Title

Investigating long-chain polyunsaturated fatty acid biosynthesis in teleost fish: Functional characterization of fatty acyl desaturase (Fads2) and Elovl5 elongase in the catadromous species, Japanese eel *Anguilla japonica*

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Abstract

The capacity for endogenous production of LC-PUFA from PUFA in euryhaline or diadromous fish is largely unknown other than for Atlantic salmon (*Salmo salar*), an anadromous species, which displays a freshwater pattern. The aim of the present study was to characterize the enzymes of the LC-PUFA pathway in Japanese eel (*Anguilla japonica*), the most important catadromous species currently being farmed. cDNAs of two key genes were cloned and functional assays showed they encoded a desaturase (Fads2) with Δ6 and Δ8 activity and an elongase (Elovl5) with activity towards C_{18} and C_{20} PUFA, with activities similar to marine fish and an Δ6/Δ8 activity ratio similar to Atlantic salmon. Furthermore, tissue distribution of the mRNA showed a clear marine pattern with highest expression in brain and eye. Phylogenetic analysis placed the eel cDNAs in line with classical taxonomy. The data suggest that diadromous species display a pattern of LC-PUFA biosynthesis capacity that likely reflects the environmental and nutritional influence of their early life stages rather than those of adult fish. Future studies aim to establish the full range of PUFA desaturases and elongases in Japanese eel and to provide further insight to the importance and relevance of LC-PUFA biosynthesis in fish species and the influence of diadromy.

Keywords

Biosynthesis; Catadromy; Elovl5; Fads2; Japanese eel; Long-chain polyunsaturated fatty acids

1. Introduction

Vertebrates, including fish, cannot synthesize polyunsaturated fatty acids (PUFA) *de novo* and so they are essential dietary nutrients (Tocher, 2010). The progressive decline in global fisheries, and increasing importance of farmed fish as the primary dietary source for humans of the beneficial n-3 long-chain (LC) PUFA (Tur et al., 2012), has prompted considerable interest in the pathways of endogenous synthesis of LC-PUFA in fish (Tocher, 2003; Turchini et al., 2010).

Dietary PUFA such as linoleic acid (LOA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3) can be converted to LC-PUFA in vertebrates, including fish, via a series of desaturation and elongation reactions. The conventionally accepted pathway for the synthesis of arachidonic acid (ARA; 20:4n-6) from LOA and eicosapentaenoic acid (EPA; 20:5n-3) from ALA requires Δ6 desaturation to 18:3n-6/18:4n-3 catalyzed by Fads2 fatty acyl desaturase, elongation to 20:3n-6/20:4n-3 by Elovl5 fatty acyl elongase, and a further Δ5 desaturation catalysed by Fads1 desaturase (Cook and McMaster, 2004). However, an alternative pathway involving initial elongation of LOA or ALA followed by Δ8 desaturation (an inherent ability of some Fads2 desaturases) may also occur (Monroig et al., 2011a). Docosahexaenoic acid (DHA; 22:6n-3) synthesis from EPA can also follow alternative pathways. For many years, the "Sprecher shunt", involving two sequential elongation steps, Δ6 desaturation and limited peroxisomal chain shortening was regarded as the vertebrate pathway (Sprecher, 2000). However, fatty acyl desaturases with Δ4 activity have now been isolated in some teleost fish indicating that the direct route, via elongation to 22:5n-3 followed by Δ4 desaturation, is also possible (Li et al., 2010; Morais et al., 2012).

The extent to which any species can convert C_{18} PUFA to LC-PUFA varies, associated with their complement of fatty acyl desaturase and elongase genes (Agaba et

al., 2004, 2005; Gregory et al., 2010; Hastings et al., 2001, 2005; Mohd-Yusof et al., 2010; Monroig et al., 2009, 2010a,b, 2011a,b, 2012; Morais et al., 2011; Tocher et al., 2006; Zheng et al., 2004, 2005, 2009). It has been generally accepted that freshwater fish species have a greater ability for conversion of C_{18} PUFA to LC-PUFA than marine species (Tocher, 2010), with the limited capacity of marine fish attributed to deficiencies in one or more key enzymes of the endogenous LC-PUFA biosynthesis pathway (Tocher, 2003, 2010). However, this generalization is complicated by the fact that many fish are actually euryhaline or diadromous. Therefore, the euryhaline marine teleost, rabbitfish *Siganus canaliculatus*, can convert C_{18} PUFA to LC-PUFA, and this activity was higher at 10 ppt salinity than that at 32 ppt salinity (Li et al., 2008). Thus, *S. canaliculatus* was the first marine teleost in which genes encoding desaturase and elongase enzymes with all the activities required for the production of DHA from C18 PUFA, had been characterized (Li et al., 2010; Monroig et al., 2012). In contrast, Atlantic salmon (*Salmo salar*), an anadromous species, living in the sea as an adult but returning to freshwater to spawn, displays a freshwater pattern (Tocher, 2003).

Whereas LC-PUFA biosynthesis and anadromy has been extensively studied in Atlantic salmon (Carmona-Antoñanzas et al., 2011; Hastings et al., 2005; Monroig et al., 2010a, 2013; Morais et al., 2009; Zheng et al., 2004, 2005), catadromous species such as anguillid eels have not been studied. The Japanese eel (*Anguilla japonica*) is one such species, spawning in the western North Pacific around the Mariana Ridge with the larvae (leptocephali) carried by the prevailing currents to East Asia where they feed and grow firstly as glass eels and then yellow eels in rivers, lakes and estuaries of Japan, Korea, China, Vietnam and the Philippines (Aida et al., 2003). After several years in freshwater, the eels mature to become silver eels that migrate to the ocean and their spawning grounds (Aida et al., 2003). The Japanese eel is a traditional food fish in East

100 Asia but wild catches are declining and it is now an important farmed species
101 accounting for the major portion of global freshwater eel production of around 260,000
102 tonnes annually (FAO, 2010).

103 Understanding the molecular basis of LC-PUFA biosynthesis and regulation in fish
104 will allow the pathway to be optimized to enable efficient and effective use of
105 sustainable plant-based alternatives in aquaculture while maintaining the n-3 LC-PUFA
106 content of farmed fish for the human consumer. The specific objectives of the present
107 study were to characterize the genes of LC-PUFA biosynthesis in the catadromous
108 species, Japanese eel, as a key step to understand the mechanisms underpinning
109 variation in the pathway among teleost fish species. In the present paper we describe the
110 cDNA cloning, functional characterization and tissue distributions of a Fads2 fatty acyl
111 desaturase and Elovl5 PUFA elongase that provide further insight of LC-PUFA
112 biosynthesis in teleost fish species.

113

114 **2. Materials and Methods**

115 *2.1 Eel samples*

116 Tissue samples from Japanese eel, *A. japonica*, were obtained from ten adult individuals
117 (body weight 380- 400 g) maintained at the facilities of Shantou Manlian Co. LTD,
118 China. The eels were sacrificed after being anaesthetized with an overdose of 3-amino-
119 benzoate methane sulphonate (MS-222) (Sigma, China), and tissues including brain,
120 eye, fat (adipose), gill, heart, intestine, kidney, muscle, esophagus and spleen were
121 sampled and immediately frozen in liquid nitrogen, then stored at -70 ºC until further
122 use.

123

124 *2.2 Cloning of putative fads2 and elovl5 from A. japonica*

125 Total RNA was extracted from eel tissues using TRIzol® Reagent (Invitrogen, USA)
126 and first strand cDNA was synthesized using random primers (FastQuant RT Kit,
127 Tiangen Biotech. Co. LTD, China). The open reading frame (ORF) fragments of the
128 desaturase and elongase cDNAs were isolated by PCR using the primers
129 AJDS1/AJDA1 (desaturase) and AJE5S1/AJE5A1 (elongase), designed on the basis of
130 published sequences of *fads2*-like and *elovl5*-like mRNAs of Japanese eel (GenBank
131 accession EU719615 and EU719614, respectively). PCR was performed using Pfu PCR
132 MasterMix (Tiangen) under the following thermal conditions: initial denaturation at 94
133 ºC for 5 min, 34 cycles of denaturation at 94 ºC for 30 s, annealing at 58 ºC for 30 s and
134 extension at 72 ºC for 2 min. The PCR products were cloned into pMD^{TM} 18-T vector
135 (TaKaRa Biotech. Co. LTD, China). The PCR fragments were sequenced at the DNA
136 Sequencing Service of the Sangon Biotech Co. LTD (China). The sequences of all PCR
137 primers used in this study are shown in Table 1.
138
139 *2.3 Phylogenetic analyses of A. japonica desaturase and elongase*
140 Phylogenetic analysis of the amino acid (aa) sequences deduced from the putative
141 desaturase and elongase cDNAs from Japanese eel and homologous genes from other
142 organisms was performed by constructing a tree using the neighbor-joining method
143 (Saitou and Nei, 1987), with confidence in the resulting tree branch topology measured
144 by bootstrapping through 10,000 iterations. All reference sequences utilized in the
145 phylogenetic analysis are shown in Table 2.
146
147 *2.4 Functional characterization of A. japonica Fads2 and Elovl5 by heterologous*
148 *expression in yeast Saccharomyces cerevisiae*

149 A cDNA synthesized with brain, liver and intestine total RNA samples was used as
150 template to amplify the ORFs of *fads2* and *elovl5*, using the Pfu PCR MasterMix
151 (Tiangen). Primers AJDS2/AJDA2 (*fads2*) and AJE5S2/AJE5A2 (*elovl5*) containing
152 restriction enzyme sites (underlined in Table 1) for *Hin*dIII (forward) and *Xba*I (reverse)
153 were used in a PCR consisting of an initial denaturing step at 94 ºC for 5 min, followed
154 by 34 cycles of denaturation at 94 ºC for 30 s, annealing at 60 ºC for 30 s, extension at
155 72 ºC for 2 min, followed by a final extension at 72 ºC for 5 min. After digestion with
156 the corresponding restriction enzymes, DNA fragments corresponding to the ORFs of
157 *A. japonica fads2* and *elovl5* were ligated into the yeast expression vector pYES2
158 (Invitrogen, UK). The recombinant plasmids pYES2-fads2 and pYES2-elovl5 were
159 obtained and used to transform yeast *S. cerevisiae* (strain InvSc1) competent cells (S.c.
160 EasyComp Transformation Kit, Invitrogen). Yeast culture and selection were according
161 to Monroig et al. (2012). Transgenic yeast expressing either the desaturase or elongase
162 were grown in the presence of potential PUFA substrates. For pYES2-fads2
163 transformed yeast, potential substrates for Δ6 (18:3n-3 and 18:2n-6), Δ8 (20:3n-3 and
164 20:2n-6), Δ5 (20:4n-3 and 20:3n-6) and Δ4 (22:5n-3 and 22:4n-6) desaturation were
165 assayed. For pYES2-elovl5 transformed yeast, PUFA substrates including 18:3n-3,
166 18:2n-6, 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3, 22:4n-6 were tested. PUFA
167 substrates were added at final concentrations of 0.5 (C_{18}), 0.75 (C_{20}) and 1.0 (C_{22}) mM
168 to compensate for decreased uptake with increased chain length (Zheng et al., 2009). A
169 control treatment consisting of yeast transformed with empty pYES2 was run under the
170 same conditions. After 2 days incubation at 30 ^{o}C, yeast cultures were harvested,
171 washed with Hank's balanced salt solution containing 1 % fatty acid-free albumin, and
172 lipid extracted by homogenization in chloroform/methanol (2:1, v/v) containing 0.01%
173 butylated hydroxytoluene (BHT) (Sigma, USA) as antioxidant (Folch et al., 1957).

174

175 *2.5 Fatty acid analysis by GC-MS*

176 Fatty acid methyl esters (FAME) from yeast total lipids were prepared, extracted and
177 purified according to methodology described by Christie (2003). Identities of fatty acids
178 (FA) were based on GC retention times and confirmed by GC-MS as described
179 previously (Hastings et al. 2001; Agaba et al. 2004). Conversion rates from PUFA
180 substrates were calculated as the proportion of exogenously added substrate FA
181 converted to desaturated or elongated FA products, [individual product area/(all
182 products areas + substrate area)] x 100.

183

184 *2.6 Tissue distribution of the A. japonica fads2 and elovl5 mRNA*

185 Tissue distributions of eel *fads2* and *elovl5* mRNA were determined by quantitative
186 real-time PCR (qPCR). Tissues investigated included brain, eye, fat (adipose), gill,
187 heart, intestine, kidney, muscle, esophagus and spleen from six individuals. Total RNA
188 was extracted using TRIzol® Reagent (Invitrogen) according to the manufacturer's
189 protocol, and 1 μg of total RNA was reverse transcribed into cDNA using random
190 hexamers (Tiangen). The qPCR analyses were performed using primers shown in Table
191 1. The relative expression of target genes were normalized with *18S* rRNA expression
192 calculated by the $2^{-\Delta\Delta Ct}$ method (Livak, Schmittgen, 2001). The qPCR amplifications
193 were carried out on a Lightcycler 480 system (Roche, Switzerland) in a final volume of
194 20 μl containing 2 μl diluted cDNA (10 ng μL^{-1}), 0.5 μM of each primer and 10 μl
195 SYBR Green I Master (Roche). Amplifications were carried out with a systematic
196 negative control (NTC: no template control, containing no cDNA). The qPCR profiles
197 contained an initial activation step at 95 °C for 5 min, followed by 40 cycles: 10 s at 95
198 °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 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

Tissue distribution results were expressed as mean normalized values (± SE) corresponding to the ratio of the copy numbers of the *fads2* and *elovl5* transcripts and the copy numbers of the reference gene, *18S* rRNA. Differences in the expression of each target cDNA (*fads2* and *elovl5*) among tissues were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test at a significance level of $P \leqslant 0.05$ (OriginPro 8.0, OriginLab Corporation, USA).

3. Results

3.1 A. japonica fads2 and elovl5 sequences and phylogenetics

In the present study, the ORFs of Japanese eel putative desaturase and elongase were cloned for functional characterization. The nucleotide sequence of the putative *fads2* ORF was 1335 bp in length encoding a putative protein of 444 aa, and that of the putative *elovl5* ORF was 885 bp in length encoding a protein of 294 aa. The eel *fads2* and *elovl5* polypeptides deduced from the ORFs obtained in the present study showed 7 and 1 aa differences, respectively, compared with previously published, non-functionally characterized, sequences ACI32415.1 and ACI32414.1 (GenBank accession numbers) (Yu et al., 2009). The newly cloned eel *fads2* and *elovl5* ORF cDNAs from the present study were deposited in GenBank with accession numbers KJ182968 and KJ182967, respectively.

223 The deduced eel Fads2 polypeptide had 68 to 98 % sequence identity with other
224 Fads2 desaturases from fish including zebrafish *Danio rerio,* common carp *Cyprinus*
225 *carpio,* rainbow trout *Oncorhynchus mykiss*, Atlantic salmon *S. salar,* masu salmon
226 *Oncorhynchus masou,* daggertooth pike conger *Muraenesox cinereus,* Atlantic cod
227 *Gadus morhua*, gilthead seabream *Sparus aurata,* European sea bass *Dicentrarchus*
228 *labrax,* cobia *Rachycentron canadum,* rabbitfish *S. canaliculatus,* nibe croaker *Nibea*
229 *mitsukurii,* Senegalese sole *Solea senegalensis*, and Southern bluefin tuna *Thunnus*
230 *maccoyii*. Lower identity scores (63 - 69 %) were obtained when the *A. japonica* Fads2
231 was compared to other Fads2-like sequences from mammals (*Homo sapiens* and *Mus*
232 *musculus*), bird (*Gallus gallus*) and amphibians (*Xenopus laevis* and *X. tropicalis*). The
233 *A. japonica* putative Fads2 showed typical Fads2 structural characteristics including
234 three histidine boxes HXXXH, HXXHH and QXXHH, common among 'front-end'
235 desaturases, a putative cytochrome b_5-like domain, and the heme-binding motif, HPGG
236 (Hashimoto et al., 2008).

237 The eel Elovl5 polypeptide had 74-81 % aa sequence identity to Elovl5 elongases of
238 other teleost fish including *D. rerio, C. carpio, S. salar, G. morhua, S. aurata, D. labrax,*
239 *R. canadum, S. canaliculatus, N. mitsukurii* and *S. senegalensis*, and 73 % and 74 %
240 identity to amphibian *X. laevis* and bird *G. gallus*, respectively. The *A. japonica* Elovl5
241 polypeptide also had the characteristic structures of the Elovl5 family including the
242 diagnostic histidine box (HXXHH), and lysine (K) and arginine (R) residues at the
243 carboxyl terminus (KKXRX), regarded as a putative endoplasmic reticulum retrieval
244 signal (Jakobsson et al., 2006).

245 The phylogenetic analysis showed all fatty acid desaturases from teleost fish as well
246 as Fads2-like proteins from other vertebrates clustered together and separately from
247 Fads1 of cartilaginous fish, amphibian, reptiles and mammals (Fig. 1). The teleost fish

248 Fads2 formed a separate group from other vertebrate Fads2 orthologues, and which was
249 subdivided into sub-clusters consisting of species largely from the same order.
250 Particularly interesting for the present study, the *A. japonica* desaturase grouped
251 together with three desaturases described for the eel-like daggertooth pike conger (*M.*
252 *cinereus*), another representative of the Anguilliforme order. The tree showing the
253 phylogenetic analysis of the newly cloned *A. japonica* elongase indicated that this gene
254 encoded a putative Elovl5 (Fig. 2). Thus, the eel elongase and those of Elovl5-like
255 proteins from fish and terrestrial vertebrates grouped together, separately from Elovl2
256 and Elovl4, other PUFA elongases with roles in the biosynthesis of LC-PUFA in
257 vertebrates including fish (Monroig et al., 2009, 2010b).

258 *3.2 Functional characterization*

259 The cloned desaturase and elongase were functionally characterized by determining the
260 FA profiles of *S. cerevisiae* transformed with pYES2 vectors containing the ORFs of *A.*
261 *japonica* desaturase (pYES2-fads2) or elongase (pYES2-elovl5) as inserts, and grown
262 in the presence of potential FA substrates. The FA composition of the control yeast
263 (transformed with empty pYES2) was characterized by having 16:0, 16:1 (16:1n-9 and
264 16:1n-7), 18:0 and 18:1n-9 as major components, as well as a single additional FA peak
265 corresponding to the exogenously added PUFA substrate (data not shown). This was
266 consistent with yeast not possessing desaturase activities towards PUFA substrates
267 (Hastings et al., 2001; Agaba et al., 2004). The FA profile of yeast transformed with
268 pYES2-fads2 showed, additionally, extra peaks when grown in the presence of 18:3n-3,
269 18:2n-6, 20:3n-3 and 20:2n-6, which corresponded to 18:4n-3, 18:3n-6, 20:4n-3 and
270 20:3n-6, respectively (Fig. 3). These data show clearly that the cloned eel Fads2 had
271 dual Δ6/Δ8 specificities, whereas Δ5 and Δ4 activities were not detected (Fig 3; Table
272 3). Functional characterization of the eel elongase confirmed the cloned cDNA encoded

a protein with Elovl5 activity. Thus, high conversion rates were obtained for C_{18} (18:4n-3 and 18:3n-6) and C_{20} (20:5n-3 and 20:4n-6) substrates, whereas no elongation activity for C_{22} substrates (22:5n-3 and 22:4n-6) was detected. In addition, the eel Elovl5 also showed relatively weak activity for the conversion of 18:3n-3 and 18:2n-6 to 20:3n-3 and 20:2n-6, respectively, providing the FA substrates of Δ8 desaturation (Fig. 4; Table 4).

3.3 Tissue expression of eel fads2 desaturase and elovl5 elongase

The tissue distributions of the Japanese eel *fads2* and *elovl5* transcripts were determined by qPCR. The highest expression of the eel *fads2* was detected in brain, followed by eye and liver (Fig. 5). The eel *elovl5* transcript was primarily expressed in brain, with liver and intestine showing the next highest expression signals (Fig. 5).

4. Discussion

The present study aimed to gain insight to the relationship between diadromy and LC-PUFA biosynthesis pathways in fish and, to this end, we investigated the molecular basis of LC-PUFA biosynthesis in the catadromous species, Japanese eel. Previously, data from the anadromous Atlantic salmon indicated that this species showed a "freshwater pattern", being able to biosynthesize LC-PUFA, EPA, ARA and DHA, from C18 PUFA precursors (Tocher et al., 2003). This was reflected at the molecular level by the presence of four distinct Fads2 desaturases with Δ6 and Δ5 activities, and Elovl5 and Elovl2 elongases in Atlantic salmon (Hastings et al., 2005; Zheng et al., 2005; Monroig et al., 2010a; Morais et al., 2009). This was consistent with the essential fatty acid (EFA) requirements of salmon that showed 18:3n-3 and 18:2n-6 were able to satisfy nutritional requirements and prevent deficiency signs (Ruyter et al., 2000). The

298 hypothesis forwarded to explain the freshwater pattern displayed by Atlantic salmon
299 was that it reflected its developmental origin with reproduction and first phase of life
300 taking place in freshwater ecosystems where LC-PUFA, especially DHA, was more
301 limiting (Leaver et al., 2008).

302 Based on the above paradigm, it might be expected that catadromous eels would
303 show a "marine pattern". However, the reported EFA requirements of Japanese eel were
304 satisfied by about 0.5 % of diet each of ALA (18:3n-3) and LOA (18:2n-6), with ALA
305 being slightly superior to LOA when supplied individually and, most relevant to the
306 present study, 1 % EPA/DHA similar to and no better than 1% ALA (Takeuchi et al.,
307 1980). Consistent with EFA requirements, an early study on European eel (*Anguilla*
308 *anguilla*) showed this similar anguillid species had the ability to desaturate and elongate
309 C18 PUFA (Kissil et al., 1987). Thus, feeding elvers for 12 weeks on a diet containing
310 corn oil (rich in LOA), in comparison to a fish oil diet, increased the proportion of ARA
311 in tissue polar lipids from 5 % to 12 % in the eels fed corn oil. This was confirmed by
312 examining the metabolic fate of [1-^{14}C]LOA given orally to eels. Seven days after
313 administration of labelled LOA, 10 % of radioactivity recovered in liver fatty acids was
314 present in trienes and tetraenes, with 4 % recovered in ARA (Kissil et al., 1987). These
315 data may be further supported by the fact that wild *A. japonica* have higher levels of
316 ARA (and LOA and ALA), and lower levels of EPA, DHA and 20:1, than farmed fish
317 (Oku et al., 2009). This may indicate active conversion of dietary LOA to ARA in wild
318 fish and that, although dietary histories were not reported, farmed eels were fed diets
319 that likely contained marine feedstuffs such as fish oil. However, other studies have
320 indicated that ARA may be essential for juvenile *A. japonica* with broken line analysis
321 suggesting a requirement level of 0.7 % of diet (Bae et al., 2010). Overall though, the

nutritional, compositional and biochemical data are consistent with anguillid eels demonstrating a freshwater pattern of EFA requirement and LC-PUFA biosynthesis.

The above provides the contextual environment within which the results of the present study must therefore be interpreted and discussed. Thus, the present study has shown that *A. japonica* possess and express genes encoding a Δ6 Fads2 desaturase and Elovl5 enzymes indicating Japanese eel have activities necessary for the conversion of ALA and LOA to 20:4n-3 and 20:3n-6, respectively. Genes or cDNAs for these activities are widely expressed in teleost fish species studied to date with both found in many species including Atlantic salmon, freshwater species such as zebrafish, rainbow trout and tilapia (*Oreochromis niloticus*), and marine fish including gilthead sea bream, Atlantic cod, turbot (*Psetta maxima*), Asian sea bass (*Lates calcarifer*), cobia, and Northern (*Thunnus thynnus*) and Southern bluefin tuna (Agaba et al., 2004, 2005; Gregory et al., 2010; Hastings et al., 2001, 2005; Mohd-Yusof et al., 2010; Morais et al., 2009, 2011; Zheng et al., 2004, 2005, 2009; Tocher et al., 2006). However, production of EPA and ARA also requires Δ5 desaturation activity (Cook and McMaster, 2004). To date a discrete, unifunctional Δ5 desaturase has only been demonstrated in Atlantic salmon (Hastings et al., 2005), with bifunctional Δ6/Δ5 desaturases described in zebrafish and rabbitfish (*Signaus canaliculatus*) (Hastings et al., 2001; Li et al., 2010). Interestingly, it was confirmed that these activities were all the products of *fads2* genes (Castro et al., 2012).

It was unclear from earlier studies whether eel have the ability to produce DHA endogenously (Kissil et al., 1987; Takeuchi et al., 1980). Depending upon the precise pathway from EPA, biosynthesis of DHA requires elongation of C_{20} and, possibly, C_{22} PUFA (Sprecher, 2000). The eel Elovl5, similar to mammalian homologues (Jakobsson et al., 2006), has the ability to elongate both C_{18} and C_{20} PUFA, but no activity towards

347 C_{22} PUFA. Thus, if the pathway to DHA in eel requires elongation of C_{22}, then an
348 Elovl2 and/or Elovl4 would be required. Elovl2 with the ability to elongate C_{20} and,
349 particularly, C_{22} PUFA, 22:5n-3 and 22:4n-6, has been demonstrated in Atlantic salmon
350 (Morais et al., 2009) and zebrafish (Monroig et al., 2009) but, to date, no *elovl2* cDNA
351 has been isolated from a marine fish species, and this had been hypothesized as
352 potentially contributing to their limited ability for DHA biosynthesis (Leaver et al.,
353 2008; Morais et al., 2009). Although attempts to clone further *fads* and *elovl* cDNAs
354 from *A. japonica* were unsuccessful (data not shown), this does not exclude the
355 possibility that further LC-PUFA biosynthetic genes are present in the genome. The
356 recent publication of the first draft of the *A. japonica* genome indicates that genomic
357 resources to provide further insight to LC-PUFA biosynthesis in Japanese eel will soon
358 be available (Henkel et al., 2012).

359 Although all the LC-PUFA biosynthetic genes or activities have yet to be
360 demonstrated, the nutritional and biochemical data suggest that eels display a freshwater
361 pattern of LC-PUFA biosynthesis. This may not require a complete paradigm shift in
362 our understanding of the evolutionary drivers underpinning LC-PUFA biosynthesis in
363 fish. One pillar in this argument has been that, compared to freshwater ecosystems, LC-
364 PUFA are readily available in marine environments, and this difference in evolutionary
365 pressure could possibly account for the apparent loss of some enzymatic activities of the
366 LC-PUFA biosynthetic pathway in marine fish. However, recent studies on the marine
367 teleosts, rabbitfish and Senegalese sole, showing the presence of Δ4 desaturases (both
368 *fads2* genes), have already suggested that this is too simplistic as other factors such as
369 trophic level and specific feeding habits might also determine the capacity of species for
370 biosynthesis of LC-PUFA (Li et al., 2010; Morais et al., 2012). Anguillid eels may also
371 support the latter as they also have unusual feeding habits in seawater. All eels are part

372 of the superorder elopomorpha that are characterized by having leptocephalus larvae
373 that are long-lived and grow much larger than larvae of other teleosts (Aida et al.,
374 2003). Although energy stores accumulated by the larvae fuel migration,
375 metamorphosis and metabolism of the glass eel stage, the diet of leptocephalus larvae is
376 poorly understood and there are few studies (Deibel et al., 2012). However, they appear
377 to feed on particulate, organic detritus termed marine snow (Aida et al., 2003), and one
378 could speculate that this diet may have relatively low levels of LC-PUFA compared to
379 the zooplankton diets of other marine teleost larvae.

380 A further activity-related characteristic of *A. japonica* Δ6 Fads2 that can be
381 compared to freshwater and marine species is the Δ8 desaturation activity. At around 5-
382 6 % conversion of 20:3n-3, the Δ8 activity of the eel Fads2 was higher than that of Δ6
383 Fads2 of freshwater species zebrafish and tilapia, which varied between 0.6 – 1.5 %,
384 and much lower than that of Δ6 Fads2 of marine species such as turbot, cod, sea bream,
385 cobia and rabbitfish that ranged from 16.6 to 31.8 % (Monroig et al., 2011). The ratio of
386 Δ6:Δ8 activities towards n-3 PUFA substrates was just under 11 in the *A. japonica*,
387 similar to that in Atlantic salmon Δ6 Fads2 desaturases (12-15), and lower than those in
388 freshwater species (22-92) and higher than the ratio in marine species (2-4) (Monroig et
389 al., 2011). Therefore, the *A. japonica* Δ6 Fads2 did not show a characteristic freshwater
390 or marine pattern and was more similar to salmon. Phylogenetic analysis of the *A.*
391 *japonica* gene products also gave no conclusive data, with the eel Δ6 Fads2 clustering
392 with the Fads2-like desaturase of a marine eel, daggertooth pike conger (*M. cinereus*),
393 and closer to salmonids and marine fish desaturases than those of freshwater species. In
394 contrast, the *A. japonica* Elovl5 clustered more closely to Elovl5 from cyprinids. The
395 tissue distribution of the *A. japonica* genes with highest expression of Δ6 Fads2 and
396 Elovl5 in brain was characteristic of marine species with cod, cobia, Asian sea bass and

meagre (*Argyrosomus regius*) all showing highest expression of LC-PUFA synthesis genes in brain (Mohd-Yusof et al., 2010; Monroig et al., 2013; Tocher et al., 2006; Zheng et al., 2009).

In conclusion, the present study has provided data confirming that *A. japonica* have two of the key enzymes (Δ6 Fads2 and Elovl5) of the LC-PUFA biosynthesis pathway, but that other activities are required for biosynthesis of EPA and ARA from C_{18} PUFA (Δ5 desaturation) and possibly also for biosynthesis of DHA from EPA (Elovl2 and/or Elovl4 elongases). Studies are ongoing with the aim of unequivocally establishing the full range of PUFA desaturases and elongases in Japanese eel and in providing further insight to the importance and relevance of LC-PUFA biosynthesis in different fish species.

Acknowledgements

We acknowledge financial support from the Major International Joint Research Project from National Natural Science Foundation of China (NSFC) (31110103913), NSFC Youth Projects (No. 31202011, 31202012), and Foundation for Distinguished Young Talents in Higher Education of Guangdong (LYM09073). Additionally, this research and OM were supported by a Marie Curie Reintegration Grant within the 7th European Community Framework Programme (PERG08-GA-2010-276916, LONGFA).

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564

565 **FIGURES**

566 Fig 1. Phylogenetic tree comparing the deduced amino acid sequence of *A. japonica*
567 desaturase with those from other vertebrates. The tree was constructed using the
568 neighbor-joining method with MEGA4. Accession numbers of the sequences are given
569 in Table 2.

570

571 Fig 2. Phylogenetic tree comparing the deduced amino acid sequence of *A. japonica*
572 elongase with those from other vertebrates. The tree was constructed using the
573 neighbor-joining method with MEGA4. Accession numbers of the sequences are given
574 in Table 2.

575

576 Fig 3. Functional characterization of the putative desaturase from Japanese eel in
577 transgenic yeast (*S. cerevisiae*). Recombinant yeast transformed with pYES2-fads2
578 were grown in the presence of Δ6 fatty acid (FA) substrates (panels A and B), and Δ8
579 substrates (panels C and D). The peaks marked as 1-4 in all panels are the main FA of *S.*
580 *cerevisiae*, namely 16:0, 16:1, 18:0 and 18:1, respectively. The peaks 5 and 7 are
581 substrates of Δ6, namely 18:3n-3 and 18:2n-6, and corresponding products are 18:4n-3
582 (6) and 18:3n-6 (8), respectively. The peaks 9 and 11 are substrates of Δ8, namely
583 20:3n-3and 20:2n-6, and corresponding products are 20:4n-3 (10) and 20:3n-6,
584 respectively.

585

586 Fig 4. Functional characterization of the putative elongase from Japanese eel in
587 transgenic yeast (*S. cerevisiae*). Recombinant yeast transformed with pYES2-elovl5
588 were grown in the presence of elongase fatty acid (FA) substrates. The peaks marked as
589 1-4 in all panels are the main FA of *S. cerevisiae*, namely 16:0, 16:1, 18:0 and 18:1,
590 respectively. The peaks 5 and 7 are putative substrates of Δ8 pathway, namely 18:3n-3
591 and 18:2n-6, and the corresponding products are 20:3n-3 (6) and 20:2n-6 (8). The peaks
592 9, 11, 13 and 15 are substrates of Elovl5, namely 18:4n-3, 18:3n-6, 20:5n-3 and 20:4n-6,
593 and the corresponding products are 20:4n-3 (10), 20:3n-6 (12), 22:5n-3 (14) and 22:4n-6
594 (16), respectively.

595

596 Fig 5. Tissue-specific expression of *fads2* (A) and *elovl5* (B) mRNA in *A. japonica*
597 examined by qPCR. Relative expression of target genes were quantified for each
598 transcript and were normalized with *18S* rRNA by $2^{-\Delta\Delta Ct}$ method. Results are means ±
599 SEM (n = 6), and different letters show significant differences ($P<0.05$) among tissues
600 as determined by one-way ANOVA followed by Tukey's multiple comparison test.

601

602 **TABLES**

603 Table 1. Primers sequences used for ORF cloning of eel *fads2* and *elovl5* and their
604 tissue expression analysis detected by qRT-PCR

Primers for ORF cloning of *fads2*	
AJDS1	5'-CAGGGAGGGAGAATAACGG-3'
AJDA1	5'-CTGAAAATTGTCATAAAGGAAG-3'
AJDS2	5'-CCGAAGCTTGAGCATAAGAGCGATGGG-3'
AJDA2	5'-GGCTCTAGAGGAGGCAGGCTTGAGG-3'
Primers for ORF cloning of *elovl5*	
AJE5S1	5'-TGGCAGTGGTTCCAAGGTT-3'
AJE5A1	5'-GTGTCAAGACAGCGAGGTTTG-3'
AJE5S2	5'-CCGAAGCTTGATGGACATGGAAATGTT-3'
AJE5A2	5'-GGCTCTAGACTCAGTCTACCCTCAGTT-3'
Primers for real-time quantitative PCR	
fads2	
AJDF3	5'-AGACCCAGCCAGTGGAGTATG-3'
AJDA3	5'-CATTGACCAGACGAGGTCCAC-3'
elovl5	
AJE5F3	5'-TGCTGTGGTCTGGCCTTGTG-3'
AJE5A3	5'-AGCCGTTCTGATGCTCTTTCC-3'
18S	
AJ18SF1	5'-TTAGTGAGGTCCTCGGATCG-3'
AJ18SA1	5'-CCTACGGAAACCTTGTTACG-3'

605 Note: The accession numbers of nucleotide sequences used for ORF cloning or qPCR of
606 *fads2* and *elovl5* were KJ182968 and KJ182967, respectively. That of *18S* rRNA was
607 FM946132.

608

609 Table 2. List of genes and the accession numbers for all the sequences used in the
610 phylogenetic analysis.

Species	Desaturase type	GenBank no.	Elongase type	GenBank no.
Siganus canaliculatus	Fads2 (Fad1)	ABR12315.2	Elovl5	ADE34561.1
	Fads2 (Fad2)	ADJ29913.1	Elovl4	ADZ73580.1
Anabas testudineus	Fads2	AFJ97304.1		
Channa striata	Fads2	ACD70298.2		
Solea senegalensis	Fads2	AEQ92868.1	Elovl5	AER58183.1
Oreochromis niloticus	Fads2	BAB62850.1		
Scophthalmus maximus	Fads2	AAS49163.1		
Lates calcarifer	Fads2	ACY25091.2	Elovl5	ACS91459.1
Rachycentron canadum	Fads2	ACJ65149.1	Elovl5	ACJ65150.1
			Elovl4	ADG59898.1
Epinephelus coioides	Fads2	ACJ26848.1		
Siniperca chuatsi	Fads2	ACH53604.1		
Sparus aurata	Fads2	ADD50000.1	Elovl5	ADD50001.1
Dicentrarchus labrax	Fads2	ACD10793.1	Elovl5	CBX53576.1
Nibea mitsukurii	Fads2	ACX54437.1	Elovl5	ACR47973.1
Argyrosomus regius	Fads2	AGG69480.1	Elovl5	AGG69479.1
Larimichthys crocea	Fads2	AFO84710.1		
Thunnus maccoyii	Fads2	ADG62353.1		
Gadus morhua	Fads2	AAY46796.1	Elovl5	ADA70325.1
Salmo salar	Fads2 (Δ6Fad_a)	NP_001165251.1	Elovl5a	NP_001117039.1
	Fads2 (Δ6Fad_b)	NP_001165752.1	Elovl5b	NP_001130024.1
	Fads2 (Δ6Fad_c)	NP_001117047.1	Elovl4	ADJ95235.1
	Fads2 (Δ5Fad)	NP_001117014.1	Elovl2	ACI62500.1
Oncorhynchus masou	Fads2	BAB63440.1		
	Fads2	ABU87822.1		
Oncorhynchus mykiss	Fads2	NP_001117759.1	Elovl5	NP_001118108.1
Muraenesox cinereus	Fads2 (Δ6_1)	AEV57604.1		
	Fads2 (Δ6_2)	AEV57605.1		
	Fads2 (Δ6_3)	AEV57606.1		
Anguilla japonica	Fads2	KJ182968	Elovl5	KJ182967
Pangasianodon hypophthalmus	Fads2	AFN21428.1		
Labeo rohita	Fads2	EF634246.2		
Cyprinus carpio	Fads2	AAG25711.1	Elovl5	AER39745.1
Danio rerio	Fads2	NP_571720.2	Elovl5	NP_956747.1
			Elovl4a	NP_956266.1
			Elovl4b	NP_957090.1
			Elovl2	AAI34116.1
Scyliorhinus canicula	Fads2	AEY94455.1		
	Fads1	AEY94454.1		
Xenopus laevis	fads2	NP_001086853.1	elovl5	Q32NI8.1
			elovl2	NP_001087564.1
Columba livia	FADS2	EMC81381.1		
Sus scrofa	FADS2	NP_001165221.1		
	FADS1	NP_001106512.1		
Papio anubis	FADS2	NP_001138559.1		
	FADS1	NP_001106097.1		
Homo sapiens	FADS2	NP_004256.1	ELOVL5	Q9NYP7.1

	FADS1	NP_037534.3	ELOVL2	Q9NXB9.2
			ELOVL4	Q9GZR5.1
Mus musculus	FADS2	NP_062673.1	ELOVL5	Q8BHI7.1
	FADS1	NP_666206.1	ELOVL2	Q9JLJ4.1
			ELOVL4	Q9EQC4.2
Rattus norvegicus	FADS2	NP_112634.1		
	FADS1	NP_445897.2		
Chelonia mydas	FADS1	EMP32024.1		

611

612

613 Table 3. Functional characterization of Japanese eel putative desaturase in yeast *S.*
614 *cerevisiae*. Results are expressed as a percentage of total fatty acid (FA) substrate
615 converted to desaturated product.

FA substrate	Product	Conversion rate (%)	Activity
18:3n-3	20:4n-3	64.8	Δ6
18:2n-6	20:3n-6	20.7	Δ6
20:3n-3	20:4n-3	6.0	Δ8
20:2n-6	20:3n-6	5.4	Δ8
20:4n-3	20:5n-3	0.0	Δ5
20:3n-6	20:4n-6	0.0	Δ5
22:5n-3	22:6n-3	0.0	Δ4
22:4n-6	22:5n-6	0.0	Δ4

616

617

618 Table 4. Functional characterization of Japanese eel Elovl5 in yeast *S. cerevisiae*.
619 Individual conversion rates were calculated according to the formula [individual
620 product area/(all products areas + substrate area)] x 100.

FA substrate	Product	Conversion rate (%)	Activity
18:3n-3	20:3n-3	10.6	C18-20
18:2n-6	20:2n-6	16.7	C18-20
18:4n-3	20:4n-3	71.1	C18-20
18:3n-6	20:3n-6	48.7	C18-20
20:5n-3	22:5n-3	30.5	C20-22
20:4n-6	22:4n-6	18.2	C20-22
22:5n-3	24:5n-3	0.0	C22-24
22:4n-6	24:4n-6	0.0	C22-24

621
622