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2 **Molecular Cloning and functional characterization of a putative**
3 ***Elov14* gene and its expression in response to dietary fatty acid**
4 **profiles in orange-spotted grouper *Epinephelus coioides***

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33 **Abstract**

34 Elongase of very long-chain fatty acids (Elovl) 4 probably plays a crucial role in
35 marine fish species, where lack of Elovl2 has been considered as one possible reason
36 for their low long-chain polyunsaturated fatty acids (LC-PUFAs) biosynthetic
37 capability. Elovl4 is the most recent member of Elovl family that has been
38 investigated in fish. Here we report the molecular cloning and functional
39 characterization of putative *elovl4* cDNA isolated from marine teleost *Epinephelus*
40 *coioides*, and its expression in response to dietary n-3 LC-PUFA and docosahexaenoic
41 acid (DHA) to eicosapentaenoic acid (EPA) ratio. The *elovl4* cDNA of grouper was
42 2341 bp including 301 bp of 5'-untranslated region (UTR), 918bp of the coding
43 region that encodes a 305 amino acids (AA) and 1122bp of 3'UTR. Heterologous
44 expression in yeast demonstrated that grouper Elovl4 could elongate saturated fatty
45 acids (FA), especially 24:0 and 26:0, up to 36:0. Also, grouper Elovl4 effectively
46 converted C20 and C22 polyunsaturated FAs to elongated polyenoic products up to
47 C36. Tissue distribution analysis revealed that Elovl4 were widely transcribed in
48 various tissues with the highest level in eye, brain and testis as described in other
49 teleosts. The transcript level of *elovl4* was significantly affected by dietary n-3
50 LC-PUFA and high LC-PUFA level repress its expression. However, the ratio of DHA
51 to EPA had no significant influence on its expression. These results may contribute to
52 better understanding the LC-PUFA biosynthetic pathway in this fish species.

53

54 **Keywords:** Elovl4; Cloning; Functional characterization; Nutrition regulation;
55 Orange spotted grouper

56

57 **Introduction**

58 Long-chain polyunsaturated fatty acids (LC-PUFAs) ($C \geq 20$) are beneficial for
59 human health, promoting the development of neuronal tissues and protect against
60 cardiovascular, immune and inflammatory condition (Salem, Litman, Kim &
61 Gawrisch 2001; Calon & Cole 2007; Eilander, Hundscheid, Osendarp, Transler &
62 Zock 2007; Ruxton, Reed, Simpson & Millington 2004). Fish species, especially
63 marine species, are the main source of LC-PUFAs for humans. However, with
64 increasing use of vegetable oils in aqua feed, the contents of docosapentaenoic acid
65 (DHA) and eicosapentaenoic acid (EPA) in farmed fish decreased significantly due to
66 the lack of LC-PUFA in vegetable oils (Lin, Liu, He, Zheng & Tian 2007; Peng, Xu,

67 Mai, Zhou, Zhang, Liufu, Zhang & Ai 2014), which may severely impact its quality
68 and value for human consumers. Therefore, the molecular mechanisms of the
69 enzymes involved in the biosynthesis of LC-PUFAs in teleost are urgently better
70 understood (Tocher 2003).

71 The biosynthesis of LC-PUFAs in vertebrates is catalyzed by fatty acyl desaturase
72 (Fads) and elongation of very long-chain fatty acids (Elovl) enzymes, which could
73 convert the dietary essential α -linolenic acid (18:3n-3) and linoleic acid (18:2n-6) to
74 LC-PUFAs, including the physiologically important DHA, EPA and arachidonic acid
75 (ARA), through consecutive desaturation and elongation reactions (Sprecher 2000;
76 Nakamura, Cho, Xu, Tang & Clarke 2001). Generally speaking, most marine fish,
77 unlike freshwater species, have low LC-PUFA biosynthetic capacity due to lack or
78 low specific enzyme activities involved in the pathway. Up to now, no $\Delta 5$ FAD cDNA
79 has been isolated from any marine fish species other than a bifunctional $\Delta 6/\Delta 5$ FAD
80 found in rabbitfish (Li, Monroig, Zhang, Wang, Zheng, Dick, You & Tocher 2010).
81 Additionally, marine fish species also appear to lack Elovl2 which could elongate C20
82 and C22 LC-PUFA and was regarded as an essential enzyme in DHA biosynthesis
83 (Monroig, Rotllant, Sánchez, Cerdá-Reverter & Tocher 2009; Morais, Monroig,
84 Zheng, Leaver & Tocher 2009). Elovl4 is the most recent member of Elovl family that
85 has been investigated in fish (Monroig, Rotllant, Cerdá-Reverter, Dick, Figueras &
86 Tocher 2010), although it has been proved to play a crucial rule in the biosynthesis of
87 both saturated and polyunsaturated very long chain fatty acids (VLC-FAs) ($C \geq 24$) in
88 mice (Cameron, Tong, Yang, Kaminoh, Kamiyah, Chen, Zeng, Chen, Luo & Zhang
89 2007). *Elovl4* cDNAs have been isolated and characterized in zebrafish (Monroig *et al.*
90 2010), Atlantic salmon (Carmona-Antoñanzas, Monroig, Dick, Davie & Tocher 2011),
91 cobia (Monroig, Webb, Ibarra-Castro, Holt & Tocher 2011) and rabbitfish (Monroig,
92 Wang, Zhang, You, Tocher & Li 2012). Zebrafish possesses two Elovl4 enzymes,
93 Elovl4a and Elovl4b. Both zebrafish Elovl4 proteins efficiently elongated saturated
94 fatty acids up to C36. However, only Elovl4b could elongate PUFA substrates to
95 corresponding elongated polyenoic products up to C36, with C20 PUFA appearing as
96 preferred substrates (Monroig *et al.* 2010). As for marine fish species, the function of
97 Elovl4 in rabbitfish and cobia were similar to Elovl4b. The ability of Elovl4 to
98 effectively elongate C22 PUFA to C24 PUFA indicates that these enzymes have the
99 potential to participate in the production of DHA, similar to Elovl2.

100 During the past decades, a number of studies have been focused on the regulation

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

111 Orange-spotted grouper, *Epinephelus coioides*, is a popular fish cultured in
112 Southeast Asia and good candidates for intensive aquaculture for their fast growth,
113 efficient feed conversion and high market value (Millamena 2002). However, only
114 few studies have been conducted to investigate the regulation of enzymes involved in
115 LC-PUFA biosynthetic pathway of grouper. Only Li, Mai, Xu, Yuan, Zhang & Ai
116 (2014) reported the expression of $\Delta 6$ FAD in response to dietary n-3 LC-PUFA.
117 Therefore, it is crucial to understand the molecular mechanisms underlying the
118 biosynthesis of PUFA in grouper, which could provide the basis for the successful
119 substitution of fish oil and maintaining high levels of n-3 LC-PUFA in the flesh.
120 Elovl4 plays a crucial role in early development of vertebrates (Monroig *et al.* 2010).
121 In the present study, grouper larvae were chosen to investigate the effect of dietary
122 fatty acid on expression of *elovl4*. The aim of the present study was conducted to
123 clone the *elovl4* cDNA, and investigate its characterization, tissue distribution and
124 mRNA expression in response to dietary fatty acid.

125

126 **Materials and methods**

127 *Experimental fish*

128 The grouper were bought from a local fish rearing farm in Yandun, Hainan, China.
129 The body mass of grouper used for cloning, the rapid amplification of cDNA ends
130 (RACE) and tissue distribution were 150.62 ± 2.35 g. The initial body weight of
131 grouper larvae used for nutritional regulation study was 70 ± 2 mg (29 Day after hatch,
132 DAH).

133

134 *Cloning and sequencing of grouper Elov14 cDNA*

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

141 First stand cDNA was synthesized using PrimeScript™ RT reagent Kit (Takara,
142 Tokyo, Japan) following the instructions. To obtain the first fragment of *elov14* cDNA
143 of grouper, degenerate polymerase chain reaction (PCR) primers were designed based
144 on highly conserved regions from *elov14* sequences of other fish (cobia, zebrafish and
145 Atlantic salmon) in Genbank and were synthesized by Biosune Biotech (Shanghai,
146 China). Two degenerate primers (Elov14-F and Elov14-R, Table 1) were designed to
147 clone a fragment within the coding region by PCR. PCR program was carried out in
148 Eppendorf Mastercycler Gradient (Eppendorf, Hamburg) and the PCR conditions
149 were: 2min at 94°C; 35cycles of 30s at 94°C, 30s at 54°C, 40s at 72°C; another 10
150 min at 72°C. The amplification products were separated by electrophoresis on a 1.5%
151 agarose gel for length difference, and then the target band was ligated into the
152 pEASY-T1 vector (TransGen Biotech, Beijing, China). The PCR fragment was
153 sequenced in Biosune Biotech (Shanghai, China) and the nucleotide sequence was
154 blasted on GenBank to confirm its high similarity with other Elov14 proteins.

155 The full-length cDNA sequence of *elov14* was obtained by 5'-and 3'-RACE using
156 the SMARTer™ RACE cDNA Amplification Kit (Clontech, CA, USA). The 3'- and
157 5'-ends cDNA templates were synthesized according to the user's manual. Four
158 gene-specific primers, Elov14-F1, Elov14-F2, Elov14-R1 and Elov14-R2 were designed
159 for the amplification of RACE cDNA fragments based on the obtained Elov14 cDNA
160 fragment (Table 1). For 3' and 5' RACE, gene-specific primers, Elov14-F2 and
161 Elov14-R1, and Universal Primer A Mix (provided in the kit) was used in first round
162 PCR. Then, nested PCR was performed with the other gene-specific primer,
163 Elov14-F1 and Elov14-R2, for 3' and 5' RACE PCRs, respectively, and a nested
164 universal primer (provided in the kit) to obtain specific PCR product. The PCR
165 products were purified, cloned into, and sequenced as described above.

166

167 *Sequence and phylogenetic analysis of Elov14*

168 Sequence alignment and analysis were conducted using the BLAST sequence
169 analysis service of National Center for Biotechnology Information
170 (<http://www.ncbi.nlm.nih.gov>). Multiple alignments of *elovl4* were performed with
171 the ClustalW Multiple Alignment program (<http://www.ebi.ac.uk/clustalw/>). The
172 deduced amino acid sequence of the newly cloned grouper *elovl4* cDNAs was aligned
173 with their corresponding orthologues from different species including human (*Homo*
174 *sapiens*, NP_073563), mouse (*Mus musculus*, NP_683743), rat (*Rattus norvegicus*,
175 NP_001178725), zebrafish (*Danio rerio*, NP_957090 and NP_956266), cobia
176 (*Rachycentron canadum*, ADG59898), Atlantic salmon (*Salmo salar*, NP_001182481).
177 Multiple sequence alignment was performed with Mega 4.0. A phylogenetic tree was
178 constructed on the basis of amino acid sequence between the grouper Elov1 cDNAs,
179 vertebrate Elov14, Elov12 and Elov15 proteins and using the neighbor joining method
180 (Saitou & Nei 1987). Confidence in the resulting phylogenetic tree branch topology
181 was measured by bootstrapping through 1000 iterations.

182

183 *Functional characterization of the grouper Elov14 in yeast*

184 The grouper Elov14 function was determined by expressing its open reading frame
185 (ORF) in *Saccharomyces cerevisiae* yeast cells as previously described (Monroig *et al.*
186 2010; Carmona-Antoñanzas *et al.* 2011). Briefly, the ORF of the grouper *elovl4* forms
187 was amplified with primers containing restriction sites (Hind III and Ecor I) (Table 1)
188 for further cloning into the yeast expression vector pYES2 (Invitrogen, Paisley, UK).
189 The purified plasmids containing the putative *elovl4* ORFs were then transformed into
190 *S. cerevisiae* competent cells InvSc1 (Invitrogen) as previously described (Hastings,
191 Agaba, Tocher, Leaver, Dick, Sargent & Teale 2001; Agaba, Tocher, Zheng, Dickson,
192 Dick & Teale 2005). One single recombinant yeast colony was grown in *S. cerevisiae*
193 minimal medium-uracil broth to produce a bulk culture required to run the functional
194 assay as follows. In order to assess the role of the grouper Elov14 in the biosynthesis of
195 very long-chain (C>24) PUFA, individual flasks of transgenic yeast were
196 supplemented with one of the following FA substrates: stearidonic acid (18:4n-3),
197 γ -linolenic acid (18:3n-6), EPA (20:5n-3), arachidonic acid (20:4n-6),
198 docosapentaenoic acid (22:5n-3), docosatetraenoic acid (22:4n-6) or DHA (22:6n-3). In
199 order to test the ability of the grouper Elov14 to elongate saturated VLC-FA, yeast
200 transformed with pYES2 containing the putative *elovl4* ORF or no insert

201 (pYES2-empty) (control) were incubated in triplicate in the absence of exogenously
202 added substrates. The VLC-FA profiles from yeast transformed with pYES2-*elovl4* and
203 pYES2-empty were then compared. Docosapentaenoic and docosatetraenoic acids
204 (>98-99 % pure) were purchased from Cayman Chemical Co. (Ann Arbor, USA) and
205 the remaining FA substrates (>99 % pure) and chemicals used to prepare the *S.*
206 *cerevisiae* minimal medium-uracil were from Sigma Chemical Co. Ltd. (Dorset, UK).
207 After 2 days, yeast were harvested and washed for further analyses. Yeast transformed
208 with pYES2 containing no insert were cultured under the same conditions as a control
209 treatment.

210

211 *Fatty acid analysis of yeast*

212 Total lipids were extracted from yeast samples and used to prepare methyl esters
213 (FAME) as described in detail previously by Monroig, Tocher, Hontoria & Navarro
214 (2013). FAME were identified and quantified after splitless injection and run in
215 temperature programming, in an Agilent 6850 Gas Chromatograph system, equipped
216 with a Sapiens-5MS (30 m ×0.25µm×0.25µm) capillary column (Teknokroma, Sant
217 Cugat del Vallés, Barcelona, Spain) coupled to a 5975 series MSD (Agilent
218 Technologies, Santa Clara, CA, USA). The elongation of endogenous substrates was
219 assessed by comparison of the areas of the FAs of control yeast with those of *Elov14*
220 transformed yeast. The elongation of exogenously added PUFA substrates (18:4n-3,
221 18:3n-6, 18:4n-3, 20:5n-3, 20:4n-6, 22:5n-3 and 22:6n-3) was calculated by the
222 stepwise proportion of substrate FA converted to elongated product as [areas of first
223 product and longer chain products / (areas of all products with longer chain than
224 substrate + substrate area)]×100.

225

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

227 For n-3 LC-PUFA level study, grouper larvae were obtained from tissue samples
228 collected for a previous publication (Li *et al.* 2014). Briefly, Triplicate groups of
229 grouper larvae (29DAH) were fed to apparent satiation six times daily for 4 weeks
230 with five isoproteic (58% crude protein) and isolipidic (16% crude lipid) diets
231 containing graded levels of n-3 LC-PUFA (0.52, 0.94, 1.57, 1.97 and 2.43%)(Table
232 2&3).

233 For DHA/EPA study, A total of 2100 larvae (29DAH, 70±2mg) were distributed

234 into 15 white plastic tanks (water volume 100L) at a stocking density of 140
235 individuals per tank. Triplicate groups of grouper larvae were fed to apparent satiation
236 six times daily for 4 weeks with five isoproteic (58% crude protein) and isolipidic
237 (16% crude lipid) diets containing graded levels of DHA/EPA (0.82, 1.28, 1.67, 2.00
238 and 2.33)(Table 4&5) and the total amount of n-3 LC-PUFA was approximately fixed
239 at 2.0% of the dry weight. Five fish in each cage were pooled into 1.5 mL tube
240 (RNase-Free, Axygen, USA), frozen in liquid nitrogen and then stored at -80°C for
241 later analysis of *Elovl4* expression.

242

243 *Real-time quantitative PCR (RT-qPCR) analysis*

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

250 The RT-qPCR was carried out in a quantitative thermal cycler
251 (Mastercyclereprealplex, Eppendorf, Germany). The amplification was performed in a
252 total volume of 25 μ L containing 2 \times SYBR[®] Premix Ex Taq[™] (Perfect Real Time)
253 (Takara, Japan), 0.5 μ L of each primer (10 μ mol L⁻¹), 1 μ L of cDNA mix. The program
254 was as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 10s, 57°C for 10s,
255 and 72°C for 20s. At the end of each PCR reaction, melting curve analysis of
256 amplification products was carried out to confirm that a single PCR product was
257 present in these reactions. Standard curves were made with five different dilutions (in
258 triplicate) of the cDNA samples and amplification efficiency was analyzed according
259 to the following equation $E=10^{(-1/Slope)}-1$. The primer amplification efficiency was
260 0.9970 for *elovl4*, 1.008 for *β -actin*. The absolute Δ CT value of the slope is 0.01,
261 which indicated that Δ CT calculation for the relative quantification of target gene could
262 be used. The expression levels of the target genes were calculated followed the
263 $2^{-\Delta\Delta t}$ method described by Livak & Schmittgen (2001).

264

265 *Statistical analysis*

266 The results were given as means \pm S.E.M. (standard error of the mean). Data from
267 each treatment were subjected to one-way ANOVA and correlation analysis where

268 appropriate using SPSS 19.0 for Windows. Tukey's multiple range test was chosen as
269 a multiple comparison test and the significance level of 5% was used. For the Elov14
270 functional characterization, the saturated VLC-FA profiles from yeast expressing the
271 *elov14* were compared to those of control yeast transformed with the empty pYES2
272 vector by a Student's t-test ($P < 0.05$).

273

274 **Result**

275 *Sequence analyses of Grouper Elov14*

276 Degenerate primers were used to amplify the PCR product of expected size (684bp)
277 from grouper liver and the deduced amino acid sequence from the 684bp product was
278 homologous to other known *elov14*. Then, two end fragments were amplified by
279 3'-RACE and 5'-RACE PCR based on the RACE technology. The complete cDNA
280 sequence of *elov14* was obtained by assembling the three fragments (1st fragment
281 and RACE products). The full-length sequence of *elov14* mRNA and the deduced
282 amino acids (AA) are shown in Fig.1. The sequences corresponding to grouper
283 elongase cDNAs (excluding the polyA tail) were 2341 bp. A 301 bp of 5'-untranslated
284 region (UTR), 918bp of the coding region that encodes a 305 AA protein (Genbank ID:
285 KF533722) and 1122bp of 3'UTR were included in the *elov14* cDNA sequence. The
286 calculated molecular mass of the protein was estimated as 35.437 KDa by using
287 Compute pI/Mw (http://web.expasy.org/compute_pi/).

288

289 *Multiple sequences alignment and phylogenetic analysis*

290 The analysis of deduced AA sequence of grouper *elov14* by BLAST indicated that
291 *elov14* in orange-spotted grouper shares sequence identity with *elov14* of other teleosts,
292 such as cobia (*R. canadum*, 94%), white-spotted rabbitfish (*Siganus. canaliculatus*,
293 95%), Atlantic salmon (*S. salar*, 86%), zebrafish (*D. rerio*, 85%), more than 60%
294 identity with *elov14* of human beings (*H. sapiens*, 65%), mouse (*M. musculus*, 65%)
295 and cattle (*B.Taurus*, 65%). However, the deduced AA of grouper *elov14* was 40%
296 identical with grouper *elov15* 36-40% identical to teleost *elov15* sequences,
297 respectively.

298 The grouper *elov14* deduced proteins contained the diagnostic histidine box
299 HXXHH motif conserved in all elongases and five membrane spanning domains. It
300 also possessed a single lysine and arginine residues at the carboxyl terminus, RXKXX
301 in *elov14* (Fig.2). The phylogenetic tree was constructed on basis of AA sequence

302 comparisons of grouper *elovl4* and other elongase from fish (zebrafish, Atlantic
303 salmon and rabbitfish) and human (Fig.3). The phylogenetic analysis showed that the
304 grouper *elovl4* clustered together with their corresponding teleost orthologues, and
305 separately from *elovl2* and *elovl5* cluster.

306

307 *Functional characterization of grouper putative Elov4*

308 The putative Elov4 of grouper was functionally characterized by determining the
309 fatty acid profiles of transformed *S. cerevisiae* with either empty pYES2 vector
310 (control) or the vector containing *elovl4* ORF inserts and grown in the presence of
311 potential FA substrates. To test the ability of grouper to elongate saturated VLC-FA,
312 transgenic yeast was grown incubated with lignoceric acid (24:0). Yeast transformed
313 with the empty vector contained measurable amounts of saturated VLC-FA, 24:0, 26:0,
314 28:0, 30:0 and 32:0 (Table 6). However, the *elovl4*-transformed yeast showed
315 decreased amounts of 24:0 and 26:0, but increased amounts of 28:0, 30:0, 32:0 and
316 34:0 (Table 6). These results confirmed that grouper Elov4 is involved in the
317 biosynthesis of saturated VLC-FA and at least 24:0, 26:0 and 28:0 may be the good
318 substrates for grouper Elov4. The role of grouper Elov4 in the biosynthesis of
319 VLC-PUFA was also investigated and transgenic yeast transformed with *elovl4* ORF
320 were incubated with C18 (18:4n-3 and 18:3n-6), C20 (20:5n-3 and 20:4n-6) and C22
321 (22:5n-3, 22:4n-6 and 22:6n-3) PUFA substrates (Table 7; Fig.4). Fatty acid
322 composition of the yeast transformed with only pYES2 shows four main fatty acid,
323 namely 16:0, 16:1n-7; 18:0 and 18:1n-9, together with whichever exogenous FA
324 added. However, GC-MS analyses confirmed that grouper Elov4 could elongate
325 PUFA to corresponding elongated polyenoic products up to C36 (Table 7; Fig.4).
326 Elov4 showed higher activity towards C20 (20:5n-3, 29.9% and 20:4n-6, 33.1%) and
327 C22 (22:5n-3, 43.8% and 22:4n-6, 51.0%) and low activity towards C18 (18:4n-3,
328 5.6% and 18:3n-6, 9.6%) (Table7). It's noteworthy that grouper Elov4 was able to
329 convert both 20:5n-3 and 22:5n-3 to 24:5n-3, the substrate for DHA biosynthesis via
330 the Sprecher shunt pathway (Sprecher 2000). However, grouper Elov4 showed
331 relative low activity towards DHA, consistent with the result in Suh & Clandinin
332 (2005).

333

334 *Tissue expression of the putative elovl4*

335 The expression level of *elovl4* varied among different tissues. The transcription of

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

341

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

343 Relative mRNA expression of *elovl4* of grouper larvae was significantly affected by
344 dietary n-3 LC-PUFA ($P < 0.05$). The relative expression of *elovl4* in the first two
345 treatments was significant higher than 2.43% n-3 LC-PUFA group. The *elovl4*
346 transcript levels were up-regulated by 0.41-fold, 0.79-fold, 0.31-fold and 0.26-fold in
347 the level of 0.52%, 0.94%, 1.57% and 1.97% treatments compared with the treatment
348 of 2.43% n-3 LC-PUFA, respectively (Fig.6a). The mRNA level of *elovl4* also showed
349 significantly negative linear relationship relative to dietary n-3 LC-PUFA with an
350 $R = 0.7467$ ($Y = -0.1351X + 1.352$). However, no significant differences were observed
351 in the expression of *Elovl4* in larvae fed diets with graded levels of DHA/EPA
352 (Fig.6b).

353

354 **Discussion**

355 *Elovl* are crucial enzymes for the condensation of activated fatty acids with
356 malonyl-CoA in the long-chain fatty acid elongation pathway (Nugteren 1965). Fish
357 *Elovl* cDNAs including *elovl5* and *elovl2* have been cloned and functionally
358 characterized from several species including freshwater species, the salmonids and
359 marine species (Agaba, Tocher, Dickson, Dick & Teale 2004; Agaba *et al.* 2005;
360 Meyer, Kirsch, Domergue, Abbadi, Sperling, Bauer, Cirpus, Zank, Moreau & Heinz
361 2004; Hastings, Agaba, Tocher, Zheng, Dickson, Dick & Teale 2004; Zheng, Ding, Xu,
362 Monroig, Morais & Tocher 2009; Mohd-Yusof, Monroig, Mohd-Adnan, Wan &
363 Tocher 2010; Morais *et al.* 2009; Monroig *et al.* 2012; Gregory, See, Gibson &
364 Schuller 2010). In recent years, *Elovl4* has been discovered in zebrafish (Monroig *et al.*
365 *et al.* 2010), Atlantic salmon (Carmona-Antoñanzas *et al.* 2011), cobia (Monroig *et al.*
366 2011) and rabbitfish (Monroig *et al.* 2012). Through the functional characterization in
367 heterologous expression in *S. cerevisiae*, the ability of *Elovl4* to effectively elongate
368 C22 PUFA made it possible to form DHA from EPA.

369 In the present study, a full-length cDNA of a putative *elovl4* was first cloned in

370 orange-spotted grouper. The deduced 305 AA showed high identity with *elovl4* of
371 other teleosts, particular cobia (*R. canadum*, 94%), white-spotted rabbitfish (*S.*
372 *canaliculatus*, 95%), Atlantic salmon (*S. salar*, 86%), zebrafish (*D. rerio*, 85%). The
373 putative grouper *elovl4* deduced proteins possessed conserved region, the diagnostic
374 histidine box HXXHH motif, conserved in all elongases and also characteristic of
375 desaturase and hydrolase enzymes containing a di-iron-oxo cluster (Fe-O-Fe), which
376 was involved in the coordination of electron reception during reactions occurring
377 during FA elongation (Jakobsson, Westerberg & Jacobsson 2006). The putative
378 grouper *elovl4* possessed an arginine residue and one lysine residue (RXKXX) in
379 position -5 and -3 from the C-terminus of the protein, respectively, which was crucial
380 for endoplasmatic reticulum retrieval signal function (Jackson, Nilsson & Peterson
381 1990). The RXKXX pattern, indicating its role in LC-PUFA synthesis (Cook &
382 McMaster 2004), is common to other teleost *elovl4* including cobia, white-spotted
383 rabbitfish, Atlantic salmon and zebrafish *elovl4a* (Monroig *et al.* 2010, 2011, 2012;
384 Carmona-Antoñanzas *et al.* 2011).

385 The phylogenetic analysis revealed that the grouper *elovl4* cDNA encodes a
386 protein more similar to the other Elov14 orthologues from teleosts and mammals, than
387 other Elov1 family in fish species, the Elov12 and Elov15. Zebrafish have two Elov14,
388 Elov14a and Elov14b, and they showed marked differences in their substrate
389 specificity (Monroig *et al.* 2011). Elov14a could only efficiently elongated saturated
390 VLC-FA up to C36, whilst Elov14b could elongate PUFA substrates to corresponding
391 elongated polyenoic products (Monroig *et al.* 2010). The teleost Elov14 proteins were
392 separated to two groups, with zebrafish Elov14a separated with Zebrafish Elov14b and
393 other teleost Elov14. Previous studies have found that the function of Elov14 in cobia,
394 white-spotted rabbitfish and Atlantic salmon was similar to Elov14b in zebrafish
395 (Monroig *et al.* 2011, 2012; Carmona-Antoñanzas *et al.* 2011). Compared with
396 Elov14a, the phylogenetic analysis indicate that the grouper Elov14 has close kinship
397 to Elov14b, which may indicate that the function of grouper Elov14 was similar to
398 Elov14b.

399 The functional characterization of grouper Elov14 was also investigated in the
400 present study and it was convinced that the function of grouper Elov14 was great
401 similarities to zebrafish Elov14b. Briefly, grouper Elov14 was efficient in the
402 biosynthesis of saturated VLC-FA, with 24:0, 26:0 and 28:0 as preferred substrates.
403 Meanwhile, grouper Elov14 also showed high efficiency in the elongation of C20

404 (20:5n-3 and 20:4n-6) and C22 (22:5n-3 and 22:4n-6) PUFA to corresponding
405 elongated polyenoic products with C36 chain-lengths. It has been reported that
406 VLC-FAs were abundant in retina (Aveldaño 1987) and brain (Robinson, Johnson &
407 Poulos 1990) and testis (Zadravec, Tvrđik, Guillou, Haslam, Kobayashi, Napier,
408 Capecchi & Jacobsson 2011), in which was consistent with the tissue distribution
409 analysis of *elovl4* mRNA transcripts. This may indicate that brain, eye and testis were
410 the prominent metabolic sites for the biosynthesis of VLC-FA. Unlike freshwater
411 species and the salmonids, marine fish species also appear to lack Elov12, regarded as
412 an essential enzyme in DHA biosynthesis (Monroig *et al.* 2009; Morais *et al.* 2009).
413 However, along with the discovery of Elov14, marine fish species could also
414 synthesize DHA from EPA through Sprecher pathway (Monroig *et al.* 2011). It was
415 found in the present study that grouper Elov14 have higher activity towards C20
416 (20:5n-3 and 20:4n-6) and C22 (22:5n-3 and 22:4n-6). In particular, grouper Elov14
417 was able to effectively convert both 20:5n-3 and 22:5n-3 to 24:5n-3, the substrate for
418 DHA biosynthesis via the Sprecher shunt pathway (Sprecher 2000). This may indicate
419 the potential role of grouper Elov14 in the biosynthesis of DHA and Elov14 could
420 compensate for the lacking of Elov12 in marine fish species.

421 The content of DHA and EPA, potentially compromising their nutritional benefit to
422 the human consumer, was reduced with the increasing use of vegetable oils, which are
423 devoid of LC-PUFA (Izquierdo, Obach, Arantzamendi, Montero, Robaina &
424 Rosenlund 2003; Torstensen, Frøyland, Ørnsrud & Lie 2004). The research on
425 regulational mechanism of the enzymes involved in LC-PUFA biosynthetic pathway
426 may make effective use of vegetable oil in aquafeeds (Tocher 2010). However, studies
427 of those enzymes of teleosts involved in LC-PUFA biosynthetic pathway remain in
428 transcriptional level, and mainly focused on Fads2, Elov15 and Elov12. As for Elov14,
429 Monroig *et al.* (2010) have studied spatial-temporal expression of zebrafish *elovl4*
430 genes. However, to the best of our knowledge, little information was available on
431 nutritional regulation of *elovl4*. In the present study, the mRNA expression of *elovl4*
432 was downregulated by dietary n-3 LC-PUFA. Meanwhile, similar results have been
433 found in the study of *elovl5* expression (Zheng *et al.* 2005; Ling *et al.* 2006; Morais *et al.*
434 *et al.* 2011). This may indicate that the nutritional regulation of *elovl4* may have some
435 characteristics similar to *elovl5*. The same changing trend of *elovl4* and *elovl5*
436 expression in response to dietary n-3 LC-PUFA may confirm the hypothesis to some
437 extent. Unlike the response to dietary n-3 LC-PUFA, the expression of *elovl4* was not

438 significantly affected by the level of DHA/EPA in the present study. In the study of
439 salmon, it was found that both DHA and EPA have similar effects on regulating the
440 expression of LXR and SREBP-1, potential regulator of fatty acid desaturase and
441 elongation ((Minghetti, Leaver & Tocher 2011). Although Elov14 has the ability to
442 elongate EPA to form DHA, the similar effect on transcription factor (LXR and
443 SREBP-1) may account for the response of *elov14* to dietary DHA/EPA and further
444 study should be conducted to investigate the specific regulation mechanism.

445 In conclusion, the cDNA of *elov14* was first cloned from orange-spotted grouper.
446 The *elov14* possessed all the features of Elovl proteins and is phylogenetically close to
447 other *elov14* orthologues of teleosts. Heterologous expression in yeast demonstrated
448 that grouper Elovl4 could elongate saturated fatty acids (FA), especially 24:0 and 26:0,
449 up to 36:0. Also, grouper Elovl4 effectively converted C20 and C22 polyunsaturated
450 FAs to elongated polyenoic products up to C36. High LC-PUFA level significant
451 decreased the expression of *elov14*, while the level of DHA/EPA have no significant
452 influence on it.

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459

460 **Conflict of Interest**

461 No conflict of interest was existed.

462

463 **Reference**

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646 **Figure legends**

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

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

658 Fig.3. Phylogenetic tree comparing the grouper *Elovl4* with elongase proteins from
659 other organisms. The tree was constructed using the Neighbor Joining method (Saitou
660 and Nei, 1987) using MEGA4. The horizontal branch length is proportional to amino
661 acid substitution rate per site. The numbers represent the frequencies (%) with which
662 the tree topology presented was replicated after 1000 iterations.

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

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

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

Fig.2

<i>Homo sapiens</i>	MGLLDSEPGSVLNVTALNDTVEFYRWTSIADKRVENWPMQSPWPTLSISTLYLLFVWLPKWKDR	70
<i>Mus musculus</i>	MGLLDSEPGSVLNAMSTAFNDTVEFYRWTSIADKRVEDWPMQSPWPTLSISTLYLLFVWLPKWKDR	70
<i>Rattus norvegicus</i>	MGLLDSEPGSVLNASTAFNDTVEFYRWTSIADKRVEDWPMQSPWPTLSISTLYLLFVWLPKWKDR	70
<i>Danio rerio Elvol4a</i>	-----MEITQHIINDTVHFYKWSLTIADKRVEKWPMMSSPLPTLAISSSYLLFLWLPKRYHQGR	59
<i>D. rerio Elvol4b</i>	-----METVHLINDSVEFYKWSLTIADKRVEKWPMMSSPLPTLAISSSYLLFLWLPKRYHQGR	59
<i>Rachycentron canadum</i>	-----MEVVIHFVNDTVEFYKWSLTIADKRVENWPMQSPWPTLAISSSYLLFLWLPKRYHQGR	59
<i>Salmo salar</i>	-----MEAVIHFVNDTVEFYKWSLTIADKRVEKWPMMSSPLPTLAISSSYLLFLWLPKRYHQGR	59
<i>Epinephelus coioides</i>	-----MEVVIHLVNDTVEFYKWSLTIADKRVENWPMQSPWPTLAISSSYLLFLWLPKRYHQGR	59
<i>Homo sapiens</i>	EPFQMRVLIIYNFQWVLLNLFIFRELFMGSYNAGYSYICQSVQYSDNVNEVRIAGALWWWYVSKGVEYL	140
<i>Mus musculus</i>	EPFQMRVLIIYNFQWVLLNLFIFRELFMGSYNAGYSYICQSVQYSDNVNEVRIAGALWWWYVSKGVEYL	140
<i>Rattus norvegicus</i>	EPFQMRVLIIYNFQWVLLNLFIFRELFMGSYNAGYSYICQSVQYSDNVNEVRIAGALWWWYVSKGVEYL	140
<i>Danio rerio Elvol4a</i>	EPFQMRKTLIIYNFQWVLLNLFIFRELFLAARAANNYSYICQPVQYSDNEVRIAGALWWWYVSKGVEYL	129
<i>D. rerio Elvol4b</i>	EPFQMRKTLIIYNFQWVLLNLFIFRELFLAARAANNYSYICQPVQYSDNVNEVRIAGALWWWYVSKGVEYL	129
<i>Rachycentron canadum</i>	QPVTDRKTLIIYNFQWVLLNLFYIAKELLIAARAAGYSYICQPVQYSDNVNEVRIAGALWWWYVSKGVEYL	129
<i>Salmo salar</i>	EPFQMRKTLIIYNFQWVLLNLFYIAKELLISARAAGYSYICQPVQYSDNVNEVRIAGALWWWYVSKGVEYL	129
<i>Epinephelus coioides</i>	QPVTDRKTLIIYNFQWVLLNLFYIAKELLISARAAGYSYICQPVQYSDNVNEVRIAGALWWWYVSKGVEYL	129
	II HXXHH	
<i>Homo sapiens</i>	DTVFFILRKKFNQVSFLHVYHHCTMFTLWWIGIKWVAGGQFFGAINNSFTHVIMYSYGLAAGFPNIQK	210
<i>Mus musculus</i>	DTVFFILRKKFNQVSFLHVYHHCTMFTLWWIGIKWVAGGQFFGAINNSFTHVIMYSYGLAAGFPNIQK	210
<i>Rattus norvegicus</i>	DTVFFILRKKFNQVSFLHVYHHCTMFTLWWIGIKWVAGGQFFGAINNSFTHVIMYSYGLAAGFPNIQK	210
<i>Danio rerio Elvol4a</i>	DTVFFILRKKFNQVSFLHVYHHCTMFTLWWIGIKWVAGGQFFGAINNSFTHVIMYSYGLAAGFPNIQK	199
<i>D. rerio Elvol4b</i>	DTVFFILRKKFNQVSFLHVYHHCTMFTLWWIGIKWVAGGQFFGAINNSFTHVIMYSYGLAAGFPNIQK	199
<i>Rachycentron canadum</i>	DTVFFILRKKFNQVSFLHVYHHCTMFTLWWIGIKWVAGGQFFGAINNSFTHVIMYSYGLAAGFPNIQK	199
<i>Salmo salar</i>	DTVFFILRKKFNQVSFLHVYHHCTMFTLWWIGIKWVAGGQFFGAINNSFTHVIMYSYGLAAGFPNIQK	199
<i>Epinephelus coioides</i>	DTVFFILRKKFNQVSFLHVYHHCTMFTLWWIGIKWVAGGQFFGAINNSFTHVIMYSYGLAAGFPNIQK	199
	III	
<i>Homo sapiens</i>	YLWWRKRYLTIIQMIQFHVITIGHTALSLEYDCPFPPKMMHWALIAAIAISFIFLFLNFYTRTYN-EPKK-PSA	278
<i>Mus musculus</i>	YLWWRKRYLTIIQMIQFHVITIGHTALSLEYDCPFPPKMMHWALIAAIAISFIFLFLNFYTRTYN-EPKK-SKT	278
<i>Rattus norvegicus</i>	YLWWRKRYLTIIQMIQFHVITIGHTALSLEYDCPFPPKMMHWALIAAIAISFIFLFLNFYTRTYN-EPKK-SKT	278
<i>Danio rerio Elvol4a</i>	YLWWRKRYLTIIQMIQFHVITIGHTALSLEYDCPFPPKMMHWALIAAIAISFIFLFLNFYTRTYN-EPKK-SKT	269
<i>D. rerio Elvol4b</i>	YLWWRKRYLTIIQMIQFHVITIGHAGHSLEYDCPFPPKMMHWALIAAIAISFIFLFLNFYTRTYN-EPKK-SKT	267
<i>Rachycentron canadum</i>	YLWWRKRYLTIIQMIQFHVITIGHAGHSLEYDCPFPPKMMHWALIAAIAISFIFLFLNFYTRTYN-EPKK-SKT	269
<i>Salmo salar</i>	YLWWRKRYLTIIQMIQFHVITIGHAGHSLEYDCPFPPKMMHWALIAAIAISFIFLFLNFYTRTYN-EPKK-SKT	269
<i>Epinephelus coioides</i>	YLWWRKRYLTIIQMIQFHVITIGHAGHSLEYDCPFPPKMMHWALIAAIAISFIFLFLNFYTRTYN-EPKK-SKT	269
	IV ER V	
<i>Homo sapiens</i>	GRT--AMNGTSSANGVS-KSEKQLMIENG-KRORNSKARGD	314
<i>Mus musculus</i>	GRT--ATNGTSSNGVN-KSEK--ALBNG-KPQRNKPKGE	312
<i>Rattus norvegicus</i>	GRT--ATNGTSSANGVN-KSEKQLVLENG-KPQRNKPKGE	314
<i>Danio rerio Elvol4a</i>	ALHNGASNGALTSSNGNTAKLEEKPAESGRRRRKRGRARD	309
<i>D. rerio Elvol4b</i>	AKS--AVNGVSMSTNG-TSKTAEVTENG-KRQKNGKHD	303
<i>Rachycentron canadum</i>	GRT--IANGTQVVTNG-HSKVEEVEDNG-KRQKGRARR	305
<i>Salmo salar</i>	AKP--VTNGVSMATNG-YNKLQDVEBNG-KRQKGRARR	306
<i>Epinephelus coioides</i>	GRT--VANGTSTVTNG-HSKVEEVEDNG-KRQKGRARR	305

Fig.3.

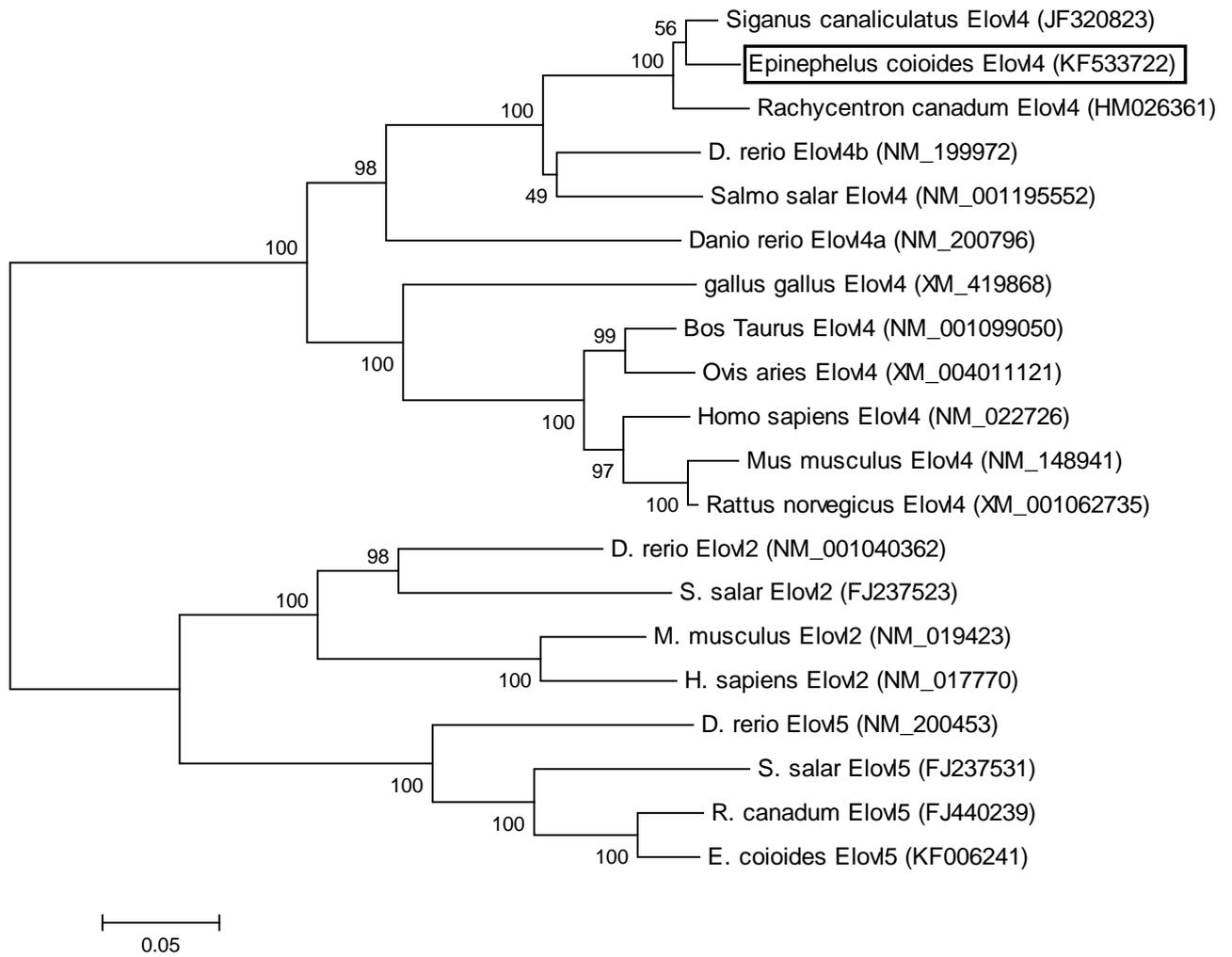


Fig.4.

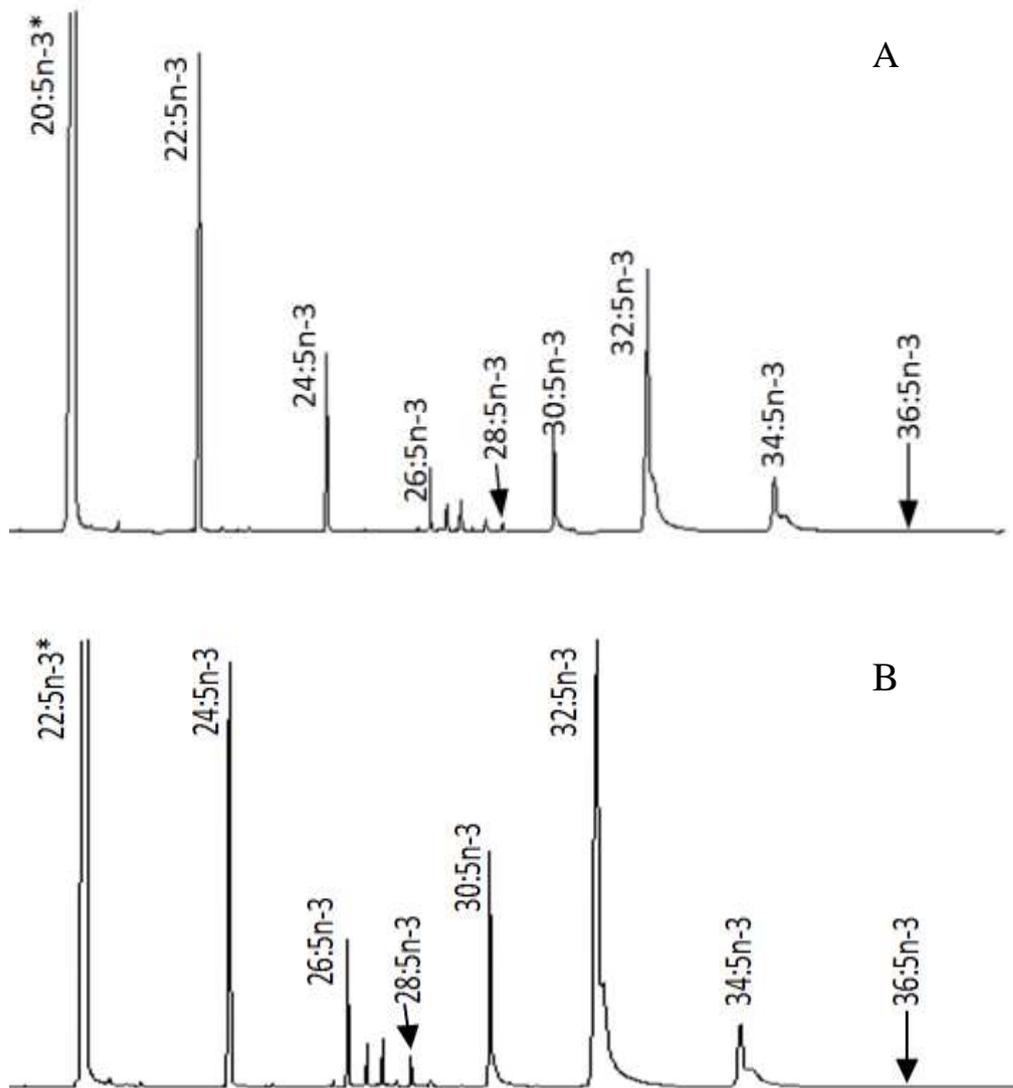


Fig.5.

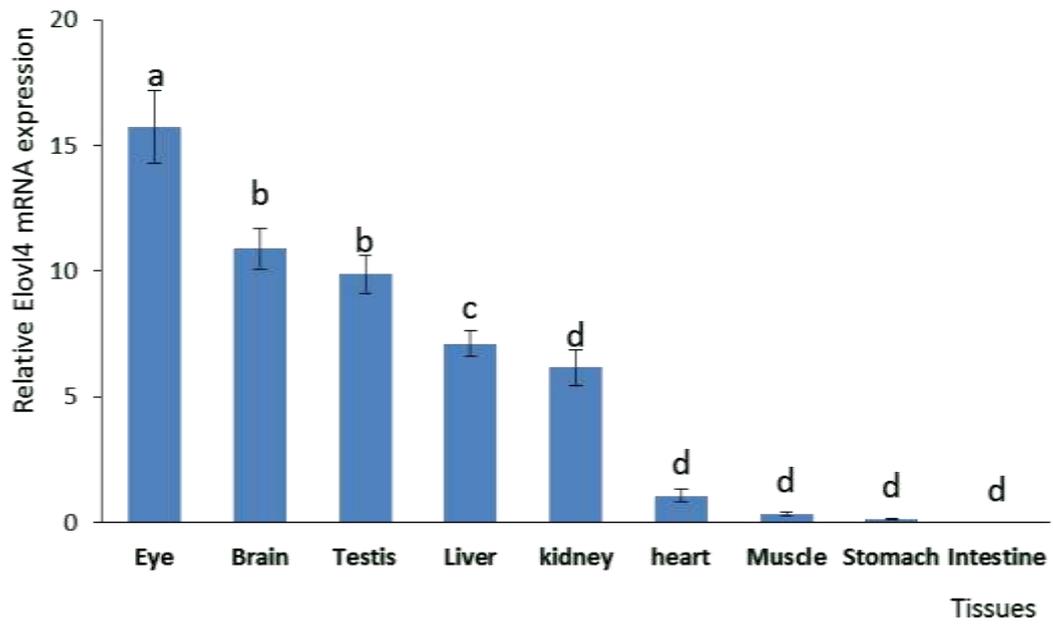


Fig.6a

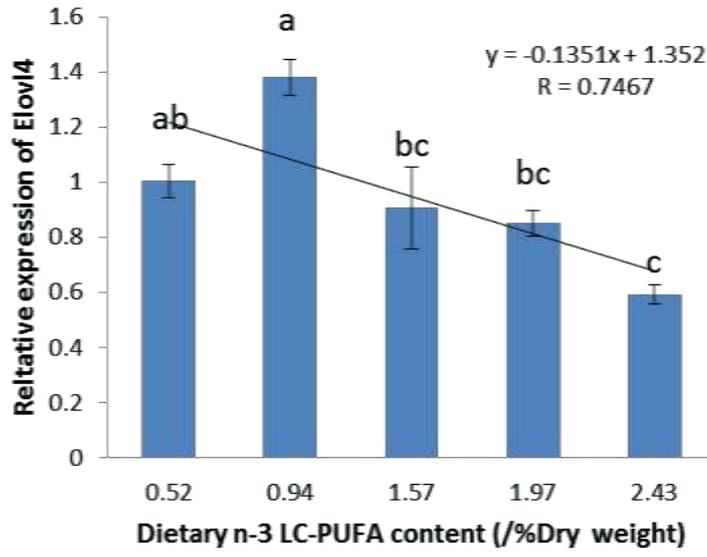
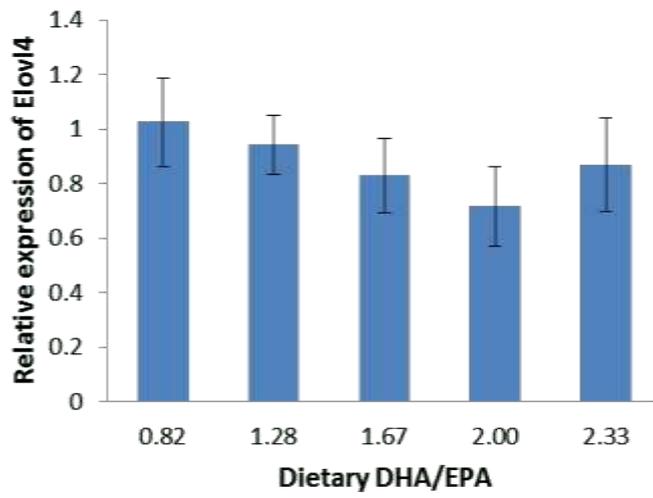


Fig.6b



Tables

Table 1. Sequences of the PCR primers used in this work.

Primer	Sequences (5'-3')	Purpose
Elov14-F	AGACAAGMGKGTGGAGAAATG	RT primer
Elov14-R	AGGATGATGAARGTGACRGCG	RT primer
Elov14-F1	CTTCCTGAGTGTATAGGGCTGGCGGTC	5'RACE primer
Elov14-F2	GCATCTGAGGTCCCAGAGCTGCCAG	5'RACE primer
Elov14-R1	GCAGGACCGCCAGCCCTATACTCAG	3'RACE primer
Elov14-R2	GGCTCTGATTGGCTACGCCGTCACCTT	3'RACE primer
gE4-HindIII-F	CCCAAGCTTATGGAGGTTGTAACACATCT	Functional characterization
gE4-EcorI-R1	CCGCTCGAGTACTCCCTTTTCGCTCGTC	Functional characterization
UPM	Long: CTAATACGACTCACTATAG GGCAAGCAGTGGTATCAACGC AGAGT Short: CTAATACGACTCACTATAGGGC	RACE method RACE method
NUP	AAGCAGTGGTATCAACGCAGAGT	RACE method
Elov14-qF	CTTTCATCATCCTCTTCGCC	RT-qPCR
Elov14-qR	TTACTCCCTTTTCGCTCGTC	RT-qPCR
β actin-F	TACGAGCTGCCTGACGGACA	RT-qPCR
β actin-R	GGCTGTGATCTCCTTCTGCA	RT-qPCR

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

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

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

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

^c Mineral premix (g kg⁻¹): Ca(H₂PO₄)₂·H₂O, 675.0; CoSO₄·4H₂O, 0.15; CuSO₄·5H₂O, 5.0; FeSO₄·7H₂O, 50.0; KCl, 50.0; KI, 0.1; MgSO₄·2H₂O, 101.7; MnSO₄·4H₂O, 18.0; NaCl, 80.0; Na₂SeO₃·H₂O, 0.05; ZnSO₄·7H₂O, 20.0.

^d Attractant(g 100g⁻¹): betaine, 50; glycine,15; alanine, 10;argine,10; taurine, 10;

inosine-5'-monophosphoric acid, 5.

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

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

^gPalmitin: Palmitic acid content, 98.7% of TFA, in the form of methylester; Shanghai Zhixin Chemical Co., Ltd., China.

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

ⁱMold inhibitor: contained 50% calcium propionic acid and 50% fumaric acid.

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

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

^aSFA: saturated fatty acids.

^bMUFA: mono-unsaturated fatty acids.

^cn-6 PUFA: n-6 poly-unsaturated fatty acids.

^dn-3 PUFA: n-3 poly-unsaturated fatty acids.

^en-3 LC-PUFA: n-3 long chain polyunsaturated fatty acids.

^fDHA/EPA: 22:6n-3/20:5n-3.

^gEPA/ARA: 20:5n-3/20:4n-6

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

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

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

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

^cMineral premix (g kg⁻¹): Ca(H₂PO₄)₂·H₂O, 675.0; CoSO₄·4H₂O, 0.15; CuSO₄·5H₂O, 5.0; FeSO₄·7H₂O, 50.0; KCl, 50.0; KI, 0.1; MgSO₄·2H₂O, 101.7; MnSO₄·4H₂O, 18.0; NaCl, 80.0; Na₂SeO₃·H₂O, 0.05; ZnSO₄·7H₂O, 20.0.

^dAttractant(g 100g⁻¹): betaine, 50; glycine, 15; alanine, 10; argine, 10; taurine, 10; inosine-5'-monophosphoric acid, 5.

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

^fEPA enriched oil: EPA content, 46.41% of TFA; DHA content, 23.66% of TFA; both in the form of triglyceride; HEBEI HAIYUAN Health biological Science and

Technology Co., Ltd., China.

^gPalmitin: Palmitic acid content, 98.7% of TFA, in the form of methylester; Shanghai Zhixin Chemical Co., Ltd., China.

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

ⁱMold inhibitor: contained 50% calcium propionic acid and 50% fumaric acid.

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

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

^a SFA: saturated fatty acids.

^b MUFA: mono-unsaturated fatty acids.

^c n-6 PUFA: n-6 polyunsaturated fatty acids.

^d n-3 PUFA: n-3 polyunsaturated fatty acids.

^e n-3 LC-UFA: n-3 long chain polyunsaturated fatty acids.

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

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

Table 6. Functional characterisation of the grouper *Elov14* 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 the empty pYES2 vector (Control) or the grouper *elov14* ORF. Results are means \pm standard deviations (N=3). Asterisks (“*”) indicate means are statistically different between treatments (Student’s *t*-test, *P*<0.05).

FA	Control	Elov14
24:0	9.1 \pm 0.4	6.9 \pm 1.2
26:0	81.5 \pm 3.1	69.5 \pm 2.5 *
28:0	7.5 \pm 2.8	20.9 \pm 0.5 *
30:0	1.5 \pm 0.1	2.4 \pm 0.8
32:0	0.3 \pm 0.2	0.4 \pm 0.2
34:0	nd	0.0 \pm 0.1

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

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

	36:5n-3	0.7	C34→36
22:4n-6	24:4n-6	51.0	C22→36
	26:4n-6	86.0	C24→36
	28:4n-6	95.6	C26→36
	30:4n-6	97.5	C28→36
	32:4n-6	61.0	C30→36
	34:4n-6	7.7	C32→36
	36:4n-6	1.2	C34→36
22:6n-3	24:6n-3	8.8	C22→34
	26:6n-3	100.0	C24→34
	28:6n-3	100.0	C26→34
	30:6n-3	75.4	C28→34
	32:6n-3	89.1	C30→34
	34:6n-3	10.8	C32→34
