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

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Abstract

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

Keywords

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

1. Introduction

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

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

teleost species (Monroig et al., 2010; Garrido et al., 2019), its more restricted tissue distribution compared to Elovl2 can still compromise the overall biosynthesis capacity of an essential nutrient such as DHA.

It is generally believed that marine fish have a limited ability for LC-PUFA biosynthesis compared to freshwater counterparts (Tocher, 2010). Loss of *fads1* (Δ5 fatty acyl desaturase) and *elovl2* had been suggested to account for the low LC-PUFA biosynthesizing capacity of marine teleosts (Castro et al., 2016). However, a recent study demonstrated that limitation in LC-PUFA biosynthesis can also be related to the number of copies of the *fads2* desaturase gene (Ishikawa et al., 2019). Interestingly, the rabbitfish *Siganus canaliculatus*, a marine herbivorous teleost that is capable of converting C_{18} PUFA to LC-PUFA, has partly overcome the above metabolic hurdle by diversifying the function of its Fads2 enzymes enabling DHA synthesis via Δ4 desaturation. This is a more direct route than the Sprecher pathway described above and, importantly, avoids the necessity for Elovl2 activity (Li et al., 2010; Monroig et al., 2012a). The diadromous species, Atlantic salmon (*Salmo salar*) has genes encoding desaturase and elongase enzymes with all the activities required for the production of DHA from C_{18} PUFA (Zheng et al., 2004, 2005; Hastings et al., 2005; Morais et al., 2009; Monroig et al., 2010; Carmona-Antonanzas et al., 2011; Oboh et al., 2017). Tocher (2003) pointed out that the pattern of LC-PUFA biosynthesis in Atlantic salmon was similar to that of freshwater fish, highlighting the influence of their early life stages in freshwater.

In contrast to diadromous salmon, we have incomplete knowledge of the LC-PUFA biosynthetic capacity of Japanese eel (*Anguilla japonica*), a typical catadromous species. An early nutrient requirement trial indicated that C_{18} PUFA could satisfy essential fatty acid (EFA) requirements of *A. japonica* (Takeuchi et al., 1980), which suggested this species had the capability for LC-PUFA biosynthesis. Direct evidence of PUFA desaturation in eel was provided by feeding ^{14}C-labeled 18:2n-6 and recovering radioactivity in trienes and tetraenes (Kissil et al., 1987). Previously, we characterized an Elovl5 with C_{18} and C_{20} PUFA elongation activities, and a Fads2 with Δ6 and Δ8 desaturase activities (Δ6/Δ8 Fads2) of *A. japonica* (Wang et al., 2014). A further study demonstrated that the *A. japonica* Fads2 can also act as a Δ6 desaturase towards 24:5n-3 (Oboh et al., 2017), a key enzymatic step in the Sprecher pathway. Recently, a *fads1* encoding an enzyme with Δ5 desaturase activity was isolated and identified from *A. japonica*, this

representing the only *fads1* found in a teleost to date (Lopes-Marques et al., 2018). Together, these studies suggest that *A. japonica* possesses a complete set of desaturase activities required for conversion of C_{18} PUFA to LC-PUFA. However, Elovl5 has only limited elongation capacity towards C_{22} PUFA (Wang et al., 2014), which suggests that Elovl2 would be required for the synthesis of DHA.

In the present study, a cDNA encoding a putative Elovl2, which catalyzes the key elongation step from C_{22} to C_{24} PUFA was cloned and functionally characterized in *A. japonica*, and its tissue gene expression pattern determined. The identification and characterization of this key activity demonstrates that Japanese eel has a complete enzyme repertoire required for the endogenous biosynthesis of DHA from C_{18} PUFA via the Sprecher pathway. These data have increased our knowledge of the diversity of LC-PUFA biosynthesis in vertebrates, and provided further insight into the regulatory mechanisms of LC-PUFA biosynthesis in teleost fish.

2. Materials and methods

2.1 Eel samples

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

2.2 Molecular cloning of elovl2 cDNA

Total RNA was extracted from eel liver using Trizol reagent (Roche, USA). Subsequently, first strand cDNA was reverse-transcribed from 1μg total RNA using FastQuant RT Kit (Tiangen Biotech Co. Ltd., China) primed with random hexamers. In order to amplify the first fragment of the *elovl2* cDNA, the degenerate primers AJE2F and AJE2R were designed on the basis of an alignment of amino acid (aa) sequences of Elovl2 proteins from zebrafish *Danio rerio* (**AAI34116.1**), cherry salmon *Oncorhynchus masou* (**AGR34076.1**), rainbow trout *Oncorhynchus mykiss* (**NP_001118108.1**) and northern pike *Esox lucius* (**XP_010884057.1**),

using the EBI ClustalW2 tool (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Table 1). The first fragment of the Japanese eel putative *elovl2* was amplified from liver cDNA by PCR (*Pfu* PCR MasterMix, Tiangen Biotech Co. Ltd., China) performed according to the following process: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. The PCR products were purified (TIANquick midi purification kit, Tiangen, China), cloned into pMD™ 18-T vector (TaKaRa Biotech Co. Ltd., China) and subsequently sequenced (Sangon Biotech Co. Ltd., China). Gene-specific primers were designed for 5′ and 3′ rapid amplification of cDNA ends (RACE) PCR (GeneRacer™ Kit, Invitrogen, USA). Sequences of all PCR primers used in the study are shown in Table 1.

2.3 Sequence and phylogenetic analysis of the A. japonica Elovl2

The deduced aa sequence of the newly cloned *elovl2*-like cDNA was aligned with orthologs from human (**NP_060240.3**), Atlantic salmon (**NP_001130025**), cherry salmon (**AGR34076.1**), rainbow trout (**AIT56593.1**), catfish *Clarias gariepinus* (**AOY10780.1**) and zebrafish (**NP_001035452.1**), using ClustalX$_2$. The aa sequence identities between the deduced Elovl2 protein from Japanese eel and other vertebrate homologs were compared using the EMBOSS Needle Pairwise Sequence Alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). A phylogenetic tree comparing the deduced aa sequence of the Japanese eel Elovl2 with Elovl proteins of birds, amphibian, reptilian, mammalian and fish (including the Agnathan *Lampetra japonicum* and teleosts), was constructed using the neighbor-joining method (Saitou and Nei, 1987) with MEGA 7.0.

2.4 Functional characterization of the A. japonica Elovl2 by heterologous expression in yeast Saccharomyces cerevisiae

Liver cDNA synthesized from total RNA was used as template to amplify the open reading frame (ORF) of the Japanese eel *elovl2* using Phusion® High-Fidelity PCR MasterMix DNA polymerase (Tiangen Biotech Co. Ltd., China). The primers AjElovl2F/AjElovl2R, containing specific restriction enzyme sites (underlined in Table 1) for *Hin*dIII (forward) and *Xba*I (reverse), were used for PCR amplification consisting of an initial denaturing step at 94 °C for 5 min,

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

2.5 Fatty acid analysis by GC–MS

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

2.6 Tissue distribution of the A. japonica elovl2 mRNA

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

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

2.7 Statistical analysis

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

3. Results

3.1. A. japonica elovl2 sequences and phylogenetic position

A 1,754-bp full-length fragment of the Japanese eel *elovl2* cDNA (excluding the polyA tail) was obtained by 5′ and 3′ RACE PCR. The sequence was deposited in GenBank with the accession number MG734863. The *elovl2*-like sequence contained an ORF of 885 bp that encodes a putative protein of 294 aa. Multiple alignment of the deduced Japanese eel Elovl2 polypeptide sequence showed approximately 73-80 % identity with Elovl2 proteins from other teleosts including zebrafish *D. rerio*, African catfish *C. gariepinus*, Atlantic salmon *S. salar*, rainbow trout *O. mykiss* and cherry salmon *O. masou*, and relative high identity to mammalian ELOVL2 proteins. The deduced Elovl2 polypeptide had 59.60 % sequence identity when compared with the Elovl5 from

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

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

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

3.2. Functional characterization of the A. japonica Elovl2

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

6, respectively (Table 2; Fig. 3). In contrast, the *A. japonica* Elovl2 showed relatively high elongase capacity towards C_{20} and C_{22} PUFA, which in all cases led to the production of C_{24} PUFA elongation products (Table 2; Fig. 3).

3.3. Tissue expression of Japanese eel elovl2 mRNA

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

4. Discussion

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

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

However, while the above may suggest there is evolutionary pressure in eels to retain the capacity for endogenous production of LC-PUFA, it is become increasingly accepted that the major influence of LC-PUFA biosynthesis pathways in teleost species is phylogenetic position (Monroig et al., 2018). *Elovl2* was reported to be lost in the Neoteleostei and has only been described in a few teleost species (Monroig et al., 2016; Ferraz et al., 2019). For some time, *elovl2* was considered as one of the genes that disappeared in the evolution of marine fish possibly as a consequence of the high content of LC-PUFA in marine environments and the resultant lack of evolutionary pressure to retain biosynthetic activities (Monroig et al., 2016; Castro et al., 2016). A recent study on the European sardine, *Sardina pilchardus*, has challenged this paradigm. Thus, *S. pilchardus*, a marine fish species, has been demonstrated to have an *elovl2* gene (Machado et al., 2018), confirming that, rather than habitat (marine vs freshwater), the phylogenetic position of *S. pilchardus* within the teleosts' tree of life, accounts for the presence of a gene that was believed to be absent in marine teleost genomes (Castro et al., 2016). Similarly, eels are part of the Elopomorpha with an evolutionary location near the base of Teleostei and thus regarded as a basal teleost. In general, basal teleosts have more conserved LC-PUFA biosynthesis pathways and have retained *elovl2* genes and, in the case of Elopomorpha, also *fads1* (Δ5 desaturase) (Castro et al., 2016; Monroig et al., 2016, 2018; Lopes-Marques et al., 2018).

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

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

In conclusion, the present study reports on the molecular cloning of a cDNA encoding an Elovl2 from Japanese eel, representing the first report of an Elovl2 in a teleost fish with a catadromous lifestyle. The present study has indicated that Japanese eel has a complete repertoire of fatty acyl desaturase and elongase enzymes enzymes required for the biosynthesis of LC-PUFA from C_{18} PUFA substrates and, specifically, that it has capability for the biosynthesis of DHA from EPA via the "Sprecher" pathway, this biosynthesis pattern maybe more similar to freshwater fish species. The highest expression of *elovl2* in adult eel was detected in brain and eyes, which was different from the pattern in freshwater and anadromous species. The results were confusing, it might be hypothesized that the LC-PUFA biosynthetic system of catadromous eels would show neither "marine pattern" nor "freshwater pattern". This expression pattern may indicate the importance of endogenous production of DHA from EPA in neural tissues in eel. These data have increased our knowledge of the diversity of LC-PUFA biosynthesis in vertebrates, and provided further insight into the regulatory mechanisms of LC-PUFA biosynthesis in teleost fish. The results provide a base for further studies aimed at the optimization and/or enhancement of endogenous production of EPA and DHA in farmed *A. japonica*, and the efficient use of sustainable plant-based oil alternatives.

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546

Legends to Figures

Fig. 1. Comparison of deduced amino acid (aa) sequences from the newly cloned Japanese eel (*Anguilla japonica,* Aj.) *elovl2*, with those from Cherry salmon (*Oncorhynchus masou*, Oma; AGR34076.1), rainbow trout (*Oncorhynchus mykiss*, Omy; AIT56593.1), Atlantic salmon (*Salmo salar*, Ss.; NP_001130025.1), African catfish (*Clarias gariepinus*, Cg.; AOY10780.1) and zebrafish (*Danio rerio*, Dr. ; AAI29269.1). Identical aa residues are shaded black and similar residues are shaded grey (alignment by ClustalX2 and colored by Genedoc). The four conserved motifs are labeled with red square frames. The conserved histidine box HXXHH is marked with "*", seven putative transmembrane domains are solid-underlined and labeled with I - VII.

Fig. 2. Phylogenetic tree comparing the deduced amino acid sequence of *Anguilla japonica* elongase with representative of PUFA elongases (Elovl2, Elovl4 and Elovl5) from other vertebrates. The tree was constructed using the neighbor-joining method in MEGA7.0. Accession numbers of sequence was labeled in bracket. Bold font and asterisk marked sequence is the cloned *elovl2* in Japanese eel.

Fig. 3. Functional characterization of the putative Elovl2 from Japanese eel in transgenic yeast *Saccharomyces cerevisiae*. Recombinant yeast transformed with pYES2-*elovl2* were grown in the presence of elongase fatty acid (FA) substrates, n-6 (A, C, E) and n-3 (B, D, F) PUFA substrates. The peaks marked as 1–4 in all panels are the main yeast endogenous FA, namely 16:0, 16:1, 18:0 and 18:1, respectively. Additionally, peaks derived from exogenously added substrates (" * ") or elongation products are indicated accordingly in panels A-E. Vertical axis, FID response; and horizontal axis, retention time.

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

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

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	CCATGTTCAACATCTGGTGGTGCGTGCT
	AjE2F2	TCCAAGCTCATTGAGTTCCTGGACACGA
	AjE2R1	GGAGGCGTGGTGGTAAACGTGCAAGAACG
	AjE2R2	TCGTGTCCAGGAACTCAATGAGCTTGGAGA
ORF cloning	AjE2S2	CCCAAGCTTTAATATGGACCAACTAGAGGCCTTTGACC
	AjE2A2	TGCTCTAGAACCCAAAACTACTGACTTTTTTGTTTGGA
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.
585
586
587
588
589

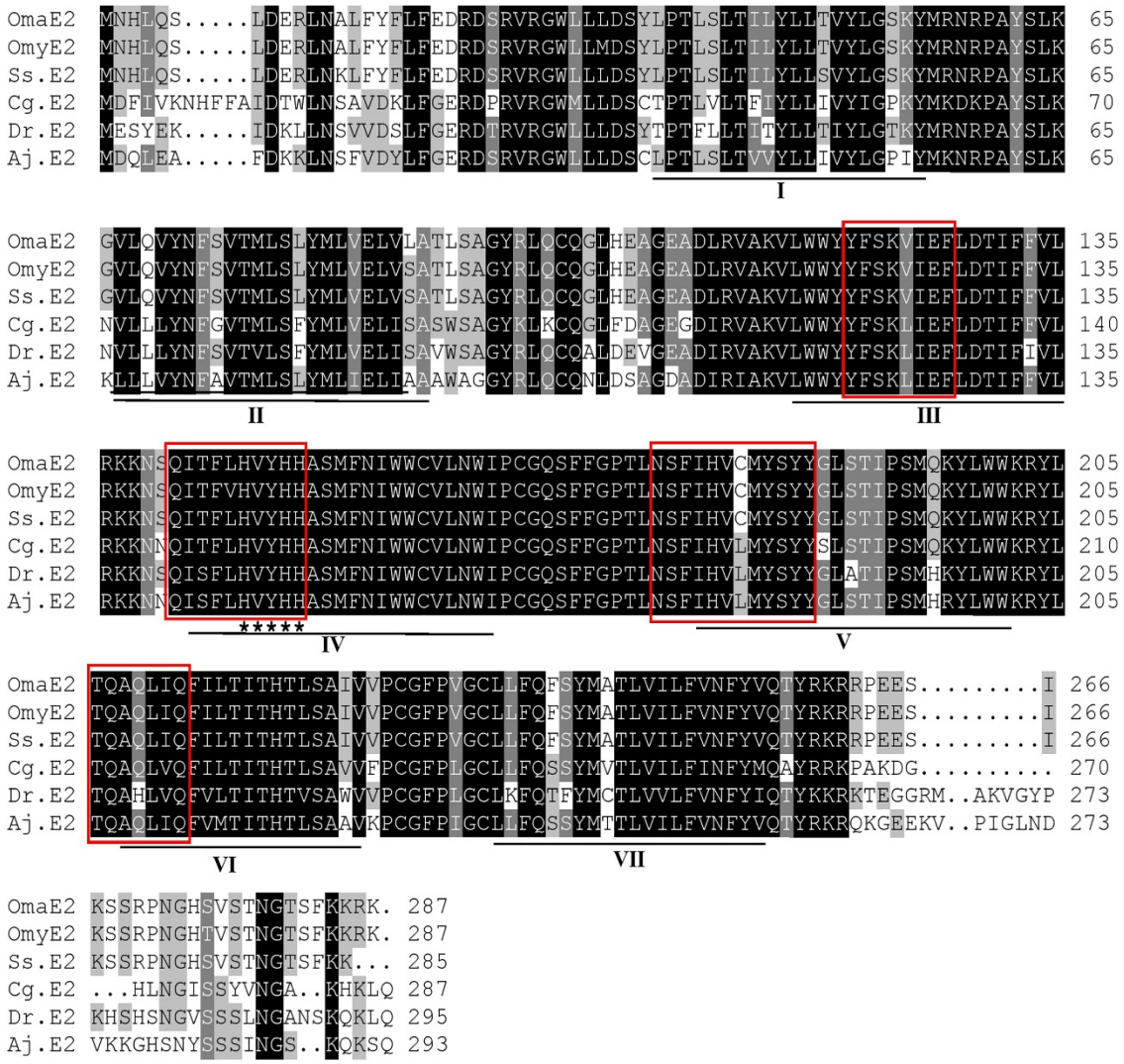
590 **Table 2.** Functional characterization of the Japanese eel Elovl2 in yeast *Saccharomyces cerevisiae.*
591 Individual conversions were calculated according to the formula [individual product area/ (all
592 products areas + substrate area)] × 100.
593

FA substrate	Product	Conversion (%)	Activity
18:3n-3	20:3n-3	ND	$C_{18}\rightarrow C_{20}$
18:2n-6	20:2n-6	ND	$C_{18}\rightarrow C_{20}$
18:4n-3	20:4n-3	6	$C_{18}\rightarrow C_{20}$
18:3n-6	20:3n-6	3	$C_{18}\rightarrow C_{20}$
20:5n-3	22:5n-3	73	$C_{20}\rightarrow C_{22}$
	24:5n-3	60	$C_{22}\rightarrow C_{24}$
20:4n-6	22:4n-6	47	$C_{20}\rightarrow C_{22}$
	24:4n-6	44	$C_{22}\rightarrow C_{24}$
22:5n-3	24:5n-3	56	$C_{22}\rightarrow C_{24}$
22:4n-6	24:4n-6	32	$C_{22}\rightarrow C_{24}$

594 ND, not detected
595

596 **Fig. 1**

597



598

599

600

601 **Fig.2.**

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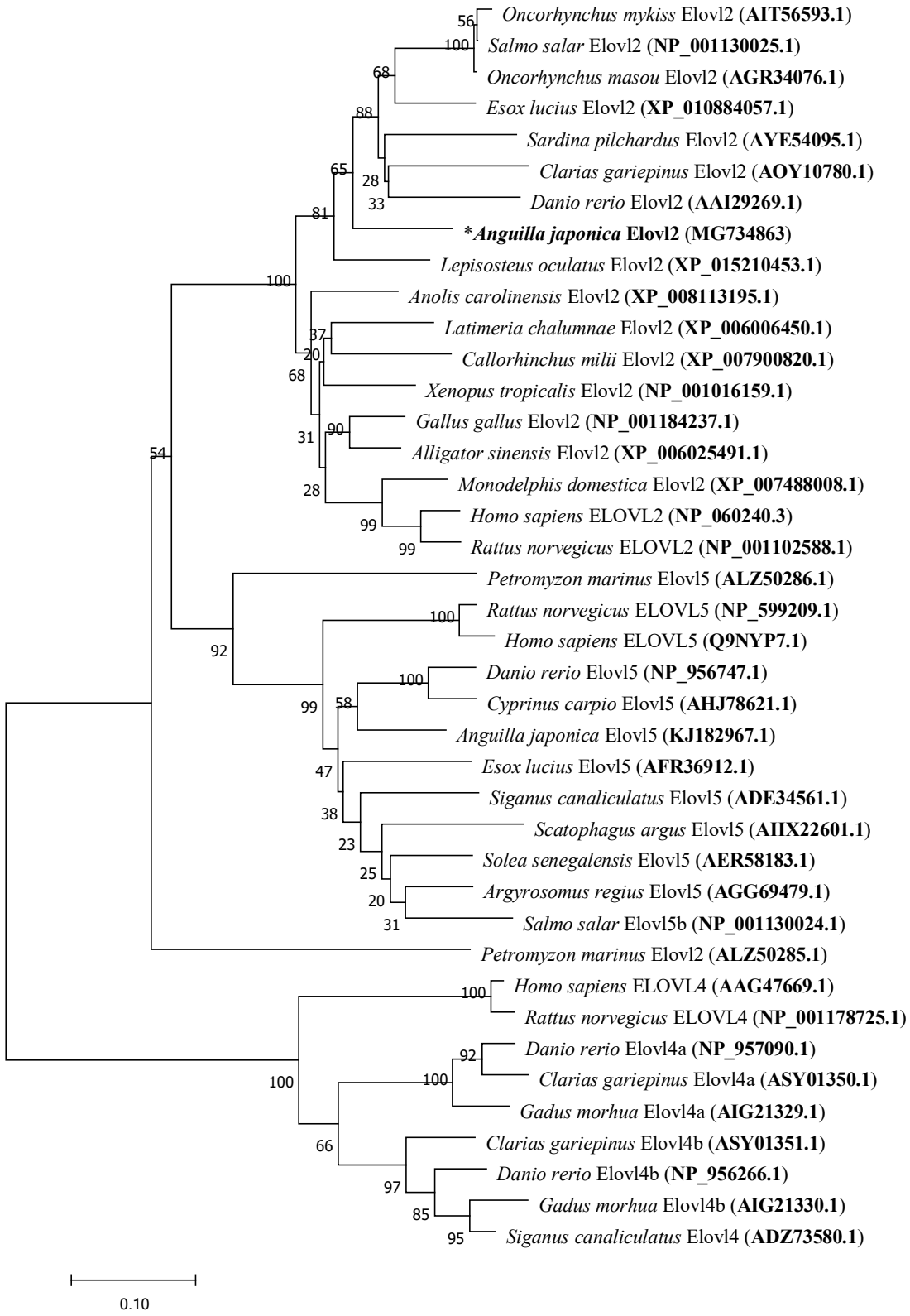
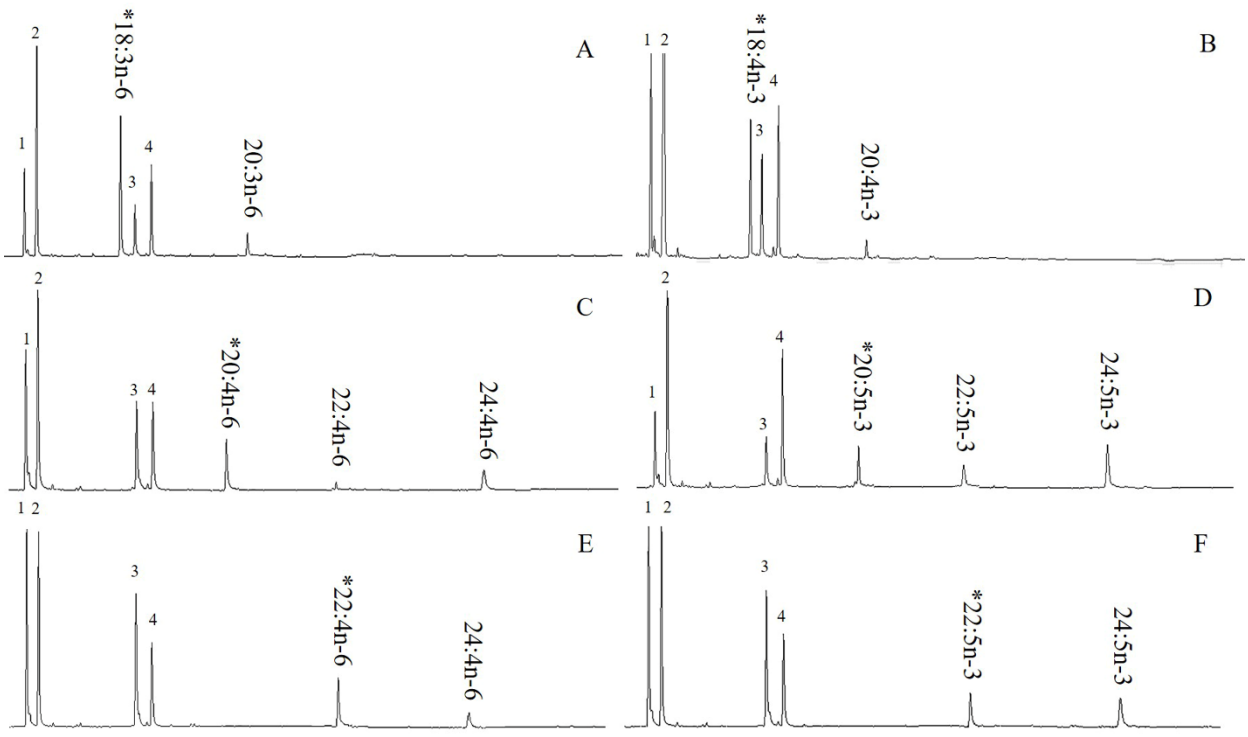

Oncorhynchus mykiss Elovl2 (**AIT56593.1**)
Salmo salar Elovl2 (**NP_001130025.1**)
Oncorhynchus masou Elovl2 (**AGR34076.1**)
Esox lucius Elovl2 (**XP_010884057.1**)
Sardina pilchardus Elovl2 (**AYE54095.1**)
Clarias gariepinus Elovl2 (**AOY10780.1**)
Danio rerio Elovl2 (**AAI29269.1**)
***Anguilla japonica* Elovl2 (MG734863)**
Lepisosteus oculatus Elovl2 (**XP_015210453.1**)
Anolis carolinensis Elovl2 (**XP_008113195.1**)
Latimeria chalumnae Elovl2 (**XP_006006450.1**)
Callorhinchus milii Elovl2 (**XP_007900820.1**)
Xenopus tropicalis Elovl2 (**NP_001016159.1**)
Gallus gallus Elovl2 (**NP_001184237.1**)
Alligator sinensis Elovl2 (**XP_006025491.1**)
Monodelphis domestica Elovl2 (**XP_007488008.1**)
Homo sapiens ELOVL2 (**NP_060240.3**)
Rattus norvegicus ELOVL2 (**NP_001102588.1**)
Petromyzon marinus Elovl5 (**ALZ50286.1**)
Rattus norvegicus ELOVL5 (**NP_599209.1**)
Homo sapiens ELOVL5 (**Q9NYP7.1**)
Danio rerio Elovl5 (**NP_956747.1**)
Cyprinus carpio Elovl5 (**AHJ78621.1**)
Anguilla japonica Elovl5 (**KJ182967.1**)
Esox lucius Elovl5 (**AFR36912.1**)
Siganus canaliculatus Elovl5 (**ADE34561.1**)
Scatophagus argus Elovl5 (**AHX22601.1**)
Solea senegalensis Elovl5 (**AER58183.1**)
Argyrosomus regius Elovl5 (**AGG69479.1**)
Salmo salar Elovl5b (**NP_001130024.1**)
Petromyzon marinus Elovl2 (**ALZ50285.1**)
Homo sapiens ELOVL4 (**AAG47669.1**)
Rattus norvegicus ELOVL4 (**NP_001178725.1**)
Danio rerio Elovl4a (**NP_957090.1**)
Clarias gariepinus Elovl4a (**ASY01350.1**)
Gadus morhua Elovl4a (**AIG21329.1**)
Clarias gariepinus Elovl4b (**ASY01351.1**)
Danio rerio Elovl4b (**NP_956266.1**)
Gadus morhua Elovl4b (**AIG21330.1**)
Siganus canaliculatus Elovl4 (**ADZ73580.1**)
0.10

603 **Fig. 3.**

604



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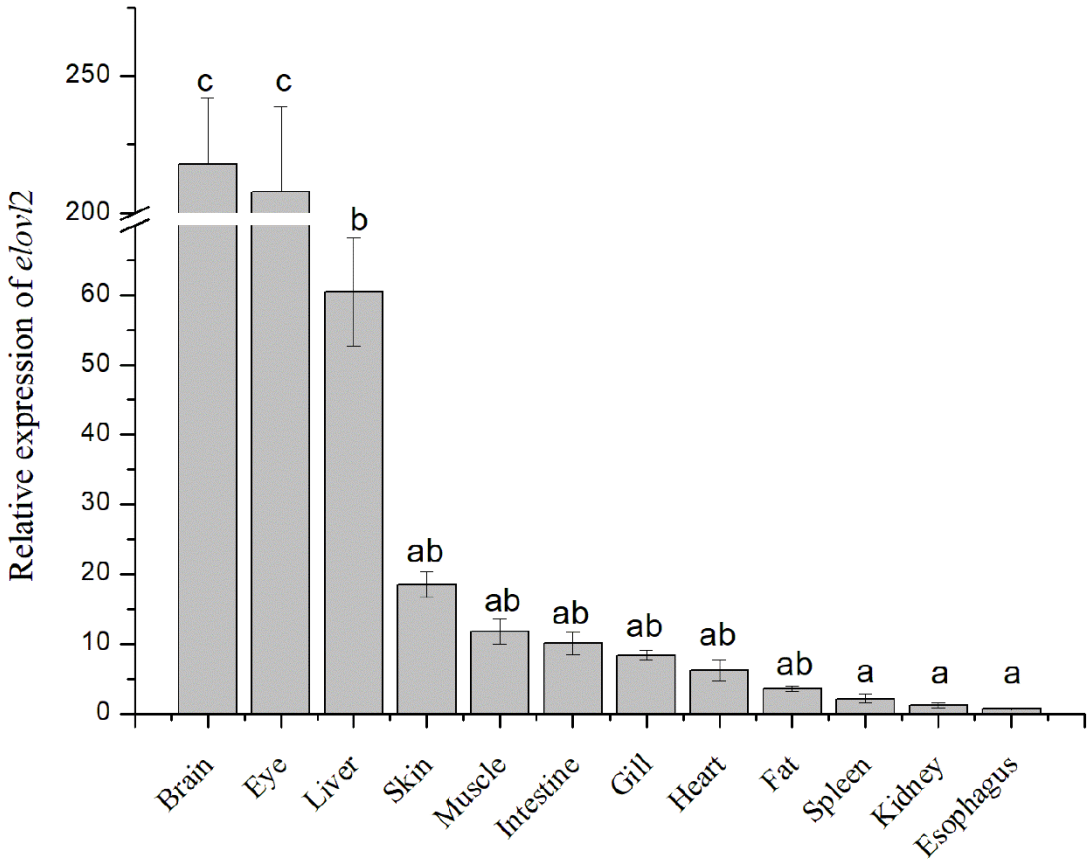
609 **Fig. 4.**


Relative expression of elovl2
250
200
60
50
40
30
20
10
0
c
c
b
ab
ab
ab
ab
ab
ab
a
a
a
Brain
Eye
Liver
Skin
Muscle
Intestine
Gill
Heart
Fat
Spleen
Kidney
Esophagus

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