

1 **Molecular and functional characterization of a putative *elovl4* gene**
2 **and its expression in response to dietary fatty acid profile in Atlantic**
3 **bluefin tuna (*Thunnus thynnus*)**

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25 **Abstract**

26 Elongation of very long-chain fatty acid 4 (Elovl4) proteins are involved in the biosynthesis of very
27 long-chain (>C₂₄) fatty acids and in many teleost fish species they are key enzymes in the pathway
28 for the production of docosahexaenoic acid (DHA; 22:6n-3) from eicosapentaenoic acid (EPA; 20:5n-
29 3). Therefore, Elovl4 may be particularly important in Atlantic bluefin tuna (ABT; *Thunnus thynnus*)
30 characterised by having high DHA to EPA ratios. The present study cloned and characterised both
31 the function and expression of an *elovl4* cDNA from ABT. The Elovl4 had an open reading frame of
32 915 base pairs encoding a putative protein of 304 amino acids. Functional characterisation
33 demonstrated that the Elovl4 enzyme had elongase activity towards all the polyunsaturated fatty acid
34 (PUFA) substrates assayed. The ABT Elovl4 contributed to DHA biosynthesis by elongation of EPA
35 and DPA to 24:5n-3, the latter being desaturated to 24:6n-3 by the action of *fads2* (Δ 6 desaturase).
36 Additionally, the ABT Elovl4 has a role in the biosynthesis of very long-chain PUFA up to C₃₄,
37 compounds of key structural roles in neural tissues such as eye and brain, which had high levels of
38 *elovl4* transcripts. Surprisingly, while the relative expression of *fads2*, required for the production of
39 DHA from EPA, was increased in liver of ABT fed a diet with reduced levels of EPA and DHA,
40 expression of *elovl4* was reduced. Results indicated that ABT has enzymes necessary for endogenous
41 production of DHA from EPA and demonstrate that Elovl4b can effectively compensate for absence
42 of Elovl2.

43

44 *Keywords:* Biosynthesis; docosahexaneonic acid; eicosapentaenoic acid; polyunsaturated fatty acids;
45 Sprecher pathway; very long-chain fatty acids.

46 **1. Introduction**

47 Atlantic bluefin tuna (ABT; *Thunnus thynnus*) is a large pelagic migratory fish species that plays
48 an important role as a top predator, influencing Atlantic and Mediterranean marine communities
49 (Shimose and Wells, 2015). Traditionally, ABT fisheries have been supplemented by farming
50 although this has actually relied on the capture of juveniles in the wild, to be fattened in so-called
51 tuna ranches (Benetti et al., 2016). In recent years, considerable efforts have been made to close the
52 life cycle of ABT (van Beijnen, 2017), and success in the production of larvae and juveniles has
53 provided the animals to enable studies into the elucidation of ideal compositions of both live feeds
54 (Betancor et al., 2017a,b) and inert weaning diets (Betancor et al., 2019). Compared to most teleost
55 fish species, tissues of ABT have high levels of the health-beneficial omega-3 long-chain (C₂₀₋₂₄)
56 polyunsaturated fatty acid (n-3 LC-PUFA), docosahexaenoic acid (DHA; 22:6n-3) and very high
57 DHA: eicosapentaenoic acid (EPA; 20:5n-3) ratios (Mourete and Tocher, 2003, 2009). This may
58 indicate a high dietary requirement for DHA, as the LC-PUFA profile of teleosts often reflects dietary
59 intake, but also may suggest preferential retention and accumulation and/or biosynthesis of DHA
60 from EPA, as tissue fatty acid compositions also reflect endogenous metabolism to some extent
61 (Tocher, 2003, 2010; Monroig et al., 2018). However, dietary DHA and EPA can currently only be
62 supplied economically by marine raw materials (fish oil and fishmeal) and, with stagnating supply
63 and increasing demand, the trend nowadays in aquafeed formulation is for the use of high levels of
64 terrestrial vegetable oils, naturally devoid of LC-PUFA, as primary lipid sources (Tocher, 2015). This
65 translates into a low dietary intake of n-3 LC-PUFA, which consequently reduces the contents of the
66 beneficial DHA and EPA in farmed fish (Sprague et al., 2016). The impacts of dietary vegetable oil
67 and low levels of dietary n-3 LC-PUFA, especially DHA, on ABT are as yet unknown (Mourete and
68 Tocher, 2009).

69 LC-PUFA can be biosynthesised from the C₁₈ PUFA, α -linolenic acid (18:3n-3) and linoleic acid
70 (18:2n-6), through enzymatic reactions mediated by fatty acyl desaturases (Fads) and elongation of
71 very long-chain fatty acid (Elovl) proteins (Castro et al., 2016; Monroig et al., 2018). Elovl have been
72 considered as rate-limiting enzymes in fatty acid synthesis, with three members identified as being
73 capable of elongating PUFA, namely Elovl2, Elovl4 and Elovl5 (Guillou et al., 2010; Jakobsson et
74 al., 2006). Most studies on teleost Elovl proteins have focussed on the characterisation of Elovl2 and
75 Elovl5 from farmed species (Monroig et al., 2016, 2018). These studies have shown that Elovl5
76 proteins are found in most teleost species and primarily elongate C₁₈ and C₂₀ PUFA, whereas Elovl2
77 acts mainly on C₂₀ and C₂₂ PUFA (Castro et al., 2016) and is absent in most ray-finned fish (Monroig
78 et al., 2016). In recent years, considerable attention has been given to the presence of Elovl4 proteins
79 in teleosts, with several farmed species shown to possess these elongases (Carmona-Antoñanzas et
80 al., 2011; Monroig et al., 2012; Kabeya et al., 2015; Jin et al., 2017; Li et al., 2017a,b; Oboh et al.,

81 2017a; Zhao et al., 2019). In mammals, Elovl4 has been demonstrated to be a critical enzyme in the
82 biosynthesis of both very long-chain (>C₂₄) saturated (VLC-SFA) and polyunsaturated fatty acids
83 (VLC-PUFA) (McMahon et al., 2007; Agbaga et al., 2008). However, in zebrafish (*Danio rerio*), the
84 first fish species in which Elovl4 was studied, two genes *elovl4a* and *elovl4b* were identified with
85 both proteins able to elongate saturated fatty acids, but only Elovl4b able to elongate PUFA (Monroig
86 et al., 2010). *In silico* searches have suggested that all teleost species likely have at least one copy of
87 both *elovl4a* and *elovl4b* (Castro et al., 2016). Importantly, teleost Elovl4b are generally able to
88 elongate 20:5n-3 (EPA) and 22:5n-3 to 24:5n-3 (Castro et al., 2016; Monroig et al., 2016), and thus
89 have the capability to play a role in the biosynthesis of DHA via the Sprecher pathway (Sprecher,
90 2000). Indeed, it has been suggested that the acquisition/retention of this ability by teleost Elovl4b
91 might compensate for the loss of Elovl2 during the evolution history of teleosts (Monroig et al., 2010;
92 2016). An early study demonstrated that ABT possessed a Fads2 with Δ 6 desaturase activity, as well
93 as an Elovl5 with the ability to elongate mainly C₁₈ and C₂₀ PUFA (Morais et al., 2011). Interestingly,
94 the ABT Elovl5 showed relatively high elongation activity towards 22:5n-3 compared to most teleost
95 Elovl5 (Morais et al., 2011), suggesting that this enzyme has some potential to contribute to the
96 Sprecher pathway as described above for Elovl4b-like proteins, in a species like ABT that lacks
97 Elovl2. Moreover, the presence or otherwise of Elovl4 in ABT was not investigated in the earlier
98 study.

99 It is known that several factors can regulate the enzymatic machinery involved in LC-PUFA
100 biosynthesis (Monroig et al., 2018). Both environmental and nutritional (diet) factors can influence
101 the expression and activity of the LC-PUFA biosynthetic enzymes (Zheng et al., 2005; Morais et al.,
102 2011; Monroig et al., 2018). Considerable research has demonstrated how dietary fatty acid profile
103 can impact the expression levels of *fads* and *elovl* genes in fish. In salmonids, an up-regulation in
104 *fads2* occurs in fish fed a diet with low levels of LC-PUFA, especially DHA (Betancor et al., 2014,
105 2015b, 2016), whereas this response is not as pronounced in carnivorous marine species (Torstensen
106 and Tocher, 2011). Few studies have evaluated the regulation of teleost *elovl4* in response to dietary
107 LC-PUFA levels (Li et al., 2017a,b; Zhao et al., 2019). Furthermore, the interrelationship between
108 the expression levels of *elovl4* with those of the different biosynthetic enzymes in the LC-PUFA
109 pathway has not been extensively studied.

110 The overarching aim of the present study is to further elucidate the biochemical mechanisms
111 underpinning the high DHA:EPA ratio in ABT, specifically investigating LC-PUFA biosynthetic
112 pathways and the production of DHA from EPA. To this end, the cDNA of an *elovl4* was cloned from
113 ABT and its tissue transcript distribution determined. We further established the function of the
114 Elovl4 in VLC-PUFA biosynthesis, and investigated the potential contribution of ABT Elovl4 and
115 Elovl5 to DHA biosynthesis *via* the Sprecher pathway. In addition, ABT juveniles were fed diets with

116 varying n-3 LC-PUFA levels to investigate the effect of dietary fatty acid composition on the
117 expression levels of the newly characterised *elovl4* as well as other genes of LC-PUFA biosynthesis
118 in ABT, namely *elovl5* and *fads2*. Taking all the data into account, the capability of ABT for the
119 biosynthesis of DHA and their potential to utilise modern, sustainable feeds rich in ingredients of
120 terrestrial origin is discussed.

121

122 **2. Materials and methods**

123 *2.1. Experimental animals*

124 All procedures were conducted in accordance with the regulations set forward by the Spanish RD
125 53/2013 (BOE 8th February 2013) and Directive 2010/63/EU of the European Parliament and the
126 Council of 22 September 2010 on the protection of animals used for scientific purposes. Additionally,
127 all experimental procedures were reviewed and approved by the Animal Welfare and Ethical Review
128 Board (AWERB) of the University of Stirling, Scotland, UK.

129 Juveniles for the nutritional trial were produced from eggs spawned in summer 2017 from captive
130 wild ABT broodstock fish maintained in a floating net cage located at El Gorguel, off the Cartagena
131 coast, SE Spain. The eggs were transferred to the Planta Experimental de Cultivos Marinos, Instituto
132 Español de Oceanografía (IEO), Puerto de Mazarrón (Murcia), Spain for hatching and initial
133 larviculture (Ortega, 2015; de la Gándara et al., 2016). Fish were weaned from the live feed stage,
134 fed gilthead sea bream (*Sparus aurata* L.) yolk sac larvae as prey, to formulated feed at 27 days after
135 hatch (dah) using a commercial diet (Magokoro®; Marubeni Nisshin Feed Co., Japan; Okada et al.,
136 2014; Kurata et al., 2015; Honryo et al., 2018) and were completely weaned by 32 dah.

137 Samples of tissues including brain, gill, heart, kidney, spleen, liver, intestine, red and white
138 muscle, adipose tissue, ovary, testis and eye used for cloning and tissue distribution of *elovl4*
139 expression were obtained from eight wild broodstock ABT (4 males and 4 females) allocated to a
140 floating cage located at El Gorguel Bay (as above) and culled for reproductive stage assessment.

141

142 *2.2. Nutritional trial*

143 A total of 184 ABT juveniles (41 dah; initial weight 3.3 ± 0.6 g) were distributed into four
144 experimental tanks (water volume 5 m³) at a stocking density of 46 individuals per tank. The fish
145 were fed *ad libitum* two diets using krill oil as the single lipid source (KO) or a blend of krill oil and
146 rapeseed oil (50:50; KORO) for 10 days. The diets were isoproteic (56 %) and isolipidic (15 %) but
147 supplied differing levels of n-3 LC-PUFA (38.4 % versus 25.5 %; Table 1). At the end of the
148 experimental trial, approximately 100 - 150 mg of liver tissue (samples of individual livers from three
149 fish per tank; six per dietary treatment) were placed in 1 ml RNAlater® (Sigma-Aldrich, Dorset, UK)

150 and processed according to manufacturer's instructions (4 °C for 24 h) before storage at -80 °C prior
151 to RNA extraction and subsequent analysis.

152

153 2.3. Tissue RNA extraction and cDNA synthesis

154 Adult ABT tissue and juvenile liver samples were homogenised in 1 ml of TriReagent® (Sigma-
155 Aldrich) RNA extraction buffer using a bead tissue disruptor (Bio Spec, Bartlesville, Oklahoma,
156 USA). Total RNA was isolated following manufacturer's instructions and quantity and quality
157 determined by spectrophotometry using a Nanodrop ND-1000 (Labtech Int., East Sussex, UK), and
158 electrophoresis using 200 ng of total RNA in a 1 % agarose gel. cDNA was synthesised using 2 µg
159 of total RNA and random primers in 20 µl reactions and the high capacity reverse transcription kit
160 without RNase inhibitor according to the manufacturer's protocol (Applied Biosystems, Warrington,
161 UK).

162

163 2.4. Molecular cloning of *elovl4*

164 Primers for cloning the cDNA open reading frame (ORF) sequence of *elovl4* were designed on
165 several ABT sequence read archive (SRA) by identifying and assembling the sequences using CAP3
166 (Huang and Madan, 1999). This was achieved by blasting the black seabream *elovl4b* sequence
167 against the available ABT transcriptomic data from SRA SRX2255758, ERX555873 and
168 ERX555874. Amplification of the first fragment of the gene, which included the ORF and parts of
169 the 5' and 3' regions was achieved by polymerase chain reaction (PCR) using cDNA synthesised from
170 adult ABT brain total RNA as template and primers designed on the 5' (UniE4F, 5'-
171 GCAGTGGTATCAACGCAGAG-3') and 3' (UniE4R, 5'-TCTCTATCCCTTCCCTCCCC-3')
172 regions of the ABT sequences obtained from SRA. PCR conditions consisted of an initial denaturation
173 step at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C
174 for 30 s, extension at 72 °C for 80 s, followed by a final extension at 72 °C for 7 min. PCR fragments
175 were purified using the Illustra GFX PCR DNA/gel band purification kit (GE Healthcare, Little
176 Chalfont, Bucks., UK), and sequenced at GATC Biotech Ltd. (Konstanz, Germany).

177

178 2.5. Sequence and phylogenetic analysis

179 The deduced amino acid (aa) sequence of the newly cloned putative ABT *elovl4* was aligned with
180 *elovl4* orthologues from a range of fish species and other vertebrates with the ClustalW tool (BioEdit
181 v7.0.9, Tom Hall, Department of Microbiology, North Carolina State University, USA). A
182 phylogenetic tree was constructed on the basis of the deduced aa sequence of ABT Elov14 and other
183 fish and vertebrate Elov14, Elov12 and Elov15 sequences using the maximum likelihood method

184 (Jones et al., 1992) with MEGA 6.0 software (<http://www.megasoftware.net/>). Confidence in the
185 resulting tree branch topology was measured using bootstrapping through 1,000 replications.

186

187 2.6. Functional characterisation of ABT *elovl4*

188 PCR fragments corresponding to the ORF of the newly cloned ABT *elovl4* cDNA were amplified
189 from cDNA synthesised from brain RNA, using the high fidelity *Pfu* DNA polymerase (Promega,
190 USA) with primers containing *Hind*III (forward) and *Xho*I (reverse) restriction sites (Table 2). PCR
191 conditions consisted of an initial denaturation at 95 °C for 2 min, followed by 32 cycles of
192 denaturation at 95 °C for 30 s, annealing at 66 °C for 30 s, extension at 72 °C for 2 min followed by
193 a final extension at 72 °C for 7 min. The DNA fragments obtained were purified as described above,
194 digested with the appropriate restriction enzymes (New England Biolabs, UK), and ligated into
195 similarly digested pYES2 expression vector (Invitrogen, UK) to produce the plasmid construct
196 pYES2-*elovl4*.

197 Yeast competent cells InvSc1 (Invitrogen) were transformed with pYES2-*elovl4* using the S.c.
198 EasyComp™ Transformation Kit (Invitrogen). Selection of yeast containing the pYES2 constructs
199 was done on *S. cerevisiae* minimal medium minus uracil (SCMM-ura) plates. One single yeast colony
200 was grown in SCMM-ura broth for 2 days at 30 °C, and subsequently subcultured in individual
201 Erlenmeyer flasks until optical density measured at a wavelength of 600 nm (OD600) reached 1, after
202 which galactose (2 %, w/v) and a PUFA substrate at a final concentration of 0.50 mM (C₁₈), 0.75 mM
203 (C₂₀) and 1.0 mM (C₂₂) were added. The fatty acid substrates included γ -linolenic acid (18:3n-6),
204 EPA (20:5n-3), arachidonic acid (20:4n-6), docosapentaenoic acid (22:5n-3), docosatetraenoic acid
205 (22:4n-6) and DHA (22:6n-3). In addition to exogenously added PUFA substrates, some Elov14 have
206 been shown to elongate saturated FA (Monroig et al., 2018). Consequently, the ability of ABT Elov14
207 to elongate yeast endogenous saturated fatty acids was investigated. For that purpose, the saturated
208 fatty acid profiles of yeast transformed with empty pYES2 vector (control) and those of yeast
209 transformed with pYES2-*elovl4* were compared after growing the yeast without addition of any
210 substrate. After 2 days, yeast were harvested, washed twice with doubled distilled water and freeze-
211 dried until further analysis. All fatty acid substrates (> 98-99 % pure) used for the functional
212 characterisation assays were obtained from Nu-Chek Prep, Inc (Elysian, MN, USA). Yeast culture
213 reagents including galactose, nitrogen base, raffinose, tergitol NP-40 and uracil dropout medium were
214 obtained from Sigma-Aldrich (Poole, UK).

215

216 2.7. Roles of ABT elongase and desaturase enzymes in DHA biosynthesis via the Sprecher pathway

217 Yeast competent cells InvSc1 (Invitrogen) were co-transformed with two different plasmid
218 constructs prepared as described below. First, the herein cloned ABT *elovl4* ORF and the previously

219 cloned ABT *elovl5* ORF were ligated into the yeast expression vector p415TEF (a centromeric
220 plasmid with a LEU2 selectable marker) to produce the constructs p415TEF-*elovl4* and p415TEF-
221 *elovl5* respectively, in which the expression of the ABT *elovl* was controlled under the yeast TEF1
222 promoter (constitutive expression). Second, the ORF of the ABT *fads2* was cloned into the episomal
223 yeast vector pYES2 to produce the constructs pYES2-*fads2*, in which the Fads expression was under
224 the control of the GAL1 promoter (inducible expression). Selection of transformant yeast containing
225 simultaneously p415TEF-*elovl* (either *elovl4* or *elovl5*) and pYES2-*fads2* was performed by growing
226 the co-transformed yeast on *S. cerevisiae* minimal medium minus uracil minus leucine
227 (SCMM-ura-leu) plates. One single colony was grown in SCMM-ura-leu broth for 24 h at 30 °C,
228 and subsequently subcultured in individual Erlenmeyer flasks at 0.1 OD600 (t_0) and supplemented
229 with either 0.5 mM Na salt of 18:3n-3 ($\Delta 6$ desaturation control) or 0.75 mM Na salt of 22:5n-3 (DPA).
230 Co-transformed yeast were then grown for 24 h ($t_0 + 24$ h) allowing the ABT Elovl (Elovl4 or Elovl5)
231 to convert the exogenously added C₂₂ substrate 22:5n-3 into its corresponding C₂₄ elongation product
232 24:5n-3. In order to test the ability of the ABT Fads2 to $\Delta 6$ desaturate 24:5n-3 synthesised by yeast,
233 expression of the ABT *fads2* was then induced ($t_0 + 24$ h) by addition of 2% galactose, after which
234 the recombinant yeast were further grown for 48 h ($t_0 + 72$ h) before collection. As positive control,
235 yeast co-transformed with *D. rerio* p415TEF-*elovl2* and ABT pYES2-*fads2* vectors were also grown
236 in SCMM-ura-leu broth as described above.

237

238 2.8. Fatty acid analysis

239 Total lipids were extracted from freeze-dried samples of yeast (Folch et al., 1957) and fatty acid
240 methyl esters (FAME) prepared as described in detail previously (Oboh et al., 2016). Preparation of
241 FAME and peak identification using Gas Chromatograph (GC) coupled with Mass Spectrometry
242 (MS) detector were performed as described by Monroig et al. (2010).

243 Briefly, the elongation of endogenous saturated fatty acids was assessed by comparison of the areas
244 of the fatty acid of control yeast with those of yeast transformed with pYES2-*elovl4*. The GC-MS
245 was operated in the electron ionisation (EI) single ion monitoring (SIM) mode. The 24:0, 26:0, 28:0,
246 30:0, 32:0, 34:0 and 36:0 response values were obtained by using the m/z ratios 382.4, 410.4, 438.4,
247 466.5, 494.5, 522.5 and 550.5, respectively. For VLC-PUFA analysis, the response values were
248 obtained by using the m/z ratios 79.1, 108.1 and 150.1 in SIM mode. As described in detail by Li et
249 al. (2017), the elongation conversions of exogenously added PUFA were calculated as [areas of first
250 product and longer chain products/(areas of all products with longer chain than substrate + substrate
251 area)] \times 100. Moreover, the ability of the ABT $\Delta 6$ Fads2 to convert 24:5n-3 to 24:6n-3 was calculated
252 as [area of 24:6n-3 / (area of 24:6n-3 + area of 24:5n-3)] \times 100, considering the area of 24:5n-3 as

253 that generated from exogenously added 22:5n-3 by either the ABT Elov15 or Elov14b in the co-
254 transformation assays (Oboh et al., 2017b).

255 2.9. qPCR analysis

256 Transcript abundance was determined by quantitative RT-PCR (qPCR) of fatty acyl elongases
257 *elovl4* and *elovl5*, and fatty acyl desaturase $\Delta 6$ *fads2*, key genes involved in the pathway for the
258 biosynthesis of LC-PUFA, particularly the production of DHA from EPA in liver, given its active
259 role in lipid metabolism. Elongation factor-1 α (*elf1 α*) and β -actin (*bactin*) were used as suitable
260 reference genes as they had been determined previously to be stable (Betancor et al., 2017a,b; 2019).
261 The cDNA was diluted 20-fold with milliQ water. The efficiency of the primers for each gene was
262 previously evaluated by serial dilutions of cDNA pooled from the samples to guarantee it was > 85
263 % for all primer pairs. Analyses by qPCR were performed using a Biometra TOptical Thermocycler
264 (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate 20 μ l reaction volumes
265 containing 10 μ l of Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific, Hemel
266 Hempstead, UK), 1 μ l of the primer corresponding to the analysed gene (10 pmol), 3 μ l of molecular
267 biology grade water and 5 μ l of cDNA (1/20 diluted). In addition, amplifications were carried out
268 with a systematic negative control (NTC, no template control) containing no cDNA. Standard
269 amplification parameters included a UDG (Uracil-DNA glycosylase) pre-treatment at 50 °C for 2
270 min, an initial denaturation step at 95 °C for 10 min, followed by 35 cycles: 15 s at 95 °C, 30 s at the
271 annealing T_m and 30 s at 72 °C. Primer sequences for genes are given in Table 2.

272

273 2.10. Statistical analysis

274 Roles of the ABT Elov14 in the elongation of saturated fatty acids were presented as mean \pm SD
275 (n = 3). Comparison of fatty acid profiles from control and yeast expressing the ABT *elovl4* were
276 compared with a Student's t-test. Tissue expression (qPCR) results were expressed as the logarithm
277 of arbitrary units after normalisation against the expression level of the housekeeping gene *ef1 α* . One
278 arbitrary unit was set at the lowest expression of the gene per each set of genes. Differences in gene
279 expression among tissues were analysed by one-way ANOVA, data not requiring any transformation.
280 Gene expression in ABT juveniles fed the two dietary treatments was compared by a Student's t-test.
281 Differences were regarded as statistically significant when P < 0.05 (Zar, 1999).

282

283 3. Results

284 3.1. Elov14 sequence and phylogenetic analysis

285 The ORF of the putative ABT *elovl4* cDNA consisted of 915 bp, encoding a protein of 304 aa.
286 Sequence analysis of the putative Elov14 protein showed that it contained the conserved histidine
287 dideoxy binding motif (HXXHH), the predicted endoplasmic reticulum (ER) retention signal with an

288 arginine (R) and lysine (K) at the carboxyl end (RXXXX), as well as several regions containing
289 similar motifs (Fig. 1).

290 We compared the deduced aa sequence of the ABT Elov14 with other fish Elov1 sequences via
291 BLASTp searches. Our results revealed that the deduced aa sequence of the ABT Elov1 showed only
292 43 % similarity with the previously described ABT Elov15 sequence (gb|ADX62355.1|). In contrast,
293 phylogenetic analysis showed that the Elov14 protein of ABT clustered with several other Elov14-like
294 sequences from teleosts, whereas a separate cluster contained those proteins from mammals and
295 cartilagenous fish (Fig. 2). It is interesting to note that, within teleosts, the herein characterised ABT
296 Elov14 grouped more closely with Elov14b-like sequences from orange spotted grouper *E. coioides*
297 (gb|AHI17192.1|; 95 %) and Nibe croaker *Nibea mitsukurii* (gb|AJD80650.1|; 94 %) (Fig. 2). These
298 results strongly suggested that the ABT *elov14* cDNA characterised here encoded an Elov14b enzyme,
299 which has been deposited in GenBank under the accession number MN171375.

300

301 3.2. Functional characterisation of ABT Elov14b

302 Functional characterisation of the ABT Elov14b protein was carried out in *S. cerevisiae* yeast cells
303 expressing the *elov14b* ORF and grown in the presence of potential fatty acid substrates. However,
304 the potential activity of the ABT Elov14b protein for the elongation of saturated fatty acids was first
305 evaluated by comparing the saturated fatty acid profiles of yeast transformed either with an empty
306 pYES2 plasmid (control) or transformed with pYES2-*elov14b* and grown in the absence of exogenous
307 fatty acid (Table 3). The results showed that pYES2-*elov14b* transformed yeast contained proportions
308 of 16:0 and 26:0 that were numerically lower (not statistically significant), and those of 28:0 and 30:0
309 that were significantly higher, than yeast transformed with empty PYES2 plasmid.

310 To determine the ability of ABT Elov14b to elongate PUFA, *S. cerevisiae* transformed with
311 pYES2-*elov14b* were grown in the presence of potential PUFA substrates (Table 4). Transgenic yeast
312 containing the *elov14b* ORF were capable of elongating exogenously added PUFA from C₁₈ to C₂₂
313 (Table 4). Thus, tetracosapentaenoic acid (24:5n-3), key intermediate in DHA biosynthesis via the
314 Sprecher pathway, can be produced from both 20:5n-3 and 22:5n-3 by Elov14b in ABT. However,
315 GC-MS analyses confirmed that even higher conversions were found for n-3 PUFA with chain
316 lengths of C₂₄ up to C₃₀ before activity declined with longer chain lengths. With n-6 PUFA, highest
317 conversions peaked at C₂₈ and declined with longer chain length. The ABT Elov14b had no activity
318 towards C₃₄ PUFA, irrespective of whether of the n-3 or n-6 series (Table 4). Additionally, yeast
319 containing empty vector and grown in the presence of the same PUFA substrates as those transformed
320 with pYES2-*elov14b* did not show any elongation activity (data not shown), in agreement with yeast
321 endogenous elongases not being able to elongate PUFA (Agaba et al., 2004).

322

323 3.3. Tissue distribution of ABT *elovl4b*

324 The transcripts for *elovl4b* were found in all of the analysed ABT tissues except the ovaries (Fig.
325 3). The highest number of mRNA copies were found in eyes, followed by brain and gills. In contrast,
326 the lowest levels of *elovl4b* expression were observed in heart and kidney.

327

328 3.4. Roles of ABT elongase and desaturase enzymes in DHA biosynthesis via the Sprecher pathway

329 The ability of ABT desaturase (*Fads2*) and elongase (*Elov14b* and *Elov15*) enzymes to operate the
330 Sprecher pathway was determined by co-transforming yeast with the ORF sequences of either *elovl4b*
331 or *elov15* and *fads2* ($\Delta 6$ desaturase). First, yeast co-transformed the ABT *elov15* and $\Delta 6$ *fads2* did not
332 contain any detectable 24:5n-3 when grown in the presence of 22:5n-3 (Table 5). This result indicated
333 that the ABT *Elov15* does not play a role in DHA biosynthesis via the Sprecher pathway. Yeast co-
334 transformed with the ABT *elovl4b* and $\Delta 6$ *fads2* were able to elongate the exogenously added 22:5n-
335 3 to 24:5n-3, confirming the activity of the ABT *elovl4b* in the constitutive expression vector
336 p415TEF (data not shown). Importantly, an additional peak corresponding to 24:6n-3 denoted a $\Delta 6$
337 desaturation of 24:5n-3 by the ABT *Fads2* (3.4 % conversion). Such $\Delta 6$ desaturation capacity was
338 also observed when ABT *Elov14b*/ABT *Fads2* co-transformed yeast were supplied with 18:3n-3,
339 which was converted to 18:4n-3 (8.7 % conversion). Overall, this demonstrates that ABT has the
340 potential to operate the Sprecher pathway by simultaneously activating the herein studied *Elov14b* to
341 produce 24:5n-3, which is subsequently converted to 24:6n-3 via a $\Delta 6$ desaturation catalysed by
342 *Fads2*. Similar results to those described above for yeast co-transformed with ABT *Elov14b*/ABT
343 *Fads2* were obtained for the elongation control yeast co-transformed with *D. rerio* *Elov12*/*T. thynnus*
344 *Fads2*, which exhibited a 6.9 % conversion of 24:5n-3 to 24:6n-3. These results suggested that both
345 the ABT *Elov14b* and the zebrafish *Elov12* are efficient in providing 24:5n-3 from the shorter-chain
346 precursor 22:5n-3.

347

348 3.5. Nutritional regulation of *elovl4b* expression: an in vivo trial

349 A trial was conducted to elucidate the nutritional regulation of *elovl4b* in juvenile ABT when
350 different levels of n-3 LC-PUFA were supplied in the diet. The experimental fish were fed either a
351 feed high (KO; 38.4 %) or low (KORO; 25.5 %) in n-3 LC-PUFA. Liver of juvenile ABT fed diet
352 KORO showed lower mRNA copy number of *elovl4b* compared to liver of fish fed diet KO ($p =$
353 0.022; Fig. 4). Additionally, expression of the $\square 6$ *fads2* fatty acyl desaturase and *elov15* elongase
354 genes so far characterised from ABT and with confirmed roles in biosynthesis of LC-PUFA (Morais
355 et al., 2011), were analysed. Low dietary n-3 LC-PUFA also led to a down-regulation in transcript
356 level of *elov15* ($p = 0.043$), whereas the expression level of *fads2* was up-regulated in fish fed KORO
357 ($p = 0.044$).

358

359 **4. Discussion**

360 In the present study, the full length cDNA sequence of a putative *elovl4b* was cloned from ABT.
361 The obtained sequence contained typical domains of Elov14 family members such as an
362 endoplasmatic reticulum retrieval signal (RXKXX) and a histidine box (HXXHH), similar to those
363 described for other teleosts Elov14 proteins (Monroig et al., 2010, 2011, 2012; Carmona-Antoñanzas
364 et al., 2011; Kabeya et al., 2015; Li et al., 2017a,b; Jin et al., 2017; Oboh et al., 2017a). The histidine
365 box is a common feature of desaturase and hydrolase enzymes in general, and in elongases is involved
366 in the coordination of electron transfer during elongation of fatty acids (Jakobsson et al., 2006).
367 Furthermore, ABT Elov14b exhibited other characteristics of microsomal membrane-bound
368 enzymes, such as multiple transmembrane regions (Jakobsson et al., 2006). Specifically, seven
369 transmembrane regions were predicted, according to the hydropathy analysis of the ABT deduced
370 Elov14 sequence. The specific number of transmembrane regions can be variable in teleost Elov14,
371 ranging from five in *E. coioides* (Li et al., 2017a), Atlantic salmon *Salmo salar* (Carmona-Antoñanzas
372 et al., 2011) and *L. crocea* (Li et al., 2017b), six in *N. mitsukurii* (Kabeya et al., 2015), to seven in
373 both African catfish *Clarius gariepinus* (Oboh et al., 2017a) and black seabream *Acanthopagrus*
374 *schlegelii* (Jin et al., 2017). Similarly, a variable number of transmembrane regions was observed
375 among Elov14 proteins in a range of other vertebrates and invertebrates in a previous study, although
376 this did not impact the strong sequence homology that, in turn, indicates substantial functional
377 conservation (Zhang et al., 2003).

378 Phylogenetic analysis showed that the newly obtained ABT Elov14b aa sequence clustered
379 together with *D. rerio* and *A. schlegelii* Elov14b sequences, and in a different branch from teleost
380 Elov14a. An *in silico* study indicated previously that most teleosts likely possess both Elov14a and
381 Elov14b (Castro et al., 2016) and, indeed, recent studies have demonstrated both isoforms in several
382 marine and freshwater teleost species (Kabeya et al., 2015; Oboh et al., 2017a; Jin et al., 2017; Yan
383 et al., 2018) as well as zebrafish (Monroig et al., 2010). A common feature of both the a and b forms
384 of Elov14 in zebrafish was the capacity to biosynthesise VLC-SFA (Monroig et al., 2010). In the
385 present study, the saturated fatty acid (i.e., precursors of VLC-SFA) profile of yeast transformed with
386 ABT *elovl4b* showed significant differences to yeast transformed with empty vector. Thus, higher
387 percentages of 28:0 and 30:0 were observed in yeast transformed with ABT *elovl4b*, which was
388 consistent with data reported for Elov14 from several other fish species including zebrafish, Atlantic
389 salmon, cobia (*Rachycentron canadum*), African catfish and orange-spotted grouper that all showed
390 28:0 as a major product of saturated fatty acid elongation by Elov14 (Monroig et al., 2010, 2011;
391 Carmona-Antoñanzas et al., 2011; Oboh et al., 2017a, Li et al., 2017a, b). This suggests that ABT
392 Elov14b have some capacity for the production of VLC-SFA.

393 Heterologous expression in yeast demonstrated that the ABT Elov14b exhibited high elongation
394 efficiencies towards exogenously added C₁₈, C₂₀ and C₂₂ PUFA substrates. Most importantly, the key
395 intermediate in the Sprecher pathway for the synthesis of DHA from EPA, 24:5n-3, was
396 biosynthesised by ABT Elov14b from both EPA and DPA. Subsequently, 24:5n-3 synthesised by the
397 action of Elov14b can be further converted to 24:6n-3 by the ABT Fads2 confirming that this enzyme
398 not only operates on C₁₈ PUFA precursors as described previously (Morais et al., 2011), but also on
399 C₂₄ substrates like 24:5n-3. Such desaturase capacity appears to be common among teleost Fads2
400 with substrate specificities other than Δ 4 desaturase (Obloh et al., 2017b). Our study enables us to
401 confirm that ABT has the enzyme machinery necessary for the endogenous production of DHA from
402 EPA and, for first time, provides molecular evidence demonstrating that Elov14b contributes to this
403 pathway, thus efficiently compensating for lack of Elov12 in most marine teleosts (Monroig et al.,
404 2010, 2011, 2012; Carmona-Antoñanzas et al., 2011; Kabeya et al., 2015; Obloh et al., 2017a; Jin et
405 al., 2017; Li et al., 2017a,b; Yan et al., 2018; Zhao et al., 2019). While endogenous production of
406 DHA is important to guarantee supply of such a physiological important compound for vertebrates,
407 this pathway may be particularly relevant in species such as ABT whose lipids are characterised by
408 having a fatty acid composition with a very high DHA:EPA ratio (Mourente and Tocher, 2003, 2009).

409 In addition to the role of ABT Elov14b in DHA biosynthesis, it is important to note that this enzyme
410 also participates in the biosynthesis of VLC-PUFA, since it was able to produce a range of polyenes
411 with chain lengths up to 34 carbons in the yeast expression system. This is largely in agreement with
412 previous studies on teleost Elov14 proteins although PUFA of up to 36 carbons have often been
413 reported (Monroig et al., 2010, 2011, 2012; Carmona-Antoñanzas et al., 2011; Kabeya et al., 2015;
414 Obloh et al., 2017a; Jin et al., 2017; Li et al., 2017a,b; Yan et al., 2018; Zhao et al., 2019). Importantly,
415 some of the VLC-PUFA detected in yeast in the present study, namely 26:6n-3, 28:6n-3, 30:6n-3,
416 32:6n-3 and 34:6n-3, were identified in retinal phosphatidylcholine (PC) of European seabass,
417 gilthead seabream, Senegalese sole and Atlantic salmon in previous studies (Garlito et al., 2019).
418 Overall, the results demonstrated the key role of teleost Elov14b in VLC-PUFA biosynthesis, a
419 metabolic pathway that is particularly active in retina, consistent with tissue expression data.

420 The mRNA copy number for *elov14b* in ABT was highest in eye, which reflected the fact that
421 retina is known to contain VLC-PUFA, primarily within PC, suggesting a very specific structural or
422 functional role for these fatty acids and, consequently, Elov14 in retinal tissue (Aveldaño, 1987).
423 Moreover, the ABT *elov14b* mRNA tissue distribution was also consistent with data obtained in other
424 teleost fish species, where photoreception/neural tissues (e.g., retina, pineal gland, brain) are
425 generally sites of high expression of *elov14b* (Monroig et al., 2010, 2011, 2012; Carmona-Antoñanzas
426 et al., 2011; Obloh et al., 2017a; Jin et al., 2017; Li et al., 2017a,b; Yan et al., 2018). However,
427 transcripts of *elov14b* were found in almost all tissues of ABT, which was similar to the expression

428 of *elovl4a*, but not *elovl4b* in zebrafish (Monroig et al., 2010). In contrast both *elovl4a* and *elovl4b*
429 were expressed in almost all tissue in African catfish, black seabream and loach (Oboh et al., 2017a;
430 Jin et al, 2017; Yan et al., 2018). Expression levels of *elovl4a* exceeded those of *elovl4b* in most
431 tissues in catfish and, to a lesser extent, in black sea bream (Oboh et al., 2017a; Jin et al, 2017). It is
432 also worth noting that *elovl4b* expression was generally low in liver of ABT, which is also a
433 characteristic shared with *elovl4b* expression in zebrafish (Monroig et al., 2010), and *elovl4b*
434 expression in several other fish species including Atlantic salmon, cobia, African catfish and black
435 seabream (Carmona-Antoñanzas et al., 2011; Monroig et al., 2011; Oboh et al., 2017a; Jin et al.,
436 2017). While the presence of *elovl4b* transcripts in tissues such as retina, pineal and testis appears
437 related to its role in VLC-PUFA biosynthesis, activity of Elovl4b in other tissues might be related
438 with its contribution to biosynthesis of LC-PUFA like DHA as described above.

439 With the continued expansion of aquaculture production, the development of more sustainable
440 feeds has become increasingly essential (Ytrestøyl et al., 2015; Shepherd et al., 2017; Tocher et al.,
441 2019). This, in turn, reduces the content of the n-3 LC-PUFA, EPA and DHA, in the feeds and the
442 resultant farmed fish products (Henriques et al., 2014; Tocher, 2015; Sprague et al., 2016). This is
443 likely to be a particular issue in a species like ABT that have high EPA and, especially, DHA contents
444 and whose nutritional quality is dependent upon high levels of these fatty acids (Mourente and
445 Tocher, 2003, 2009). In general, low dietary levels of LC-PUFA have been shown to up-regulate the
446 expression of *fads2* desaturases in teleosts as a mechanism to enhance the biosynthesis of EPA and
447 DHA when fed diets with low levels of LC-PUFA (Leaver et al., 2008; Torstensen and Tocher, 2011).
448 Consistent with this, the transcript level of ABT *fads2* ($\Delta 6$ desaturase) was up-regulated in ABT fed
449 the diet with lower content of n-3 LC-PUFA (diet KORO) in the present study. Perhaps surprisingly,
450 higher dietary levels of n-3 LC-PUFA (diet KO) tended to increase the transcript copy numbers of
451 both *elovl4b* and *elovl5* elongases in liver of ABT. In contrast, relative expression of *elovl4b* mRNA
452 in visceral mass was reduced in orange-spotted grouper fed diets with graded increased levels of n-3
453 LC-PUFA (Li et al., 2017a), in liver of large yellow croaker fed high dietary n-3 LC-PUFA (Li et al.,
454 2017b), and in liver of rainbow trout fed a diet high in soybean oil but not linseed oil (Zhao et al.,
455 2019). Similarly, in the study in orange-spotted grouper, relative expression of *elovl4* tended to
456 decrease as dietary DHA:EPA ratio increased (Li et al., 2017a). While the above three studies
457 investigated the nutritional regulation of *elovl4b*-like genes, regulation of *elovl4a* by dietary PUFA
458 has been only reported in loach *M. anguillicaudatus*, where *elovl4a* was up-regulated in fin cell
459 cultures supplemented with 18:2n-6 and 18:3n-3 (Yan et al., 2018). Overall, studies reporting the
460 expression of fatty acid elongases in fish species in response to dietary levels of LC-PUFA have
461 yielded inconsistent results (Monroig et al., 2018) and, while some studies have shown nutritional
462 regulation, many others have not (Leaver et al., 2008; Tocher, 2010; Torstensen and Tocher, 2011).

463 In conclusion, the present study demonstrated that ABT, *T. thynnus*, possess an Elovl4b with roles
464 in the biosynthesis of VLC-PUFA up to 34 carbons, compounds of key structural roles in neural
465 tissues such as eye (retina) with high presence of *elovl4b* transcripts. Moreover, the ABT Elovl4b
466 contributes to the DHA biosynthesis by elongation of EPA and DPA to 24:5n-3, the latter being
467 desaturated to 24:6n-3 by the action of the ABT $\Delta 6$ Fads2. These results confirm that ABT has the
468 enzyme machinery necessary for the endogenous production of DHA from EPA and demonstrate that
469 Elovl4b can effectively compensate for absence of Elovl2 in many teleost species.

470

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479

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646 of fatty acyl desaturase and elongase genes in liver of Atlantic salmon (*Salmo salar*). *Biochim.*
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650 **Table 1.** Total lipid fatty acid composition (percentage of total fatty acids) of the experimental diets
 651 with higher (KO) and lower (KORO) levels of n-3 long-chain polyunsaturated fatty acids used in the
 652 feeding trial with juvenile Atlantic bluefin tuna.

	KO	KORO
14:0	6.8	3.6
16:0	16.5	12.1
18:0	4.3	4.2
Total SFA ¹	28.3	20.6
16:1n-7	4.9	3.0
18:1n-9	10.9	27.0
18:1n-7	4.6	3.9
20:1n-9	2.2	2.5
Total MUFA ²	26.0	39.7
18:2n-6	2.2	7.8
20:4n-6	0.6	0.5
Total n-6 PUFA ³	3.4	8.7
18:3n-3	0.9	3.9
18:4n-3	2.0	1.1
20:4n-3	0.4	0.3
20:5n-3	13.3	7.7
22:5n-3	2.7	2.0
22:6n-3	22.0	15.5
Total n-3 PUFA ⁴	41.5	30.5
Total PUFA	45.8	39.7
Total n-3 LC-PUFA	38.4	25.5
n-3/n-6	12.2	3.5
DHA/EPA	1.6	2.0

653 Results are means of duplicate analyses. ¹Totals include 15:0, 20:0, 22:0 and 24:0; ²Totals include
 654 16:1n-9, 18:1n-11, 20:1n-7, 22:1 isomers and 24:1; ³Totals include 18:3n-6, 20:2n-6, 22:4n-6 and
 655 22:5n-6; ⁴Totals include 20:3n-3 and 22:3n-3; DHA, docosaheptaenoic acid; EPA, eicosapentaenoic
 656 acid; KO, diet formulated with 15 % lipid as krill oil; KORO, diet formulated with 15 % lipid
 657 formulated with krill oil and rapeseed oil (1:1, v/v); MUFA, monounsaturated fatty acid; PUFA,
 658 polyunsaturated fatty acid; SFA, saturated fatty acid.

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661 **Table 2.** Sequences of primers used for cloning, functional characterisation and quantitative RT-PCR
 662 (qPCR).

Primer	Sequences (5'-3')	Purpose
<i>UniE4b-F</i>	GCAGTGGTATCAACGCAGAG	First fragment isolation
<i>UniE4b-R</i>	TCTCTATCCCTTCCCTCCCC	
<i>Ttelovl4bv-F</i>	CCC <u>AAGCTT</u> AAGATGGAGGCTGTAACACA	Functional characterisation
<i>Ttelovl4bv-R</i>	CCG <u>CTCGAGT</u> TACTCCTTTTTTCGCTCTTC	
<i>Ttelovl5v-F</i>	CCC <u>AAGCTT</u> AAAATGGAGACTTTCAATTATAAACTGAACA	
<i>Ttelovl5v-R</i>	CCG <u>CTCGAGT</u> CAATCCACCCGCAGTTTCT	
<i>Tfadsv-F</i>	CCG <u>GAGCTC</u> AATATGGGTGGTGGAGGCCAGC	Gene expression (qPCR)
<i>Tfadsv-R</i>	CCG <u>CTCGAGT</u> CATTTATGAAGATATGCATC	
<i>elovl4-F</i>	ATCCAGTTCCACGTGACCAT	
<i>elovl4-R</i>	CCATAGAGGTGCCGTTTGTG	
<i>elovl5-F</i>	CCACGCTAGCATGCTGAATA	
<i>elovl5-R</i>	ATGGCCATATGACTGCACAC	
<i>fads2d6-F</i>	CCGTGCACTGTGTGAGAAAC	
<i>fads2d6-R</i>	CAGTGTAAGCGATAAAATCAGCTG	
<i>ef1a-F</i>	CCCCTGGACACAGAGACTTC	
<i>ef1a-R</i>	GCCGTTCTTGGAGATACCAG	
<i>βactin-F</i>	ACCCACACAGTGCCCATCTA	
<i>βactin-R</i>	TCACGCACGATTTCCCTCT	

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667 **Table 3.** Saturated fatty acid profiles (percentage of total fatty acids) of yeast *Saccharomyces*
668 *cerevisiae* transformed with either the empty pYES2 vector (Control) or the Atlantic bluefin tuna
669 *elovl4* ORF (Elov14b). Results are means \pm SD (n = 3). Statistical differences observed between
670 treatments (Student t-test, P < 0.05) are indicated with an asterisk.

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	Control		Elov14b	
14:0	1.2	\pm 0.7	1.4	\pm 1.3
15:0	0.7	\pm 0.1	0.6	\pm 0.2
16:0	48.0	\pm 5.2	45.8	\pm 3.4
18:0	34.5	\pm 3.6	34.2	\pm 3.2
20:0	0.6	\pm 0.1	0.7	\pm 0.1
22:0	0.8	\pm 0.2	0.9	\pm 0.1
24:0	0.9	\pm 0.1	0.9	\pm 0.1
26:0	12.6	\pm 2.6	12.3	\pm 2.9
28:0	0.5	\pm 0.1	2.7*	\pm 1.0
30:0	0.1	\pm 0.0	0.3*	\pm 0.1

Table 4. Functional characterisation of Elovl4b elongase of Atlantic bluefin tuna by heterologous expression in the yeast *Saccharomyces cerevisiae*. Data are presented as the percentage conversions of polyunsaturated fatty acid (FA) substrates. Individual conversions were calculated according to the formula [areas of first product and longer chain products / (areas of all products with longer chain than substrate + substrate area)] × 100.

FA substrate	Product	% Conversion	Elongation
18:3n-6	20:3n-6	9.5	C18→36
	22:3n-6	24.9	C20→36
	24:3n-6	43.3	C22→36
	26:3n-6	100	C24→36
	28:3n-6	100	C26→36
	30:3n-6	77.0	C28→36
	32:3n-6	20.5	C30→36
	34:3n-6	n.d.	C32→36
	36:3n-6	n.d.	C34→36
20:5n-3	22:5n-3	18.2	C20→36
	24:5n-3	49.1	C22→36
	26:5n-3	62.7	C24→36
	28:5n-3	93.4	C26→36
	30:5n-3	99.4	C28→36
	32:5n-3	92.0	C30→36
	34:5n-3	18.5	C32→36
	36:5n-3	n.d.	C34→36
20:4n-6	22:4n-6	22.5	C20→36
	24:4n-6	56.0	C22→36
	26:4n-6	65.7	C24→36
	28:4n-6	91.1	C26→36
	30:4n-6	97.0	C28→36
	32:4n-6	66.2	C30→36
	34:4n-6	4.6	C32→36
	36:4n-6	n.d.	C34→36
22:5n-3	24:5n-3	24.1	C22→36
	26:5n-3	100	C24→36
	28:5n-3	66.1	C26→36
	30:5n-3	99.3	C28→36
	32:5n-3	88.6	C30→36
	34:5n-3	16.6	C32→36
	36:5n-3	n.d.	C34→36
22:4n-6	24:4n-6	12.4	C22→36
	26:4n-6	54.4	C24→36
	28:4n-6	85.8	C26→36
	30:4n-6	96.5	C28→36
	32:4n-6	56.2	C30→36
	34:4n-6	3.5	C32→36
	36:4n-6	n.d.	C34→36
22:6n-3	24:6n-3	0.7	C22→36
	26:6n-3	100	C24→36
	28:6n-3	100	C26→36
	30:6n-3	100	C28→36
	32:6n-3	25.6	C30→36
	34:6n-3	6.2	C32→36

Table 5. Roles of the Atlantic bluefin tuna (ABT) Elov15 and Elov14b elongases and Fads2 fatty acyl desaturase in DHA biosynthesis via the Sprecher pathway. Fatty acid conversions were calculated as the percentage of 24:5n-3 desaturated to 24:6n-3 as $[\text{area of } 24:6n-3 / (\text{area of } 24:6n-3 + \text{area of } 24:5n-3)] \times 100$. Conversions of 18:3n-3 to 18:4n-3 (control for $\Delta 6$ desaturation) are also indicated. In order to normalise the percentage conversions, ratios between the activities on 24:5n-3 and those on 18:3n-3 (“ $\Delta_{24:5n-3} / \Delta_{18:3n-3}$ ”) are also presented for each co-transformation assay. Conversions detected for the elongation control consisting of the *Danio rerio* Elov12 (ZF Elov12) co-expressed with the ABT Fads2 are also indicated.

	% Conversion		
	18:3n-3 → 18:4n-3	24:5n-3 → 24:6n-3	$\Delta_{24:5n-3} / \Delta_{18:3n-3}$
ABT Elov15 / ABT Fads2	12.7	n.d.	0.00
ABT Elov14b / ABT Fads2	8.7	3.4	0.39
ZF Elov12 / ABT Fads2	9.0	6.9	0.77

n.d., Not detected

Legends to Figures

Fig. 1. ClustalW amino acid alignment of the deduced Elov14 protein of Atlantic bluefin tuna (*Thunnus thynnus*) with Elov14 proteins from other fish species including *Danio rerio* (Elov14a, gb|NP_957090.1|; Elov14b, gb |NP_956266.1|), *Rachycentron canadum* Elov14b (gb|HM026361|), *Nibea mitsukurii* (gb|AJD80650.1|) and *Salmo salar* Elov14b (gb |HM208347|), as well as those of mammals, *Mus musculus* (gb |AAG47667.1|) and *Homo sapiens* (gb|NP_073563.1|). Identical residues are shaded black and similar residues (based on the Blosum62 matrix, using ClustalW default parameters) are shaded grey. Indicated are four (i–iv) conserved motifs of Elov1 enzymes: (i) KXXEXXDT, (ii) QXXFLHXXHH, (iii) NXXXHXXMYXYY and (iv) TXXQXXQ, as well as the putative endoplasmic reticulum (ER) retrieval signal RXXXX at the C-terminus (Zhang et al., 2003).

Fig. 2. Phylogenetic tree comparing the deduced amino acid sequence of Elov14 of Atlantic bluefin tuna (*Thunnus thynnus*; highlighted in bold) with Elov12, Elov14 and Elov15 sequences from a range of vertebrates. The tree was constructed using the Maximum Likelihood method and JTT matrix-based model (Jones et al., 1992) using MEGA 6.0 software with a Kimura 2-parameter substitution model. The tree is drawn to scale, with branch lengths measured in the number of is proportional to amino acid substitution rate per site. The *Mortierella alpina* PUFA elongase was included in the analysis as outgroup sequence to construct the rooted tree.

Fig. 3. Distribution of *elov14* transcript in tissues of Atlantic bluefin tuna as determined by qPCR. Values correspond to the log-normalised (*ef1 α*) relative expression (RE) of the target gene in each tissue. For comparison, the expression level in ovary, which was the lowest, was defined as 1 before the expression values were then log transformed. The results represent the average of eight individuals (n = 8; 4 males and 4 females; between 200 – 250 kg total weight and 10 to 15 years old) with standard error (SEM), other than for ovary and testis (n = 4). Values with different superscript letters are significantly different (ANOVA; P < 0.05). A, adipose tissue; B, brain; E, eye; G, gills; H, heart; I, intestine; K, kidney; L, liver; O, ovary; R, red muscle; S, spleen; T, testis; W, white muscle.

Fig. 4. Effect of diet on the expression of fatty acyl elongases *elov14* and *elov15*, and fatty acyl desaturase \square 6 *fads2* in liver of Atlantic bluefin tuna (*Thunnus thynnus* L.) juveniles fed diets with higher (KO) or lower (KORO) levels of n-3 long-chain polyunsaturated fatty acids. Values are normalised expression ratios with the expression level in fish fed KO set to 1 and are means \pm SD of six individuals (n = 6). Values with different superscript letters are significantly different (Student t-

test; $P < 0.05$). KO, diet with 15 % lipid supplied by krill oil; KORO, diet with 15 % lipid supplied by krill oil and rapeseed oil (1:1; v/v).

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          10      20      30      40      50      60
Thunnus thynnus Elov14  MEAVGHFVNDTFEYRWSLTIADKRVEWRFMMSSPPTLISL LYLLEF
Nibea mitsukurii Elov14 -----MEAVGHFVNDTFEYRWSLTIADKRVEWRFMMSSPPTLISL LYLLEF
Rachycentron canadum Elov14 -----MEAVGHFVNDTFEYRWSLTIADKRVEWRFMMSSPPTLISL LYLLEF
Danio rerio Elov14a     -----MEIQCHINDTFEYRWSLTIADKRVEWRFMMSSPPTLISL LYLLEF
Danio rerio Elov14b     -----MEAVHLMNDSVEYRWSLTIADKRVEWRFMMSSPPTLISL LYLLEF
Salmo salar Elov14      -----MEAVGHFVNDTFEYRWSLTIADKRVEWRFMMSSPPTLISL LYLLEF
Mus musculus ELOVL4     MGLLDSEPGSVLNAMSTAFNDFEYRWSLTIADKRVEWRFMMSSPPTLISL LYLLEF
Homo sapiens ELOVL4     MGLLDSEPGSVLNVSTALNDFEYRWSLTIADKRVEWRFMMSSPPTLISL LYLLEF

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          10      20      30      40      50      60
Thunnus thynnus Elov14  MEAVGHFVNDTFEYRWSLTIADKRVEWRFMMSSPPTLISL LYLLEF
Nibea mitsukurii Elov14 -----MEAVGHFVNDTFEYRWSLTIADKRVEWRFMMSSPPTLISL LYLLEF
Rachycentron canadum Elov14 -----MEAVGHFVNDTFEYRWSLTIADKRVEWRFMMSSPPTLISL LYLLEF
Danio rerio Elov14a     -----MEIQCHINDTFEYRWSLTIADKRVEWRFMMSSPPTLISL LYLLEF
Danio rerio Elov14b     -----MEAVHLMNDSVEYRWSLTIADKRVEWRFMMSSPPTLISL LYLLEF
Salmo salar Elov14      -----MEAVGHFVNDTFEYRWSLTIADKRVEWRFMMSSPPTLISL LYLLEF
Mus musculus ELOVL4     MGLLDSEPGSVLNAMSTAFNDFEYRWSLTIADKRVEWRFMMSSPPTLISL LYLLEF
Homo sapiens ELOVL4     MGLLDSEPGSVLNVSTALNDFEYRWSLTIADKRVEWRFMMSSPPTLISL LYLLEF

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          130      140      150      160      170      180
Thunnus thynnus Elov14  EVRIAGALWWMYISRGVELDIVFFIIRKKRNQVSPFLVYHHCTMFLWVIGIKWVGGQ
Nibea mitsukurii Elov14 -----EVRIAGALWWMYISRGVELDIVFFIIRKKRNQVSPFLVYHHCTMFLWVIGIKWVGGQ
Rachycentron canadum Elov14 -----EVRIAGALWWMYISRGVELDIVFFIIRKKRNQVSPFLVYHHCTMFLWVIGIKWVGGQ
Danio rerio Elov14a     EVRIAGALWWMYISRGVELDIVFFIIRKKRNQVSPFLVYHHCTMFLWVIGIKWVGGQ
Danio rerio Elov14b     EVRIAGALWWMYISRGVELDIVFFIIRKKRNQVSPFLVYHHCTMFLWVIGIKWVGGQ
Salmo salar Elov14      EVRIAGALWWMYISRGVELDIVFFIIRKKRNQVSPFLVYHHCTMFLWVIGIKWVGGQ
Mus musculus ELOVL4     EVRIAGALWWMYISRGVELDIVFFIIRKKRNQVSPFLVYHHCTMFLWVIGIKWVGGQ
Homo sapiens ELOVL4     EVRIAGALWWMYISRGVELDIVFFIIRKKRNQVSPFLVYHHCTMFLWVIGIKWVGGQ

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          190      200      210      220      230      240
Thunnus thynnus Elov14  SFFGADNSIHWLVMYFYYGLAAGPQMKRYLWKKYITIIQMIGPHVTIGHAGHSLYTS
Nibea mitsukurii Elov14 -----SFFGADNSIHWLVMYFYYGLAAGPQMKRYLWKKYITIIQMIGPHVTIGHAGHSLYTS
Rachycentron canadum Elov14 -----SFFGADNSIHWLVMYFYYGLAAGPQMKRYLWKKYITIIQMIGPHVTIGHAGHSLYTS
Danio rerio Elov14a     SFFGADNSIHWLVMYFYYGLAAGPQMKRYLWKKYITIIQMIGPHVTIGHAGHSLYTS
Danio rerio Elov14b     SFFGADNSIHWLVMYFYYGLAAGPQMKRYLWKKYITIIQMIGPHVTIGHAGHSLYTS
Salmo salar Elov14      SFFGADNSIHWLVMYFYYGLAAGPQMKRYLWKKYITIIQMIGPHVTIGHAGHSLYTS
Mus musculus ELOVL4     SFFGADNSIHWLVMYFYYGLAAGPQMKRYLWKKYITIIQMIGPHVTIGHAGHSLYTS
Homo sapiens ELOVL4     SFFGADNSIHWLVMYFYYGLAAGPQMKRYLWKKYITIIQMIGPHVTIGHAGHSLYTS

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          250      260      270      280      290      300
Thunnus thynnus Elov14  CFFPRWMAALIGYATPIILPNNFYHAYRRPSSACKRGR--AANGSMVING-HSK
Nibea mitsukurii Elov14 -----CFFPRWMAALIGYATPIILPNNFYHAYRRPSSACKRGR--AANGSMVING-HSK
Rachycentron canadum Elov14 -----CFFPRWMAALIGYATPIILPNNFYHAYRRPSSACKRGR--AANGSMVING-HSK
Danio rerio Elov14a     CFFPRWMAALIGYATPIILPNNFYDYRRQPRRDKPRALHNGANGALTVSSNGYAK
Danio rerio Elov14b     CFFPRWMAALIGYATPIILPNNFYDYRRQPR--LKENSS--AANGSMSTNG-YSK
Salmo salar Elov14      CFFPRWMAALIGYATPIILPNNFYDYRRTPRSARKYAR--VTVNGSMATNG-YNK
Mus musculus ELOVL4     CFFPRWMAALIGYATPIILPNNFYDYRRQPR--LKENSS--AANG--ISSNG-VNK
Homo sapiens ELOVL4     CFFPRWMAALIGYATPIILPNNFYDYRRQPR--LKENSS--AANG--ISSNG-VSK

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          310      320
Thunnus thynnus Elov14  EEE--VEENG-KRQKKCRARKE
Nibea mitsukurii Elov14 -----EEE--VEENG-KRQKKCRARKE
Rachycentron canadum Elov14 -----EEE--VEENG-KRQKKCRARKE
Danio rerio Elov14a     EEE--KFAESGRRRRKCRARKE
Danio rerio Elov14b     TAE--VTEENG-KRQKKCRARKE
Salmo salar Elov14      LOD--VEENGKRQKKCRARKE
Mus musculus ELOVL4     SEK--ALENG-KRQKKCRARKE
Homo sapiens ELOVL4     SEKQIMTEENG-KRQKKCRARKE

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