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

N. Y. Mohd-Yusof[a, *], O. Monroig[b], A. Mohd-Adnan[a, c], K.-L. Wan[a, c], D. R. Tocher[b]

[a]School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor DE, Malaysia

[b]Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK

[c]Malaysia Genome Institute, Heliks Emas Block, UKM-MTDC Technology Centre, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor DE, Malaysia

* *Corresponding author: Tel. +603 89267446; Fax, +603 89267972; Email, yuziana@gmail.com.*

Abstract *Lates calcarifer*, commonly known as the Asian sea bass or barramundi, is an interesting species that has great aquaculture potential in Asia including Malaysia and also Australia. We have investigated essential fatty acid metabolism in this species, focusing on the endogenous highly unsaturated fatty acid (HUFA) synthesis pathway using both biochemical and molecular biological approaches. Fatty acyl desaturase (Fad) and elongase (Elovl) cDNAs were cloned and functional characterization identified them as Δ6 Fad and Elovl5 elongase enzymes, respectively. The Δ6 Fad was equally active towards 18:3n-3 and 18:2n-6, and Elovl5 exhibited elongation activity for C_{18-20} and C_{20-22} elongation and a trace of C_{22-24} activity. The tissue profile of gene expression for Δ*6 fad* and *elovl*5 genes, showed brain to have the highest expression of both genes compared to all other tissues. The results of tissue fatty acid analysis showed that the brain contained more docosahexaenoic acid (DHA, 22:6n-3) than flesh, liver and intestine. The HUFA synthesis activity in isolated hepatocytes and enterocytes using [1-^{14}C]18:3n-3 as substrate was very low with the only desaturated product detected being 18:4n-3. These findings indicate that *L. calcarifer* display an essential fatty acid pattern similar to other marine fish in that they appear unable to synthesize HUFA from C_{18} substrates. High expression of Δ6 *fad* and *elovl5* genes in brain may indicate a role for these enzymes in maintaining high DHA levels in neural tissues through conversion of 20:5n-3.

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

Introduction

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

Fish is well recognized as a good source of protein and beneficial lipids, especially n-3 highly unsaturated fatty acids (HUFA) (Ackman 2005). Despite being the source of n-3 HUFA for human consumers, marine fish themselves require dietary HUFAs to satisfy their essential fatty acid requirements for optimal growth and survival (Tocher 2003). Inadequate levels of n-3HUFA, primarily eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), increased the incidence of illness and reduced the survival and growth rates in some marine fish species (Dendrinos and Thorpe 1987; Sorgeloos et al. 1998; Watanabe et al. 1989; Dhert et al. 1990). Rimmer et al. (1994) reported mortalities among *L. 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 Δ6 desaturation, followed by elongation and Δ5 desaturation (Cook and McMaster 2004).
85 Then EPA is converted to DHA by another path involving two further elongation steps, a Δ6
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*

cDNAs have been cloned from both fresh and marine water fish including Δ6 desaturases from common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) (Seiliez et al. 2001), a bifunctional Δ6/Δ5 Fad from freshwater zebrafish (*Danio rerio*) (Hastings et al. 2001), and Δ6 and Δ5 desaturase from Atlantic salmon (Hastings et al. 2005; Zheng et al. 2005). In true marine fish such as gilthead sea bream (*Sparus aurata*), turbot (*Psetta maxima*) and cod (*Gadus morhua*), only Δ6 *fad* cDNAs have been identified so far (Seiliez et al. 2003; Zheng et al. 2004; Tocher et al. 2006). Similarly, *elovl* cDNAs have been cloned from freshwater species, including zebrafish, common carp, tilapia (*Oreochromis niloticus*), Atlantic salmon and rainbow trout, as well from marine fish including gilthead sea bream, turbot and cod (Agaba et al. 2005; Hastings et al. 2005; Morais et al. 2009). To date, similar studies have been performed in two euryhaline tropical fish, finding genes encoding Δ6 Fad in white-spotted spinefoot (*Siganus canaliculatus*) both Δ6 Fad and Elovl5 in cobia (*Rachycentron canadum*) (Li et al. 2008; Zheng et al. 2009).

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

Materials and methods

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 m^2 (220 L) tanks supplied with
115 dechlorinated mains freshwater at 26 ± 2 ^oC 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 ^{o}C to 150 ^{o}C at
143 40 ^{o}C min^{-1} and then to 230 °C at 2.0 ^{o}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

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

Functional characterization of *fad* and *elovl* cDNAs by heterologous expression in *Saccharomyces cerevisiae*

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

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

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

For assay of HUFA biosynthesis, livers and intestines were carefully dissected from twelve 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 cm^2 tissue culture flasks and incubated
212 at 22 °C for 1 h with 0.5 μCi of either [1-^{14}C]18:3n-3 or [1-^{14}C]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

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

Results

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

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

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

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

sequence contained two major domains, namely cytochrome b_5 (PF00173) and Fad type 1 (PF00487), and transmembrane regions (Fig. 1). Pairwise comparison of the translated amino acids sequences showed the *L. calcarifer* Fad shared 90 % identity to the Δ6 Fad of cobia, 86 % identity to those of gilthead sea bream and European sea bass, 76-77 % identity to salmonid Fads and 70 % identity to the zebrafish Δ6/Δ5 Fad (Table 3). This was reflected in phylogenetic analysis that clustered the *L. calcarifer* Fad closest to cobia and other marine fish, and more distantly from salmonids and freshwater species (Fig. 2).

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

Functional characterization of *L. calcarifer* Fad and Elovl

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

18:4n-3 and 18:3n-6, respectively (Figs. 4B and C). In contrast, when yeasts transformed with *fad* CDS were grown in the presence of 20:4n-3 and 20:3n-6, no desaturated products were observed (Figs. 4D and E). Similarly, no desaturated products were observed when transformed yeast were grown in the presence of 22:5n-3 or 22:4n-6 (data not shown). These data indicated that the *L. calcarifer* Fad had Δ6 activity and that when expressed in yeast it showed only slight higher affinity towards the n-3 substrate with a conversion rate of 32 % for the desaturation of 18:3n-3 compared to 28 % for desaturation of 18:2n-6 (Table 5).

When yeast transformed with the *L. calcarifer elovl* CDS was grown in the presence of 18:4n-3 and 18:3n-6, additional peaks were observed corresponding to their immediate elongation products, 20:4n-3 and 20:3n-6, and the further elongated products, 22:4n-3 and 22:3n-6 (Fig. 5B shows trace for incubation with 18:4n-3). Transformed yeast grown in presence of 20:5n-3 and 20:4n-6, showed additional peaks corresponding to their immediate elongation products, 22:5n-3 and 22:4n-6, and the further elongated products, 24:5n-3 and 24:4n-6 (Fig. 5C shows n-3 series). Transformed yeast grown with 22:5n-3 and 22:4n-6 also displayed additional minor peaks for the elongation products, 24:5n-3 and 24:4n-6, respectively (Fig. 5D shows n-3 series). Note, elongation products 18:1n-7, 20:1n-9 and 20:1n-7 from the endogenous fatty acids 16:1n-7, 18:1n-9 and 18:1n-7, respectively, were also detected (Fig. 5B-D). These data indicated that the *L. calcarifer* elongase displayed a pattern most similar to Elovl5 with similar activity towards C18 and C20 PUFA and much lower towards C22 (Table 6).

HUFA biosynthesis pathway in hepatocytes and enterocytes

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

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

Tissue expression of *L. calcarifer Δ6 fad* and *elovl5* genes

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

Discussion

In the present study, both biochemical and molecular biology approaches were used to provide data to investigate EFA metabolism and the HUFA synthesis pathway in *L. calcarifer*. Similar studies have provided useful information in understanding the production 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 Δ6 Fad, an
346 elongase and Δ5 Fad (Tocher 2003). Production of DHA from EPA involves two further
347 elongations, a Δ6 desaturation and a peroxisomal chain shortening (Sprecher 2000). A Δ6 Fad
348 has been found in many fish species, whereas Δ5 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 Δ6 Fad and an
351 Elovl5 elongase, respectively. The *L. calcarifer* Δ6 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 C_{18} substrates, but was more active towards n-3 C_{20}
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 C_{18-20} 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 C_{20} and C_{22} HUFAs compared to
371 C_{18} 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;

Tocher and Dick 1999, 2001). In the present study, we used short-term primary cultures of hepatocyte and enterocyte cells prepared by collagenase digestion. The isolated cells were attached to a plastic surface, but there was no growth or division over the time course of the experiment, less than 6 hours (Buzzi et al. 1996, 1997). This type of cell preparation retains their differentiated phenotype and will reflect most of the biological and physiological mechanism *in vivo* compared to established cell lines. This strategy has limitations, as equivalent neural cell preparation cannot be isolated from the brain, a potentially important tissue in HUFA metabolism (Bell et al. 1994; Tocher et al. 1996). The data from *L. calcarifer* hepatocytes and enterocytes showed the activity was very low, the only products confirmed being 18:4n-3, the product of Δ6 Fad activity, and a trace of its elongation product 20:3n-3. In absolute terms, the activities in *L. calcarifer* are very much lower than the activities measured in salmon (Zheng et al. 2005b; Tocher et al. 2002). Thus, the rates of desaturation of [1-^{14}C]18:3n-3 in *L. calcarifer* hepatocytes and enterocytes were approximately 0.8 pmol.h^{-1}.mg protein^{-1} and 0.4 pmol.h^{-1}.mg protein^{-1}, respectively. These rates were intermediate between those found earlier for Atlantic salmon and Atlantic cod, with *L. calcarifer* hepatocytes and enterocytes showing higher Δ6 desaturation activity than cod, but lower than that of salmon (Tocher et al. 2006).

The relatively low activity in liver and intestine was perhaps not unexpected based on previous data with marine fish (Tocher et al. 2006), and was also supported by the gene expression data for Δ*6 fad* and *elovl5* in *L. calcarifer*. Copy number of both Δ*6 fad* and *elovl5* mRNA were low in most tissues including liver and intestine, except for brain where relevant levels of transcripts were found. This was also the pattern observed in other marine fish, such as cod and cobia, with *fad* and *elovl5* expression highest by far in brain (Tocher et al. 2006; Zheng et al. 2009). In contrast, both Δ*6* and Δ*5 fad* and PUFA elongases are expressed to the 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 *Δ5 fad* and possibly *elovl2* genes. Although only *Δ6 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

analyzed resembled a vegetable oil-type diet utilized for nutritional trial/experiment in previous studies with twice as much C_{18} PUFA compared to HUFA (Tocher et al. 2006). The results of the present trial suggest this may not be ideal if *L. calcarifer* are "biochemically" marine fish, lacking a complete HUFA synthesis pathway. However, the DHA contents in the tissues of the *L. calcarifer* fingerlings in the present trial suggest they were not adversely affected.

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

Acknowledgements

This project was supported by the Genomics and Molecular Biology Initiatives Programme of the Malaysia Genome Institute, Ministry of Science, Technology and Innovation Malaysia (Project No. 07-05-16-MGI-GMB009). The authors would like to acknowledge technical support provided by Nik Daud Nik Sin from the Fisheries Research Institute, Malaysia, and Nial Auchinachie at the tropical aquarium and James Dick in the nutrition lab in the Institute of Aquaculture, University of Stirling, UK. OM was supported by a EU Marie Curie Intra-European Fellowship (PIEF-GA-2008-220929, COBIAGENE).

451 **References**

452 Ackman, R.G. (1980) Fish Lipids, Part 1. In: Connell JJ (ed) Advances in Fish Science and
453 Technology, Fishing News Books, Farnham, pp 87–103

454 Ackman RG (2005) Fatty acids in fish and shellfish. In: Ching KC (ed) Fatty acids in foods
455 and their health implications, CRC Press, New York, pp 155-186

456 Agaba MK, Tocher DR, Dickson CA, Zheng X, Dick JR, Teale AJ (2005) Cloning and
457 functional characterisation of polyunsaturated fatty acid elongases from marine and
458 freshwater teleost fish. Comp Biochem Physiol 142B: 342-352

459 Allen GR (1989) Freshwater Fishes of Australia. T.F.H. Publications, New Jersey, pp 240

460 Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search
461 tool. J Mol Biol 215: 403-410

462 Awang A (1987) Sea bass (*Lates calcarifer*) larvae and fry production in Malaysia. In:
463 Copland JW, Grey DL (ed) Management of wild and cultured sea bass/ barramundi
464 *(Lates calcarifer)*, Ruskin Press, Melbourne, pp 144-147

465 Bell JG, Tocher DR, Sargent JR (1994) Effects of supplementation with 20:3(n-6), 20:4(n-6)
466 and 20:5(n-3) on the production of prostaglandin E and F of the 1-, 2- and 3-series in
467 turbot (*Scophthalmus maximus*) brain astrogial cells in primary culture. Biochim
468 Biophys Acta 1211: 335-342

469 Bell G, Torstensen B, Sargent J (2005) Replacement of marine fish oils with vegetable oils in
470 feeds for farmed salmon. Lipid Technol. 17, 7-11

471 Boonyaratpalin M, Suraneiranat P, Tunpibal T (1998) Replacement of fish meal with various
472 types of soybean products in diets for the Asian seabass, *Lates calcarifer*. Aquaculture
473 161: 67-78

474 Brodtkorb T, Rosenlund G, Lie Ø (1997) Effects of dietary levels of 20:5n–3 and 22:6n–3 on
475 tissue lipid composition in juvenile Atlantic salmon, *Salmo salar*, with emphasis on
476 brain and eye Aquacult Nutr 3: 175-187
477 Buzzi M, Henderson RJ, Sargent JR (1996) The desaturation and elongation of linoleic acid
478 and eicosapentaenoic acid by hepatocytes and liver microsomes from rainbow trout
479 (*Oncorhynchus mykiss*) fed diets containing fish oil or olive oil. Biochim Biophys Acta
480 1299:235-244
481 Buzzi M, Henderson RJ, Sargent JR (1997) Biosynthesis of docosahexaenoic acid in trout
482 hepatocytes proceeds via 24-carbon intermediates. Comp Biochem Physiol 116B: 263-
483 267
484 Caballero MJ, Izquierdo MS, Kjorsvik E, Fernandez AJ, Rosenlund G (2004) Histological
485 alterations in the liver of sea bream, *Sparus aurata L.*, caused by short or long-term
486 feeding with vegetable oils. Recovery of normal morphology after feeding fish oil as the
487 sole lipid source. J Fish Dis 27: 531-541
488 Cook, HW, McMaster, RCR. (2004) Fatty acid desaturation and chain elongation in
489 eukaryotes. In: D. E. Vance, J. E. (eds), Biochemistry of Lipids, Lipoproteins and
490 Membranes (4th Edition), Elsevier, Amsterdam, pp 181-204
491 Dendrinos P, Thorpe JP (1987) Experiments on the artificial regulation amino acid and fatty
492 acid contents of food organisms to meet the assessed nutritional requirements of larval,
493 post-larval and juvenile Dover sole [*Solea solea* (L.)]. Aquaculture 61: 121-154
494 Dhert P, Lavens P, Duray M, Sorgeloos P (1990) Improved larval survival at metamorphosis
495 of Asian seabass (*Lates calcarifer*) using ω3-HUFA-enriched live food. Aquaculture 90:
496 63-74
497 Ewing B, Hillier L, Wendl MC, Green P (1998) Base-calling of automated sequencer traces
498 using Phred I: Accuracy assessment. Genome Res 8: 175-185

499 Ewing B, Green P (1998) Base-calling of automated sequencer traces using Phred II: Error
500 probabilities. Genome Res 8: 186-194
501 Federal of Agriculture Marketing (FAMA) (2005) Analisis industri ikan siakap, FAMA,
502 Selangor, pp 4-24
503 Ghioni C, Tocher DR, Sargent JR (1997) The effect of culture on morphology, lipid and fatty
504 acid composition, and polyunsaturated fatty acid metabolism of rainbow trout
505 (*Oncorhynchus mykiss*) skin cells. Fish Physiol Biochem 16: 499-513
506 Ghioni C, Tocher DR, Bell MV, Dick JR, Sargent JR (1999) Low C18 to C20 fatty acid
507 elongase activity and limited conversion of stearidonic acid, 18:4n−3, to
508 eicosapentaenoic acid, 20:5n−3, in a cell line from the turbot, *Scophthalmus maximus*.
509 Biochim Biophys Acta 1437: 170-181
510 Green E (1999) Documentation for Phrap and CrossMatch (Versuion 0.990319). Available at
511 http://www.phrP.ORG/PHRp.docs/phrap. Accessed 31 May 2009
512 Grey DL (1987) An overview of *Lates calcarifer* in Australia and Asia. In: Copland JW, Grey
513 DL (ed) Management of wild and cultured sea bass/barramundi (*Lates calcarifer*),
514 Ruskin Press, Melbourne, pp 15-21
515 Hastings N, Agaba M, Tocher DR, Leaver MJ, Dick JR, Sargent JR, Teale AJ (2001) A
516 vertebrate fatty acid desaturase with delta 5 and delta 6 activities. Proc Natl Acad Sci
517 USA 98: 14304-14309
518 Hastings N, Agaba MK, Tocher DR, Zheng X, Dickson CA, Dick JR, Teale AJ (2005)
519 Molecular cloning and functional characterization of fatty acyl desaturase and elongase
520 cDNAs involved in the production of eicosapentaenoic and docosahexaenoic acids from
521 α-linolenic acid in Atlantic salmon (*Salmo salar*). Mar Biotechnol 6: 463-474
522 Izquierdo MS, Montero D, Robaina L, Caballero MJ, Rosenlund G, Ginés R (2005)
523 Alterations in fillet fatty acid profile and flesh quality in gilthead seabream (*Sparus*

524 *aurata*) fed vegetable oils for a long term period. Recovery of fatty acid profiles by
525 fish oil feeding. Aquaculture 250: 431-444
526 Li Y-Y, HuC.-B, Zheng Y-J, Xia X-A, Xu W-J, Wang S-Q, Chen W-Z, Sun Z-W, Huang J-
527 H (2008) The effects of dietary fatty acids on liver fatty acid composition and Δ6-
528 desaturase expression differ with ambient salinities in *Siganus canaliculatus*. Comp
529 Biochem Physiol 151B: 183-190
530 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin
531 Phenol Reagent. J Biol Chem 193: 265-275
532 Merrick JR, Schmida GE (1984) Australian Freshwater Fishes - Biology and Management.
533 Macquarie University, North Ryde, pp 409
534 Monroig Ó., Rotllant J, Sánchez E, Cerdá-Reverter JM, Tocher DR (2009) Expression patterns
535 of genes of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis during
536 embryonic development of zebrafish *Danio rerio*. Biochim Biophys Acta, in press.
537 Morais S, Monroig O, Zheng X, Leaver MJ, Tocher DR (2009) Highly unsaturated fatty acid
538 synthesis in Atlantic salmon: isolation of genes of fatty acyl elongases and
539 characterisation of ELOVL5-and ELOVL2-like elongase cDNAs. Mar Biotechnol 11:
540 627-639
541 Mourente G (2003) Accumulation of DHA (docosahexaenoic acid; 22:6n−3) in larval and
542 juvenile fish brain. In: Browman H, Skiftesvik AB (ed) The Big Fish Bang. Institute
543 of Marine Research, Bergen, pp 239-248
544 Mourente G, Dick JR, Bell JG, Tocher DR (2005a) Effect of partial substitution of dietary
545 fish oil by vegetable oil on desaturation and oxidation of [1-^{14}C]18:3n-3 and [1-
546 ^{14}C]20:5n-3 in hepatocytes and enterocytes of European seabass (*Dicentrarchus labrax*
547 L.). Aquaculture 248: 173-186

548 Mourente G, Good JE, Bell JG (2005b) Partial substitution of fish oil with rapeseed oil,
549 linseed oil and olive oil in diets for European sea bass (*Dicentrarchus labrax* L.):
550 effects on flesh fatty acid composition, plasma prostaglandins E2 and F2?, immune
551 function and effectiveness of a fish oil finishing diet. Aquacult Nutr 11: 25-40
552 Mourente G, Good JE, Thompson KD, Bell JG (2007) Effects of partial substitution of dietary
553 fish oil with blends of vegetable oils, on blood leukocyte fatty acid compositions,
554 immune function and histology in European sea bass, (*Dicentrarchus labrax* L.). Br J
555 Nutr 98: 770-779
556 Quevillon E, Silventoinen V, Pillai S, Harte N, Mulder N, Apweiler R, Lopez R (2005)
557 InterProScan: protein domains identifier. Nucleic Acids Res 33: W116-W120
558 Rimmer MA, Reed AW, Levitt MS, Lisle AT (1994) Effect of nutritional enhancement of live
559 food organisms on growth and survival of barramundi, *Lates calcarifer* (Bloch), larvae.
560 Aquacult Fish Manage 25: 143-156
561 Saitou N, Nei M (1987) The neighbor-joining method. A new method for reconstructing
562 phylogenetic trees. Mol Biol Evol 4:406–425
563 Sargent JR, Bell MV, Tocher DR (1993) Docosahexaenoic acid and the development of brain
564 and retina in fish. In: Drevon CA, Baksaas I, Krokan HE (ed) Omega-3 Fatty Acids:
565 Metabolism and Biological Effects, Birkhäuser, Basel, pp 139-149
566 Sargent JR, Tocher DR, Bell JG (2002) The lipids. In: Halver JE, Hardy RW (ed) Fish
567 Nutrition, 3rd Edition, Academic Press, San Diego, pp 181-257
568 Seiliez I, Panserat S, Corraze G, Kaushik S, Bergot P (2001) Cloning, tissue distribution and
569 nutritional regulation of a Δ6-desaturase-like enzyme in rainbow trout. Comp Biochem
570 Physiol 130B: 83-93

571 Seiliez I, Panserat S, Corraze G, Kaushik S, Bergot P (2003) Cloning and nutritional
572 regulation of a Δ6-desaturase-like enzyme in marine teleost gilthead seabream (*Sparus*
573 *aurata*). Comp Biochem Physiol 135B: 449-460
574 Sprecher H (2000) Metabolism of highly unsaturated n−3 and n−6 fatty acids. Biochim
575 Biophys Acta 1486: 219-231
576 Sorgeloos P, Leger P, Lavens P (1998) Improved larval rearing of European and Asian
577 seabass, seabream, mahi-mahi, siganid and milkfish using enrichment diets for
578 *Brachiomus* and *Artemia*. World Aquac 19: 78-79
579 Stubhaug I, Tocher DR, Bell JG, Dick JR, Torstensen BE (2005) Fatty acid metabolism in
580 Atlantic salmon (*Salmo salar* L.) hepatocytes, and influence of dietary vegetable oil.
581 Biochim Biophys Acta 1734: 277-288
582 Tocher DR (2003) Metabolism and functions of lipids and fatty acids in teleost fish. Rev Fish
583 Sci 11: 107-184
584 Tocher DR, Dick JR (1999) Polyunsaturated fatty acid metabolism in a cell sulture model of
585 essential fatty acid deficiency in a freshwater fish, carp (*Cyprinus carpio*). Fish Physiol
586 Biochem 21: 257-267
587 Tocher DR, Dick JR (2001) Effects of essential fatty acid deficiency and supplementation of
588 docosahexaenoic acid (DHA; 22:6n-3) on cellular and membrane fatty acid
589 compositions and fatty acyl desaturation in a cell culture model. Prostaglandins
590 Leukotrienes Essent Fatty Acids 64: 11-22
591 Tocher DR, Harvie DG (1998) Fatty acid compositions of major phosphoglycerides from fish
592 neural tissue: (n-3) and (n-6) polyunsaturated fatty acids in rainbow trout (*Salmo*
593 *gairdneri*) and cod (*Gadus marhua*) brains and retinas. Fish Physiol Biochem 5: 229-
594 239

595 Tocher DR, Sargent JR (1990) Effect of temperature on the incorporation into phospholipid
596 classes and the metabolism via desaturation and elongation of (n−3) and (n−6)
597 polyunsaturated fatty acids in fish cells in culture. Lipids 25: 435-442
598 Tocher DR, Agaba M, Hastings N, Teale AJ (2003) Biochemical and molecular studies of the
599 polyunsaturated fatty acid desaturation pathway in fish. In: Browman HI, Skiftesvik AB
600 (ed) The Big Fish Bang. Proceedings of the 26th Annual Larval Fish Conference.
601 Institute of Marine Research, Bergen, pp 211 – 227
602 Tocher DR, Bell JG, Sargent JR (1996) Production of eicosanoids derived from 20:4n-6 and
603 20:5n-3 in primary cell culture of turbot (*Scophthalmus maximus*) brain astrocytes in
604 response to platelet activating factor, substance P and interleukin-1β. Comp Biochem
605 Physiol 115B: 215-222
606 Tocher DR, Carr J, Sargent JR (1989) Polyunsaturated fatty acid metabolism in cultured cell
607 lines : Differential metabolism of (n-3) and (n-6) series acids by cultured cells
608 originating from a freshwater teleost fish and from a marine teleost fish. Comp Biochem
609 Physiol 94B: 367-374
610 Tocher DR, Fonseca-Madrigal J, Bell JG, Dick JR, Henderson RJ, Sargent JR (2002) Effects
611 of diets containing linseed oil on fatty acid desaturation and oxidation in hepatocytes
612 and intestinal enterocytes in Atlantic salmon (*Salmo salar*). Fish Physiol Biochem 26:
613 157-170
614 Tocher DR, Zheng X, Schlechtriem C, Hastings N, Dick JR, Teale AJ (2006) Highly
615 unsatrurated fatty acid synthesis in marine fiesh: Cloning, functional characterization,
616 and nutritional regulation of fatty acyl Δ6 desaturase of Atlantic cod (*Gadus morhua*
617 L.). Lipids 41: 1003-1016
618 Torstensen BE, Bell JG, Rosenlund G, Henderson RJ, Graff IE, Tocher DR, Lie Ø, Sargent
619 JR (2005) Tailoring of Atlantic salmon (*Salmo salar L.*) flesh lipid composition and

620 sensory quality by replacing fish oil with a vegetable oil blend. J Agricult Food Chem
621 53: 10166-10178
622 Turchini GM, Torstensen BE, Ng WK (2009) Fish oil replacement in finfish nutrition. Rev
623 Aquac 1: 10-57
624 Uauy R, Hoffman DR, Peirano P, Birch DG, Birch EE (2001) Essential fatty acids in visual
625 and brain development Lipids 36: 885-895
626 Walford J, Lim TM, Lam TJ (1990) Replacing live foods with encapsulated diets in the
627 rearing of seabass (*Lates calcarifer*) larvae: do the larve ingest and digest protein-
628 membrane microcapsules? Aquaculture 92: 225 – 235
629 Watanabe T, Izquierdo MS, Takeuchi T, Satoh S, Kitajama C (1989) Comparison between
630 eicosapentaneoic and docosahexaenoic acids in terms of essential fatty acid efficacy in
631 larval red seabream. Nippon Suisan Gakk 55: 1635-1640
632 Whelan, J. A., Russell, N. B, Whelan,M. A. 2003. A method for the absolute quantification of
633 cDNA using real-time PCR. J Immunol Methods 278: 261-269
634 Williams KC, Barlow CG, Rodgers L, Hockings I, Agrcopra C, Ruscoe I (2003) Asian
635 seabass *Lates calcarifer* perform well when fed pelleted diets in high protein and lipid.
636 2003. Aquaculture 225: 191-206
637 Wilson R, Sargent JR (1992) High-resolution separation of polyunsaturated fatty acids by
638 argentation thin-layer chromatography, J Chromatogr 623: 403–407
639 Zheng X, Seiliez I, Hastings N, Tocher DR, Panserat S, Dickson CA (2004) Characterization
640 and comparison of fatty acyl Δ6 desaturase cDNAs from freshwater and marine teleost
641 fish species. Comp Biochem Physiol 139B: 269-279
642 Zheng X, Tocher DR, Dickson CA, Dick JR, Bell JG, Teale AJ (2005a) Highly unsaturated
643 fatty acid synthesis in vertebrates: New insights with the cloning and characterization of
644 a Δ6 desaturase of Atlantic salmon. Lipids 40: 13-24

645 Zheng X, Torstensen BE, Tocher DR, Dick JR, Henderson RJ, Bell JG (2005b)
646 Environmental and dietary influences on highly unsaturated fatty acid biosynthesis and
647 expression of fatty acyl desaturase and elongase genes in liver of Atlantic salmon
648 (*Salmo salar*). Biochim Biophys Acta 1734:13-24
649 Zheng X, Ding Z, Xu Y, Monroig O, Morais S, Tocher DR (2009) Physiological roles of fatty
650 acyl desaturases and elongases in marine fish: Characterisation of cDNAs of fatty acyl
651 Δ6 desaturase and elovl5 elongase of cobia (*Rachycentron canadum*). Aquaculture 290:
652 122-131
653

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.

Fig. 4 Functional characterization of the *L. calcarifer* Fad in transgenic yeast (*S. cerevisiae*). Fatty acids were extracted from yeast transformed with pYES2 vector containing CDS of the putative *fad* cDNA and grown in the presence of Δ6 substrates: 18:3n-3 (B) and 18:2n-6 (C), and Δ5 substrates 20:4n-3 (D) and 20:3n-6 (E). Panel A represents a negative control with yeast transformed with empty vector and cultured with 18:3n-3. The first four peaks in all the panels are the main endogenous fatty acids of *S. cerevisiae*, identified as 16:0 (1), 16:1n-7 (2), 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 exogenously added fatty acid substrates 18:3n-3, 18:2n-6, 20:4n-3 and 20:3n-6, respectively. Peak 6 (B) and peak 8 (C) were identified as the desaturation products, 18:4n-3 and 18:3n-6, respectively. Vertical axis, FID response; horizontal axis, retention time.

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

Fig. 6 Desaturation of [1-^{14}C]18:3n-3 (upper panel) and [1-^{14}C]20:5n-3 (lower panel) in hepatocytes and enterocytes from *L. calcarifer*. Results represent the rates conversion (pmol h^{-1} mg^{-1} tissue protein) of ^{14}C-labelled substrate to desaturated/elongated products and are means ± 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 (± 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.

709

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	CCCGGTACCAGGATGGGAGGTGGAGGC
LCDVR1	CCGCTCGAGTCATTTATGGAGATATGCATCG
LCEVF1	CCCAAGCTTAAAATGGAGACCTTCAATCATAAACTG
LCEVR1	CCGCTCGAGTCAATCCACCCTCAGTTTCTT
qLcDesF	TTAATTCCCTTTGCCGTATTTAAA
qLcDesR	AAGAAATCCTGCACAGAATCTGAA
qLcEloF	ATGGTCACGCTCATTATCCTTTT
qLcEloR	AGCATTGGGTGGCGGTTTC

710

711

712

713

714

715

716

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

718

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

720 [a]Includes 20:0 and 22:0.

721 [b]Includes 20:1, 22:1 and 24:1.

722

722

723 **Table 3** 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

725 Data are percentages of amino acid residues that are identical.

726

727

728

729

Table 4 Identity matrix showing the results of a pair-wise comparison between the identities of the amino acid sequenced of fish elongases.

	Cobia Elovl5	Turbot Elovl5	Nile tilapia Elovl5	Atlantic salmon Elovl5	Atlantic cod Elovl5	African catfish Elovl5	Zebrafish Elovl5	Zebrafish Elovl2	Atlantic salmon Elovl2
Barramundi Elovl5	95	89	88	82	78	75	77	53	56
Cobia Elovl5		89	87	73	79	76	77	52	55
Turbot Elovl5			82	79	75	74	74	53	54
Nile tilapia Elovl5				78	73	73	74	53	57
Atlantic salmon Elovl5					77	77	75	54	52
Atlantic cod Elovl5						74	71	53	50
African catfish Elovl5							79	52	55
Zebrafish Elovl5								54	52
Zebrafish Elovl2									75

Data are percentages of amino acid residues that are identical.

743 **Table 5** Functional characterisation of the *L. calcarifer* Δ6 Fad.

Fatty acid substrate	Product	Conversion (%)	Activity
18:3n-3	18:4n-3	32.0	Δ6
18:2n-6	18:3n-6	28.3	Δ6
20:4n-3	20:5n-3	ND	Δ5
20:3n-6	20:4n-6	ND	Δ5
22:5n-3	22:6n-3	ND	Δ4
22:4n-6	22:5n-6	ND	Δ4

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

752 ND, not detected.

753

754

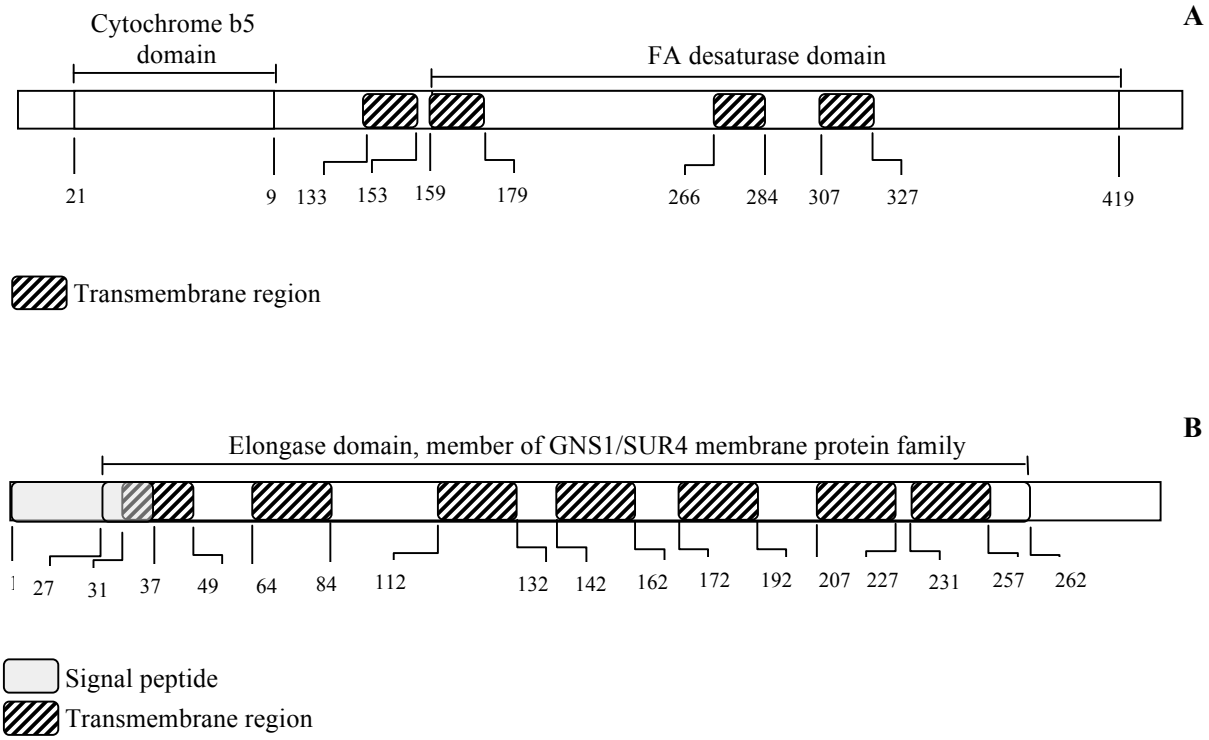
Table 6 Functional characterisation of *L. calcarifer* Elovl5 elongase. Results are expressed as a percentage of total fatty acid substrate converted to elongated product.

Fatty acid substrate	Product	Conversion (%)	Activity
18:4n-3	20:4n-3	45.7	C18 → C20
	22:4n-3	21.9	C20 → C22
	24:4n-3	ND	C22 → C24
	Total	67.6	
18:3n-6	20:3n-6	51.6	C18 → C20
	22:3n-6	21.5	C20 → C22
	24:3n-6	1.3	C22 → C24
	Total	74.4	
20:5n-3	22:5n-3	72.6	C20 → C22
	24:5n-3	3.1	C22 → C24
	Total	75.7	
20:4n-6	22:4n-6	62.6	C20 → C22
	24:4n-6	2.5	C22 → C24
	Total	65.1	
22:5n-3	24:5n-3	1.6	C22 → C24
22:4n-6	24:4n-6	2.9	C22 → C24

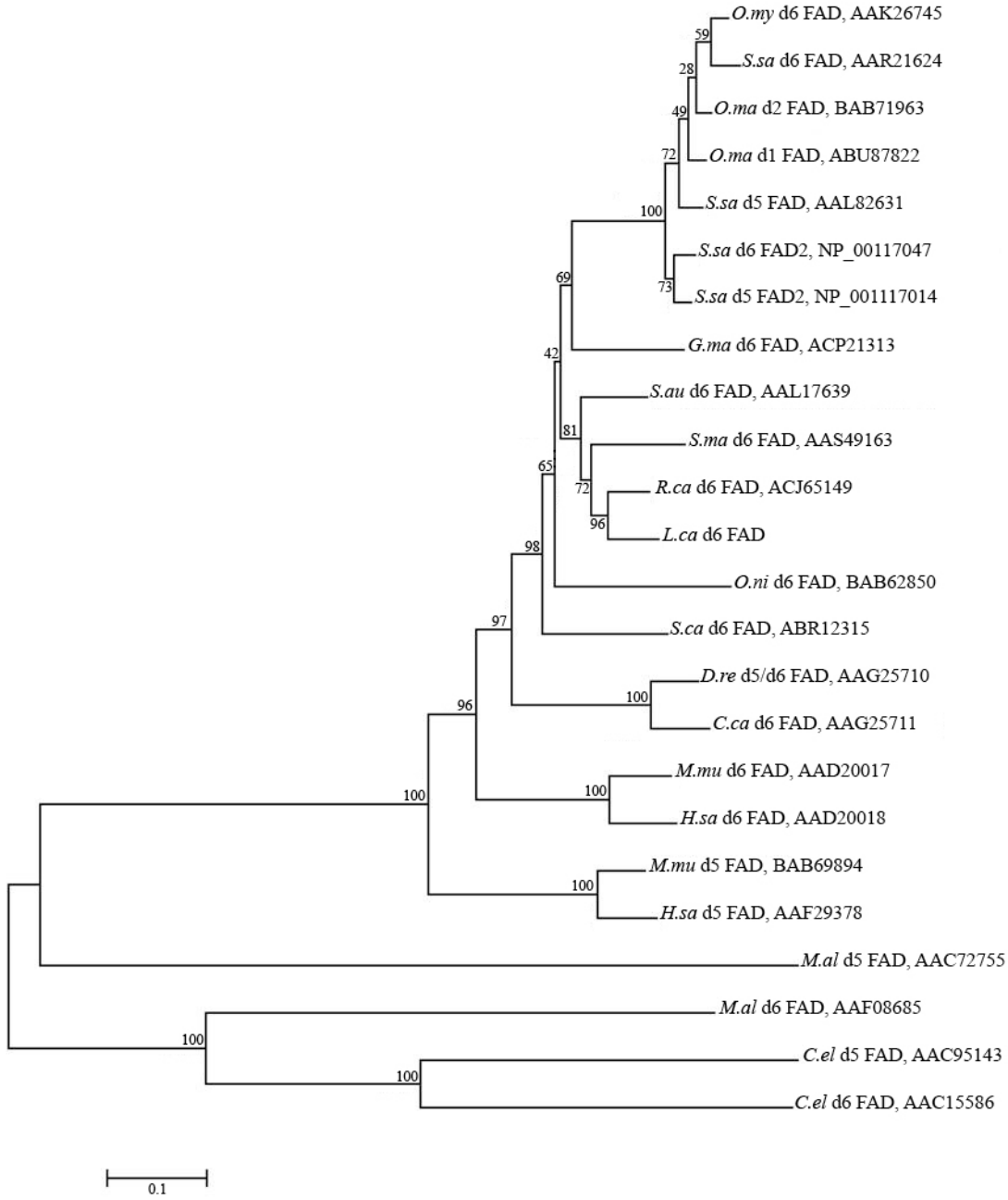
Results are expressed as a percentage of total fatty acid substrate converted to elongated products. Percentage of stepwise conversion into intermediary products of the elongation pathway is also shown.

ND, not detected.

Fig. 1


A
Cytochrome b5 domain
FA desaturase domain
21
9
133
153
159
179
266
284
307
327
419
Transmembrane region
B
Elongase domain, member of GNS1/SUR4 membrane protein family
1
27
31
37
49
64
84
112
132
142
162
172
192
207
227
231
257
262
Signal peptide
Transmembrane region

796 Fig.2



797

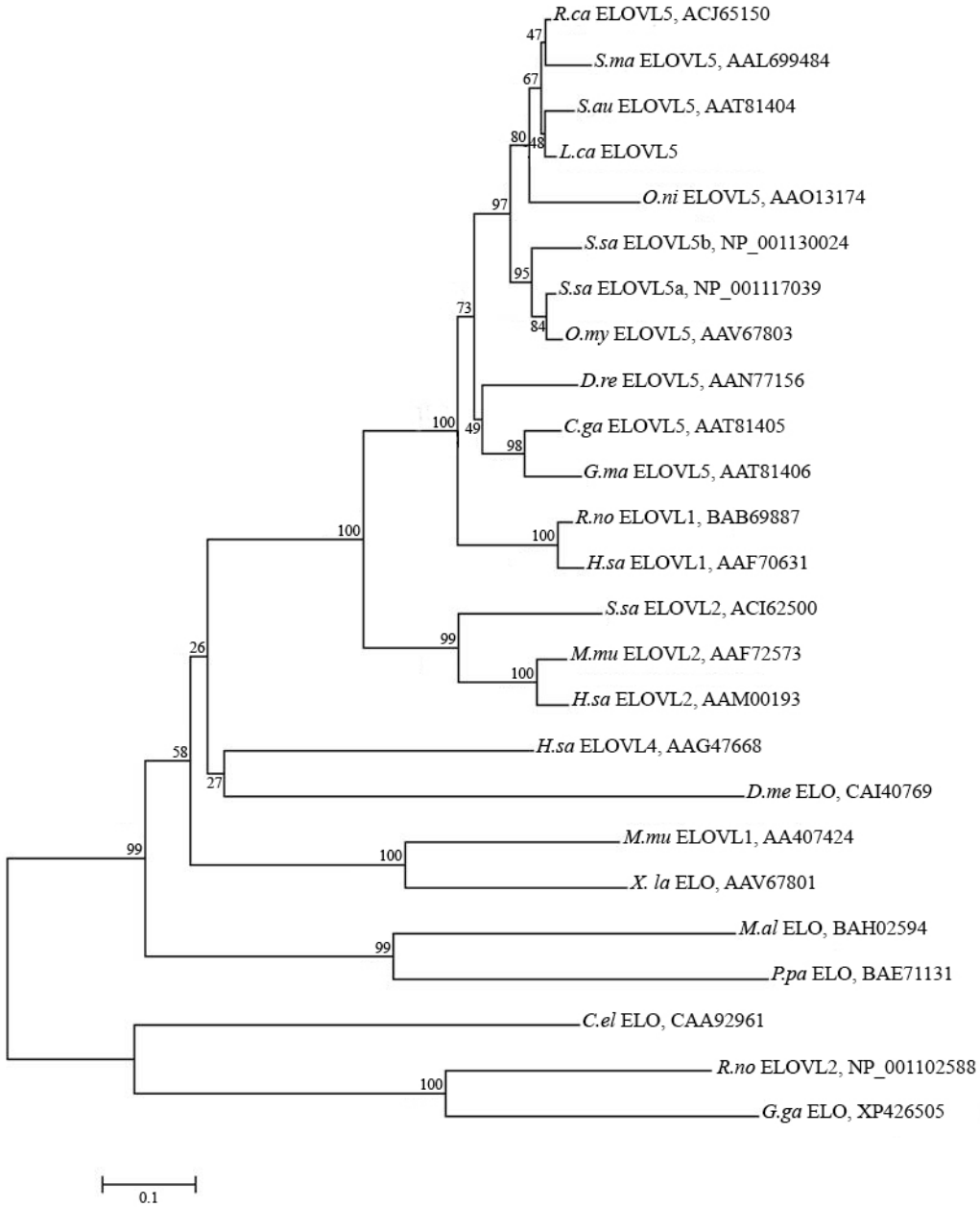
798

799

800

801

802 Fig.3



803

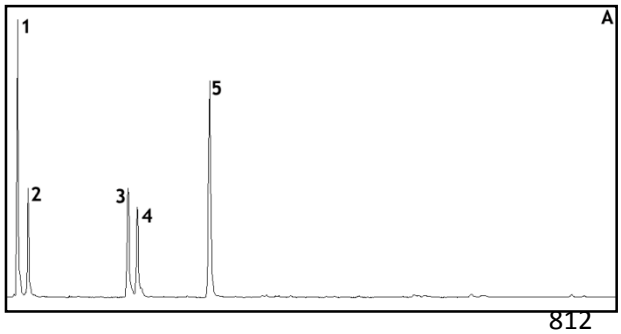
804

805

806

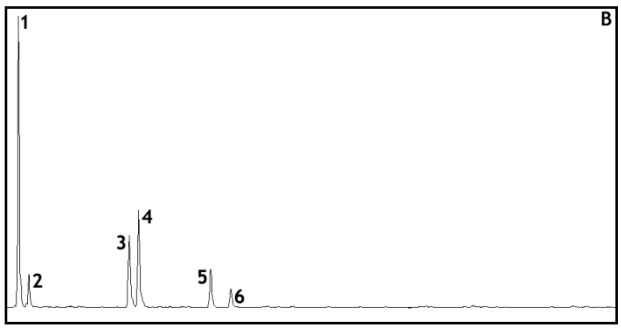
807

808 Fig.4



812

813

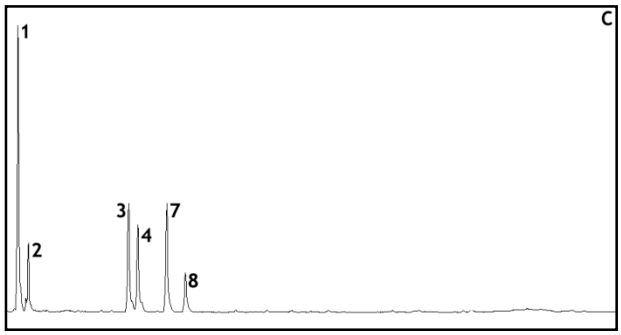


814

815

816

817

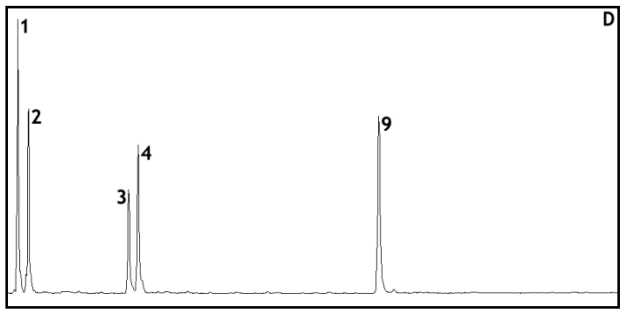


818

819

820

821

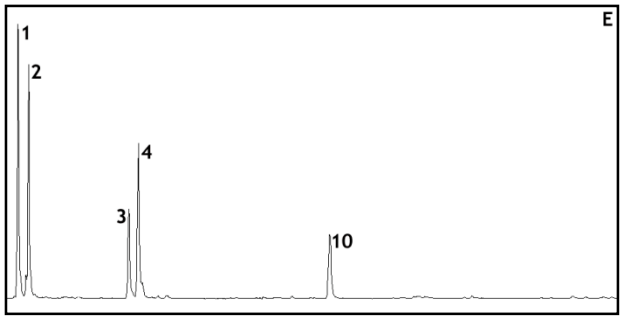


822

823

824

825



826

827 Fig.5.

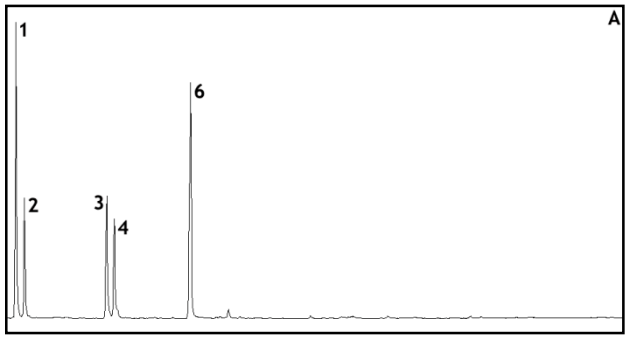
828

829

830

831

832

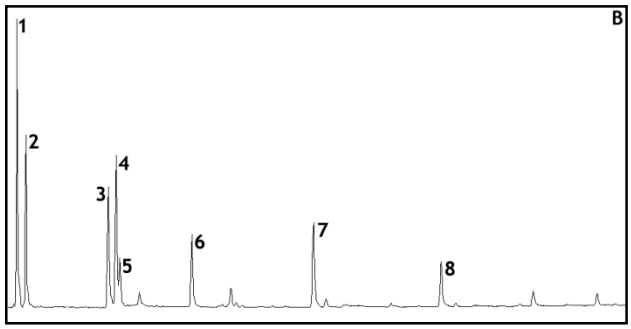


833

834

835

836

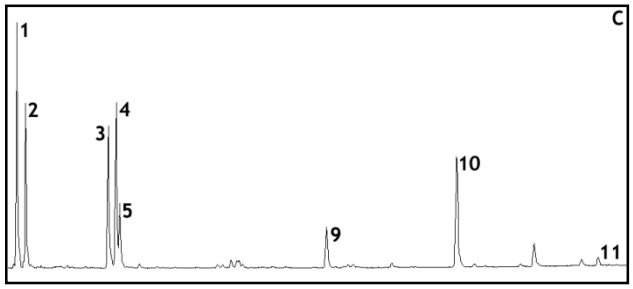


837

838

839

840

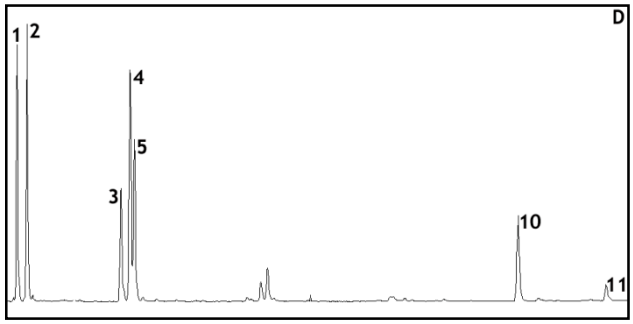


841

842

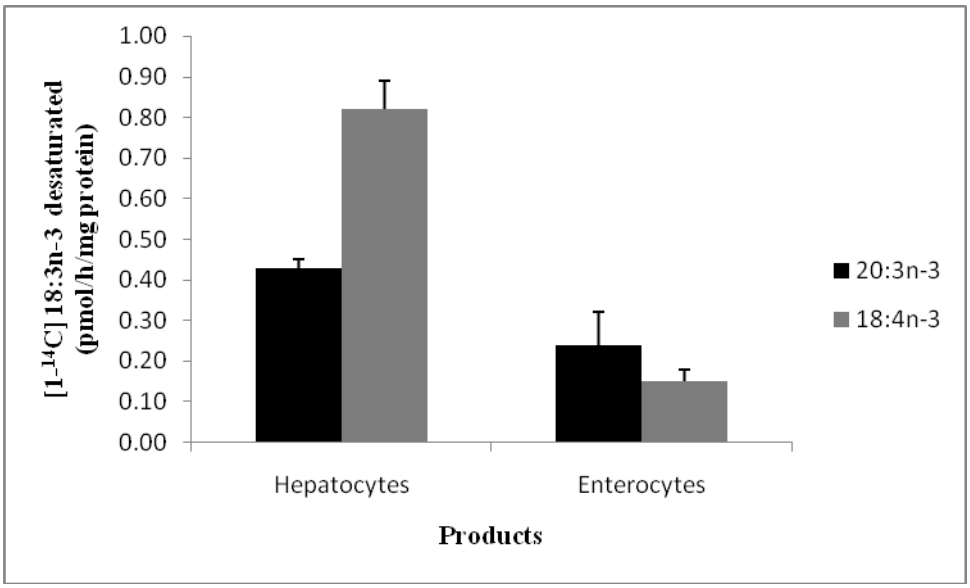
843

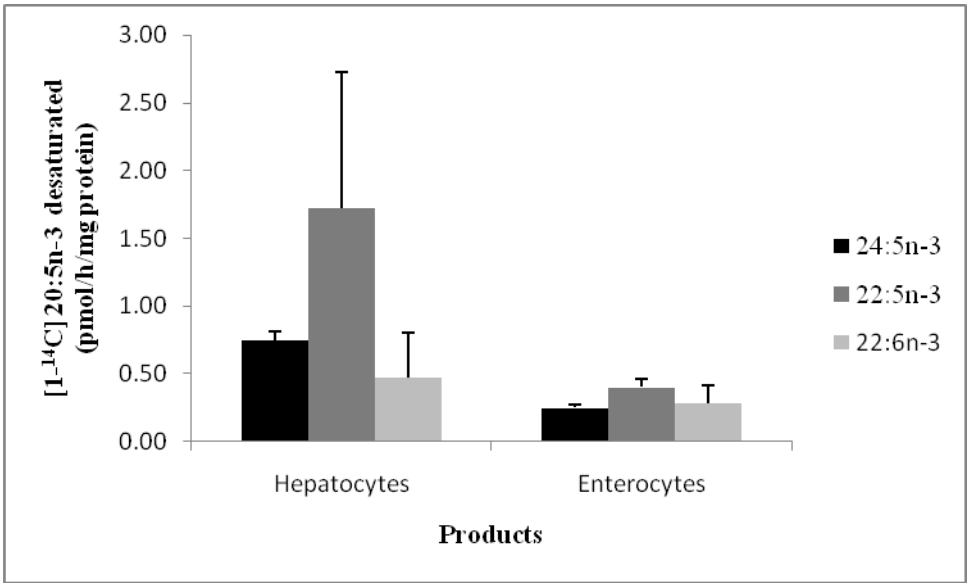
844



845

Fig. 6.


[1-14C] 18:3n-3 desaturated (pmol/h/mg protein)
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Hepatocytes
Enterocytes
Products
20:3n-3
18:4n-3


[1-14C] 20:5n-3 desaturated (pmol/h/mg protein)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Hepatocytes
Enterocytes
Products
24:5n-3
22:5n-3
22:6n-3

854 Fig.7.

855

856