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1 The catadromous teleost *Anguilla japonica* has a complete enzymatic  
2 repertoire for the biosynthesis of docosahexaenoic acid from  $\alpha$ -linolenic  
3 acid: Cloning and functional characterization of an Elovl2 elongase

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31 **Abstract**

32       The Japanese eel *Anguilla japonica* is a catadromous fish species with considerable farming  
33 scale. Previous studies showed that dietary  $\alpha$ -linolenic acid (18:3n-3) and linoleic acid (18:2n-6)  
34 satisfied essential fatty acid requirements in eel, which suggested that Japanese eel should have a  
35 complete pathway for the biosynthesis of long-chain polyunsaturated fatty acids (LC-PUFA).  
36 However, existing knowledge was insufficient to explain the molecular basis of LC-PUFA  
37 biosynthetic capacity in eel. In order to further characterize this pathway in eel, a full-length cDNA  
38 of a putative fatty acyl elongase was isolated, with the ORF encoding a protein with 294 amino  
39 acids. The putative elongase displayed high homology to Elovl2 of other teleosts. Functional  
40 characterization by heterologous expression in yeast showed the protein product of the cDNA had  
41 high activity towards C<sub>20</sub> and C<sub>22</sub> PUFA substrates and low activity towards C<sub>18</sub> PUFA substrates,  
42 characteristic of Elovl2 elongases. Tissue distribution of the *elovl2* mRNA showed highest  
43 expression in brain and eyes, which was different from freshwater and anadromous species. This  
44 may reflect an important role for this enzyme in the *in situ* endogenous biosynthesis of  
45 docosahexaenoic acid (DHA) in neural tissues in eel. This is the first report of an Elovl2 in a  
46 catadromous teleost and demonstrates that Japanese eel has a complete enzyme repertoire required  
47 for the endogenous biosynthesis of DHA via the Sprecher pathway. These data have increased our  
48 knowledge of the diversity of LC-PUFA biosynthesis in vertebrates, and provided further insight  
49 into the regulatory mechanisms of LC-PUFA biosynthesis in teleost fish.

50 **Keywords**

51 Japanese eel; Anadromous species; Long-chain polyunsaturated fatty acids; Elongation;  
52 Biosynthesis.

53

## 54 **1. Introduction**

55 Long-chain ( $\geq C_{20-24}$ ) polyunsaturated fatty acids (LC-PUFA) such as arachidonic acid (ARA,  
56 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are  
57 important compounds to maintain health and physiological functions in humans and other  
58 vertebrates (Delgado-Lista et al., 2012; Muhlhausler and Ailhaud, 2013). In addition to dietary  
59 input, vertebrates can also obtain LC-PUFA via endogenous production (biosynthesis) from  $C_{18}$   
60 polyunsaturated fatty acid (PUFA) precursors including linoleic acid (LA, 18:2n-6) and  $\alpha$ -  
61 linolenic acid (ALA, 18:3n-3), through a series of consecutive desaturation and elongation  
62 reactions (Guillou et al., 2010; Castro et al., 2016). Fish are the primary source of the health-  
63 promoting n-3 LC-PUFA, EPA and DHA, in the human food basket (Bell and Tocher, 2009;  
64 Tocher, 2009; Tur et al., 2012) and this has prompted interest in understanding the mechanisms  
65 by which fish, particularly farmed species, produce and accumulate these fatty acids in edible  
66 parts. The ability of fish to biosynthesize LC-PUFA from  $C_{18}$  PUFA precursors varies among  
67 species (Garrido et al., 2019), with the variability accounted for by the complement and function  
68 of genes encoding two types of enzymes with key roles in LC-PUFA biosynthesis, namely fatty  
69 acyl desaturases (Fads) and elongation of very long-chain fatty acid (Elovl) proteins (Castro et al.,  
70 2016).

71 Previous studies have shown that vertebrates possess three members of the Elovl protein  
72 family with roles in PUFA elongation, namely Elovl2, Elovl4 and Elovl5 that differ in their fatty  
73 acid (FA) substrate specificities (Castro et al., 2016; Monroig et al., 2018). Elovl5 has a preference  
74 for  $C_{18}$  and  $C_{20}$  PUFA, whereas Elovl2 is predominantly involved in elongation of  $C_{20}$  and  $C_{22}$   
75 PUFA. Consequently, Elovl2, by elongating 22:5n-3 to 24:5n-3, plays a pivotal role in DHA  
76 biosynthesis through the so-called “Sprecher pathway” (Sprecher, 2000) with the elongation  
77 product (24:5n-3) subsequently desaturated to 24:6n-3 prior to being chain-shortened to DHA  
78 (22:6n-3) in peroxisomes (Guillou et al., 2010; Castro et al., 2016). Elovl4 participates in the  
79 elongation of very long-chain ( $C \geq 24$ ) PUFA substrates found in retina and testis (McMahon et al.,  
80 2007; Agaba et al., 2010; Monroig et al., 2010; Santiago Valtierra et al., 2018), although studies  
81 have shown that teleost Elovl4 are also involved in LC-PUFA biosynthesis since they also  
82 elongate of  $C_{18}$ ,  $C_{20}$  and  $C_{22}$  PUFA substrates (Castro et al., 2016). While such elongation capacity  
83 of teleost Elovl4 has been hypothesized to compensate for the absence of an *elovl2* gene in many

84 teleost species (Monroig et al., 2010; Garrido et al., 2019), its more restricted tissue distribution  
85 compared to Elov12 can still compromise the overall biosynthesis capacity of an essential nutrient  
86 such as DHA.

87 It is generally believed that marine fish have a limited ability for LC-PUFA biosynthesis  
88 compared to freshwater counterparts (Tocher, 2010). Loss of *fads1* ( $\Delta 5$  fatty acyl desaturase) and  
89 *elov12* had been suggested to account for the low LC-PUFA biosynthesizing capacity of marine  
90 teleosts (Castro et al., 2016). However, a recent study demonstrated that limitation in LC-PUFA  
91 biosynthesis can also be related to the number of copies of the *fads2* desaturase gene (Ishikawa et  
92 al., 2019). Interestingly, the rabbitfish *Siganus canaliculatus*, a marine herbivorous teleost that is  
93 capable of converting C<sub>18</sub> PUFA to LC-PUFA, has partly overcome the above metabolic hurdle  
94 by diversifying the function of its Fads2 enzymes enabling DHA synthesis via  $\Delta 4$  desaturation.  
95 This is a more direct route than the Sprecher pathway described above and, importantly, avoids  
96 the necessity for Elov12 activity (Li et al., 2010; Monroig et al., 2012a). The diadromous species,  
97 Atlantic salmon (*Salmo salar*) has genes encoding desaturase and elongase enzymes with all the  
98 activities required for the production of DHA from C<sub>18</sub> PUFA (Zheng et al., 2004, 2005; Hastings  
99 et al., 2005; Morais et al., 2009; Monroig et al., 2010; Carmona-Antonanzas et al., 2011; Oboh et  
100 al., 2017). Tocher (2003) pointed out that the pattern of LC-PUFA biosynthesis in Atlantic salmon  
101 was similar to that of freshwater fish, highlighting the influence of their early life stages in  
102 freshwater.

103 In contrast to diadromous salmon, we have incomplete knowledge of the LC-PUFA  
104 biosynthetic capacity of Japanese eel (*Anguilla japonica*), a typical catadromous species. An early  
105 nutrient requirement trial indicated that C<sub>18</sub> PUFA could satisfy essential fatty acid (EFA)  
106 requirements of *A. japonica* (Takeuchi et al., 1980), which suggested this species had the  
107 capability for LC-PUFA biosynthesis. Direct evidence of PUFA desaturation in eel was provided  
108 by feeding <sup>14</sup>C-labeled 18:2n-6 and recovering radioactivity in trienes and tetraenes (Kissil et al.,  
109 1987). Previously, we characterized an Elov15 with C<sub>18</sub> and C<sub>20</sub> PUFA elongation activities, and a  
110 Fads2 with  $\Delta 6$  and  $\Delta 8$  desaturase activities ( $\Delta 6/\Delta 8$  Fads2) of *A. japonica* (Wang et al., 2014). A  
111 further study demonstrated that the *A. japonica* Fads2 can also act as a  $\Delta 6$  desaturase towards  
112 24:5n-3 (Oboh et al., 2017), a key enzymatic step in the Sprecher pathway. Recently, a *fads1*  
113 encoding an enzyme with  $\Delta 5$  desaturase activity was isolated and identified from *A. japonica*, this

114 representing the only *fads1* found in a teleost to date (Lopes-Marques et al., 2018). Together, these  
115 studies suggest that *A. japonica* possesses a complete set of desaturase activities required for  
116 conversion of C<sub>18</sub> PUFA to LC-PUFA. However, Elov15 has only limited elongation capacity  
117 towards C<sub>22</sub> PUFA (Wang et al., 2014), which suggests that Elov12 would be required for the  
118 synthesis of DHA.

119 In the present study, a cDNA encoding a putative Elov12, which catalyzes the key elongation  
120 step from C<sub>22</sub> to C<sub>24</sub> PUFA was cloned and functionally characterized in *A. japonica*, and its tissue  
121 gene expression pattern determined. The identification and characterization of this key activity  
122 demonstrates that Japanese eel has a complete enzyme repertoire required for the endogenous  
123 biosynthesis of DHA from C<sub>18</sub> PUFA via the Sprecher pathway. These data have increased our  
124 knowledge of the diversity of LC-PUFA biosynthesis in vertebrates, and provided further insight  
125 into the regulatory mechanisms of LC-PUFA biosynthesis in teleost fish.

126

## 127 **2. Materials and methods**

### 128 *2.1 Eel samples*

129 Ten adult Japanese eel *A. japonica* fed on commercial eel feed containing 54.2 % protein and  
130 7.3 % lipid were obtained from a commercial fish farm in Chenghai district, Shantou, China. Fish  
131 were anaesthetized and euthanized with an overdose of 2-phenoxyethanol (Sigma, China) and  
132 brain, eye, liver, skin, white muscle, intestine, heart, gill, spleen, heart, kidney and esophagus and  
133 adipose tissue were collected. Tissue samples were immediately frozen in liquid nitrogen, and  
134 subsequently stored at -80 °C until further analysis.

135

### 136 *2.2 Molecular cloning of elov12 cDNA*

137 Total RNA was extracted from eel liver using Trizol reagent (Roche, USA). Subsequently,  
138 first strand cDNA was reverse-transcribed from 1 µg total RNA using FastQuant RT Kit (Tiangen  
139 Biotech Co. Ltd., China) primed with random hexamers. In order to amplify the first fragment  
140 of the *elov12* cDNA, the degenerate primers AJE2F and AJE2R were designed on the basis of an  
141 alignment of amino acid (aa) sequences of Elov12 proteins from zebrafish *Danio rerio*  
142 (AAI34116.1), cherry salmon *Oncorhynchus masou* (AGR34076.1), rainbow trout  
143 *Oncorhynchus mykiss* (NP\_001118108.1) and northern pike *Esox lucius* (XP\_010884057.1),

144 using the EBI ClustalW2 tool (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) (Table 1). The first  
145 fragment of the Japanese eel putative *elovl2* was amplified from liver cDNA by PCR (*Pfu* PCR  
146 MasterMix, Tiangen Biotech Co. Ltd., China) performed according to the following process:  
147 initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at  
148 59 °C for 30 s and extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. The  
149 PCR products were purified (TIANquick midi purification kit, Tiangen, China), cloned into  
150 pMD™ 18-T vector (TaKaRa Biotech Co. Ltd., China) and subsequently sequenced (Sangon  
151 Biotech Co. Ltd., China). Gene-specific primers were designed for 5' and 3' rapid amplification  
152 of cDNA ends (RACE) PCR (GeneRacer™ Kit, Invitrogen, USA). Sequences of all PCR primers  
153 used in the study are shown in Table 1.

154

### 155 2.3 Sequence and phylogenetic analysis of the *A. japonica* *Elov12*

156 The deduced aa sequence of the newly cloned *elovl2*-like cDNA was aligned with orthologs  
157 from human (NP\_060240.3), Atlantic salmon (NP\_001130025), cherry salmon (AGR34076.1),  
158 rainbow trout (AIT56593.1), catfish *Clarias gariepinus* (AOY10780.1) and zebrafish  
159 (NP\_001035452.1), using ClustalX<sub>2</sub>. The aa sequence identities between the deduced *Elov12*  
160 protein from Japanese eel and other vertebrate homologs were compared using the EMBOSS  
161 Needle Pairwise Sequence Alignment tool ([http://www.ebi.ac.uk/Tools/psa/emboss\\_needle/](http://www.ebi.ac.uk/Tools/psa/emboss_needle/)). A  
162 phylogenetic tree comparing the deduced aa sequence of the Japanese eel *Elov12* with *Elov1*  
163 proteins of birds, amphibian, reptilian, mammalian and fish (including the Agnathan *Lampetra*  
164 *japonicum* and teleosts), was constructed using the neighbor-joining method (Saitou and Nei,  
165 1987) with MEGA 7.0.

166

### 167 2.4 Functional characterization of the *A. japonica* *Elov12* by heterologous expression in yeast 168 *Saccharomyces cerevisiae*

169 Liver cDNA synthesized from total RNA was used as template to amplify the open reading  
170 frame (ORF) of the Japanese eel *elovl2* using Phusion® High-Fidelity PCR MasterMix DNA  
171 polymerase (Tiangen Biotech Co. Ltd., China). The primers AjElov12F/AjElov12R, containing  
172 specific restriction enzyme sites (underlined in Table 1) for *Hind*III (forward) and *Xba*I (reverse),  
173 were used for PCR amplification consisting of an initial denaturing step at 94 °C for 5 min,

174 followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at  
175 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. DNA fragments were purified  
176 (E.Z.N.A. Gel Extraction Kit, Omega, USA), digested with the corresponding restriction  
177 endonucleases (New England Biolabs, Inc., USA) and ligated into similarly restricted yeast  
178 expression vector pYES2 (Invitrogen, UK). The recombinant plasmids (High Pure Plasmid  
179 Isolation Kit, Roche, USA) containing the putative *elovl2* ORF (pYES2-*elovl2*) were used to  
180 transform *Saccharomyces cerevisiae* (strain INVSc1) competent cells (S. c. EasyComp™  
181 Transformation Kit, Invitrogen). Yeast culture and selection were according to Monroig et al.  
182 (2012a). Recombinant yeast expressing *elovl2* was supplemented with potential PUFA substrates  
183 for fatty acyl elongases, namely 18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3  
184 and 22:4n-6. The PUFA substrates were added at final concentrations of 0.5, 0.75 and 1.0 mM for  
185 C<sub>18</sub>, C<sub>20</sub> and C<sub>22</sub>, respectively, to compensate for the decreased uptake with increased chain length  
186 (Lopes-Marques et al., 2017). A control treatment consisting of yeast transformed with empty  
187 pYES2 was run under the same conditions. After 2 days of incubation at 30 °C and continuous  
188 agitation, yeast cultures were harvested, washed with Hank's balanced salt solution containing 1 %  
189 fatty acid-free albumin, and homogenized in chloroform/methanol (2:1, v/v) containing 0.01 %  
190 butylated hydroxytoluene (BHT; Sigma, USA) as antioxidant.

191

## 192 2.5 Fatty acid analysis by GC-MS

193 Total lipid was extracted from yeast according to Folch et al. (1957) and fatty acid methyl  
194 esters were prepared and purified according to method described by Christie (2003). The identities  
195 of fatty acids were confirmed by gas chromatography (GC) coupled with a mass spectrometer  
196 (GC-MS) (2010-ultra, Shimadzu, Japan) as described previously (Hastings et al., 2001; Agaba et  
197 al., 2004). Conversions of PUFA substrates were calculated as the proportion of exogenously  
198 added substrate FA converted to elongated FA products, as [individual product area/ (all products  
199 areas + substrate area)] × 100.

200

## 201 2.6 Tissue distribution of the *A. japonica elovl2* mRNA

202 Tissue distribution of *elovl2* mRNA was determined by quantitative real-time PCR (qPCR).  
203 Total RNA was extracted using TRIzol® Reagent (Roche, Switzerland) according to the



204 manufacturer's protocol, and 1 µg of total RNA was reverse-transcribed into cDNA using random  
205 hexamers (Applied Biosystems, USA). The qPCR analyses were performed using the primers  
206 shown in Table 1. The relative expression of *elovl2* was normalized with 18S rRNA expression  
207 calculated by the  $2^{-\Delta\Delta C_t}$  method (Livak and Schmittgen, 2001). The qPCR amplifications were  
208 carried out on a LightCycler® 480 System (Roche, Switzerland), in a final volume of 20 µL  
209 containing 2 µL of diluted cDNA, 0.5 µM of each primer and 10 µL of SYBR Green I Master Mix  
210 (Roche). Amplifications were carried out with a systematic negative control containing no cDNA  
211 (NTC, no template control). The qPCR profiles contained an initial activation step at 95 °C for 5  
212 min, followed by 40 cycles: 10 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C. After the amplification  
213 phase, a dissociation curve of 0.5 °C increments from 65 °C to 95 °C was performed, enabling  
214 confirmation of the amplification of a single product in each reaction. No primer-dimer formation  
215 occurred in the NTC.

216

## 217 2.7 Statistical analysis

218 Results of the tissue distribution analyses are expressed as mean normalized values ± SEM  
219 (n = 6) corresponding to the ratio of the copy numbers of the *elovl2* transcripts and the copy  
220 numbers of the reference gene, 18S rRNA. Differences in the expression of *elovl2* among tissues  
221 were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple  
222 comparison test at a significance level of  $P \leq 0.05$  (OriginPro 8.0, OriginLab Corporation, USA).

223

## 224 3. Results

### 225 3.1. *A. japonica elovl2* sequences and phylogenetic position

226 A 1,754-bp full-length fragment of the Japanese eel *elovl2* cDNA (excluding the polyA tail)  
227 was obtained by 5' and 3' RACE PCR. The sequence was deposited in GenBank with the accession  
228 number MG734863. The *elovl2*-like sequence contained an ORF of 885 bp that encodes a putative  
229 protein of 294 aa. Multiple alignment of the deduced Japanese eel Elov12 polypeptide sequence  
230 showed approximately 73-80 % identity with Elov12 proteins from other teleosts including  
231 zebrafish *D. rerio*, African catfish *C. gariepinus*, Atlantic salmon *S. salar*, rainbow trout *O. mykiss*  
232 and cherry salmon *O. masou*, and relative high identity to mammalian ELOVL2 proteins. The  
233 deduced Elov12 polypeptide had 59.60 % sequence identity when compared with the Elov15 from

234 Japanese eel (GenBank accession number KJ182967) (Wang et al., 2014).

235 The deduced polypeptide sequence of the Japanese eel Elovl2 contained four conserved  
236 motifs: KXXE/DXXDT, QXXFLHXYHH (containing the diagnostic histidine box (HXXHH)  
237 conserved in all members of the Elovl family), NXXHXXMYXYY and TXXQXXQ (indicated  
238 by boxes in Fig. 1). The sequence also possessed lysine (K) or arginine (R) residues near the  
239 carboxyl terminus, a feature regarded as putative endoplasmic reticulum (ER) retrieval signals  
240 (Agaba et al., 2005; Jakobsson et al., 2006). The Japanese eel putative Elovl2 protein sequence  
241 was predicted by TMHMM Server v. 2.0 to contain seven transmembrane regions, I-VII (marked  
242 with a solid underline in Fig. 1).

243 A phylogenetic tree was constructed based on the aa sequences of the deduced Japanese eel  
244 Elovl2 and representatives of all three PUFA Elovl protein families (Elovl2, Elovl4 and Elovl5)  
245 from a variety of animal species. The phylogenetic analysis showed that the Japanese eel Elovl2-  
246 deduced polypeptide sequence clustered together with other vertebrate Elovl2 orthologs, and more  
247 distantly from clusters containing Elovl4 and Elovl5 sequences, the latter including the *A.*  
248 *japonica* Elovl5 characterized previously (Wang et al., 2014) (Fig. 2). These results confirmed  
249 that the newly cloned *A. japonica* elongase is an ortholog of *elovl2*.

250

### 251 3.2. Functional characterization of the *A. japonica* Elovl2

252 The Japanese eel Elovl2 was functionally characterized by determining the FA profiles of *S.*  
253 *cerevisiae* transformed with pYES2 vector containing *elovl2* cDNA ORF as insert (pYES2-*elovl2*),  
254 and grown in the presence of C<sub>18</sub> (18:2n-6, 18:3n-3, 18:4n-3 and 18:3n-6), C<sub>20</sub> (20:5n-3 and 20:4n-  
255 6) and C<sub>22</sub> (22:5n-3 and 22:4n-6) PUFA substrates. The FA composition of control yeast  
256 (transformed with empty pYES2) was characterized by having 16:0, 16:1n-7, 18:0 and 18:1n-9,  
257 abundant FA in wild type yeast (Hastings et al., 2001). An additional FA peak was found to  
258 correspond to the exogenously added PUFA substrate (data not shown). This result was consistent  
259 with yeast not possessing elongase activities towards PUFA substrates in *S. cerevisiae* (Agaba et  
260 al., 2004; Hastings et al., 2005). Yeast transformed with pYES2-*elovl2* were able to elongate  
261 several PUFA substrates that were supplied exogenously. The Japanese eel Elovl2 showed low  
262 capacity to elongate C<sub>18</sub> PUFA substrates, with no activity towards 18:3n-3 and 18:2n-6 and  
263 relatively low conversions towards 18:4n-3 and 18:3n-6 that were elongated to 20:4n-3 and 20:3n-

264 6, respectively (Table 2; Fig. 3). In contrast, the *A. japonica* Elovl2 showed relatively high  
265 elongase capacity towards C<sub>20</sub> and C<sub>22</sub> PUFA, which in all cases led to the production of C<sub>24</sub> PUFA  
266 elongation products (Table 2; Fig. 3).

267

### 268 3.3. Tissue expression of Japanese eel *elovl2* mRNA

269 Determination of tissue distribution of *elovl2* mRNA by qPCR showed the Japanese eel  
270 *elovl2* had widespread expression with all tissues analyzed showing *elovl2* transcripts (Fig. 4).  
271 The highest expression of *elovl2* was detected in the brain and eye followed by liver.

272

## 273 4. Discussion

274 Functional characterization of the putative elongase of *A. japonica* by heterologous  
275 expression in *S. cerevisiae* confirmed that it was an Elovl2 and able to efficiently elongate C<sub>20</sub>  
276 (20:5n-3 and 20:4n-6) and C<sub>22</sub> (22:5n-3 and 22:4n-6) PUFA substrates and, to a much lower extent,  
277 C<sub>18</sub> (18:4n-3 and 18:3n-6) substrates. Compared with other mammalian and teleost orthologs,  
278 these results were similar to observations for Elovl2 proteins of Atlantic salmon, zebrafish,  
279 tambaqui and mouse (Leonard et al., 2000; Monroig et al., 2009; Morais et al., 2009; Ferraz et al.,  
280 2019), but different to the Elovl2 of rainbow trout, human and rat, which showed no activity  
281 towards C<sub>18</sub> PUFA substrates (Leonard et al., 2002; Gregory et al., 2011; Gregory and James,  
282 2014). While the ability of Elovl2 to elongate C<sub>18</sub> PUFA in some species might be accounted for  
283 its shared evolutionary origin with Elovl5 (Monroig et al., 2016), it is clear that both C<sub>20</sub> and C<sub>22</sub>  
284 PUFA are preferred elongation substrates for Elovl2. Such elongation capacity enables Elovl2  
285 enzymes to produce 24:5n-3 from both EPA (20:5n-3) and DPA (22:5n-3). Indeed, the *A. japonica*  
286 Elovl2 characterized in the present study was able to elongate EPA and DPA to a relatively high  
287 extent in comparison to Elovl2 from Atlantic salmon and zebrafish (Monroig et al., 2009; Morais  
288 et al., 2009), although lower when compared with that of rainbow trout (Gregory and James, 2014).  
289 Similar to other species, the activity of Japanese eel Elovl2 towards n-3 PUFA substrates were  
290 generally higher than those towards n-6 PUFA substrates. This is consistent with previous findings  
291 indicating that, generally, the enzymes involved in LC-PUFA biosynthesis from LA and ALA act  
292 on both n-3 and n-6 series fatty acids, with a general preference for n-3 PUFA (Tocher et al., 1998;  
293 Monroig et al., 2018). In mammalian and fish, elongases involved in LC-PUFA biosynthesis are

294 generally more efficient in elongating n-3 rather than n-6 HUFA substrates (Inagaki et al., 2000;  
295 Leonard et al., 2002; Morais et al., 2009; Monroig et al., 2009; Gregory and Jame., 2014; Oboh  
296 et al., 2016). However, some species eg. *Octopus vulgaris* elongase appeared to exhibit higher  
297 elongation rates towards n-6 compared to n-3 substrates because of the particularly important  
298 physiological roles of ARA in the common octopus (Monroig et al., 2012b; Milou et al., 2006).  
299 The substrate preference might reflect the different requirement for physiological functions of n-  
300 3 and n-6 PUFA in different animals (Monroig et al., 2012b). Thus, the substrate preference of  
301 Japanese eel *Elovl2* to n-3 PUFA reflects an important physiological role of n-3 PUFA especially  
302 DHA, in this fish.

303         Based on our previous studies, we considered that the ability of *A. japonica* to biosynthesize  
304 DHA from C<sub>18</sub> PUFA may be restricted at the step of conversion DPA to 24:5n-3 (Wang et al.,  
305 2014). The present results provide new data for Japanese eel that enable us to confirm that this  
306 species has all the enzyme activities required, not only for the biosynthesis of EPA and ARA from  
307 18:3n-3 and 18:2n-6, respectively, but also for the production of DHA from EPA. Therefore, the  
308 nutritional and biochemical evidence available now suggests that *A. japonica*, a catadromous fish  
309 species, has a similar pattern of LC-PUFA biosynthesis to freshwater and salmonid fish, which  
310 generally possess complete pathways for the biosynthesis of LC-PUFA from C<sub>18</sub> PUFA (Takeuchi  
311 et al., 1980; Chow et al., 2010; Tocher, 2010; Wang et al., 2014; Ferraz et al., 2019). In recent  
312 years, researchers have postulated that various confounding factors including habitat, trophic level  
313 and ecology, feeding habits, and diadromy are all potential drivers underpinning the presence  
314 and/or modulating the activity of enzymes involved in LC-PUFA biosynthesis and, consequently,  
315 the capacity for LC-PUFA biosynthesis in fish species (Bell and Tocher, 2009; Castro et al., 2016;  
316 Monroig et al., 2016). In this respect, life cycle and feeding habits may play a role in eels. In the  
317 oceanic larval phase, the long-lived leptocephali are believed to feed primarily on organic detritus  
318 termed ‘marine snow,’ which is nutritionally poor and low in LC-PUFA compared with the  
319 zooplankton diets of other marine teleost larvae (Man and Hodgkiss, 1981; Aida et al., 2003;  
320 McKinnon, 2006; Deibel et al., 2012). After metamorphosis, glass eels migrate into freshwater  
321 that is relatively poor in DHA (Leaver et al., 2008) for development into elvers and adults. It has  
322 also been reported that, during migration back to the oceanic spawning grounds, silver eels may  
323 not assimilate any nutrition (Chow et al., 2010). Therefore, the life cycle of eels may suggest that

324 endogenous production of LC-PUFA would be required to compensate for generally low dietary  
325 input.

326 However, while the above may suggest there is evolutionary pressure in eels to retain the  
327 capacity for endogenous production of LC-PUFA, it is become increasingly accepted that the  
328 major influence of LC-PUFA biosynthesis pathways in teleost species is phylogenetic position  
329 (Monroig et al., 2018). *Elovl2* was reported to be lost in the Neoteleostei and has only been  
330 described in a few teleost species (Monroig et al., 2016; Ferraz et al., 2019). For some time, *elovl2*  
331 was considered as one of the genes that disappeared in the evolution of marine fish possibly as a  
332 consequence of the high content of LC-PUFA in marine environments and the resultant lack of  
333 evolutionary pressure to retain biosynthetic activities (Monroig et al., 2016; Castro et al., 2016).  
334 A recent study on the European sardine, *Sardina pilchardus*, has challenged this paradigm. Thus,  
335 *S. pilchardus*, a marine fish species, has been demonstrated to have an *elovl2* gene (Machado et  
336 al., 2018), confirming that, rather than habitat (marine vs freshwater), the phylogenetic position  
337 of *S. pilchardus* within the teleosts' tree of life, accounts for the presence of a gene that was  
338 believed to be absent in marine teleost genomes (Castro et al., 2016). Similarly, eels are part of  
339 the Elopomorpha with an evolutionary location near the base of Teleostei and thus regarded as a  
340 basal teleost. In general, basal teleosts have more conserved LC-PUFA biosynthesis pathways and  
341 have retained *elovl2* genes and, in the case of Elopomorpha, also *fads1* ( $\Delta 5$  desaturase) (Castro et  
342 al., 2016; Monroig et al., 2016, 2018; Lopes-Marques et al., 2018).

343 Previously we showed that *elovl5* expression was highest in brain, and the highest expression  
344 of *fads2* mRNA was in brain and in the eye in adult *A. japonica* (Wang et al., 2014). The present  
345 study showed that *elovl2* gene was also predominantly expressed in the eyes and brain, and to a  
346 lesser extent liver in adult *A. japonica*. In contrast, *elovl2* expression was highest in liver and  
347 intestine in teleosts such as zebrafish, Atlantic salmon and African catfish (Monroig et al., 2009;  
348 Morais et al., 2009; Oboh et al., 2016). While the precise reason for this difference in *elovl2*  
349 expression pattern is unknown, it may be related to the natural diet of eels, which may be  
350 particularly limited in terms of DHA, not simply in the DHA-poor freshwater environment, but  
351 also in the marine stages due to the feeding habits of leptocephali and silver eels as described  
352 above. As DHA is particularly functionally important in neural tissues, and accumulated in brain  
353 and eye/retinal membranes, the tissue distribution of the key genes of the LC-PUFA biosynthesis

354 pathway may reflect the importance of endogenous synthesis of DHA from EPA in these tissues  
355 (Tocher, 2003, 2010; Leaver et al., 2008; Bell and Tocher, 2009).

356 In conclusion, the present study reports on the molecular cloning of a cDNA encoding an  
357 Elov12 from Japanese eel, representing the first report of an Elov12 in a teleost fish with a  
358 catadromous lifestyle. The present study has indicated that Japanese eel has a complete repertoire  
359 of fatty acyl desaturase and elongase enzymes enzymes required for the biosynthesis of LC-PUFA  
360 from C<sub>18</sub> PUFA substrates and, specifically, that it has capability for the biosynthesis of DHA from  
361 EPA via the “Sprecher” pathway, this biosynthesis pattern maybe more similar to freshwater fish  
362 species. The highest expression of *elov12* in adult eel was detected in brain and eyes, which was  
363 different from the pattern in freshwater and anadromous species. The results were confusing, it  
364 might be hypothesized that the LC-PUFA biosynthetic system of catadromous eels would show  
365 neither “marine pattern” nor “freshwater pattern”. This expression pattern may indicate the  
366 importance of endogenous production of DHA from EPA in neural tissues in eel. These data have  
367 increased our knowledge of the diversity of LC-PUFA biosynthesis in vertebrates, and provided  
368 further insight into the regulatory mechanisms of LC-PUFA biosynthesis in teleost fish. The  
369 results provide a base for further studies aimed at the optimization and/or enhancement of  
370 endogenous production of EPA and DHA in farmed *A. japonica*, and the efficient use of  
371 sustainable plant-based oil alternatives.

372

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379

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546

547 **Legends to Figures**

548

549 **Fig. 1.** Comparison of deduced amino acid (aa) sequences from the newly cloned Japanese eel  
550 (*Anguilla japonica*, Aj.) *elovl2*, with those from Cherry salmon (*Oncorhynchus masou*, Oma;  
551 AGR34076.1), rainbow trout (*Oncorhynchus mykiss*, Omy; AIT56593.1), Atlantic salmon (*Salmo*  
552 *salar*, Ss.; NP\_001130025.1 ), African catfish (*Clarias gariepinus*, Cg.; AOY10780.1) and  
553 zebrafish (*Danio rerio*, Dr. ; AAI29269.1). Identical aa residues are shaded black and similar  
554 residues are shaded grey (alignment by ClustalX2 and colored by Genedoc). The four conserved  
555 motifs are labeled with red square frames. The conserved histidine box HXXHH is marked with  
556 “\*”, seven putative transmembrane domains are solid-underlined and labeled with I - VII.

557

558 **Fig. 2.** Phylogenetic tree comparing the deduced amino acid sequence of *Anguilla japonica*  
559 elongase with representative of PUFA elongases (Elov12, Elov14 and Elov15) from other  
560 vertebrates. The tree was constructed using the neighbor-joining method in MEGA7.0. Accession  
561 numbers of sequence was labeled in bracket. Bold font and asterisk marked sequence is the cloned  
562 *elovl2* in Japanese eel.

563

564 **Fig. 3.** Functional characterization of the putative Elov12 from Japanese eel in transgenic yeast  
565 *Saccharomyces cerevisiae*. Recombinant yeast transformed with pYES2-*elovl2* were grown in the  
566 presence of elongase fatty acid (FA) substrates, n-6 (A, C, E) and n-3 (B, D, F) PUFA substrates.  
567 The peaks marked as 1–4 in all panels are the main yeast endogenous FA, namely 16:0, 16:1, 18:0  
568 and 18:1, respectively. Additionally, peaks derived from exogenously added substrates (“ \* ”) or  
569 elongation products are indicated accordingly in panels A-E. Vertical axis, FID response; and  
570 horizontal axis, retention time.

571

572 **Fig. 4.** Tissue-specific expression of *elovl2* mRNA in *Anguilla japonica* examined by qPCR.  
573 Relative expression of target genes were quantified for each transcript and were normalized with  
574 ribosomal 18S rRNA by  $2^{-\Delta\Delta Ct}$  method. Absolute copy numbers of target genes were quantified  
575 for each transcript and were normalized by absolute levels of 18S RNA. Results are means  $\pm$  SEM  
576 (n = 6), and different letters show significant differences ( $P < 0.05$ ) among tissues as determined

577 by one-way ANOVA followed by Tukey's multiple comparison test.

578

579 **Table 1.** Primers used for cDNA cloning or determining gene expression of *Anguilla japonica*  
580 elongases.  
581

Aim	primer	Primer sequence (5'- 3')
First fragment cloning	AJE2F	GGYTACCGKCTGCAGTGTCA
	AJE2R	ATCCAGTTGAGCACGCACHA
RACE PCR cloning	AjE2F1	CCATGTTCAACATCTGGTGGTGC GTGCT
	AjE2F2	TCCAAGCTCATTGAGTTCCTGGACACGA
	AjE2R1	GGAGGCGTGGTGGTAAACGTGCAAGAACG
	AjE2R2	TCGTGTCCAGGAACTCAATGAGCTTGGAGA
ORF cloning	AjE2S2	CCCAAGCTTTAATATGGACCAACTAGAGGCCTTTGACC
	AjE2A2	TGCTCTAGAACCCAAA ACTACTGACTTTTTTTGTTTGGGA
qPCR	qE2S1	CAAAGTACTGTGGTGGTACTACTT
	qE2A1	GGTAAACGTGCAAGAACGAAAT
	18sF1	TTAGTGAGGTCCTCGGATCG
	18sA1	CCTACGGAAACCTTGTTACG

582 Note: The gene sequences information for *elovl2* first fragment cloning was shown in the materials and  
583 methods content. The accession number of nucleotide sequence used for RACE PCR and ORF cloning or  
584 qPCR of *elovl2* was MG735863. That of 18S rRNA was FM946132.

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586  
587  
588  
589

590 **Table 2.** Functional characterization of the Japanese eel Elovl2 in yeast *Saccharomyces cerevisiae*.  
 591 Individual conversions were calculated according to the formula [individual product area/ (all  
 592 products areas + substrate area)] × 100.

593

FA substrate	Product	Conversion (%)	Activity
18:3n-3	20:3n-3	ND	C <sub>18</sub> →C <sub>20</sub>
18:2n-6	20:2n-6	ND	C <sub>18</sub> →C <sub>20</sub>
18:4n-3	20:4n-3	6	C <sub>18</sub> →C <sub>20</sub>
18:3n-6	20:3n-6	3	C <sub>18</sub> →C <sub>20</sub>
20:5n-3	22:5n-3	73	C <sub>20</sub> →C <sub>22</sub>
	24:5n-3	60	C <sub>22</sub> →C <sub>24</sub>
20:4n-6	22:4n-6	47	C <sub>20</sub> →C <sub>22</sub>
	24:4n-6	44	C <sub>22</sub> →C <sub>24</sub>
22:5n-3	24:5n-3	56	C <sub>22</sub> →C <sub>24</sub>
22:4n-6	24:4n-6	32	C <sub>22</sub> →C <sub>24</sub>

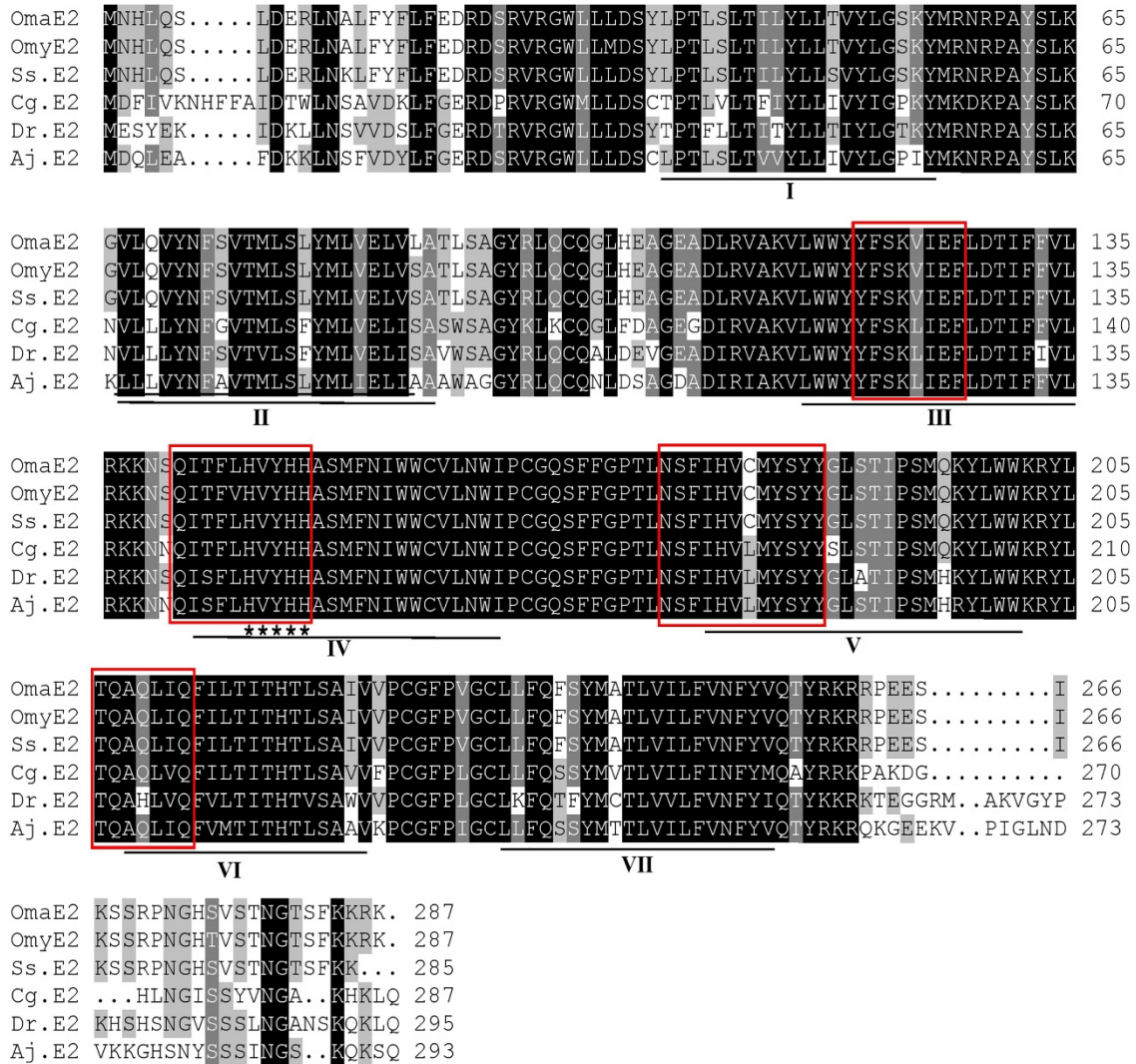
594 ND, not detected

595



596 Fig. 1

597



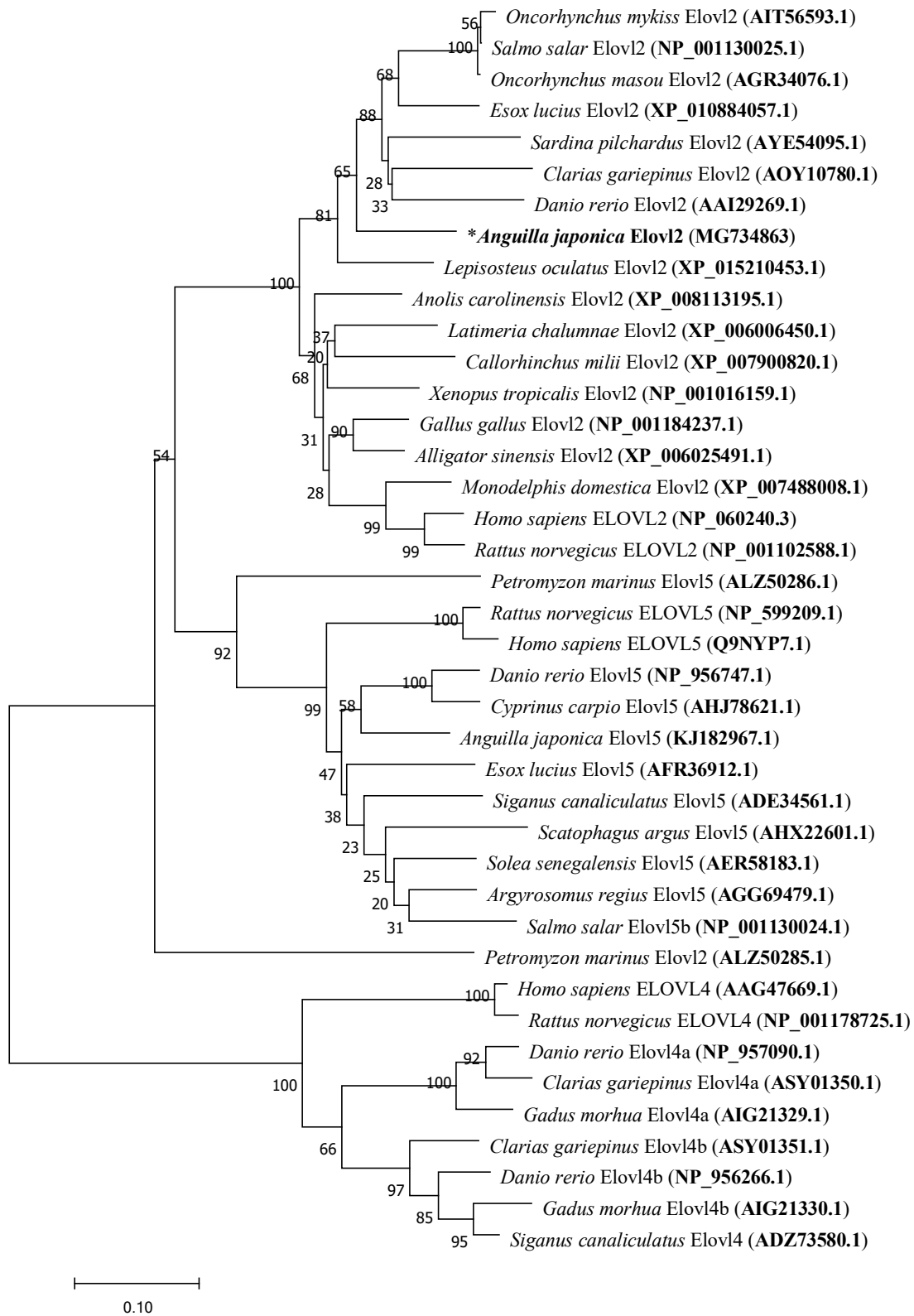
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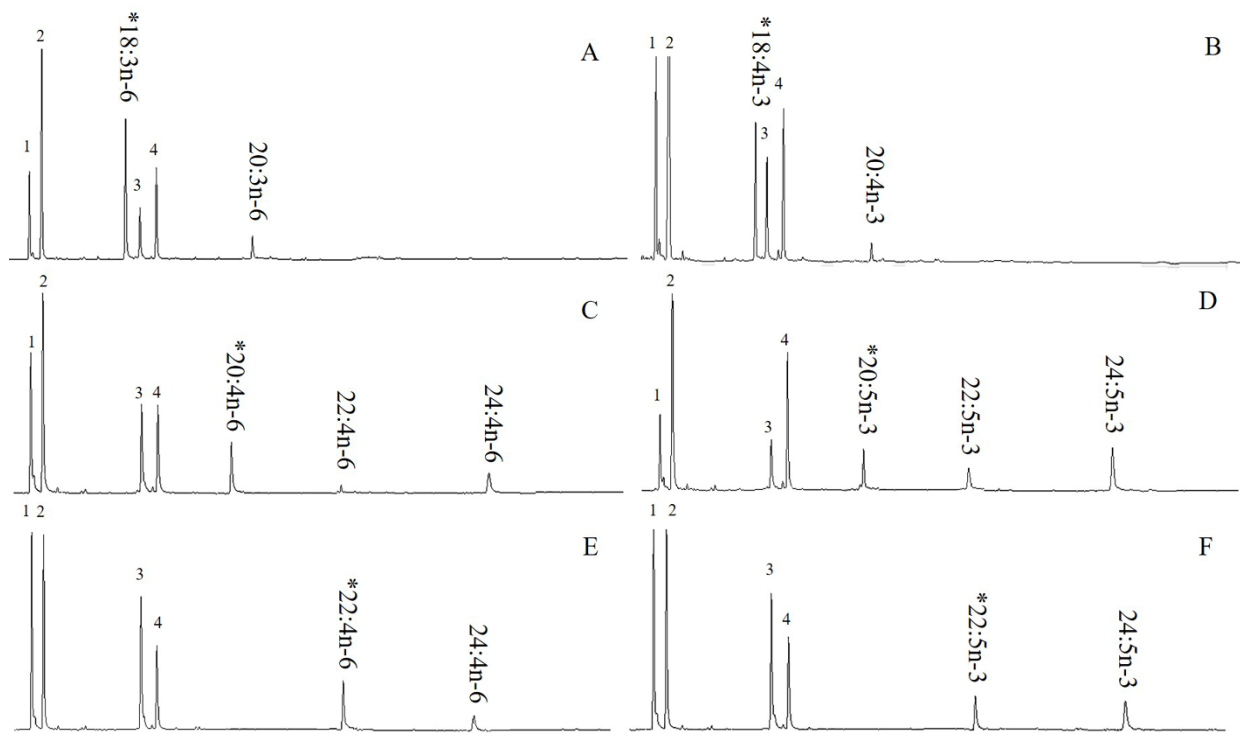
601 Fig.2.

602



603 **Fig. 3.**

604



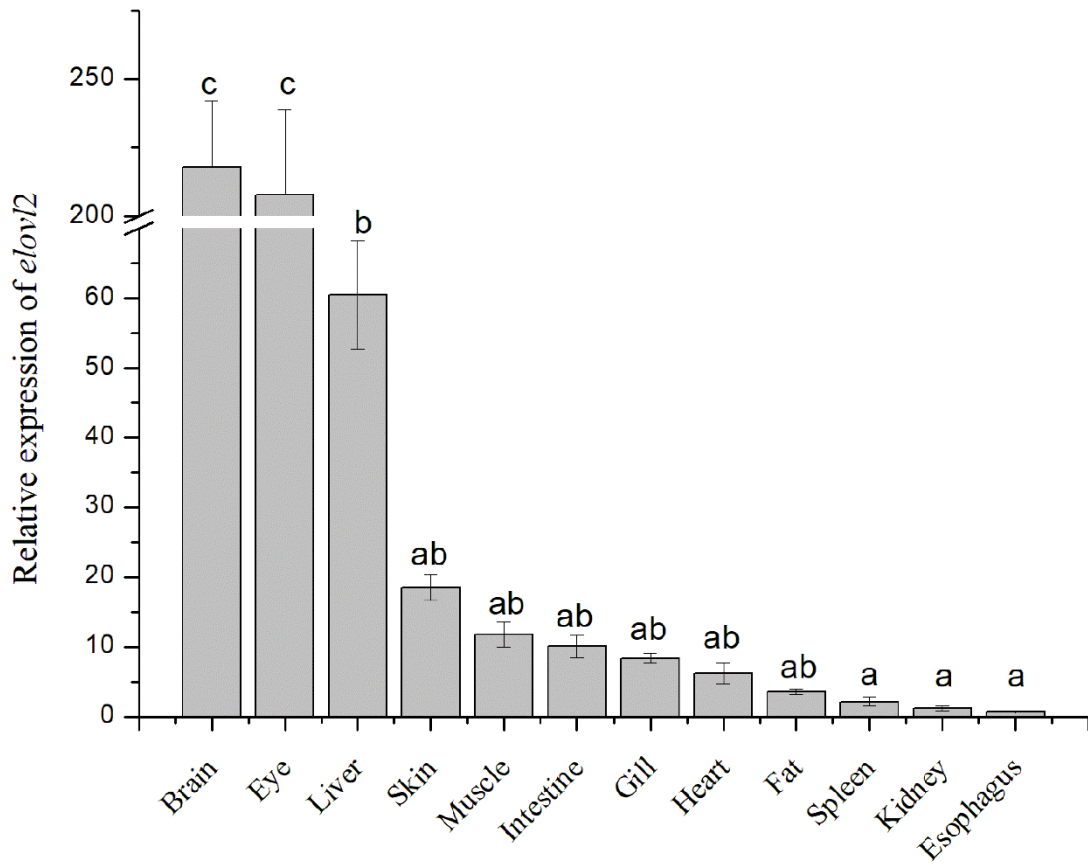
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609 Fig. 4.



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