saccharomyces cerevisiae*

169   PCR fragments corresponding to the open reading frame (ORF) of the putative *elovl4*

170 elongase were amplified from cobia brain cDNA using the high fidelity Pfu Turbo DNA

171 polymerase (Stratagene, Agilent Technologies, Cheshire, UK). A two-round PCR

172 approach was used with the first round performed with specific primers COBE4U5F

173 and COBE4U3R (Table 1). PCR conditions consisted of an initial denaturing step at 95

174 ºC for 2 min, followed by 32 cycles of denaturation at 95ºC for 30 s, annealing at 57 ºC

175 for 30 s, extension at 72 ºC for 1 min 45 s, followed by a final extension at 72 ºC for 5

176 min. First round PCR products were used as template for the nested PCR with thermal

177 conditions described above, and with primers containing restriction sites (underlined in

178 Table 1) COBE4VF (*Hin*dIII) and COBE4VR (*Xho*I). The DNA fragments were then

179 digested with the corresponding restriction endonucleases (New England BioLabs,

180 Herts, UK) and ligated into a similarly restricted pYES2 yeast expression vector

181 (Invitrogen, Paisley, UK). The purified plasmids (GenElute™ Plasmid Miniprep Kit,

182 Sigma) containing the putative *elovl4* ORF were then used to transform *S. cerevisiae*

183 competent cells (S.c. EasyComp Transformation Kit, Invitrogen). Transformation and

184 selection of yeast with recombinant pYES2-*elovl4* plasmids and yeast culture were

185 performed as described in detail previously (Agaba et al., 2004). Briefly, cultures of

186 recombinant yeast were grown in *S. cerevisiae* minimal medium$^{-uracil}$ supplemented with

187 one of the following fatty acid (FA) substrates: lignoceric acid (24:0), eicosapentaenoic

188 acid (20:5n-3), arachidonic acid (20:4n-6), docosapentaenoic acid (22:5n-3),

189 docosatetraenoic acid (22:4n-6) or docosahexaenoic acid (22:6n-3). Docosapentaenoic

190 and docosatetraenoic acids (> 98 – 99 % pure) were purchased from Cayman Chemical

191 Co. (Ann Arbor, USA) and the remaining FA substrates (> 99 % pure) and chemicals

192 used to prepare the *S. cerevisiae* minimal medium-uracil were from Sigma Chemical

193 Co. Ltd. (Dorset, UK). Lignoceric acid was dissolved in α-cyclodextrin (Singh and

194 Kishimoto, 1983) at 5 μM and added to the yeast cultures at a final concentration of 0.6

195 μM, whereas PUFA substrates were added at final concentrations of 0.75 (C_{20}) and 1.0

196 (C_{22}) mM. After 2 days, yeast were harvested and washed for further analyses. Yeast

197   transformed with pYES2 containing no insert were cultured under the same conditions

198   as a control treatment.

199

200   *2.5. Yeast FAME analysis by GC-MS*

201   Total lipids were extracted by homogenization of yeast samples in

202   chloroform/methanol (2:1, v/v) containing 0.01% BHT as antioxidant (Folch et al.,

203   1957). Fatty acid methyl esters (FAME) were subsequently prepared, extracted and

204   purified (Christie, 2003), and identified and quantified using a gas chromatograph

205   (GC8000) coupled to an MD800 mass spectrometer (ThermoFisher Scientific, Hemel

206   Hempstead, UK), and using the methodology described by Monroig et al. (2010).

207   Elongation rates from PUFA substrates were calculated by the proportion of substrate

208   FA converted to elongated FA product as [product area/(product area + substrate area)]

209   x 100. Conversion rates from 24:0 were not calculated as yeast endogenously contains

210   several of the FA involved in the elongation pathway. Instead, contents of individual

211   saturated FA $\geq C_{24}$ from *elovl4*-transformed yeast were calculated and compared to

212   control yeast.

213

214   *2.6. Expression of fad, elovl5 and elovl4 genes during cobia early development and*

215   *elovl4 tissue distribution*

216   To study the expression of the target genes during embryonic development of cobia,

217   pools of ~50 embryos were collected from a single spawn at 0, 3, 6, 12, 18, 24, 36, 48,

218   60, 72 and 84 hours post-fertilization (hpf). This time window encompasses the entire

219   embryogenesis of cobia (Faulk et al., 2007), herein referred to as the period between the

220   zygote stage and the oesophagus opening (Gatesoupe et al., 2001). Total RNA was

221   extracted using Tri Reagent (Sigma) according to the manufacturer's protocol, and 1 μg

222   of total RNA reverse transcribed into cDNA (Verso™ cDNA kit, ABgene) primed with

223   random hexamers. Expression of *fad*, *elovl5* and *elovl4* transcripts during embryonic

224   development was determined by reverse transcriptase PCR (RT-PCR) with an initial

225   denaturing step at 95 ºC for 2 min, followed by 35 cycles of denaturation at 95 ºC for 30

226   s, annealing at 56 ºC for 30 s, extension at 72 ºC for 40 s, and a final extension at 72 ºC

227   for 5 min using primers shown in Table 1. Expression of the housekeeping gene *β-actin*

228   was determined to check the efficiency of cDNA synthesis and the cDNA integrity.

229   Expression of the cobia putative *elovl4* was measured in adult tissues by RT-PCR.

230   Total RNA from pituitary gland, brain, liver, anterior intestine, eye, kidney, red and

231   white muscle, ovary, testis, gill, spleen, skin, stomach, pyloric caeca and spinal cord

232   was extracted as described above, and 1 μg of total RNA reverse transcribed into

233   cDNA. Primers used for expression of *elovl4* and *β-actin* were as described for the

234   embryo samples.

235

236   *2.7. Fatty acid analyses of cobia embryos*

237   In order to monitor the changes in FA composition during early development, pools

238   of ~200 embryos were sampled at different stages (0, 24, 48 and 72 hpf). Total lipid

239   extraction and FAME preparation were performed as described above for yeast samples.

240   FAME were analysed by gas chromatography and flame ionization detection as

241   described previously (Tocher et al., 2010).

242

243   **3. Results**

244   *3.1. Cobia Elovl4 sequence and phylogenetics*

245   A 2290-bp (excluding polyA tail) full-length cDNA sequence was obtained by 5' and

246   3' RACE PCR and deposited in the GenBank database under the accession number

247   HM026361. It contains an ORF of 918 bp encoding a putative protein of 305 AA,

248   sharing 42.1 % AA sequence identity with the previously described cobia Elovl5-like

249   elongase (Zheng et al., 2009). Cobia putative Elovl4 possesses the histidine dideoxy

250   binding motif HXXHH, and the putative endoplasmic reticulum (ER) retrieval signal

251   with an arginine (R) and a lysine (K) residues at the carboxyl terminus, RXKXX (Fig.

252   2) (Jakobsson et al., 2006). By sequence comparison with a mouse ELOVL4 (Zhang et

253   al., 2003), five putative transmembrane-spanning domains, containing hydrophobic AA

254   stretches, can be predicted (Fig. 2).

255   The deduced AA sequence from the Elovl4 cDNA predicts a protein that is 62.7 -

256   63.2 % identical to several mammalian ELOVL4-like elongases including human,

257   mouse and rat, and 62.3 - 63.2 % identical to predicted Elovl4 proteins from birds.

258   When the AA sequence of cobia Elovl4 was compared to fish Elovl4s, high identity

259   scores were found with *Tetraodon nigroviridis* Elovl4 (91.5 % identical) and zebrafish

260   Elovl4b (82.0 % identical), whereas lower identity scores were observed when

261   compared with zebrafish Elovl4a (70 % identity) and *Takifugu rubripes* (66.4 %

262   identity). Differentiation among fish Elovl4 proteins is also reflected in the phylogenetic

263   analysis. Although all fish Elovl4 proteins grouped with the mammalian and bird

264   orthologues, and separately from other members of the Elovl family such as Elovl2 and

265   Elovl5, two clusters appeared to exist, with cobia Elovl4 grouping with zebrafish

266   Elovl4b and pufferfish *Tetraodon nigroviridis* predicted Elovl4, and more distantly a

267   group including zebrafish Elovl4a and *Takifugu rubripes* Elovl4 (Fig.3).

268

269   *3.2. Functional characterization*

270   The cobia putative Elovl4 elongase was functionally characterized by determining the

271   FA profiles of transgenic *S. cerevisiae* containing the cobia *elovl4* cDNA ORF and

272   grown in the presence of potential FA substrates. In order to test the ability of cobia

273   Elovl4 to elongate saturated VLC-FA, yeast transformed with pYES2 containing the

274   putative *elovl4* ORF or no insert (control) were incubated with lignoceric acid (24:0)

275   (Table 2; Fig. 4). The results confirm that cobia Elovl4 is involved in the biosynthesis of

276   saturated VLC-FA. Thus, control yeast transformed with empty vector and incubated in

277   the presence of 24:0 contained measurable amounts of 24:0 (10.2 % of total saturates $\geq$

278   C_{24}), 26:0 (79.7 %) and 28:0 (7.9 %), with traces of 30:0 and 32:0 (Table 2). In contrast,

279   *elovl4*-transformed yeast showed a different profile of saturated VLC-FAs $\geq$ C_{24}

280   compared to control yeast, with decreased contents of 24:0 and 26:0, and concomitant

281   increased levels of 28:0 (4.3-fold), 30:0 (9.7-fold) and 32:0 (4.1-fold) (Table 2). These

282   results suggest that at least 24:0, 26:0 and 28:0 are good substrates for cobia Elovl4.

283   In order to test the role of cobia Elovl4 in the biosynthesis of VLC-PUFA, transgenic

284   yeast transformed with Elovl4 ORF were incubated with C_{20} (20:5n-3 and 20:4n-6) and

285   C_{22} (22:5n-3, 22:4n-6 and 22:6n-3) PUFA substrates (Table 3; Fig. 5). The FA

286   composition of the yeast transformed with pYES2 vector containing no insert (control)

287   is characterized by having only 16:0, 16:1n-7, 18:0 and 18:1n-9, together with

288   whichever exogenous FA was added, consistent with *S. cerevisiae* possessing no PUFA

289   elongase activity (Agaba et al., 2004). GC-MS analyses revealed that cobia Elovl4

290   elongated 20:5n-3 and 20:4n-6 with conversions of 33 % and almost 55 %, respectively

291   (Table 3). Cobia Elovl4 also showed high activity towards the C_{22} substrates, 22:5n-3

292   and 22:4n-6, with conversions of 34 % and 41 %, respectively (Table 3). Fatty acids

293   produced by *elovl4*-transformed yeast incubated with PUFA included polyenes up to

294   C_{36}, with C_{32} PUFA consistently being the most abundant products (Table 3; Fig. 5). It

295   is noteworthy that cobia Elovl4 was able to convert both 20:5n-3 and 22:5n-3 to 24:5n-

296   3, the C_{24} substrate for $\Delta 6$ Fad in DHA (22:6n-3) synthesis. However, in contrast cobia

297   Elovl4 showed very little activity towards DHA itself, which was only marginally

298   converted to longer products (Table 3).

299

300   *3.3. Temporal expression patterns of fad, elovl5 and elovl4*

301   Temporal expression of *fad*, *elovl5* and *elovl4* was studied by RT-PCR using cDNA

302   samples obtained from embryos at different developmental stages from 0 to 84 hpf (Fig.

303   6). Transcripts of the three target genes were detected from the zygote stage (0 hpf),

304   indicating that mRNA transcripts of these genes are transferred maternally (Monroig et

305   al., 2009, 2010). Although comparisons of transcript levels from RT-PCR analyses have

306   to be made with caution, some temporal patterns can be predicted in the expression of

307   the target genes. The three target genes showed low expression at the beginning of the

308   experimental period, with a noticeable signal increase from 18 hpf (*elovl4*) and 36 hpf

309   ($\Delta6fad$ and *elovl5*) onwards. The expression of the housekeeping gene *β-actin* remained

310   constant during early development of cobia.

311   Adult tissue distribution of *elovl4* mRNA transcript was determined by RT-PCR (Fig.

312   7). The results revealed that cobia *elovl4* was expressed in most of the tissues analyzed,

313   with eye (probably retina), brain and pituitary gland showing high expression signals.

314   Only low expression of *elovl4* was detected in liver and no expression was detected in

315   pyloric caeca, two major metabolic sites in the biosynthesis of $C_{18\text{-}22}$ LC-PUFA in fish

316   (Fig. 7).

317

318   *3.4. Fatty acid composition of cobia embryos*

319   Overall activity of the LC-PUFA synthesis pathway during cobia embryogenesis was

320   estimated by analyzing the FA composition (% of total FA) of embryos collected at

321   different developmental stages (Table 4). The percentages of C_{18} PUFA precursors,

322   18:3n-3 and 18:2n-6, were generally constant over the time-course of cobia

323   embryogenesis. The effects of embryogenic development on LC-PUFA levels were

324   variable depending upon the actual fatty acid. For instance, the proportion of DHA, the

325   most abundant LC-PUFA in cobia embryos, appeared to initially decrease and then

326   increase in later development without any clear trend or obvious pattern. Note that,

327   unlike transgenic yeast samples from Elovl4 functional characterization (see above),

328   VLC-FA could not be determined in the embryo samples. Whereas FA up to C_{22} are

329   present in measurable amounts in lipids, VLC-FAs selectively accumulate in specific

330   lipid classes of certain tissues such as retina, brain and gonads (Poulos, 1995), which are

331   not fully developed in embryonic stages. Therefore, the analysis of these compounds

332   requires large samples of specific tissues and thus is impractical in fish embryos.

333

334   **4. Discussion**

335   *Elovl* cDNAs including Elovl5- and Elovl2-like elongases have been cloned and

336   functionally characterized from several fish including freshwater, salmonid and marine

337   species (Agaba et al., 2004, 2005; Zheng et al., 2005; Monroig et al., 2009; Morais et

338   al., 2009; Gregory et al., 2010; Mohd-Yusof et al., 2010). More recently Elovl4 proteins

339   from zebrafish have been investigated, representing the first non-human Elovl4-like

340   proteins that have been functionally characterized (Monroig et al., 2010). *ELOVL4* was

341   first identified as a gene causing a dominant form of Stargardt-like macular dystrophy in

342   humans (Bernstein et al., 2001; Zhang et al., 2001). Its localization in the ER (Grayson

343   et al., 2005), the site of long-chain FA synthesis, its AA sequence similarities with other

344   elongase family proteins and its high expression levels in tissues having high

345   requirements for VLC-FAs, suggested a role for ELOVL4 in FA biosynthesis. The

346   actual function of ELOVL4, however, was recently confirmed by Agbaga et al. (2008)

347   who demonstrated that the human enzyme participates in the biosynthesis of VLC-FAs

348   including both saturated and polyunsaturated FAs. Whereas saturated VLC-FAs play

349   essential structural functions in the maintenance of skin permeability in mammals

350   (Uchida et al., 2008), the functions of the very long-chain polyunsaturated fatty acids

351   (VLC-PUFAs) appear to be related to their unusually long aliphatic chains (C_{24}-C_{38})

352   and the consequent characteristic that some VLC-PUFA possess by combining the

353   properties of saturated fatty acid in the proximal end with those of PUFA in the distal

354   end (Agbaga et al., 2008). Thus, VLC-PUFA are compounds uniquely found in specific

355   lipid molecules of retina (Alvedaño, 1987, 1988), brain (Robinson et al., 1990), and

356   testis (Furland et al., 2003, 2007a,b).

357   Cobia Elovl4 exhibits characteristic features of microsomal-bound enzymes including

358   a single histidine box redox centre motif, a canonical ER retention signal and multiple

359   transmembrane regions (Jakobsson et al., 2006; Molday et al., 2010). Phylogenetic

360   analysis suggested that the newly cloned *elovl4* cDNA encodes a protein more similar to

361   other Elovl4 proteins from mammals, birds and fish, in comparison to other fish Elovl

362   proteins including the previously characterized cobia Elovl5 (Zheng et al., 2009). This

363   is in agreement with Elovl4 proteins being highly conserved through evolution (Lagali

364   et al., 2003). Interestingly, fish Elovl4s themselves clustered in two separate groups

365   with zebrafish Elovl4a and *Takifugu rubripes* Elovl4 in one group, and zebrafish

366   Elovl4b, *Tetraodon nigroviridis* Elovl4 and cobia Elovl4, in the other. The clustering

367   pattern observed for fish Elovl4s is consistent with members of the two groups having

368   different functions.

369   The functional analysis of cobia Elovl4 revealed great similarities with the formerly

370   characterized zebrafish Elovl4b (Monroig et al., 2010). Whereas zebrafish Elovl4a was

371   only able to produce saturated VLC-FA, zebrafish Elovl4b, and now also cobia Elovl4,

15

372   are efficient in the synthesis of both saturated VLC-FA and VLC-PUFA up to C_{36}.

373   Decreased proportions of 26:0 and concomitant increased levels of 28:0 and 30:0 in

374   transgenic yeast expressing cobia *elovl4* indicate its involvement in the biosynthesis of

375   28:0 and 30:0 from 26:0. This is in agreement with the conversions shown by

376   mammalian ELOVL4 using genetically engineered mice (Cameron et al., 2007; Li et

377   al., 2007a,b; Vasireddy et al., 2007) and human cell lines not naturally expressing

378   *ELOVL4* (Agbaga et al., 2008). Additionally, the cobia Elovl4 elongated C_{20} and C_{22}

379   PUFA substrates that were converted to polyenes up to C_{36} of the n-3 and n-6 series.

380   These compounds are relatively abundant in specific lipid classes of tissues including

381   retina, testis and brain of vertebrates including fish (Poulos, 1995). Although VLC-FA

382   were not measured in cobia tissues for this study, the presence of *elovl4* mRNA

383   transcripts in some of those tissues including eye and brain suggests that these are also

384   metabolic sites for VLC-FA biosynthesis in fish. These findings highlight the

385   importance that the study of VLC-FA and their biosynthesis might have in farmed fish

386   in which altered visual acuity (critical in visual predators such as most cultured fish

387   species) and disruptions of brain functioning can jeopardize normal development of

388   fish. Also interesting is the fact that cobia Elovl4 appears to be highly expressed in

389   pituitary gland (hypophysis). Although it is well known that vertebrate brain regions

390   including pituitary gland accumulate LC-PUFA (Carrié et al., 2000), the presence of

391   *elovl4* mRNA indicates that an active biosynthesis of VLC-FAs, probably

392   polyunsaturated acyl chains, may occur in fish hypophysis.

393   DHA is one of the most abundant LC-PUFAs in tissues such as eye, brain and

394   gonads, the likely reason why Elovl4, highly expressed in these tissues, was initially

395   believed to be involved in the biosynthesis of DHA in mammals. Several studies,

396   however, have shown that mammalian Elovl4 does not directly participate in DHA

397 biosynthesis, but acts on longer ($> C_{26}$) polyunsaturated substrates (Molday et al.,

398 2010). The efficiency of cobia Elovl4 for the conversion of 22:5n-3 to 24:5n-3 suggests

399 that, in contrast to mammalian orthologues, some fish Elovl4s have a potential role in

400 the biosynthesis of DHA via the so-called Sprecher Pathway, in which 24:5n-3 is the

401 substrate for Δ6 Fad producing 24:6n-3, which is subsequently chain-shortened to DHA

402 (Sprecher, 2000). Whereas Elovl4 encountered in other marine fish genomes including

403 fugu *Takifugu rubripes* and stickleback *Gasterosteus aculeatus* have not been

404 functionally characterized, our results on cobia Elovl4 confirm that marine fish possess

405 Elovl4 involved in DHA biosynthesis that may act to compensate for the apparent lack

406 of Elovl2-like proteins. Interestingly such a role in DHA production predicted for cobia

407 Elovl4 is in contrast to the elongation activity shown by this protein on DHA itself.

408 Despite its activity towards similar substrates such as 22:5n-3, Elovl4 did not show

409 much activity towards DHA which was only marginally elongated. This is in agreement

410 with functional analysis of zebrafish Elovl4s (Monroig et al., 2010), and studies in

411 mammals where retina preparations showed active elongation of radiolabeled 22:5n-3,

412 whereas DHA remained virtually unmodified and was, in contrast, directly esterified

413 into phospholipids without further metabolism (Rotstein et al., 1996; Suh and

414 Clandinin, 2005).

415 Early developmental stages of organisms including fish show high requirements for

416 LC-PUFA to support the formation of specific tissues where they are selectively

417 accumulated in particular lipid classes (Tocher, 2003). Whereas deposition of

418 preformed essential LC-PUFA in the embryo has been proven to depend on broodstock

419 diet (Rodríguez et al., 1998; Mazorra et al., 2003; Izquierdo et al., 2001) and genetic

420 makeup (Pickova et al., 1997), the ability of fish embryonic stages to endogenously

421 biosynthesize essential LC-PUFA has remained unexplored. Based on the key elongase

and desaturase mRNA levels and the dynamics of FA biosynthesis products investigated in a recent study (Monroig et al., 2009), we predicted that zebrafish embryos are capable of LC-PUFA biosynthesis during early developmental stages. Similarly, cobia embryos also express a $\Delta6fad$ and the elongases *elovl4* and *elovl5*, all proved to participate in the biosynthesis pathway of LC-PUFA (Zheng et al., 2009). However, endogenous biosynthesis does not appear to be functional during the initial 24 h for cobia embryogenesis, with very low expression signals of $\Delta6fad$ and *elovl5* during this period. This argument is in part supported by the depletion of LC-PUFA, especially DHA and to a lesser extent EPA, during very early embryogenesis, when the expected utilization of saturated and monounsaturated FAs for energy supply would perhaps produce an increase in the relative amounts of DHA, which is preferentially retained in lipid cell membranes (Tocher et al., 1985; Fraser et al., 1988). The increased expression signals for *elovl4* (18 hpf) and $\Delta6fad$ and *elovl5* (36 hpf) suggest a potential activation of parts of the LC-PUFA biosynthetic pathway at later stages of cobia embryogenesis, possibly to fulfil the requirements of DHA necessary for the developing neuronal tissues. However, this was not clearly reflected in embryonic DHA levels and further experiments using embryonic cell culture preparations incubated with radiolabeled substrates are required to clarify how the increased expression of LC-PUFA biosynthetic genes affects enzymatic activities in the LC-PUFA biosynthetic pathway.

In conclusion, the present investigation demonstrates that cobia express an Elovl4-like protein with high similarity to other Elovl4 orthologues from vertebrates and whose function differs from that of the previously characterized Elovl5 in this species. Cobia Elovl4 is able to elongate both saturated and polyunsaturated substrates to products up to C_{36}. Notably, cobia Elovl4 can participate in the biosynthesis of DHA. Our results also demonstrate the presence of *elovl4*, *elovl5* and $\Delta6fad$ transcripts during

447 embryogenesis suggesting that parts of the LC-PUFA synthesis pathway may be

448 activated during development of embryos of marine fish species.

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457

458 **6. References**

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657

658   **Figure captions**

659   Fig. 1. Biosynthesis pathways of long-chain polyunsaturated fatty acids from C_{18}

660   precursors 18:3n-3 and 18:2n-6. Enzymatic activities shown in the scheme are predicted

661   from heterologous expression in *Saccharomyces cerevisiae* of genes isolated from fish

662   species. Dotted arrows indicate conversions only reported by a $\Delta 4$ desaturase from

663   rabbitfish *Siganus canaliculatus*.

664   *Conversion only reported for zebrafish Elovl4b (Monroig et al., 2010).

665

666   Fig. 2. ClustalW2 amino acid alignment of cobia Elovl4 with human ELOVL4

667   (gb|NP_073563.1|) and fish Elovl4-like proteins including *T. rubripes*

668   (emb|ENSTRUT00000011027|), zebrafish (gb|NM_200796| and gb|NM_199972|), and

669   *T. nigrov*iridis (emb|CAG01780|). Identical residues are shaded black and similar

670   residues (based on the Gonnet matrix, using ClustalW2 default parameters) are shaded

671   grey. Indicated are the conserved histidine box motif HXXHH, five (I-V) putative

672   membrane-spanning domains, and the putative endoplasmic reticulum (ER) retrieval

673   signal (Zhang et al., 2003).

674

675   Fig. 3. Phylogenetic tree comparing the putative cobia Elovl4, with other Elovl4

676   orthologues and Elovl2- and Elovl5-like elongases. The tree was constructed using the

677   Neighbour Joining method (Saitou and Nei, 1987) using MEGA4. The horizontal

678   branch length is proportional to amino acid substitution rate per site. The numbers

679   represent the frequencies (%) with which the tree topology presented was replicated

680   after 1000 iterations.

681

682   Fig. 4. Role of cobia Elovl4 in the biosynthesis of saturated very long-chain fatty acids

683   (VLC-FA). Yeast (*S. cerevisiae*) transformed with empty pYES2 vector (A) or pYES2

684   containing the ORF of *elovl4* (B) as insert were grown in the presence of lignoceric acid

685   (24:0), and the fatty acid composition was determined. Substrate 24:0 ("*") and its

686   corresponding elongated products are indicated accordingly. Vertical axis, MS

687   response; horizontal axis, retention time.

688

689   Fig. 5. Role of cobia Elovl4 in the biosynthesis of very long-chain fatty acids (VLC-

690   PUFA). Yeast (*S. cerevisiae*) transformed with pYES2 vector containing the ORF of

691   *elovl4* as insert were grown in the presence of PUFA substrates 22:5n-3 (A) and 22:4n-6

692   (B), and the fatty acid composition was determined. Substrates ("*") and their

693   corresponding elongated products are indicated accordingly. Vertical axis, MS

694   response; horizontal axis, retention time.

695

696   Fig. 6. RT-PCR analyses of the temporal expression patterns of the previously cloned

697   Δ6*fad* and *elvol5* (Zheng et al., 2009), and the newly isolated *elovl4* during cobia early

698   development (0 to 84 hpf). hpf, hours post-fertilization; NTC, no template control.

699

700   Fig. 7. RT-PCR analyses showing the spatial expression of *elovl4* in cobia adults.

701   Expression of the housekeeping gene *β-actin* is also shown.

702

Table 1. Sequence of the primer pairs used, size of the fragment produced and accession number of the sequence used as reference for primer design, for *elovl4* ORFs cloning and reverse transcriptase PCR (RT-PCR) performed in cobia embryos and adult tissues.

Aim	Transcript	Primer	Primer sequence	Fragment	Accession No[1].
ORF cloning	*elovl4*	COBE4U5F	5'-TGAGAGGAGCAGGGCATCAA-3'	1082 bp	HM026361
		COBE4U3R	5'-TCCTTCCCTACCCTCCATCCT-3'		
		COBE4VF	5'-CCCAAGCTTAGGATGGAGGTTGTAACACAT-3'	946 bp	
		COBE4VR	5'-CCGCTCGAGTCTTCCTTCTTTACTCCCT-3'		
RT-PCR	*Δ6fad*	COBDESF	5'-ATCTGTTTCCTACGATGCCA-3'	531 bp	FJ440238
		COBDESR	5'-AGCTGGGATTGTCAGGGTAA-3'		
	elovl5	COBELO5F	5'-GGTGGTACTACTTCTCCAAGC-3'	594 bp	FJ440239
		COBELO5R	5'-CCTAGCAGCATTTGCTAACAC-3'		
	elovl4	COBELO4F	5'-TGCCTGTACCTGCTCTTCCT-3'	446 bp	HM026361
		COBELO4R	5'-GCCAGGCCATAGTAACCGTA-3'		
	ß-actin	COBACTF	5'-GATCCTGACAGAGCGTGG-3'	132 bp	EU266539
		COBACTR	5'-GAAGAGGAGGAGGCAGC-3'		

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

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Table 2. Functional characterisation of cobia 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 $\geq$ 24
found in yeast transformed with either cobia *elovl4*
ORF or empty pYES2 vector (Control).

FA	Control	Elovl4
24:0*	10.2	5.8
26:0	79.7	41.8
28:0	7.9	33.9
30:0	1.5	14.5
32:0	0.7	2.9
34:0	0.0	0.8
36:0	0.0	0.2

* Lignoceric acid used as exogenously added substrate.

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Table 3. Functional characterisation of cobia Elovl4 elongase: conversions on polyunsaturated fatty acid (FA) substrates. Results are expressed as a percentage of total FA substrate converted to elongated product. Percentage of stepwise conversion into intermediary products of the elongation pathway is also shown.

FA substrate	Product	Elovl4	Activity
20:5n-3	22:5n-3	9.7	C20→22
	24:5n-3	3.7	C22→24
	26:5n-3	0.4	C24→26
	28:5n-3	0.2	C26→28
	30:5n-3	1.5	C28→30
	32:5n-3	12.0	C30→32
	34:5n-3	5.5	C32→34
	36:5n-3	0.2	C34→36
	Total	33.1	
20:4n-6	22:4n-6	11.1	C20→22
	24:4n-6	6.5	C22→24
	26:4n-6	1.0	C24→26
	28:4n-6	0.5	C26→28
	30:4n-6	6.4	C28→30
	32:4n-6	23.4	C30→32
	34:4n-6	5.3	C32→34
	36:4n-6	0.5	C34→36
	Total	54.6	
22:5n-3	24:5n-3	3.9	C22→24
	26:5n-3	0.5	C24→26
	28:5n-3	0.1	C26→28
	30:5n-3	1.9	C28→30
	32:5n-3	18.3	C30→32
	34:5n-3	9.0	C32→34
	36:5n-3	0.3	C34→36
	Total	34.1	
22:4n-6	24:4n-6	2.9	C22→24
	26:4n-6	0.6	C24→26
	28:4n-6	0.2	C26→28
	30:4n-6	4.9	C28→30
	32:4n-6	25.2	C30→32
	34:4n-6	6.4	C32→34
	36:4n-6	0.7	C34→36
	Total	40.9	
22:6n-3	24:6n-3	1.3	C22→24
	26:6n-3	0.0	C24→26
	28:6n-3	0.0	C26→28
	30:6n-3	0.2	C28→30
	32:6n-3	2.3	C30→32
	34:6n-3	0.6	C32→34
	36:6n-3	0.0	C34→36
	Total	4.3	

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Table 4. Fatty acid composition of cobia embryos at different stages of development. Results are expressed in percentage of total fatty acids.

Fatty acid	0 hpf	24 hpf	48 hpf	72 hpf
14:0	1.5	1.4	1.2	1.3
15:0	0.3	0.3	0.3	0.3
16:0	17.6	18.4	17.1	19.5
18:0	4.3	5.0	4.9	6.4
20:0	0.1	0.2	0.2	0.3
Total saturated	23.7	25.4	23.9	27.8
16:1n-9	0.3	0.4	0.3	0.3
16:1n-7	5.7	5.7	5.2	5.1
18:1n-9	12.9	13.3	12.7	13.5
18:1n-7	3.5	3.6	3.5	3.8
20:1[1]	0.9	1.1	1.3	1.0
22:1[2]	0.3	0.6	0.4	0.3
24:1n-9	0.3	0.5	0.4	0.4
Total monounsaturated	23.9	25.1	23.8	24.4
C_{16} PUFA	0.6	0.6	1.0	0.6
18:2n-6	2.6	2.6	2.6	2.6
18:3n-6	0.2	0.2	0.2	0.2
20:2n-6	0.1	0.2	0.2	0.2
20:3n-6	0.4	0.2	0.3	0.4
20:4n-6	2.6	2.5	2.9	3.1
22:4n-6	0.2	0.2	0.2	0.2
22:5n-6	0.6	0.5	0.6	0.6
Total n-6 PUFA	6.8	6.4	7.0	7.3
18:3n-3	0.5	0.5	0.5	0.5
18:4n-3	0.6	0.5	0.5	0.4
20:3n-3	0.1	0.1	0.1	0.1
20:4n-3	0.5	0.5	0.5	0.4
20:5n-3	10.5	9.5	9.7	8.6
22:5n-3	1.2	2.3	2.4	2.2
22:6n-3	26.0	22.2	25.0	23.7
Total n-3 PUFA	39.5	35.6	38.7	35.9

[1] predominantly n-9 isomer; [2] predominantly n-11 isomer; PUFA, polyunsaturated fatty acid; hpf, hours post-fertilisation

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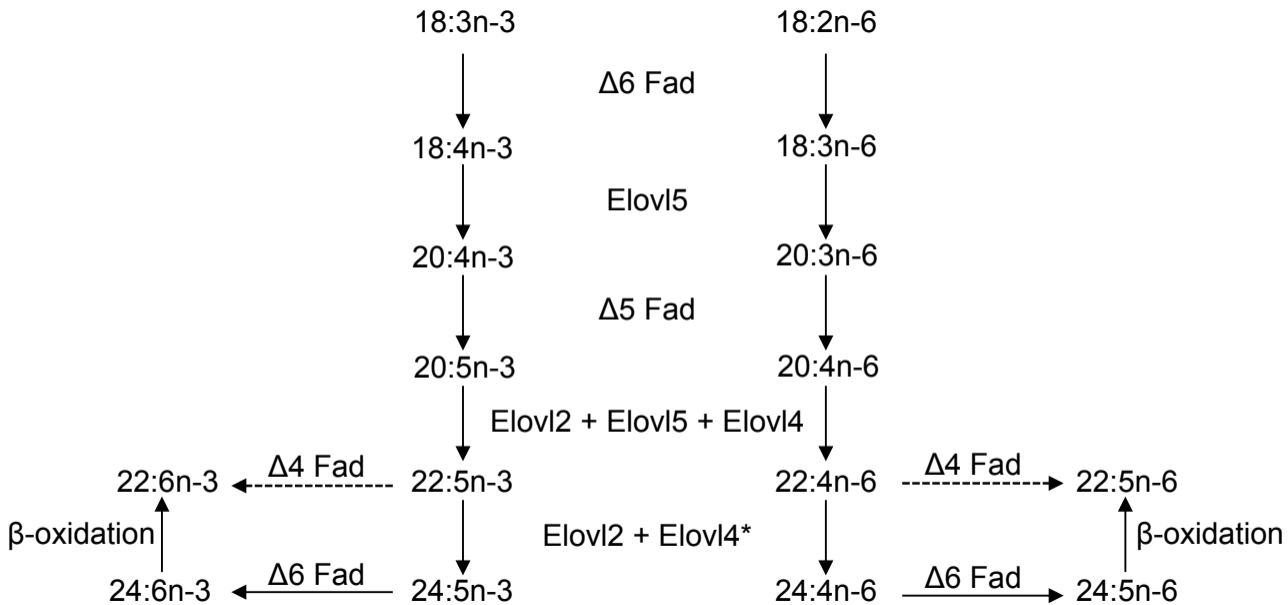
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Danio rerio Elovl4a     -----------MEIIQHIINDTVHFYKWSLTIADKRVEKWPLMDSPLPTLAISSSYLLFL  49
D. rerio Elovl4b        -----------METVVHLMNDSVEFYKWSLTIADKRVEKWPMMSSPLPTLGISVLYLLFL  49
Tetraodon nigroviridis  -----------MEVVTHFVNDTVEFYKWSLTIADKRVENWPMMSSPIPTLVISCLYLFFL  49
Rachycentron canadum    -----------MEVVTHFVNDTVEFYKWSLTIADKRVENWPMMASPLPTLAISCLYLLFL  49
                                                                        I

Homo sapiens            WLGPKWMKDREPFQMRLVLIIYNFGMVLLNLFIFRELFMGSYNAGYSYICQSVDYSNNVH 120
Takifugu rubripes       WLGPKYMKNREPFQLRKTLIVYNFSMVFLNFFIFKELFMAARAAKYSYICQRVDYSDDPN 109
Danio rerio Elovl4a     WLGPKYMQGREPFQLRKTLIIYNFSMVILNFFIFKELFLAARAANYSYICQPVDYSDDPN 109
D. rerio Elovl4b        WAGPLYMQNREPFQLRKTLIVYNFSMVLLNFYICKELLLGSRAAGYSYLCQPVNYSNDVN 109
Tetraodon nigroviridis  WAGPRYMQDRQPYTLRKTLIVYNFSMVLNFYIAKELLLGSRAAGYSYLCQPVNYSNDVN 109
Rachycentron canadum    WVGPRYMQDRQPYTLRRTLIVYNFSMVLNFYIAKELLIATRAAGYSYLCQPVNYSNDVN 109
                                                  II

                                                              HXXHH
Homo sapiens            EVRIAAALWWYFVSKGVEYLDTVFFILRKKNNQVSFLHVYHHCTMFTLWWIGIKWVAGGQ 180
Takifugu rubripes       EVRVAGALWWYFISKGIEYLDTVFFILRKKFSQVTFLHVYHHCTMFTLWWIGIKWVAGGQ 169
Danio rerio Elovl4a     EVRVAAALWWYFISKGVEYLDTVFFILRKKFNQISFLHVYHHCTMFTLWWIGIKWVAGGQ 169
D. rerio Elovl4b        EVRIASALWWYYISKGVEFLDTVFFIMRKKFNQVSFLHVYHHCTMFILWWIGIKWVPGGQ 169
Tetraodon nigroviridis  EVRIASALWWYYISKGVEFLDTVFFILRKKFTQVSFLHVYHHCTMFILWWIGIKWVPGGQ 169
Rachycentron canadum    EVRIASALWWYYISKGVEFLDTVFFILRKKFNQVSFLHVYHHCTMFILWWIGIKWVPGGQ 169

Homo sapiens            AFFGAQLNSFIHVIMYSYYGLTAFGPWIQKYLWWKRYLTMLQLIQFHVTIGHTALSLYTD 240
Takifugu rubripes       SFFGAHMNAAIHVLMYLYYGLASCGPKIQKYLWWKKYLTIIQMVQFHVTIGHTALSLYVN 229
Danio rerio Elovl4a     SFFGAHMNAAIHVLMYLYYGLAAFGPKIQKFLWWKKYLTIIQMVQFHVTIGHTALSLYSD 229
D. rerio Elovl4b        SFFGATINSGIHVLMYGYYGLAAFGPKIQKYLWWKKYLTIIQMIQFHVTIGHAAHSLYTG 229
Tetraodon nigroviridis  SFFGATINSSIHVLMYGYYGLAALGPQMQRYLWWKKYLTIIQMIQFHVTIGHAGHSLYTG 229
Rachycentron canadum    AFFGATINSSIHVLMYGYYGLAALGPQMQKYLWWKKYLTIIQMIQFHVTIGHAGHSLYTG 229
                                     III                                 IV

Homo sapiens            CPFPKWMHWALIAYAISFIFLFLNFYIRTYKEP--KKPKAG--------KTAMNGISANG 290
Takifugu rubripes       CDFPHWMHYSLICYAITFIVLFGNFYYQTYRRQQPRRDASSSKAAKAVANGALNGLSRNA 289
Danio rerio Elovl4a     CPFPKWMHWCLIGYALTFIILFGNFYYQTYRRQ-PRRDKP-----RALHNGASNGALTSS 283
D. rerio Elovl4b        CPFPAWMQWALIGYAVTFIILFANFYYQTYRRQP--RLKTA--------KSAVNGVSMST 279
Tetraodon nigroviridis  CPFPTWMQWALIGYAVTFIILFANFYYHAYRRKPSSKQKGG--------KNITNGNTAVT 281
Rachycentron canadum    CPFPCWMQWALIGYAVTFIILFANFYYHAYRGKPSSSQKGG--------KPIANGTSVVT 281
                                  V

                                                        ER
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Takifugu rubripes       NGAAVMGGKDEKPQENSGRRKRKGRAKRD 318
Danio rerio Elovl4a     NGNTAK--LEEKPAE-SGRRRRKGRAKRD 309
D. rerio Elovl4b        NGTSKT----AEVTENGK-KQKKGKGKHD 303
Tetraodon nigroviridis  NGHSNA----EEEEDGKKRQKKGRAKRE 306
Rachycentron canadum    NGHSKV----EEVEDNGK-RQKKGRAKRE 305
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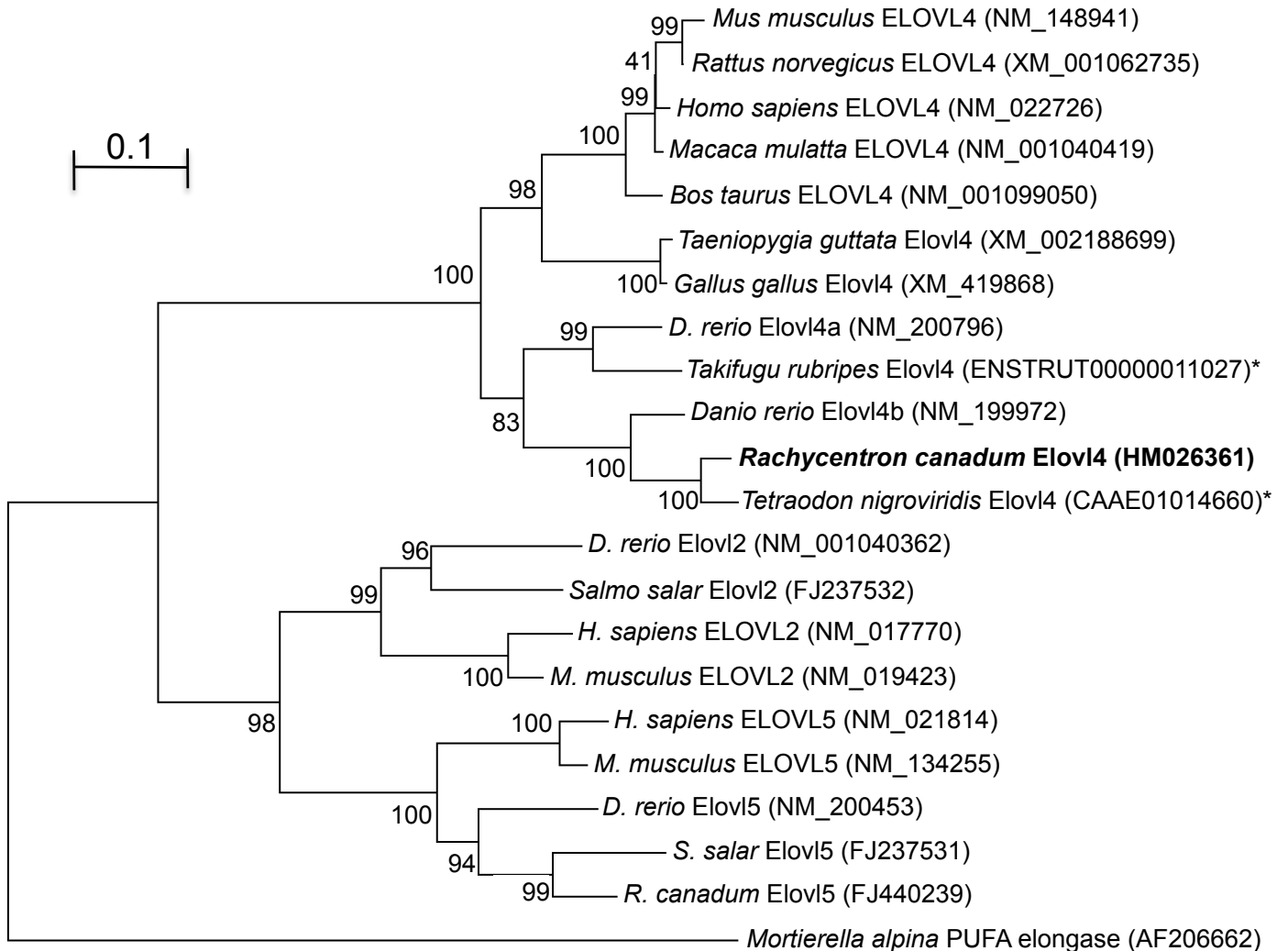
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99 *Mus musculus* ELOVL4 (NM_148941)

41 *Rattus norvegicus* ELOVL4 (XM_001062735)

99 *Homo sapiens* ELOVL4 (NM_022726)

100 *Macaca mulatta* ELOVL4 (NM_001040419)

98 *Bos taurus* ELOVL4 (NM_001099050)

Taeniopygia guttata Elovl4 (XM_002188699)

100 *Gallus gallus* Elovl4 (XM_419868)

100

99 *D. rerio* Elovl4a (NM_200796)

Takifugu rubripes Elovl4 (ENSTRUT00000011027)*

83 *Danio rerio* Elovl4b (NM_199972)

100 **Rachycentron canadum Elovl4 (HM026361)**

100 *Tetraodon nigroviridis* Elovl4 (CAAE01014660)*

96 *D. rerio* Elovl2 (NM_001040362)

99 *Salmo salar* Elovl2 (FJ237532)

H. sapiens ELOVL2 (NM_017770)

100 *M. musculus* ELOVL2 (NM_019423)

98

100 *H. sapiens* ELOVL5 (NM_021814)

M. musculus ELOVL5 (NM_134255)

D. rerio Elovl5 (NM_200453)

100

94 *S. salar* Elovl5 (FJ237531)

99 *R. canadum* Elovl5 (FJ440239)

Mortierella alpina PUFA elongase (AF206662)

	0	3	6	12	18	24	36	48	60	72	84	NTC
Δ6fad												
elovl5												
elovl4												
β-actin												