

1 **Investigation of highly unsaturated fatty acid metabolism in the**
2 **Asian sea bass, *Lates calcarifer***

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16

16 **Abstract** *Lates calcarifer*, commonly known as the Asian sea bass or barramundi, is an
17 interesting species that has great aquaculture potential in Asia including Malaysia and also
18 Australia. We have investigated essential fatty acid metabolism in this species, focusing on
19 the endogenous highly unsaturated fatty acid (HUFA) synthesis pathway using both
20 biochemical and molecular biological approaches. Fatty acyl desaturase (Fad) and elongase
21 (Elovl) cDNAs were cloned and functional characterization identified them as $\Delta 6$ Fad and
22 Elovl5 elongase enzymes, respectively. The $\Delta 6$ Fad was equally active towards 18:3n-3 and
23 18:2n-6, and Elovl5 exhibited elongation activity for C₁₈₋₂₀ and C₂₀₋₂₂ elongation and a trace of
24 C₂₂₋₂₄ activity. The tissue profile of gene expression for $\Delta 6$ *fad* and *elovl5* genes, showed
25 brain to have the highest expression of both genes compared to all other tissues. The results of
26 tissue fatty acid analysis showed that the brain contained more docosahexaenoic acid (DHA,
27 22:6n-3) than flesh, liver and intestine. The HUFA synthesis activity in isolated hepatocytes
28 and enterocytes using [1-¹⁴C]18:3n-3 as substrate was very low with the only desaturated
29 product detected being 18:4n-3. These findings indicate that *L. calcarifer* display an essential
30 fatty acid pattern similar to other marine fish in that they appear unable to synthesize HUFA
31 from C₁₈ substrates. High expression of $\Delta 6$ *fad* and *elovl5* genes in brain may indicate a role
32 for these enzymes in maintaining high DHA levels in neural tissues through conversion of
33 20:5n-3.

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36 **Keywords:** Fatty acid; Metabolism; Desaturase; Elongase; cDNA; Functional
37 characterization.

38 **Introduction**

39 *Lates calcarifer* also known as the Asian sea bass or barramundi is one of the most
40 commercially valuable cultured species in Southeast Asia. It satisfies the important criteria of
41 being a fast growing and durable fish with good tasting flesh, which promises that it will be
42 the most cultured fish species in Malaysia. It grows rapidly, reaching a marketable size of 600
43 g in six months and, as a euryhaline fish, it has the ability to adapt to a wide range of salinity.
44 It has good market value and there is high demand for live or chilled fish not only in
45 restaurants and hotels, but also domestically (Awang 1987). To fulfil the market demands and
46 fully exploit its great potential, there is expansion in *L. calcarifer* aquaculture throughout
47 Malaysia. However, there are disadvantages that hinder the establishment of high-throughput
48 production for *L. calcarifer* including the high input cost. Compared to other countries like
49 Thailand and Indonesia, the production cost for *L. calcarifer* in Malaysia is still high at
50 between 15 – 20 %, with the high cost of fish meal and oil based feeds being the primary
51 factor (FAMA 2005). Although some nutritional aspects of *L. calcarifer* have been
52 investigated, lipid nutrition has not been intensively studied (Walford et al. 1990; Rimmer et
53 al. 1994; Boonyaratpalin et al. 1998; Williams et al. 2003).

54 Fish is well recognized as a good source of protein and beneficial lipids, especially n-3
55 highly unsaturated fatty acids (HUFA) (Ackman 2005). Despite being the source of n-3
56 HUFA for human consumers, marine fish themselves require dietary HUFAs to satisfy their
57 essential fatty acid requirements for optimal growth and survival (Tocher 2003). Inadequate
58 levels of n-3HUFA, primarily eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic
59 acid (DHA, 22:6n-3), increased the incidence of illness and reduced the survival and growth
60 rates in some marine fish species (Dendrinis and Thorpe 1987; Sorgeloos et al. 1998;
61 Watanabe et al. 1989; Dhert et al. 1990). Rimmer et al. (1994) reported mortalities among *L.*
62 *calcarifer* larvae fed on untreated brine shrimp, and improvement of survival and growth of *L.*

63 *calcarifer* larvae fed on nutritionally enriched brine shrimp. With the increasing health
64 awareness among humans, fish oil, the major source of n-3 HUFAs is in high demand, and
65 this has now become an issue in fish nutrition and aquaculture (Turchini et al. 2009). Thus, it
66 has dictated that more sustainable oil sources must be explored to replace dietary fish oil, and
67 currently the best candidates are vegetable oils (Sargent et al. 2002). However, the effective
68 use of vegetable oils to replace fish oil in formulated diets may vary among fish species
69 depending on the efficiency of the endogenous HUFA biosynthesis pathway in the fish, which
70 is also influenced of several factors including developmental stage, ecological and feeding
71 habitats (Tocher 2003). Feeding marine fish with diets containing vegetable oils can reduce
72 their growth performance and lead to potential health issues including fat deposition in the
73 liver and suppression of immune function (Caballero et al. 2004; Bell et al. 2005; Mourente et
74 al. 2005a; 2005b; 2007). Importantly, it also lowers the n-3 HUFA content of the flesh,
75 compromising the nutritional quality to human consumers that is so crucial to maintain while
76 utilizing plant-based feed formulations (Bell et al. 2005, Izquierdo et al. 2005; Torstensen et
77 al. 2005). Therefore, it is important to have knowledge of the activity of the endogenous
78 HUFA biosynthesis pathway and the key enzymes involved in effort to optimize the activity
79 of the pathway and direct the formulation of alternative plant-based diets for each individual
80 species (Tocher 2003).

81 HUFA biosynthesis involves a series of fatty acyl desaturase (Fad) and elongase of very long
82 fatty acids (Elovl) enzymes. For the production of DHA, its main precursor, the C18
83 polyunsaturated fatty acid (PUFA), α -linolenic acid (ALA, 18:3n-3) is transformed to EPA by
84 Δ 6 desaturation, followed by elongation and Δ 5 desaturation (Cook and McMaster 2004).
85 Then EPA is converted to DHA by another path involving two further elongation steps, a Δ 6
86 desaturation and a peroxisomal chain shortening process (Sprecher 2000). Investigations of
87 HUFA biosynthesis mechanisms in fish have been performed in various species, and *fad*

88 cDNAs have been cloned from both fresh and marine water fish including $\Delta 6$ desaturases
89 from common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) (Seiliez et al.
90 2001), a bifunctional $\Delta 6/\Delta 5$ Fad from freshwater zebrafish (*Danio rerio*) (Hastings et al.
91 2001), and $\Delta 6$ and $\Delta 5$ desaturase from Atlantic salmon (Hastings et al. 2005; Zheng et al.
92 2005). In true marine fish such as gilthead sea bream (*Sparus aurata*), turbot (*Psetta maxima*)
93 and cod (*Gadus morhua*), only $\Delta 6$ *fad* cDNAs have been identified so far (Seiliez et al. 2003;
94 Zheng et al. 2004; Tocher et al. 2006). Similarly, *elovl* cDNAs have been cloned from
95 freshwater species, including zebrafish, common carp, tilapia (*Oreochromis niloticus*),
96 Atlantic salmon and rainbow trout, as well from marine fish including gilthead sea bream,
97 turbot and cod (Agaba et al. 2005; Hastings et al. 2005; Morais et al. 2009). To date, similar
98 studies have been performed in two euryhaline tropical fish, finding genes encoding $\Delta 6$ Fad in
99 white-spotted spinefoot (*Siganus canaliculatus*) both $\Delta 6$ Fad and Elov15 in cobia
100 (*Rachycentron canadum*) (Li et al. 2008; Zheng et al. 2009).

101 The present study investigated HUFA biosynthesis in *L. calcarifer* as a platform towards
102 understanding the mechanisms underpinning the effects of substituting dietary fish oil with
103 vegetable oil. Thus, the fatty acid compositions of a current commercial diet and *L. calcarifer*
104 tissues were determined, followed by cloning, functional characterization and tissue
105 expression profile of cDNAs involved in HUFA biosynthesis, and direct assay of the pathway
106 in hepatocytes and enterocytes.

107

108 **Materials and methods**

109

110 Fish rearing

111 Juvenile *L. calcarifer* reared in brackish water within floating cage with salinity average of
112 ~25 ppt and at temperature of ~28°C at Leguna Semerak, Kelantan, Malaysia, were used to
113 obtain tissue RNA for the cloning of expressed *fad* and *elovl* genes. For all other studies, the
114 *L. calcarifer* were fingerlings (~ 100 g) held in 1 m² (220 L) tanks supplied with
115 dechlorinated mains freshwater at 26 ± 2 °C and with a 12L:12D photoperiod at the Institute
116 of Aquaculture, University of Stirling, UK. All the fish were fed a 3 mm commercial diet for
117 *L. calcarifer* with 44 % protein (fish, shrimp, wheat and soybean meals) and 10 % lipid
118 (Grobest Group, Australia).

119 Bioinformatic analyses

120 All primers were designed using PrimerPremier software version 5.0. Phred program (Ewing
121 et al. 1998; Ewing and Green 1998) was used to assess quality of the raw chromatogram for
122 sequencing data, and CrossMatch (Green 1999) analysis was performed to remove vector
123 sequences. Sequences were aligned automatically by CLUSTALX but manually edited,
124 aligned and assembled with the Bioedit program. For gene identification, sequence homology
125 was searched against the GenBank database using the BLASTN and BLASTX programs
126 (Altschul et al. 1990). Amino acid sequences were also analyzed using InterProScan
127 (Quevillon et al. 2005), to search protein homology against interrogated databases.
128 Phylogenetic analyses were carried out based on amino acid sequences using the Neighbour
129 Joining method (Saitou and Nei 1987) and the trees were constructed using CLUSTALX and
130 NJPLOT.

131

132 Determination of fatty acid compositions of diet and *L. calcarifer* tissues

133 Samples of brain, liver, intestine and flesh were collected from four fish for lipid and fatty
134 acid analyses. Diet samples and tissues were weighed, and lipid extracted by homogenization

135 in chloroform/methanol (2:1, v/v) containing 0.01 % butylated hydroxytoluene (BHT) as
136 antioxidant, as described previously (Tocher and Harvie 1988). Fatty acid methyl esters
137 (FAMES) were prepared from 1 mg portions of total lipid by acid-catalyzed
138 transesterification, extracted and purified by thin layer chromatography (TLC), all as
139 described previously (Hastings et al. 2001). FAMES were separated and quantified by gas-
140 liquid chromatography (Carlo Erba Vega 8160, Milan, Italy) using a 30 m x 0.32 mm i.d.
141 capillary column (CP Wax 52CB, Chrompak, London, UK) and on-column injection.
142 Hydrogen was used as carrier gas and temperature programming was from 50 °C to 150 °C at
143 40 °C min⁻¹ and then to 230 °C at 2.0 °C min⁻¹. Individual FAMES were identified by
144 comparison with known standards and by reference to published data (Ackman 1980; Tocher
145 and Harvie 1988). Data were collected and processed using Chromcard for Windows (version
146 1.19).

147

148 Cloning of putative fatty acid desaturase and elongase

149 Immediately after sacrifice, samples of brain and liver tissue were dissected and placed
150 directly into TRI Reagent® (Molecular Research, Cincinnati, OH, USA) for immediate
151 extraction of RNA according to manufacturer's instructions. Based on alignment of sequences
152 existing in the GenBank database, degenerate primers, Fish-Desat-F and Fish-Desat-R were
153 designed for *Fad* (Table 1). Similarly, Fish-Elong-R was designed for *Elovl* and used with
154 *Elo1A*, a forward degenerate primer used previously by Hastings et al. 2005. Using liver total
155 RNA, PCR amplifications were performed to obtain *fad* and *elovl* cDNA fragments using
156 Access RT-PCR System (Promega, Madison, WI, USA). A fragment for *fad* was obtained
157 under the following PCR conditions: initial denaturation at 94°C for 2 min, 35 cycles of
158 denaturation at 94°C for 30 s, annealing at 65°C for 30 s and extension at 72°C for 2 min. The

159 PCR conditions for Elov1 were similar except annealing was at 57°C for 30 s and extension at
160 72°C was for 1 min. The PCR products were cloned into the pTZ57R/T vector accordingly to
161 the protocol of InsT/Aclone™ PCR Product Cloning kit (Fermentas, Glen Burnie, MD, USA).
162 Gene specific primers for *fad* and *elov1* were designed for Rapid Amplification of cDNA Ends
163 (RACE) PCR using FirstChoice RLM-RACE kit (Ambion, Inc., Austin, TX, USA) (Table 1).
164 A nested 5'RACE PCR approach was applied using the same program for both PCR steps and
165 both genes. The PCR conditions were as following: initial denaturation at 95°C for 2 min, 33
166 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 2
167 min. For *fad*, 3'RACE PCR used the following: initial denaturation at 94°C for 2 min, 35
168 cycles of denaturation at 94°C for 30 s, annealing at 66°C for 30 s and extension at 72°C for 2
169 min. For *elov1*, nested 3'RACE PCR used identical conditions except annealing was at 55°C
170 for 30 s. The RACE-PCR products were cloned into the pBluescript KS II+ vector
171 (Stratagene, La Jolla, CA, USA) for sequencing using DTCS kit and the CEQ™ 8800 Genetic
172 Analysis System (Beckman Coulter Inc., Fullerton, CA, USA). To obtain full-length cDNA
173 sequences, Bioedit program was utilized to assemble and align all the sequences from RT-
174 PCR, 5' and 3' RACE PCR.

175

176 Functional characterization of *fad* and *elov1* cDNAs by heterologous expression in
177 *Saccharomyces cerevisiae*

178 Appropriate primers for putative *fad* and *elov1* coding regions (CDS) were designed for use
179 with the pYES2 expression vector (Invitrogen, Paisley, UK). For *fad*, the forward primer
180 LCDVF1 contained a *Kpn* I restriction site and the reverse primer LCDVR1 contained an *Xho*
181 I restriction site (Table 1). Primer pair for *elov1* which was LCEVF1 and LCEVR1 contained
182 restriction sites of *Hind* III and *Xho* I, respectively. PCR was performed using high fidelity

183 PfuTurbo® DNA polymerase (Stratagene, USA) and amplification involved an initial
184 denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s,
185 annealing at 60°C for 30 s and extension at 72°C for 1.5 min. Subsequently, the PCR
186 fragments were restricted and ligated into the expression vector. Ligated products were
187 transformed into TOP10F' *E. coli* competent cells (Invitrogen, UK) and recombinant
188 plasmids, pYES2-LcDes and pYES2-LcElo were extracted using GenElute Plasmid Miniprep
189 Kit (Sigma, Poole, UK). The plasmids were transformed into the yeast *S. cerevisiae* strain
190 InvSc1 using the S.c.EasyComp Transformation Kit (Invitrogen, UK). Selection of yeasts
191 containing pYES2-LcDes and pYES2-LcElo was carried out on *S. cerevisiae* minimal
192 medium minus uracil (SC-U). The transformant yeasts were cultured in SC-U broth with
193 galactose induction of gene expression as described previously (Hastings et al., 2001). To
194 assay *fad*, cultures were supplemented with one of the following fatty acid substrates: 18:3n-
195 3, 18:2n-6, 20:4n-3, 20:3n-6, 22:5n-3 and 22:4n-6. For *elovl* assay, the following substrates
196 were supplemented, 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3 and 22:4n-6. After three
197 days incubation, yeast cells were harvested, washed, dried, lipid extracted, and FAMES
198 prepared, extracted, purified and analysed as described previously (Hastings et al. 2001).
199 Conversion of fatty acid substrates to desaturated or elongated products was calculated from
200 the GC chromatograms as percentage of product area per total of product and substrate areas
201 (Hastings et al. 2005).

202

203 Determining the activity of the HUFA synthesis pathway in isolated hepatocytes and
204 enterocytes

205 For assay of HUFA biosynthesis, livers and intestines were carefully dissected from twelve
206 fish (6 pools of 2 fish) to produce six hepatocyte and six enterocyte preparations. Each pool of

207 tissues was chopped, incubated with collagenase and cells sieved through 100 µm nylon
208 gauze as described previously (Mourente et al. 2005). One hundred µL of each cell
209 preparation was taken for protein determination by the method of Lowry et al. (1951),
210 following incubation with 1 M NaOH/0.25 % (w/v) SDS for 1 h at 60 °C. For each cell
211 preparation, two 5 ml portions were dispensed into 25 cm² tissue culture flasks and incubated
212 at 22 °C for 1 h with 0.5 µCi of either [1-¹⁴C]18:3n-3 or [1-¹⁴C]20:5n-3, added as complexes
213 with fatty acid free-bovine serum albumin (BSA) (Ghioni et al. 1997). After incubation, cells
214 were harvested, washed and lipid extracted as described previously (Mourente et al. 2005).
215 Total lipid was transmethylated and FAMES prepared as described above. FAMES were
216 separated by argentation (silver nitrate) TLC (Wilson and Sargent 1992), radiolabelled
217 FAMES located on the plate by autoradiography, and quantified by liquid scintillation after
218 scraping from the TLC plates (Stubhaug et al. 2005).

219

220 Tissue expression profile of *fad* and *elovl* genes

221 Expression of the target genes was measured in tissues by quantitative real-time PCR (qPCR).
222 Samples of ten different tissues including brain, gill, heart, intestine, kidney, liver, spleen,
223 adipose, red and white muscle, were dissected from four individual fingerlings for RNA
224 extraction using UltraTurrax® homogenizer in Trizol® reagent (Gibco, Invitrogen, USA).
225 Synthesis of first strand cDNA was performed using Verso™ cDNA kit (Thermo Scientific,
226 Waltham, MA, USA). Primer pairs of qDF1-qDR1 and qEF2-qER2 were designed for *fad* and
227 *elovl* with fragment sizes of 156 bp and 197 bp, respectively (Table 1). Amplification of
228 cDNA templates and DNA standards was carried out using Absolute™ QPCR SYBR Green
229 Mix (Thermo Scientific, USA) under the following conditions: initial denaturation at 95°C for
230 15 min, 40 cycles of denaturation at 95°C for 15 s, annealing at 62°C for 15 s and extension at

231 72°C for 15 s. Thermal cycling and fluorescence detection were conducted in the Quantica
232 system (Techne Incorporated, Barloworld Scientific Ltd, Stone, UK), and gene expression
233 levels quantified based on statistical evaluation, one-way analysis of variance (ANOVA)
234 followed by Duncan HSD test (P<0.05) was performed to compare the expression level
235 among tissue samples (SPSS, Chicago, USA).

236

237 **Results**

238 Fatty acid composition of *L. calcarifer* diet and tissues

239 The commercial *L. calcarifer* diet contained 28 % saturated fatty acids, predominantly 16:0
240 (19 %), around 35 % monounsaturated fatty acids, mainly 18:1n-9 (24 %), 22 % n-6 PUFA
241 that was almost all 18:2n-6 (20 %), and 15 % n-3 PUFA with two-thirds being EPA and DHA
242 in a 1:1 ratio (Table 2). Similarly, the fatty acid compositions of the *L. calcarifer* tissues were
243 dominated by 16:0, 18:1n-9 and 18:2n-6 (Table 2). All tissues contained 30 – 33 % total
244 saturated fatty acids, except for brain with significant differences in the unsaturated fatty
245 acids compared to the other tissues. Thus, brain was characterized by having lower levels of
246 16:1n-7, 18:1n-9, and total monounsaturated fatty acids, and very much lower 18:2n-6 and
247 total n-6 PUFA, and lower EPA compared to all other tissues. In contrast, DHA was at least
248 two-fold higher and n-3:n-6 ratio at least 3-fold higher in brain and, arachidonic acid (ARA;
249 20:4n-6) was also highest in brain, besides flesh (Table 2).

250 Sequence analyses of cloned *L. calcarifer fad* and *elovl* cDNAs

251 The cDNA sequence for *L. calcarifer* putative FAD contained 181 bp of 5'
252 untranslated region (UTR), 1338 bp of CDS, which coded 445 amino acids (GenBank
253 accession no. GQ214179) and 456 bp of 3' UTR. InterProScan analysis showed the protein

254 sequence contained two major domains, namely cytochrome b₅ (PF00173) and Fad type 1
255 (PF00487), and transmembrane regions (Fig. 1). Pairwise comparison of the translated amino
256 acids sequences showed the *L. calcarifer* Fad shared 90 % identity to the Δ6 Fad of cobia, 86
257 % identity to those of gilthead sea bream and European sea bass, 76-77 % identity to
258 salmonid Fads and 70 % identity to the zebrafish Δ6/Δ5 Fad (Table 3). This was reflected in
259 phylogenetic analysis that clustered the *L. calcarifer* Fad closest to cobia and other marine
260 fish, and more distantly from salmonids and freshwater species (Fig. 2).

261 The putative Elovl mRNA obtained from *L. calcarifer* was shown to be 1264 bp in length
262 with 885 bp CDS, encoding a protein of 294 amino acids (GenBank accession no.
263 GQ214180) flanked by 164 bp of 5' and 215 bp of 3' UTR. InterProScan analysis showed the
264 protein sequence belonged to the family of GNS1/SUR4 membrane proteins (PF01151), and
265 revealed a signal peptide and multiple transmembrane regions (Fig. 1). The *L. calcarifer*
266 putative elongase CDS shared from 95% identity with cobia Elovl5 to 75-77 % identity with
267 Elovl5s from catfish and zebrafish, but only 53-56 % identity with Elovl2s from salmon and
268 zebrafish (Table 4). Consistent with this, phylogenetic analysis showed that the *L. calcarifer*
269 Elovl clustered closest to Elovl5s of gilthead sea bream and other marine species such as
270 cobia and turbot, and distance from Elovl5s from salmonid and other freshwater fish, and
271 further still from Elovl2s from salmon and mammals (Fig.3).

272

273 Functional characterization of *L. calcarifer* Fad and Elovl

274 As negative controls, the fatty acid composition of yeast transformed with the empty
275 vector showed 16:0, 16:1n-7, 18:0 and 18:1n-9, the four main fatty acids normally found in *S.*
276 *cerevisiae* (Hastings et al. 2001), along with exogenously derived fatty acids (Figs. 4A and
277 5A). When yeast transformed with the *fad* CDS was grown in the presence of 18:3n-3 and
278 18:2n-6, two additional peaks were observed corresponding to their desaturated products,

279 18:4n-3 and 18:3n-6, respectively (Figs. 4B and C). In contrast, when yeasts transformed with
280 *fad* CDS were grown in the presence of 20:4n-3 and 20:3n-6, no desaturated products were
281 observed (Figs. 4D and E). Similarly, no desaturated products were observed when
282 transformed yeast were grown in the presence of 22:5n-3 or 22:4n-6 (data not shown). These
283 data indicated that the *L. calcarifer* Fad had $\Delta 6$ activity and that when expressed in yeast it
284 showed only slight higher affinity towards the n-3 substrate with a conversion rate of 32 %
285 for the desaturation of 18:3n-3 compared to 28 % for desaturation of 18:2n-6 (Table 5).

286 When yeast transformed with the *L. calcarifer elovl* CDS was grown in the presence
287 of 18:4n-3 and 18:3n-6, additional peaks were observed corresponding to their immediate
288 elongation products, 20:4n-3 and 20:3n-6, and the further elongated products, 22:4n-3 and
289 22:3n-6 (Fig. 5B shows trace for incubation with 18:4n-3). Transformed yeast grown in
290 presence of 20:5n-3 and 20:4n-6, showed additional peaks corresponding to their immediate
291 elongation products, 22:5n-3 and 22:4n-6, and the further elongated products, 24:5n-3 and
292 24:4n-6 (Fig. 5C shows n-3 series). Transformed yeast grown with 22:5n-3 and 22:4n-6 also
293 displayed additional minor peaks for the elongation products, 24:5n-3 and 24:4n-6,
294 respectively (Fig. 5D shows n-3 series). Note, elongation products 18:1n-7, 20:1n-9 and
295 20:1n-7 from the endogenous fatty acids 16:1n-7, 18:1n-9 and 18:1n-7, respectively, were
296 also detected (Fig. 5B-D). These data indicated that the *L. calcarifer* elongase displayed a
297 pattern most similar to Elov15 with similar activity towards C18 and C20 PUFA and much
298 lower towards C22 (Table 6).

299

300 HUFA biosynthesis pathway in hepatocytes and enterocytes

301 Activity of HUFA biosynthesis pathway was investigated by measuring the recovery of
302 radioactivity in the summed desaturated or/and elongated products of [$1-^{14}\text{C}$] 18:3n-3 (Fig. 6).
303 Data essentially showed that radioactivity was only recovered in 18:4n-3, as the only major

304 desaturated product, and 20:3n-3, the dead-end elongation product, when *L. calcarifer*
305 hepatocytes or enterocytes were incubated with [1-¹⁴C]18:3n-3 (Fig.6, upper). The rate of
306 conversion of the radiolabelled substrate into elongated product, 20:3n-3 and desaturated
307 product, 18:4n-3, were higher in hepatocytes than in enterocytes. The conversion rates of
308 18:3n-3 to 18:4n-3 and 20:3n-3 in hepatocytes was 0.82 pmol.h⁻¹.mg protein⁻¹ and 0.43
309 pmol.h⁻¹.mg protein⁻¹, respectively, whereas in enterocytes the conversion rate to 18:4n-3 and
310 20:3n-3 was 0.15 and 0.24 pmol.h⁻¹.mg protein⁻¹, respectively. When incubated with [1-
311 ¹⁴C]20:5n-3, radioactivity was primarily recovered in the elongation products 22:5n-3 and
312 24:5n-3, but conversion to DHA was also observed (Fig.6, lower).

313

314 Tissue expression of *L. calcarifer* $\Delta 6$ *fad* and *elovl5* genes

315 The expression of *L. calcarifer* $\Delta 6$ *fad* and *elovl5* in ten different tissues was expressed as
316 mean of absolute copy number (\pm SEM) of $\Delta 6$ *fad* and *elovl5* transcripts in 25 ng of total
317 RNA (Whelan et al. 2003) (Fig. 7). Both $\Delta 6$ *fad* and *elovl5* mRNAs were highly expressed in
318 the brain, and in liver at only a low level. Desaturase mRNA was also detected at high levels
319 in kidney and intestine with low expression in heart and white muscle. Elongase transcripts
320 were abundant in intestine and kidney compared to adipose, gill and spleen.

321

322 **Discussion**

323

324 In the present study, both biochemical and molecular biology approaches were used to
325 provide data to investigate EFA metabolism and the HUFA synthesis pathway in *L.*
326 *calcarifer*. Similar studies have provided useful information in understanding the production
327 of HUFA in fish species (Tocher et al. 2003, 2006). All the fish used in the study were fed a

328 standard commercial *L. calcarifer* diet. The high contents of 18:2n-6, 18:1n-9 and, to a lesser
329 extent, 18:3n-3, and the low n-3:n-6 PUFA ratio (0.7) indicated that the diet was formulated
330 with a relatively high proportion of plant products and, indeed, the fatty acid composition
331 resembled that of a diet formulated with vegetable oil used in a previous study of the
332 nutritional regulation of fatty acyl desaturase and HUFA synthesis in Atlantic cod (Tocher et
333 al. 2006). The fatty acid composition of the diet was clearly reflected in the fatty acid
334 compositions of the *L. calcarifer* tissues. However, despite being fed this diet, the flesh still
335 contained 17 % n-3 HUFA, which contrasts with diet that was supplying only 11 % n-3
336 HUFA. Therefore there was evidence that *L. calcarifer* were able to maintain levels of DHA
337 above the dietary level. The results from the present study suggest this is unlikely to be due to
338 synthesis of DHA from 18:3n-3, rather it must be due to very effective selective retention
339 rather than synthesis (Tocher 2003). However, all tissues had higher DHA levels than in diet
340 whereas EPA was lower than in the diet. This could also simply reflect differential oxidation
341 rates, but perhaps conversion of EPA to DHA is also a factor.

342 As an initial step to investigate the HUFA biosynthesis pathway in *L. calcarifer*, it was
343 essential to identify key enzymes involved. A series of Fad and Elovl enzymes are required
344 for the production of HUFA from C18 PUFA (Sprecher 2000). This production of EPA or
345 arachidonic acid (ARA, 20:4n-6) from 18:3n-3 or 18:2n-6, respectively, requires $\Delta 6$ Fad, an
346 elongase and $\Delta 5$ Fad (Tocher 2003). Production of DHA from EPA involves two further
347 elongations, a $\Delta 6$ desaturation and a peroxisomal chain shortening (Sprecher 2000). A $\Delta 6$ Fad
348 has been found in many fish species, whereas $\Delta 5$ Fad has only been isolated in salmon and
349 zebrafish. In the present study, we successfully cloned cDNAs of a fatty acyl desaturase and
350 an elongase from *L. calcarifer* which, when expressed in yeast, resulted to be $\Delta 6$ Fad and an
351 Elovl5 elongase, respectively. The *L. calcarifer* $\Delta 6$ Fad was almost equally active towards
352 18:3n-3 and 18:2n-6 whereas most fish desaturases have displayed a preference towards the

353 n-3 PUFA (Hastings et al. 2001; Zheng et al. 2004, 2005a; Tocher et al. 2006). The *L.*
354 *calcarifer* Elovl5 displayed elongation of n-3 and n-6, primarily of C18 and C20. *L. calcarifer*
355 Elovl5 showed a preference for n-6 C₁₈ substrates, but was more active towards n-3 C₂₀
356 substrates. This was similar to the activities of Elovl5 elongases from other marine fish like
357 turbot, sea bream and cobia that were more active towards EPA compared to ARA (Zheng et
358 al. 2009). In contrast, Elovl5 elongases cloned from freshwater and diadromous species were
359 more active on n-3 PUFA irrespective of chain length (Agaba et al. 2005). Previous studies of
360 HUFA synthesis in fish cell lines suggested C₁₈₋₂₀ elongase activity was limited in turbot cells
361 (Ghioni et al. 1999). The cloning and functional characterization of Elovl5 elongases from *L.*
362 *calcarifer*, cobia, sea bream and turbot would indicate that marine fish in general do not lack
363 the gene for C18-20 elongase (Agaba et al. 2005; Zheng et al. 2009). However, it is reported
364 that it is relatively common for enzymes in the desaturation/elongation pathway to be absent
365 or down-regulated in long established cell lines (Ghioni et al. 1999). It was suggested that
366 there has been evolutionary adaptation to the availability of HUFA in the natural diet of fish
367 species. In fresh water omnivorous fish, there could be a demand for HUFA biosynthesis to
368 produce EPA and DHA, whereas in carnivorous marine fish, there is less requirements for the
369 production of HUFA because of the n-3 HUFA-rich diet. Zebrafish and salmon also express a
370 further elongase, Elovl2 that has high specificity towards C₂₀ and C₂₂ HUFAs compared to
371 C₁₈ PUFA (Morais et al. 2009; Monroig et al. 2009). No homologues of Elovl2 have been
372 found in genomes of the marine fish stickleback, pufferfish and medaka, all species of the
373 order of Acanthopterygii that mostly inhabit the marine ecosystem, another possible
374 molecular mechanism underlying their low HUFA biosynthesis capability (Morais et al.
375 2009).

376 Biochemical studies of HUFA biosynthesis have been advanced by the use of cell cultures
377 that have elucidated key parts of the pathway (Tocher et al. 1989; Tocher and Sargent 1990;

378 Tocher and Dick 1999, 2001). In the present study, we used short-term primary cultures of
379 hepatocyte and enterocyte cells prepared by collagenase digestion. The isolated cells were
380 attached to a plastic surface, but there was no growth or division over the time course of the
381 experiment, less than 6 hours (Buzzi et al. 1996, 1997). This type of cell preparation retains
382 their differentiated phenotype and will reflect most of the biological and physiological
383 mechanism *in vivo* compared to established cell lines. This strategy has limitations, as
384 equivalent neural cell preparation cannot be isolated from the brain, a potentially important
385 tissue in HUFA metabolism (Bell et al. 1994; Tocher et al. 1996). The data from *L. calcarifer*
386 hepatocytes and enterocytes showed the activity was very low, the only products confirmed
387 being 18:4n-3, the product of $\Delta 6$ Fad activity, and a trace of its elongation product 20:3n-3. In
388 absolute terms, the activities in *L. calcarifer* are very much lower than the activities measured
389 in salmon (Zheng et al. 2005b; Tocher et al. 2002). Thus, the rates of desaturation of [1-
390 ^{14}C]18:3n-3 in *L. calcarifer* hepatocytes and enterocytes were approximately $0.8 \text{ pmol.h}^{-1}.\text{mg}$
391 protein^{-1} and $0.4 \text{ pmol.h}^{-1}.\text{mg protein}^{-1}$, respectively. These rates were intermediate between
392 those found earlier for Atlantic salmon and Atlantic cod, with *L. calcarifer* hepatocytes and
393 enterocytes showing higher $\Delta 6$ desaturation activity than cod, but lower than that of salmon
394 (Tocher et al. 2006).

395 The relatively low activity in liver and intestine was perhaps not unexpected based on
396 previous data with marine fish (Tocher et al. 2006), and was also supported by the gene
397 expression data for $\Delta 6$ *fad* and *elovl5* in *L. calcarifer*. Copy number of both $\Delta 6$ *fad* and *elovl5*
398 mRNA were low in most tissues including liver and intestine, except for brain where relevant
399 levels of transcripts were found. This was also the pattern observed in other marine fish, such
400 as cod and cobia, with *fad* and *elovl5* expression highest by far in brain (Tocher et al. 2006;
401 Zheng et al. 2009). In contrast, both $\Delta 6$ and $\Delta 5$ *fad* and PUFA elongases are expressed to the
402 greatest extent in liver, intestine and also brain (Zheng et al. 2005b). It has been speculated

403 that the high expression of *fad* and *elovl* in fish brain is related to the important role of DHA
404 in brain (Tocher et al. 2006; Zheng et al. 2009). This has been further supported by the data
405 on *L. calcarifer* that shows a high concentration of DHA in brain, despite the fish being fed a
406 diet that was clearly high in plant products and 18:2n-6, and the very specific expression of
407 FAD and Elovl in the brain. It is well established that in fish larvae, the levels and ratios of
408 dietary ARA, EPA DHA are important particularly to support the growth of brain and retina
409 for proper development of cognitive and visual systems (Uauy et al. 2001; Sargent et al. 1993;
410 Brodtkorb et al. 1997). Thus the role of Fad and Elovl enzymes in neural tissues is possibly to
411 ensure sufficient DHA despite fluctuations in dietary EPA and DHA levels, particularly at
412 crucial times in development such as larval development (Mourente 2003). Recently, high
413 expression of *fad* and *Elovl* genes was noted in the head region of developing zebrafish
414 embryos (Monroig et al. 2009).

415 *L. calcarifer* is known to be a euryhaline or catadromous fish and, in Malaysia, this fish
416 species is reared in the marine environment with a salinity range of 30 to 32 ppt. In nature,
417 wild adult *L. calcarifer* travel to estuaries for spawning and the newly-hatched larvae grow in
418 brackish water before moving back into salt water. However in Australia, *L. calcarifer*
419 originate and return to their original river systems after migration to the estuaries to breed
420 (Grey 1987; Allen 1989; Merrick and Schmida 1984). The present study on Malaysian *L.*
421 *calcarifer* that had been adapted to freshwater, showed an overall HUFA synthesis pathway
422 as previously described in other marine fish species such as Atlantic cod and sea bream
423 (Tocher et al. 2006). It would be interesting to determine if fresh water *L. calcarifer* display a
424 different pattern of HUFA biosynthesis. This would require the *L. calcarifer* genome to
425 contain $\Delta 5$ *fad* and possibly *elovl2* genes. Although only $\Delta 6$ *fad* and *elovl5* were cloned in the
426 present study, exhaustive study with more specific primers was not performed and so the
427 presence or absence of these other genes cannot be confirmed. However, the commercial diet

428 analyzed resembled a vegetable oil-type diet utilized for nutritional trial/experiment in
429 previous studies with twice as much C₁₈ PUFA compared to HUFA (Tocher et al. 2006). The
430 results of the present trial suggest this may not be ideal if *L. calcarifer* are “biochemically”
431 marine fish, lacking a complete HUFA synthesis pathway. However, the DHA contents in the
432 tissues of the *L. calcarifer* fingerlings in the present trial suggest they were not adversely
433 affected.

434 Overall, the investigation of the HUFA synthesis pathway in *L. calcarifer*, based on data
435 from biochemical and molecular approaches, showed clear biological correlations. This
436 knowledge will be good platform for further work on the optimal EFA requirements and
437 functional roles of HUFA in this species. It is still a need to study the pathway at a cellular or
438 molecular level especially in understanding the gene regulation of the key enzymes for the
439 pathway. Reliable information on that aspect will aid in better diet formulation for improving
440 survival rate especially among the larvae and to maintain flesh quality for consumers.

441

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653 **Legends to figures**

654 **Fig. 1** Schematics of protein sequences for Fad (445 amino acids) and Elovl (294 amino
655 acids) from *L. calcarifer* based on InterProScan analysis. Numbers represent the position in
656 the amino acid sequence.

657 **Fig. 2** Phylogenetic tree of fatty acyl desaturases from *L. calcarifer* (*L.ca*) and other fish
658 species: rainbow trout *O. mykiss* (*O.my*), Atlantic salmon *S. salar* (*S.sa*), cherry salmon *O.*
659 *masou* (*O.ma*), Atlantic cod *G. marhua* (*G.ma*), gilthead seabream *S. aurata* (*S.au*); turbot, *S.*
660 *maximus* (*S.ma*); cobia, *R. canadum* (*R.ca*); Nile tilapia, *O. niloticus* (*O.ni*); white-spotted
661 spinefoot, *S. canaliculatus* (*S.ca*); zebrafish, *D. rerio* (*D.re*); common carp, *C. carpio* (*C.ca*),
662 mammals: mouse, *M. musculus* (*M.mu*); human, *H. sapiens* (*H.sa*), fungus: *M. alpina* (*M.al*)
663 and nematode: *C. elegans* (*C.el*). The horizontal branch length is proportional to amino acid
664 substitution per site. The numbers represent the frequencies with which the tree topology
665 presented here was replicated after 1000 bootstrap iterations.

666 **Fig. 3** Phylogenetic tree of fatty acyl elongase from *L. calcarifer* (*L.ca*) with other fish
667 species: cobia, *R. canadum* (*R.ca*); turbot, *S. maximus* (*S.ma*); gilthead seabream, *S. aurata*
668 (*S.au*); Nile tilapia, *O. niloticus* (*O.ni*); Atlantic salmon, *S. salar* (*S.sa*); rainbow trout, *O.*
669 *mykiss* (*O.my*); zebrafish, *D. rerio* (*D.re*); catfish, *C. gariepinus* (*C.ga*); Atlantic cod, *G.*
670 *marhua* (*G.ma*), mammals: mouse, *M. musculus* (*M.mu*); human, *H. sapiens* (*H.sa*), rat, *R.*
671 *norvegicus* (*R.no*), insect: fruitfly, *D. melanogaster* (*D.me*), fungus: *M. alpina* (*M.al*),
672 nematode: *C. elegans* (*C.el*), amphibia: toad, *X. laevis* (*X.la*), avian: red jungle fowl, *G. gallus*
673 (*G.ga*) and moss, *P. patens* (*P.pa*). The horizontal branch length is proportional to amino acid
674 substitution per site. The numbers represent the frequencies with which the tree topology
675 presented here was replicated after 1000 bootstrap iterations.

676 **Fig. 4** Functional characterization of the *L. calcarifer* Fad in transgenic yeast (*S. cerevisiae*).
677 Fatty acids were extracted from yeast transformed with pYES2 vector containing CDS of the
678 putative *fad* cDNA and grown in the presence of $\Delta 6$ substrates: 18:3n-3 (B) and 18:2n-6 (C),
679 and $\Delta 5$ substrates 20:4n-3 (D) and 20:3n-6 (E). Panel A represents a negative control with
680 yeast transformed with empty vector and cultured with 18:3n-3. The first four peaks in all the
681 panels are the main endogenous fatty acids of *S. cerevisiae*, identified as 16:0 (1), 16:1n-7 (2),
682 18:0 (3) and 18:1n-9 (4). Peak 5 (A and B), peak 7 (C), peak 9 (D) and peak 10 (E) are the
683 exogenously added fatty acid substrates 18:3n-3, 18:2n-6, 20:4n-3 and 20:3n-6, respectively.
684 Peak 6 (B) and peak 8 (C) were identified as the desaturation products, 18:4n-3 and 18:3n-6,
685 respectively. Vertical axis, FID response; horizontal axis, retention time.

686 **Fig. 5** Functional characterization of the *L. calcarifer* putative Elovl in transgenic yeast (*S.*
687 *cerevisiae*). Fatty acids were extracted from yeast transformed with pYES2 vector containing
688 CDS of the putative *elovl* cDNA grown in the presence of 18:4n-3 (B), 20:5n-3 (C) and
689 22:5n-3 (D). Panel A represents a negative control with yeast transformed with empty vector
690 and cultured with 18:4n-3. The first four peaks in all the panels are the main endogenous fatty
691 acids of *S. cerevisiae*, 16:0 (1), 16:1n-7 (2), 18:0 (3) and 18:1n-9 (4). Peak 5 (B and C)
692 corresponds to 18:1n-7 arising from the elongation of the yeast endogenous 16:1n-7 and the
693 remaining peaks correspond to the exogenously added fatty acids and their elongation
694 products which are 18:4n-3 (6), 20:4n-3 (7), 22:4n-3 (8), 20:5n-3 (9), 22:5n-3 (10) and 24:5n-
695 3 (11). Other minor peaks (not labeled) result from the elongation of endogenous fatty acids.
696 Vertical axis, FID response; horizontal axis, retention time.

697 **Fig. 6** Desaturation of [1-¹⁴C]18:3n-3 (upper panel) and [1-¹⁴C]20:5n-3 (lower panel) in
698 hepatocytes and enterocytes from *L. calcarifer*. Results represent the rates conversion (pmol
699 h⁻¹ mg⁻¹ tissue protein) of ¹⁴C-labelled substrate to desaturated/elongated products and are
700 means \pm S.D (n=4).

701 **Fig. 7** Tissue distribution of *Δ6 fad* and *elovl5* genes in *L. calcarifer*. Transcript (mRNA)
702 copy numbers were determined by quantitative real-time PCR (qPCR) as described in the
703 Methods section. Results expressed as means of absolute copy number (\pm SEM) of *fad* and
704 *elovl5* transcripts in 25 ng of total RNA. Lowercase (desaturase) and uppercase (elongase)
705 letters show significant differences ($P < 0.05$) among tissues as determined by one-way
706 ANOVA followed by Duncan HSD test (SPSS, Chicago, USA). A, adipose tissue; B, brain;
707 G, gill; H, heart; I, intestine; K, kidney; L, liver; RM, red muscle; S, spleen; RW, white
708 muscle.

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709 **Table 1** List of PCR primers used in this study

Primer ID	Sequence (5'→ 3')
Fish-Desat-F	TACACCTGGGAYGAGGTSCAGAYBCAC
Fish-Desat-R	AGGTGTCCYCTGAACCAGTCGTTGAAG
Fish-Elong-R	ASSACYTGGARGAAGCTGTTDAYGG
Elo1A	CCTGTGTGGTAYTAYTT
LcDes-5GSP-Outer	GGGATGAAAGGCAGTGAACG
LcDes-5GSP-Inner	TGATGACACGAAACCCTCCC
LcElo-5GSP-Outer	TTCTCAAATGTCAATCCACCCTCAGTT
LcElo-5GSP-Inner	ATCACGACGTGGACGAAGCT
LcDes-3GSP	ACCGCTGCTCATTCCAGTTTTCTTCC
LcElo-3GSP	GTTTATGGACACCTTCTTCTT
LCDVF1	CCC <u>GGTACC</u> AGGATGGGAGGTGGAGGC
LCDVR1	CCG <u>CTCGAGT</u> CATTTATGGAGATATGCATCG
LCEVF1	CCC <u>AAGCTT</u> AAAAATGGAGACCTTCAATCATAAACTG
LCEVR1	CCG <u>CTCGAGT</u> CAATCCACCCTCAGTTTTCTT
qLcDesF	TTAATTCCCTTTGCCGTATTTAAA
qLcDesR	AAGAAATCCTGCACAGAATCTGAA
qLcEloF	ATGGTCACGCTCATTATCCTTTT
qLcEloR	AGCATTGGGTGGCGGTTTC

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716 **Table 2** Fatty acid compositions (% total fatty acids) of commercial diet and brain, liver, intestine and flesh of
 717 *L. calcarifer*

Fatty acid	Diet		Brain		Liver		Intestine		Flesh	
14:0	2.6	± 0.1	1.4	± 0.3	4.5	± 0.5	4.7	± 0.5	2.9	± 0.3
15:0	0.2	± 0.0	0.2	± 0.0	0.4	± 0.0	0.4	± 0.0	0.3	± 0.0
16:0	19.4	± 0.4	18.4	± 1.6	19.4	± 0.6	18.5	± 0.4	19.5	± 0.6
18:0	5.3	± 0.1	12.4	± 2.1	5.3	± 0.2	5.8	± 0.2	7.4	± 0.6
Total saturates ^a	27.9	± 0.6	33.2	± 3.4	30.1	± 1.1	30.0	± 1.0	30.7	± 1.0
16:1n-9	0.2	± 0.1	0.8	± 1.4	0.1	± 0.1	0.2	± 0.2	0.1	± 0.1
16:1n-7	3.8	± 0.1	2.7	± 1.5	5.3	± 0.3	5.6	± 0.4	3.9	± 0.5
18:1n-9	23.8	± 0.6	16.0	± 7.8	21.1	± 0.7	21.8	± 1.0	19.6	± 1.1
18:1n-7	2.7	± 0.3	1.6	± 0.7	2.8	± 0.1	2.8	± 0.1	2.6	± 0.1
Total monounsaturates ^b	34.7	± 0.4	23.9	± 8.1	32.9	± 0.3	33.9	± 0.7	28.7	± 1.7
18:2n-6	20.2	± 0.2	5.4	± 0.9	16.7	± 0.9	17.7	± 0.4	14.8	± 1.4
18:3n-6	0.1	± 0.0	0.2	± 0.0	0.6	± 0.2	0.4	± 0.1	0.3	± 0.0
20:2n-6	0.2	± 0.0	0.2	± 0.0	0.4	± 0.0	0.3	± 0.0	0.3	± 0.0
20:3n-6	0.1	± 0.0	0.4	± 0.1	0.3	± 0.1	0.3	± 0.0	0.3	± 0.0
20:4n-6	0.6	± 0.0	2.1	± 0.3	1.1	± 0.0	1.0	± 0.1	2.7	± 0.6
22:4n-6	0.1	± 0.0	0.3	± 0.0	0.2	± 0.0	0.2	± 0.0	0.3	± 0.0
22:5n-6	0.1	± 0.0	0.2	± 0.0	0.3	± 0.0	0.3	± 0.1	0.7	± 0.2
Total n-6 PUFA	21.6	± 0.2	8.8	± 0.7	19.6	± 1.0	20.2	± 0.4	19.5	± 0.7
18:3n-3	2.5	± 0.0	0.6	± 0.1	1.9	± 0.2	1.9	± 0.2	1.2	± 0.2
18:4n-3	0.8	± 0.0	0.3	± 0.0	0.8	± 0.0	0.7	± 0.0	0.4	± 0.1
20:3n-3	0.0	± 0.0	0.0	± 0.0	0.1	± 0.0	0.1	± 0.0	0.0	± 0.0
20:4n-3	0.2	± 0.0	0.2	± 0.0	0.3	± 0.0	0.3	± 0.0	0.3	± 0.0
20:5n-3	5.2	± 0.1	2.4	± 0.1	4.1	± 0.2	3.7	± 0.0	4.4	± 0.3
22:4n-3	0.0	± 0.0	0.0	± 0.0	0.0	± 0.0	0.0	± 0.0	0.0	± 0.0
22:5n-3	0.7	± 0.1	1.8	± 0.2	2.2	± 0.2	2.1	± 0.2	2.4	± 0.2
22:6n-3	5.2	± 0.3	20.6	± 3.4	6.9	± 0.4	5.6	± 0.4	10.2	± 1.6
Total n-3 PUFA	14.7	± 0.6	25.8	± 3.2	16.3	± 0.5	14.5	± 0.7	18.9	± 1.8
Total PUFA	37.4	± 0.5	43.0	± 4.8	37.0	± 1.0	36.2	± 0.9	40.6	± 1.6
(n-3)/(n-6)	0.7	± 0.0	2.9	± 0.6	0.8	± 0.1	0.7	± 0.1	1.0	± 0.1

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719 Results are expressed as percentage of total FA and are means ± SD (n=4). PUFA, Polyunsaturated fatty acids.

720 ^aIncludes 20:0 and 22:0.

721 ^bIncludes 20:1, 22:1 and 24:1.

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Table 23 Identity matrix showing the results of a pair-wise comparison between the identities of the amino acid sequenced of fish
724 desaturases.

	Cobia Δ6	European sea bass Δ6	Gilthead seabream Δ6	Atlantic cod Δ6	Atlantic salmon Δ6	Atlantic salmon Δ5	Zebrafish Δ5/Δ6	Nile tilapia	Rainbow trout Δ6	Cherry salmon Δ6
Barramundi Δ6	90	86	86	79	77	77	70	77	77	76
Cobia Δ6		88	87	80	77	78	71	77	76	76
European sea bass Δ6				82	78	78	67	76	77	76
Gilthead seabream Δ6					76	76	70	72	77	77
Atlantic cod Δ6						91	66	71	94	76
Atlantic salmon Δ6							65	72	92	92
Atlantic salmon Δ5								63	66	94
Zebrafish Δ5/Δ6									72	66
Nile tilapia										71
Rainbow trout Δ6										96

Data 725 percentages of amino acid residues that are identical.

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Table 29 Identity matrix showing the results of a pair-wise comparison between the identities of the amino acid sequenced of fish elongases.

	Cobia Elov15	Turbot Elov15	Nile tilapia Elov15	Atlantic salmon Elov15	Atlantic cod Elov15	African catfish Elov15	Zebrafish Elov15	Zebrafish Elov12	Atlantic salmon Elov12
Barramundi Elov15	95	89	88	82	78	75	77	53	56
Cobia Elov15		89	87	73	79	76	77	52	55
Turbot Elov15			82	79	75	74	74	53	54
Nile tilapia Elov15				78	73	73	74	53	57
Atlantic salmon Elov15					77	77	75	54	52
Atlantic cod Elov15						74	71	53	50
African catfish Elov15							79	52	55
Zebrafish Elov15								54	52
Zebrafish Elov12									75

Data 731 percentages of amino acid residues that are identical.

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743 **Table 5** Functional characterisation of the *L. calcarifer* $\Delta 6$ Fad.

Fatty acid substrate	Product	Conversion (%)	Activity	744
18:3n-3	18:4n-3	32.0	$\Delta 6$	745
18:2n-6	18:3n-6	28.3	$\Delta 6$	746
20:4n-3	20:5n-3	ND	$\Delta 5$	747
20:3n-6	20:4n-6	ND	$\Delta 5$	748
22:5n-3	22:6n-3	ND	$\Delta 4$	749
22:4n-6	22:5n-6	ND	$\Delta 4$	750

751 Results are expressed as a percentage of total fatty acid substrate converted to desaturated products.

752 ND, not detected.

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754 **Table 6** Functional characterisation of *L. calcarifer* Elovl5 elongase. Results are expressed as a
 755 percentage of total fatty acid substrate converted to elongated product.

Fatty acid substrate	Product	Conversion (%)	Activity	756
18:4n-3	20:4n-3	45.7	C18 → C20	757
	22:4n-3	21.9	C20 → C22	
	24:4n-3	ND	C22 → C24	758
	Total	67.6		
18:3n-6	20:3n-6	51.6	C18 → C20	759
	22:3n-6	21.5	C20 → C22	760
	24:3n-6	1.3	C22 → C24	
	Total	74.4		761
20:5n-3	22:5n-3	72.6	C20 → C22	
	24:5n-3	3.1	C22 → C24	762
	Total	75.7		763
20:4n-6	22:4n-6	62.6	C20 → C22	
	24:4n-6	2.5	C22 → C24	764
	Total	65.1		765
22:5n-3	24:5n-3	1.6	C22 → C24	766
22:4n-6	24:4n-6	2.9	C22 → C24	767
				768

769 Results are expressed as a percentage of total fatty acid substrate converted to elongated products. Percentage
 770 of stepwise conversion into intermediary products of the elongation pathway is also shown.

771 ND, not detected.

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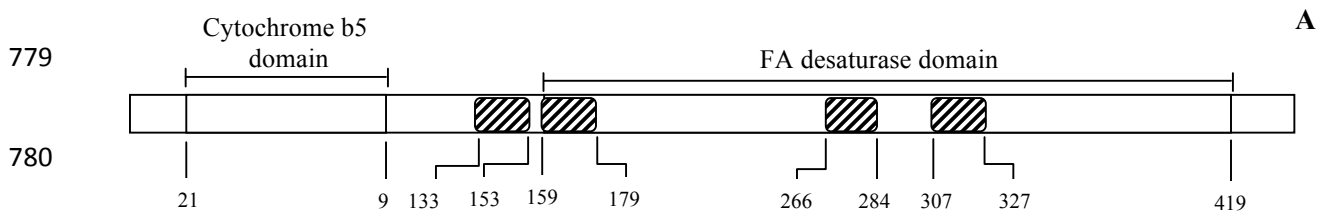
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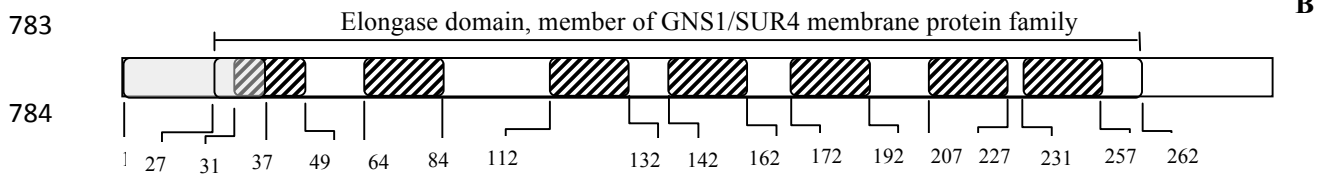
778 Fig. 1




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 Transmembrane region


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 Signal peptide

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 Transmembrane region

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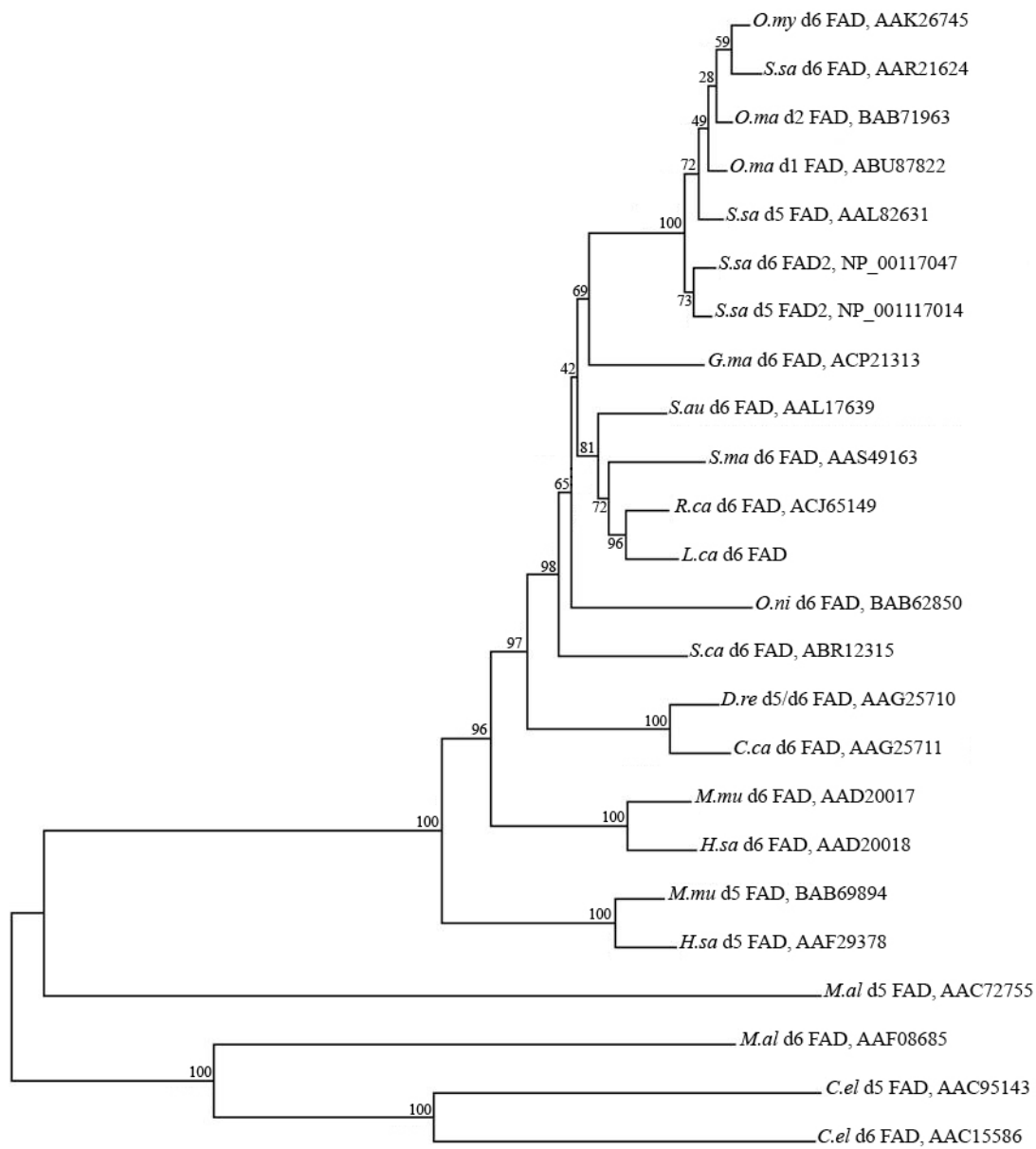
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796 Fig.2



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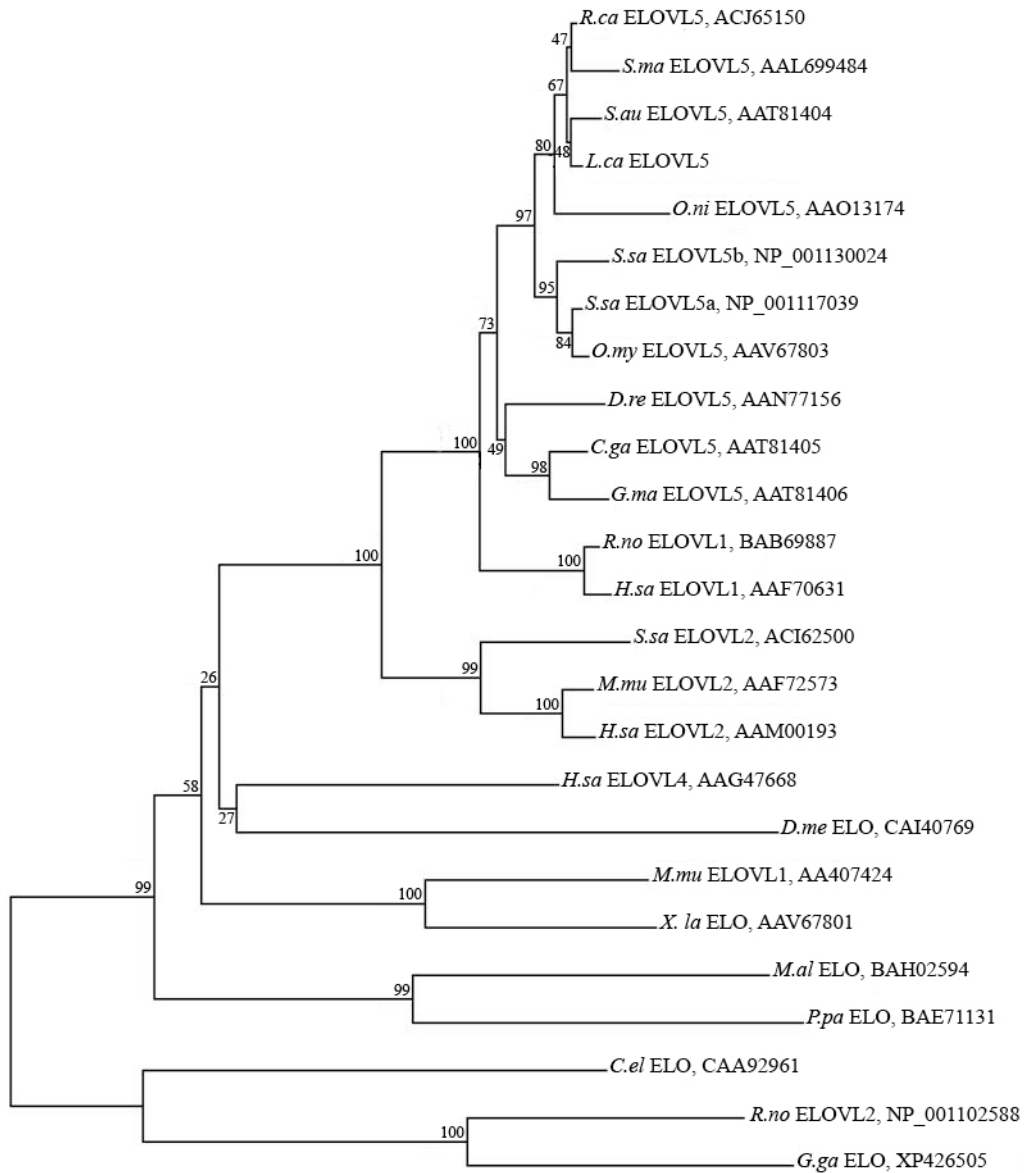
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802 Fig.3



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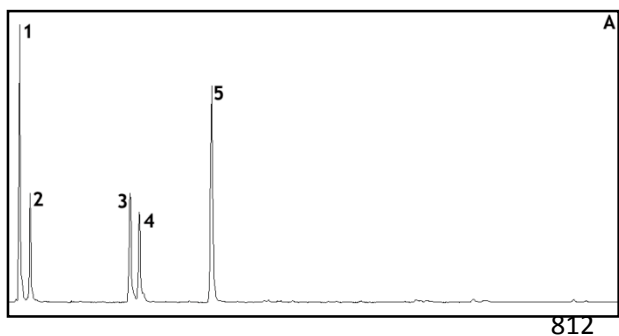
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808 Fig.4

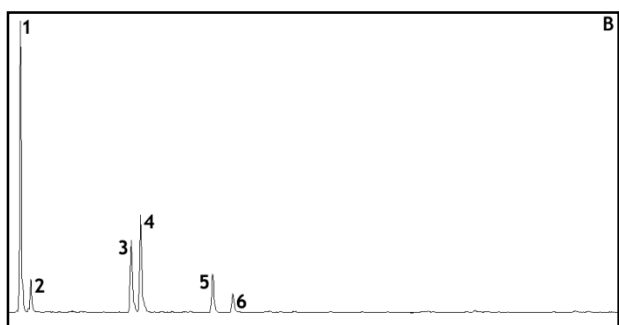


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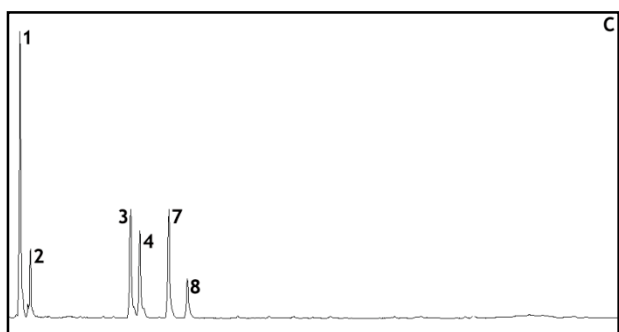


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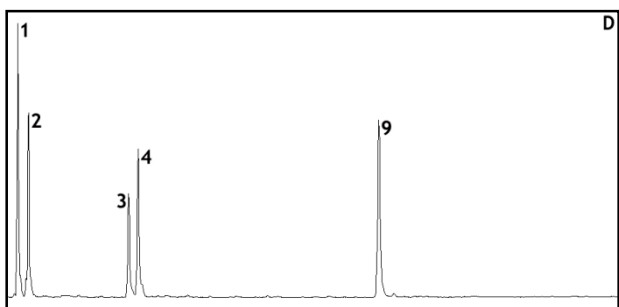


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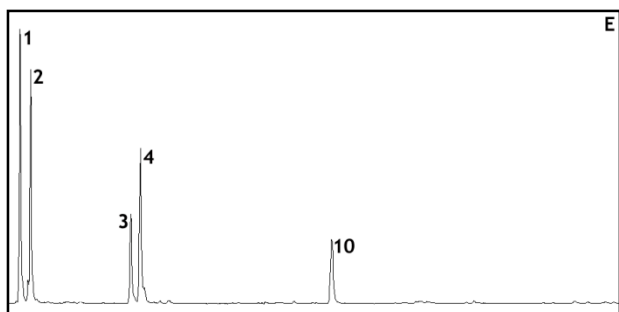
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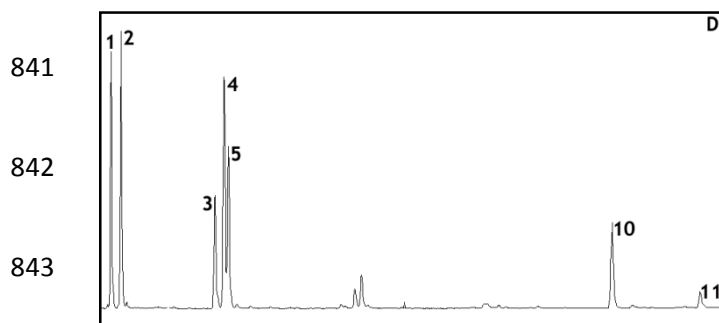
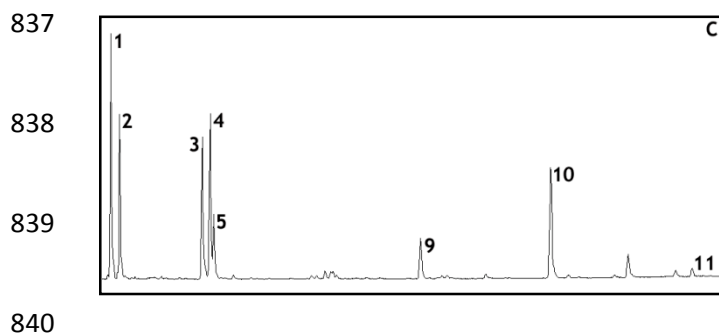
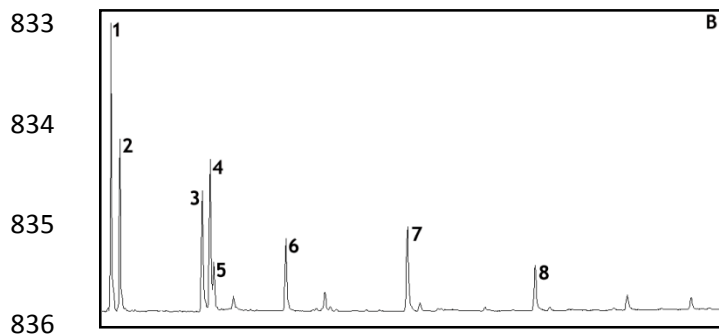
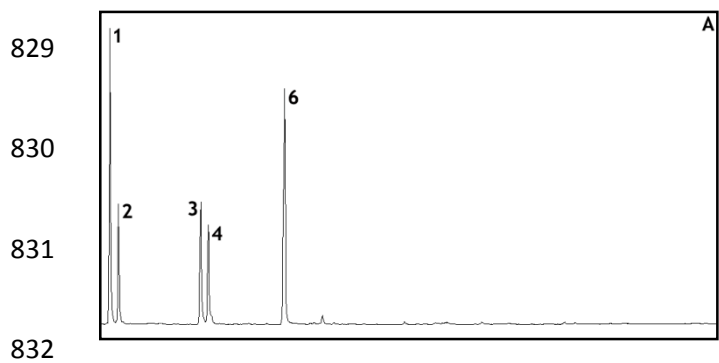
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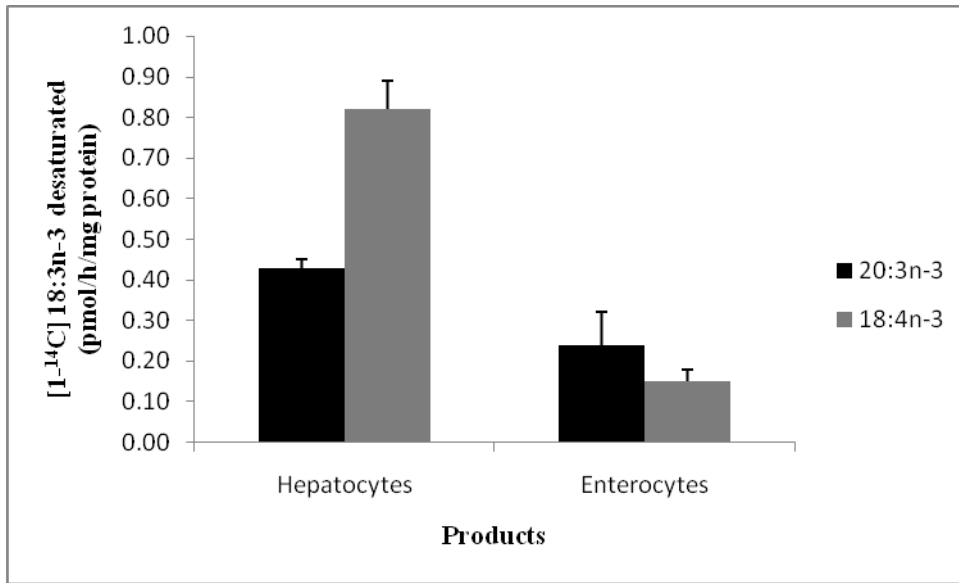


827 Fig.5.

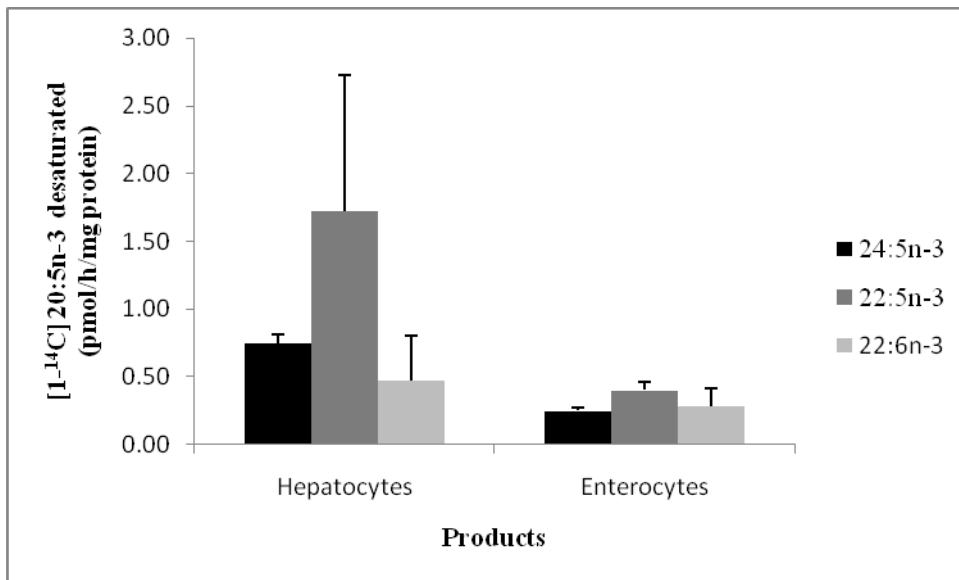
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846 Fig. 6.



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854 Fig.7.

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