

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

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**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  $\Delta 6$  Fad and Elovl5 elongase enzymes, respectively. The  $\Delta 6$  Fad was equally active towards 18:3n-3 and 18:2n-6, and Elovl5 exhibited elongation activity for C<sub>18-20</sub> and C<sub>20-22</sub> elongation and a trace of C<sub>22-24</sub> activity. The tissue profile of gene expression for  $\Delta 6$  *fad* and *elovl5* 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-<sup>14</sup>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<sub>18</sub> substrates. High expression of  $\Delta 6$  *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 (Dendrinis 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.*

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

HUFA biosynthesis involves a series of fatty acyl desaturase (Fad) and elongase of very long fatty acids (Elovl) enzymes. For the production of DHA, its main precursor, the C18 polyunsaturated fatty acid (PUFA),  $\alpha$ -linolenic acid (ALA, 18:3n-3) is transformed to EPA by  $\Delta$ 6 desaturation, followed by elongation and  $\Delta$ 5 desaturation (Cook and McMaster 2004). Then EPA is converted to DHA by another path involving two further elongation steps, a  $\Delta$ 6 desaturation and a peroxisomal chain shortening process (Sprecher 2000). Investigations of 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  $\Delta 6$  desaturases from common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) (Seiliez et al. 2001), a bifunctional  $\Delta 6/\Delta 5$  Fad from freshwater zebrafish (*Danio rerio*) (Hastings et al. 2001), and  $\Delta 6$  and  $\Delta 5$  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  $\Delta 6$  *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  $\Delta 6$  Fad in white-spotted spinefoot (*Siganus canaliculatus*) both  $\Delta 6$  Fad and Elov15 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

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

#### Bioinformatic analyses

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

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

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

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

#### Cloning of putative fatty acid desaturase and elongase

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

tissues was chopped, incubated with collagenase and cells sieved through 100 µm nylon gauze as described previously (Mourente et al. 2005). One hundred µL of each cell preparation was taken for protein determination by the method of Lowry et al. (1951), following incubation with 1 M NaOH/0.25 % (w/v) SDS for 1 h at 60 °C. For each cell preparation, two 5 ml portions were dispensed into 25 cm<sup>2</sup> tissue culture flasks and incubated at 22 °C for 1 h with 0.5 µCi of either [1-<sup>14</sup>C]18:3n-3 or [1-<sup>14</sup>C]20:5n-3, added as complexes with fatty acid free-bovine serum albumin (BSA) (Ghioni et al. 1997). After incubation, cells were harvested, washed and lipid extracted as described previously (Mourente et al. 2005). Total lipid was transmethylated and FAMES prepared as described above. FAMES were separated by argentation (silver nitrate) TLC (Wilson and Sargent 1992), radiolabelled FAMES located on the plate by autoradiography, and quantified by liquid scintillation after scraping from the TLC plates (Stubhaug et al. 2005).

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

Expression of the target genes was measured in tissues by quantitative real-time PCR (qPCR). Samples of ten different tissues including brain, gill, heart, intestine, kidney, liver, spleen, adipose, red and white muscle, were dissected from four individual fingerlings for RNA extraction using UltraTurrax® homogenizer in Trizol® reagent (Gibco, Invitrogen, USA). Synthesis of first strand cDNA was performed using Verso™ cDNA kit (Thermo Scientific, Waltham, MA, USA). Primer pairs of qDF1-qDR1 and qEF2-qER2 were designed for *fad* and *elovl* with fragment sizes of 156 bp and 197 bp, respectively (Table 1). Amplification of cDNA templates and DNA standards was carried out using ABsolute™ QPCR SYBR Green Mix (Thermo Scientific, USA) under the following conditions: initial denaturation at 95°C for 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<sub>5</sub> (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  $\Delta 6$  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 Elov15 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 [ $1\text{-}^{14}\text{C}$ ] 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-<sup>14</sup>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<sup>-1</sup>.mg protein<sup>-1</sup> and 0.43 pmol.h<sup>-1</sup>.mg protein<sup>-1</sup>, respectively, whereas in enterocytes the conversion rate to 18:4n-3 and 20:3n-3 was 0.15 and 0.24 pmol.h<sup>-1</sup>.mg protein<sup>-1</sup>, respectively. When incubated with [1-<sup>14</sup>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* $\Delta 6$ *fad* and *elovl5* genes

The expression of *L. calcarifer*  $\Delta 6$  *fad* and *elovl5* in ten different tissues was expressed as mean of absolute copy number ( $\pm$  SEM) of  $\Delta 6$  *fad* and *elovl5* transcripts in 25 ng of total RNA (Whelan et al. 2003) (Fig. 7). Both  $\Delta 6$  *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

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

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

n-3 PUFA (Hastings et al. 2001; Zheng et al. 2004, 2005a; Tocher et al. 2006). The *L. calcarifer* Elovl5 displayed elongation of n-3 and n-6, primarily of C<sub>18</sub> and C<sub>20</sub>. *L. calcarifer* Elovl5 showed a preference for n-6 C<sub>18</sub> substrates, but was more active towards n-3 C<sub>20</sub> substrates. This was similar to the activities of Elovl5 elongases from other marine fish like turbot, sea bream and cobia that were more active towards EPA compared to ARA (Zheng et al. 2009). In contrast, Elovl5 elongases cloned from freshwater and diadromous species were more active on n-3 PUFA irrespective of chain length (Agaba et al. 2005). Previous studies of HUFA synthesis in fish cell lines suggested C<sub>18-20</sub> elongase activity was limited in turbot cells (Ghioni et al. 1999). The cloning and functional characterization of Elovl5 elongases from *L. calcarifer*, cobia, sea bream and turbot would indicate that marine fish in general do not lack the gene for C<sub>18-20</sub> elongase (Agaba et al. 2005; Zheng et al. 2009). However, it is reported that it is relatively common for enzymes in the desaturation/elongation pathway to be absent or down-regulated in long established cell lines (Ghioni et al. 1999). It was suggested that there has been evolutionary adaptation to the availability of HUFA in the natural diet of fish species. In fresh water omnivorous fish, there could be a demand for HUFA biosynthesis to produce EPA and DHA, whereas in carnivorous marine fish, there is less requirements for the production of HUFA because of the n-3 HUFA-rich diet. Zebrafish and salmon also express a further elongase, Elovl2 that has high specificity towards C<sub>20</sub> and C<sub>22</sub> HUFAs compared to C<sub>18</sub> PUFA (Morais et al. 2009; Monroig et al. 2009). No homologues of Elovl2 have been found in genomes of the marine fish stickleback, pufferfish and medaka, all species of the order of Acanthopterygii that mostly inhabit the marine ecosystem, another possible molecular mechanism underlying their low HUFA biosynthesis capability (Morais et al. 2009).

Biochemical studies of HUFA biosynthesis have been advanced by the use of cell cultures 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  $\Delta 6$  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- $^{14}\text{C}$ ]18:3n-3 in *L. calcarifer* hepatocytes and enterocytes were approximately 0.8 pmol.h<sup>-1</sup>.mg protein<sup>-1</sup> and 0.4 pmol.h<sup>-1</sup>.mg protein<sup>-1</sup>, respectively. These rates were intermediate between those found earlier for Atlantic salmon and Atlantic cod, with *L. calcarifer* hepatocytes and enterocytes showing higher  $\Delta 6$  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  $\Delta 6$  *fad* and *elovl5* in *L. calcarifer*. Copy number of both  $\Delta 6$  *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  $\Delta 6$  and  $\Delta 5$  *fad* and PUFA elongases are expressed to the greatest extent in liver, intestine and also brain (Zheng et al. 2005b). It has been speculated

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

*L. calcarifer* is known to be a euryhaline or catadromous fish and, in Malaysia, this fish species is reared in the marine environment with a salinity range of 30 to 32 ppt. In nature, wild adult *L. calcarifer* travel to estuaries for spawning and the newly-hatched larvae grow in brackish water before moving back into salt water. However in Australia, *L. calcarifer* originate and return to their original river systems after migration to the estuaries to breed (Grey 1987; Allen 1989; Merrick and Schmida 1984). The present study on Malaysian *L. calcarifer* that had been adapted to freshwater, showed an overall HUFA synthesis pathway as previously described in other marine fish species such as Atlantic cod and sea bream (Tocher et al. 2006). It would be interesting to determine if fresh water *L. calcarifer* display a different pattern of HUFA biosynthesis. This would require the *L. calcarifer* genome to contain  $\Delta 5$  *fad* and possibly *elovl2* genes. Although only  $\Delta 6$  *fad* and *elovl5* were cloned in the present study, exhaustive study with more specific primers was not performed and so the 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<sub>18</sub> 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.

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## Legends to figures

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

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

**Fig. 3** Phylogenetic tree of fatty acyl elongase from *L. calcarifer* (*L.ca*) with other fish species: cobia, *R. canadum* (*R.ca*); turbot, *S. maximus* (*S.ma*); gilthead seabream, *S. aurata* (*S.au*); Nile tilapia, *O. niloticus* (*O.ni*); Atlantic salmon, *S. salar* (*S.sa*); rainbow trout, *O. mykiss* (*O.my*); zebrafish, *D. rerio* (*D.re*); catfish, *C. gariepinus* (*C.ga*); Atlantic cod, *G. marhua* (*G.ma*), mammals: mouse, *M. musculus* (*M.mu*); human, *H. sapiens* (*H.sa*), rat, *R. norvegicus* (*R.no*), insect: fruitfly, *D. melanogaster* (*D.me*), fungus: *M. alpina* (*M.al*), nematode: *C. elegans* (*C.el*), amphibia: toad, *X. laevis* (*X.la*), avian: red jungle fowl, *G. gallus* (*G.ga*) and moss, *P. patens* (*P.pa*). The horizontal branch length is proportional to amino acid substitution per site. The numbers represent the frequencies with which the tree topology 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  $\Delta 6$  substrates: 18:3n-3 (B) and 18:2n-6 (C), and  $\Delta 5$  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  $\pm$  S.D (n=4).

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

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

Primer ID	Sequence (5'→ 3')
Fish-Desat-F	TACACCTGGGAYGAGGTSCAGAYBCAC
Fish-Desat-R	AGGTGTCCYCTGAACCAAGTCGTTGAAG
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> CAATCCACCCTCAGTTTCTT
qLcDesF	TTAATTCCCTTTGCCGTATTTAAA
qLcDesR	AAGAAATCCTGCACAGAATCTGAA
qLcEloF	ATGGTCACGCTCATTATCCTTTT
qLcEloR	AGCATTGGGTGGCGGTTTC

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**Table 2** Fatty acid compositions (% total fatty acids) of commercial diet and brain, liver, intestine and flesh of *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 <sup>a</sup>	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 <sup>b</sup>	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

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

<sup>a</sup>Includes 20:0 and 22:0.

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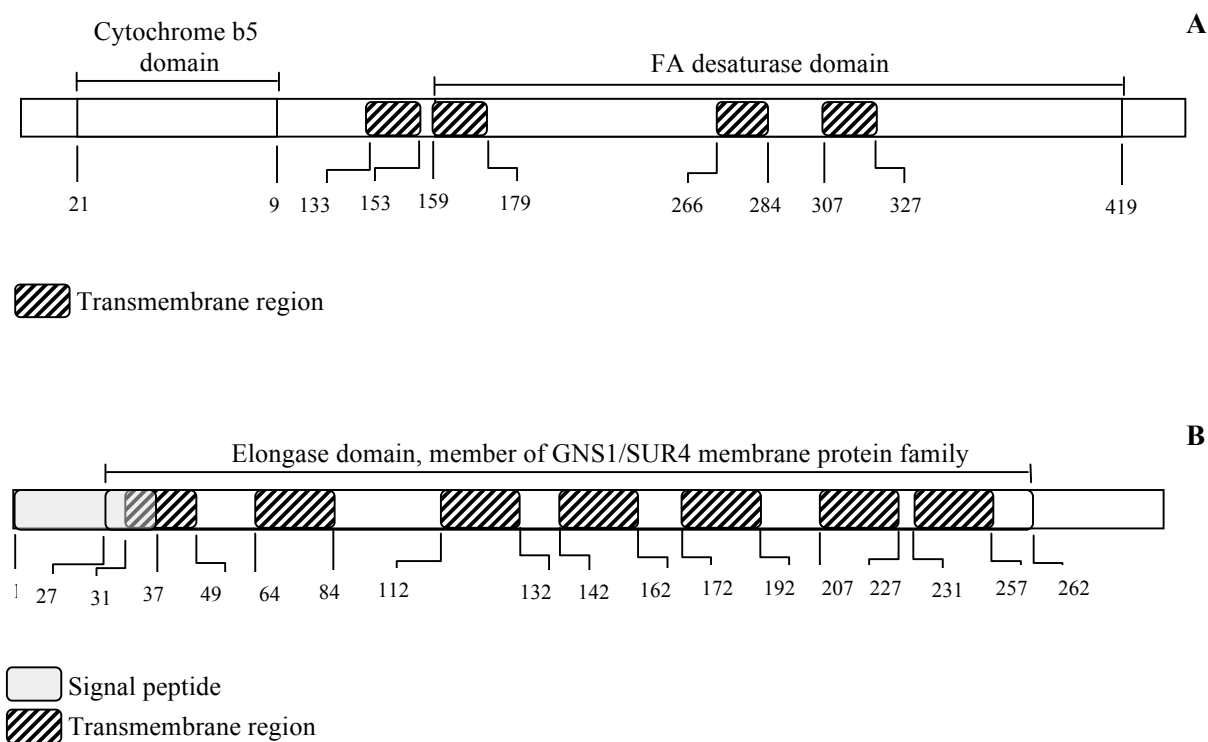
**Table 6** Functional characterisation of *L. calcarifer* Elovl5 elongase. Results are expressed as a percentage of total fatty acid substrate converted to elongated product.

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

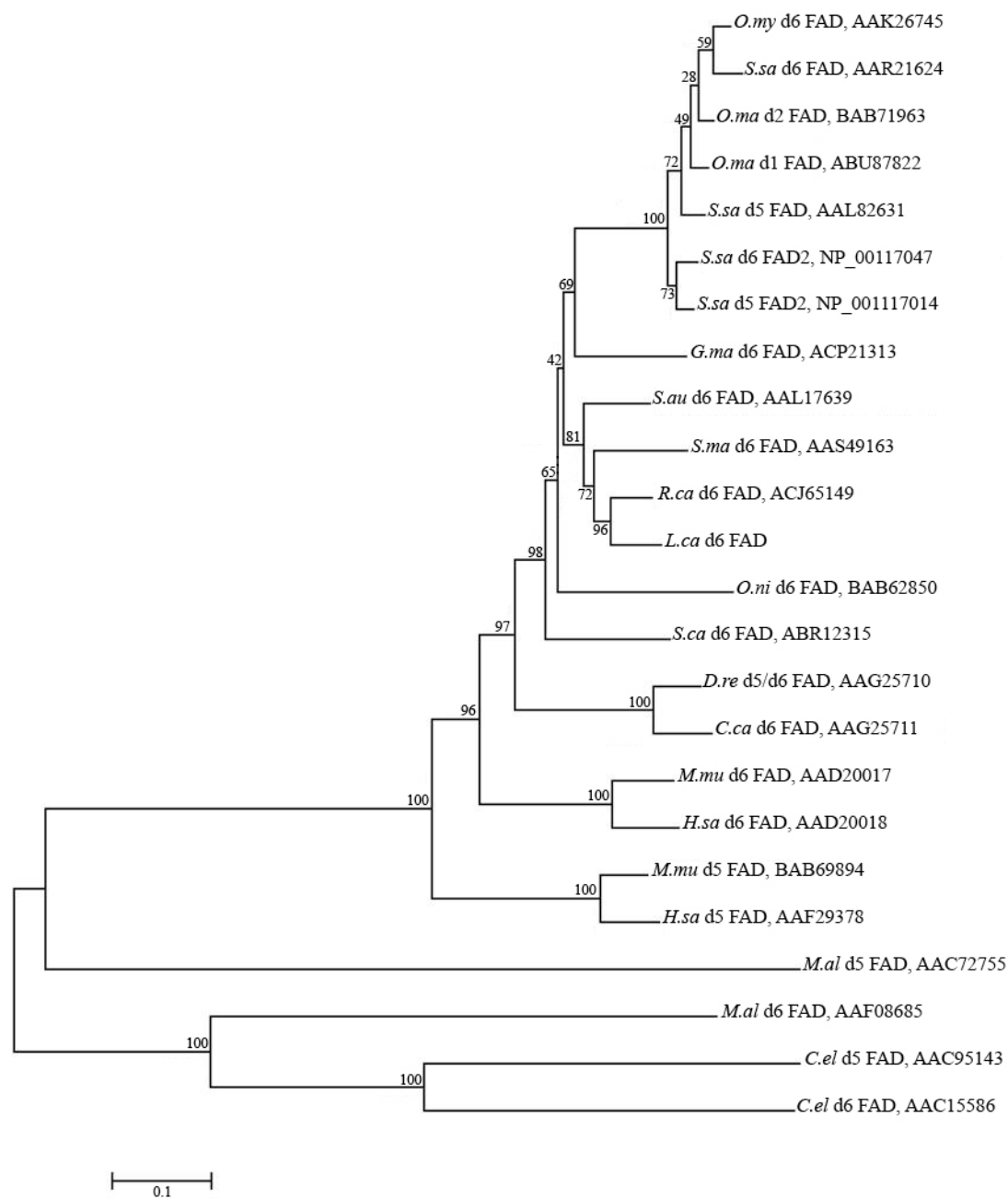
Results are expressed as a percentage of total fatty acid substrate converted to elongated products. Percentage of stepwise conversion into intermediary products of the elongation pathway is also shown.

ND, not detected.

Fig. 1



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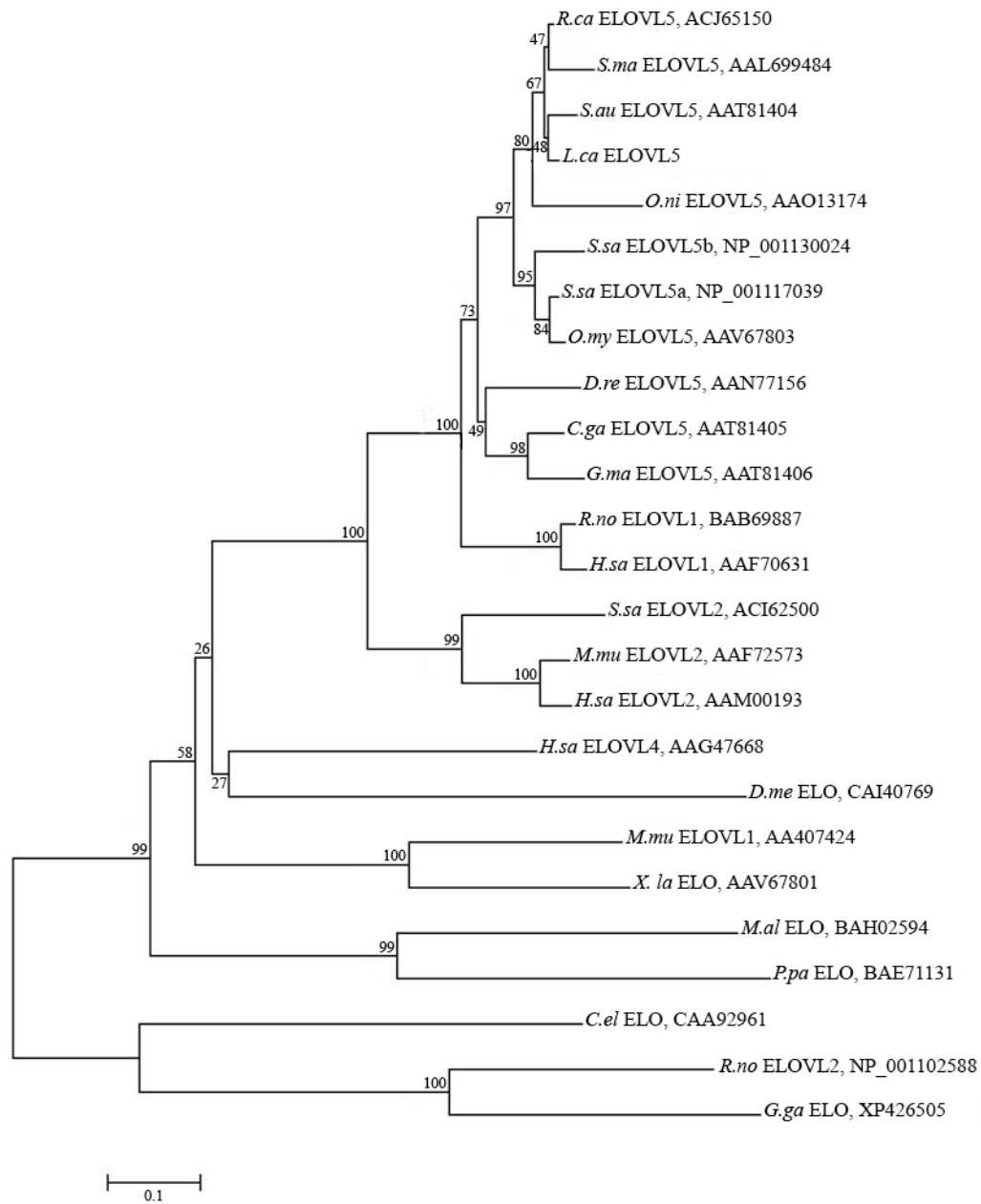
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802 Fig.3



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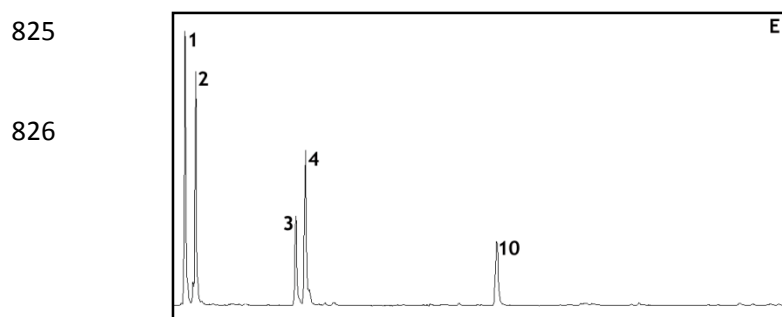
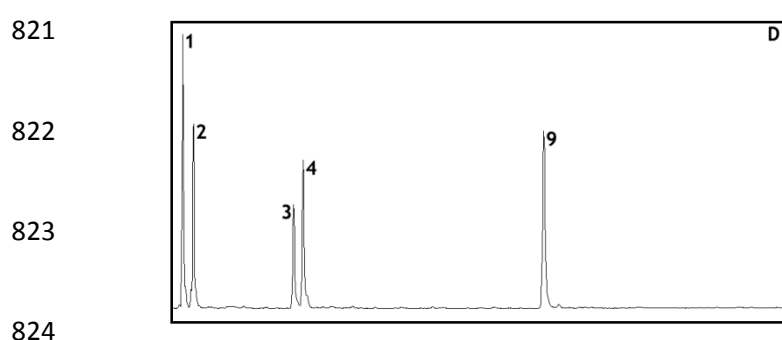
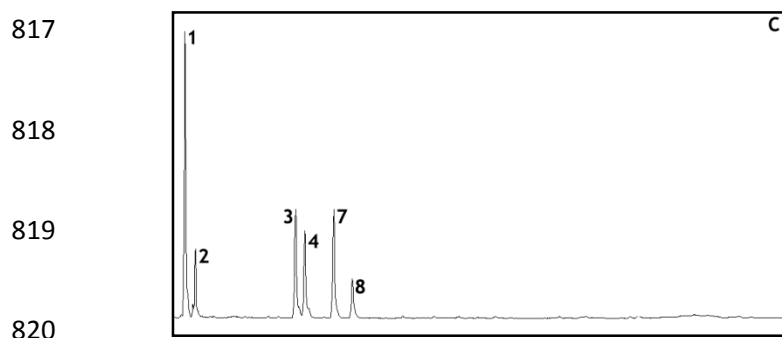
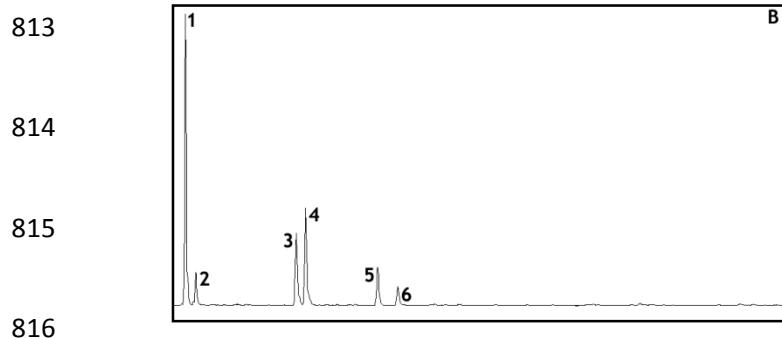
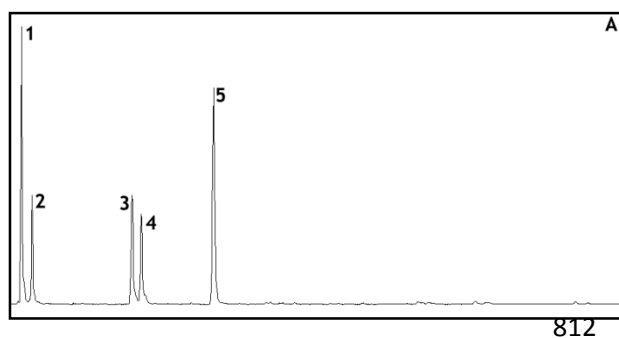
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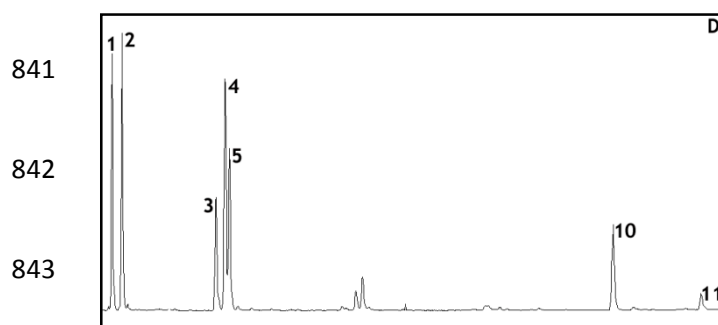
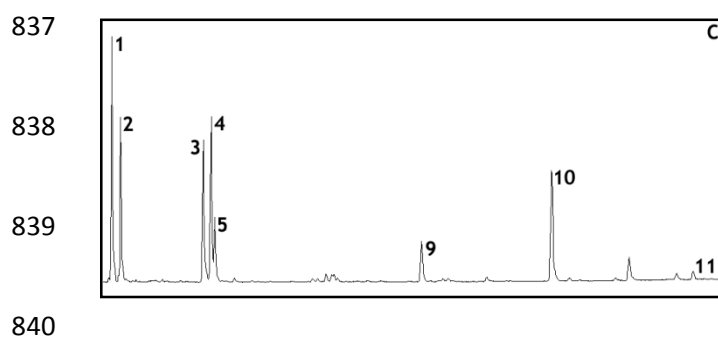
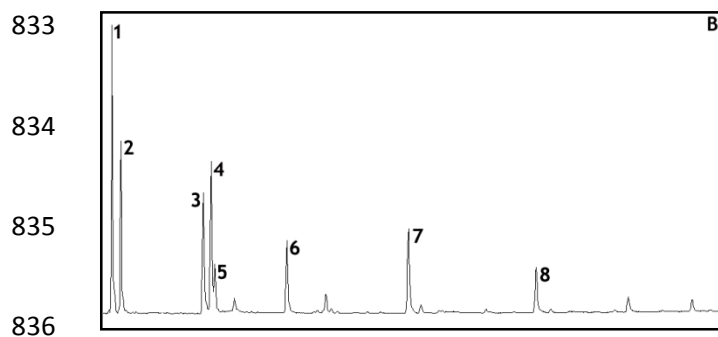
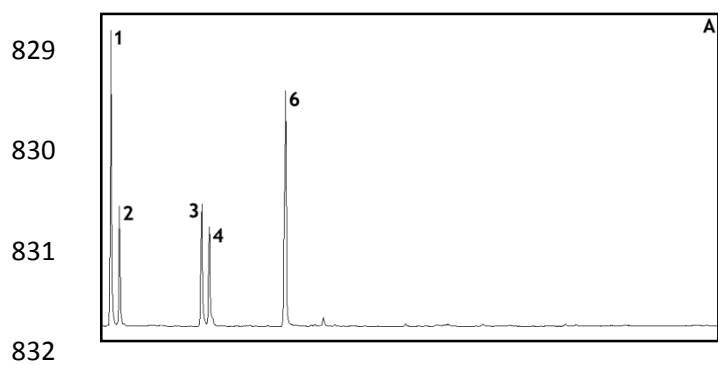


808 Fig.4



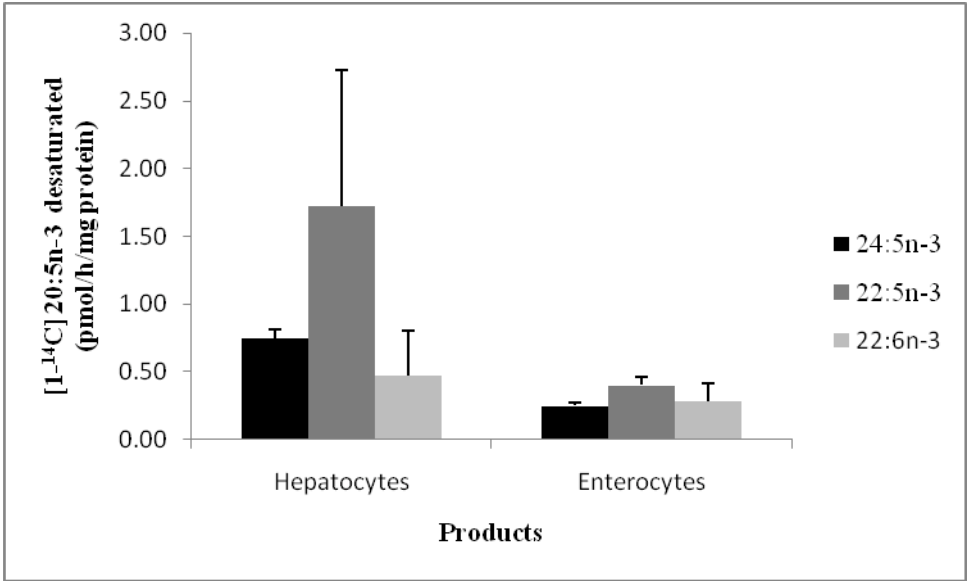
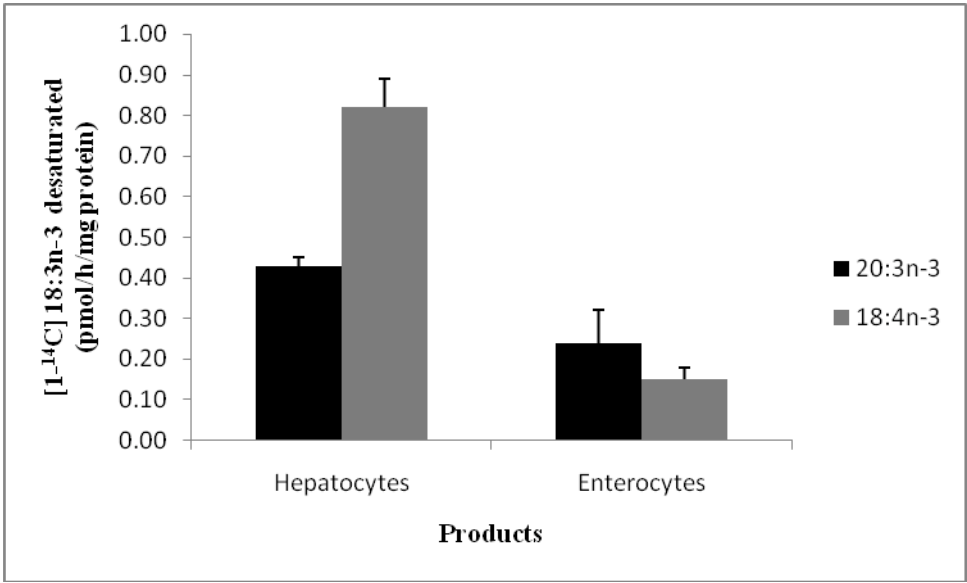
827 Fig.5.

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846 Fig. 6.



854 Fig.7.

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