Title

Expression of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis genes during zebrafish *Danio rerio* early embryogenesis

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Summary

Long-chain polyunsaturated fatty acids (LC-PUFAs) are essential in important physiological processes, many of which are particularly vital during embryonic development. This study investigated the expression of genes encoding enzymes involved in LC-PUFA biosynthesis, namely fatty acyl desaturase (Fad) and Elovl5- and Elovl2-like elongases, during early embryonic development of zebrafish. Firstly, zebrafish *elovl2* cDNA was isolated and functionally characterised in yeast, showing high specificity towards C20 and C22 PUFAs, compared to C18 substrates. Secondly, spatial-temporal expression for *elovl2* and the previously cloned *fad* and *elovl5* were studied during zebrafish early embryonic development. Temporal expression shows that all three genes are expressed from the beginning of embryogenesis (zygote), suggesting maternal mRNA transfer to the embryo. However, a complete activation of the biosynthetic pathway seems to be delayed until 12 hpf, when noticeable increases of *fad* and *elovl2* transcripts were observed, in parallel with high docosahexaenoic acid levels in the embryo. Spatial expression was studied by whole-mount *in situ* hybridization in 24 hpf embryos, showing that *fad* and *elovl2* are highly expressed in the head area where neuronal tissues are developing. Interestingly, *elovl5* shows specific expression in the pronephric ducts, suggesting an as yet unknown role in fatty acid metabolism during zebrafish early embryonic development. The yolk syncytial layer also expressed all three genes, suggesting an important role in remodelling of yolk fatty acids during zebrafish early embryogenesis. Tissue distribution in zebrafish adults demonstrates that the target genes are expressed in all tissues analysed, with liver, intestine and brain showing the highest expression.

Introduction

Long-chain polyunsaturated fatty acids (LC-PUFAs) are essential compounds that play key roles in numerous metabolic and physiological processes ensuring normal cellular function. Some LC-PUFAs, including arachidonic (20:4n-6, ARA) and eicosapentaenoic (20:5n-3, EPA) acids, are precursors of eicosanoids, biologically active compounds that modulate physiological processes including inflammation, reproduction and hemostasis [1]. Increased dietary levels of n-3 LC-PUFAs including EPA and docosahexaenoic acid (DHA, 22:6n-3) have being described as health promoters related to cardiovascular, immune, and inflammatory conditions [2,3]. Additionally LC-PUFAs are constituents of cell membrane phospholipids, determining in part fluidity, and activity of membrane proteins and enzymes involved in transport and signal transduction [4]. This is critical in neuronal tissues where a unique degree of fluidity and compressibility of cell membranes is provided by DHA-rich phospholipids that enable rapid conformational changes required for neurotransmission and photoreception [5].

The biosynthesis of LC-PUFAs in vertebrates involves consecutive desaturation and elongation reactions that convert the essential fatty acids (EFAs) 18:3n-3 (α-linolenic acid) and 18:2n-6 (linoleic acid) to longer-chain, more unsaturated fatty acids (FAs) of the same series, including EPA, DHA and ARA (Fig. 1, [6,7]). Two types of enzymes are responsible for these conversions, namely fatty acyl desaturases (Fad) and elongases of very long fatty acids (Elovl). The former introduce a double bond in the fatty acyl chain at C6 (Δ6 Fad) or C5 (Δ5 Fad) from the carboxyl group. On the other hand, Elovl account for the condensation of activated FAs with malonyl-CoA in the FA elongation pathway. Several members of the Elovl family are involved in PUFA biosynthesis in mammals, those being Elovl5 with substrate specificity for

C18 FAs and Elovl2 for C20 and C22 [8,9]. Additionally, Elovl4 has been speculated to participate in the elongation steps required for synthesis of DHA in mammalian retina [9].

The importance of LC-PUFA in developing organisms is illustrated by their accretion in neuronal tissues during embryogenesis [10-15]. Additionally, deficient production of LC-PUFAs during development can cause neuromuscular defects, cuticle abnormalities, reduced brood size, and altered biological rhythms in *Caenorhabditis elegans* mutants that lack *fat-3*, the gene for Δ6 desaturase [16]. In mammals, it has been suggested that LC-PUFAs are preferentially delivered from the mother to the fetus by transfer across the placenta since fetal LC-PUFA biosynthetic capacity appears to be limited [12,17]. In oviparous organisms such as avians, FAs present in yolk in the form of triacylglycerol or phospholipid molecules are absorbed into the yolk sac membrane for delivery into the embryonic circulation and utilisation for energy, membrane biogenesis, and fat deposition [18]. Amounts of LC-PUFAs deposited by the hen are insufficient to fulfil the requirements of the embryo, and therefore biosynthesis of LC-PUFA by the chicken embryo is, contrary to human fetus, very active in order to compensate such a deficiency [19,20].

In fish, studies have demonstrated that supply of LC-PUFAs to embryos is greatly influenced by the diet of broodstock [21,22], and that suboptimal levels of LC-PUFA delivered to larvae may compromise ability to capture prey in herring (*Clupea harengus*) [23], delay response to visual stimuli in gilthead sea bream (*Sparus aurata*) [24], and impair schooling behaviour in yellowtail (*Seriola quiqueradiata*) [25,26] and Pacific threadfin (*Polydactylus sexfilis*) [27]. Despite the known importance of LC-PUFA supply during embryonic development and their proven selective accumulation in certain lipid classes [28], little is known about the capability of fish

embryos for endogenous biosynthesis to supplement preformed LC-PUFA present in the yolk.

Significant progress has been made in characterising the desaturases and elongases involved in LC-PUFA synthesis in fish including freshwater [29-33] and marine species [34-38]. Zebrafish (*Danio rerio*), a popular model organism in vertebrate developmental biology, has recently been used to study aspects of lipid metabolism [39-42]. Two enzymes involved in LC-PUFA biosynthesis have been characterised in zebrafish, a Fad with dual Δ5/Δ6 activity unique among vertebrates [43], and an elongase with high specificity towards C18 and, to a lesser extent, C20 PUFA [30], similar to elongases found in several other fish species [31-32]. Recently, a cDNA for a second elongase was isolated from salmon and shown to have high specificity towards C20 and C22 PUFA [33].

The present study aimed to investigate the expression of Fad and Elovl enzymes involved in LC-PUFA biosynthesis during early development of zebrafish. Firstly, we isolated and functionally characterised a second zebrafish elongase cDNA important in the biosynthesis of DHA. Secondly, the spatial-temporal expression pattern of the newly cloned elongase, together with the previously isolated Fad [43] and elongase [30], was investigated during zebrafish embryogenesis. Expression of these three enzymes enable zebrafish to synthesise all LC-PUFA from C18 EFA, and therefore zebrafish are an excellent model to study early developmental regulation of LC-PUFA synthesis in vertebrates.

Materials and methods

Fish maintenance

Adult AB wild-type zebrafish strain were maintained at the facilities of the Instituto de Investigaciones Marinas (IIM-CSIC) as described previously [44]. Zebrafish embryos collected from mating of single broodstock couples were isolated and raised at 28.5ºC and staged according to the number of hours post-fertilization (hpf) [43]. For whole-mount *in situ* hybridization analyses, dechorionated embryos were fixed overnight at 4 ºC in 4 % paraformaldehyde in 1xPBS, washed in PBS, and dehydrated through a methanol series, and stored at -20 ºC in 100 % methanol. To inhibit embryo pigmentation, embryo medium was supplemented with 0.003 % 1-phenyl-2-thiourea (PTU, Sigma, Alcobendas, Spain) [44].

Zebrafish Elovl2: cloning and functional characterization by heterologous expression in Saccharomyces cerevisiae

PCR fragments corresponding to the ORF of the putative Elovl2 elongase (gb|NP_001035452|) were amplified from zebrafish liver cDNA using specific primers containing restriction sites (underlined) – Elovl2VF (CCCAAGCTTAGGATGGAATCATATGAAAAAATTGATAAG; *Hin*dIII) and Elovl2VR (CCGCTCGAGTCACTGTAGCTTCTGTTTGGAG; *Xho*I). PCR was performed using the high fidelity PfuTurbo® DNA polymerase (Stratagene, Agilent Technologies, Cheshire, UK), with an initial denaturing step at 95 ºC for 2 min, followed by 35 cycles of denaturation at 95ºC for 30 s, annealing at 57 ºC for 30 s, extension at 72 ºC for 1 min 10 s, followed by a final extension at 72 ºC for 5 min. The DNA fragments were then digested with the corresponding restriction endonucleases (New England BioLabs, Herts, UK) and ligated into a similarly restricted pYES2 yeast expression vector (Invitrogen, Paisley, UK). The purified plasmids (GenElute™ Plasmid Miniprep Kit, Sigma) containing the putative Elovl2

ORF were then used to transform *S. cerevisiae* competent cells (S.c. EasyComp Transformation Kit, Invitrogen). Transformation and selection of yeast with recombinant pYES2-*elovl2* plasmids, yeast culture and FA analysis was performed as described in detail previously [28,41,44]. Briefly, cultures of recombinant yeast were grown in *S. cerevisiae* minimal medium$^{-uracil}$ supplemented with one of the following FA substrates: stearidonic acid (18:4n-3), γ-linolenic acid (18:3n-6), EPA (20:5n-3), ARA (20:4n-6), docosapentaenoic acid (22:5n-3) or docosatetraenoic acid (22:4n-6). Docosapentaenoic and docosatetraenoic acids (>98-99% pure) were purchased from Cayman Chemical Co. (Ann Arbor, USA) and the remaining FA substrates (>99% pure) and chemicals used to prepare the *S. cerevisiae* minimal medium$^{-uracil}$ were from Sigma Chemical Co. Ltd. (Dorset, UK). FAs were added to the yeast cultures at final concentrations of 0.5 (C18), 0.75 (C20) and 1.0 (C22) mM. After 2-days, yeast were harvested and washed, and lipid extracted by homogenization in chloroform/methanol (2:1, v/v) containing 0.01% BHT as antioxidant. FA methyl esters were prepared, extracted, purified, and analysed by GC in order to calculate the proportion of substrate FA converted to elongated FA product as [product area/(product area +substrate area)] x 100. Identities of FA peaks were based on GC retention times and confirmed by GC-MS as described previously [30,43].

Sequence and phylogenetic analysis of Elovl2

The amino acid (AA) sequence deduced from the zebrafish Elovl2 cDNA (gb|NP_001035452|) was compared with human (gb|NP_060240|), mouse (gb|NP_062296|) and rat (gb|NP_001102588|) ELOVL2s, amphibian *Xenopus laevis* (gb|NP_001087564|) and X. *tropicalis* (gb|NP_001016159|) Elovl2s, bird *Taenopygia guttata* (gb|XP_002186815.1|) and *Gallus gallus* (gb|XP_418947|) predicted Elovl2-

like proteins, and salmon Elovl2 (gb|FJ237532|) using the EMBOSS Pairwise Alignment Algorithms tool (http://www.ebi.ac.uk/Tools/emboss/align/). A phylogenetic tree was constructed on the basis of the AA sequence alignments between the putative zebrafish Elovl2, Elovl2 orthologs and Elvol5 proteins, and using the Neighbour Joining method [47]. Confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping through 1000 iterations.

Temporal expression of fad, elovl5, elovl2 during zebrafish ontogeny

To study the expression of the target genes during the embryonic development of zebrafish, total RNA was extracted from pools of 20-30 embryos collected at 0, 3, 6, 9, 12, 14, 24, 48, and 72 hpf using Tri Reagent (Sigma) according to manufacturer's protocol. Five µg of total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase first strand cDNA synthesis kit (Promega, Madison, USA). Qualitative expression of *fad*, *elovl5* and *elovl2* transcripts during embryonic development was determined by reverse transcriptase PCR (RT-PCR) on cDNA samples, with an initial denaturing step at 95 ºC for 2 min, followed by 35 cycles of denaturation at 95 ºC for 30 s, annealing at 60 ºC for 30 s, and extension at 72 ºC for 1 min 40 s, followed by a final extension at 72 ºC for 5 min. Expression of *β-actin* was also determined as reference gene [48]. Primers used for RT-PCR on embryos cDNA samples are shown in Table 1.

Spatial expression of fad, elovl5, elovl2, whole-mount in situ hybridization

To examine the spatial expression of zebrafish *fad*, *elovl5* and *elovl2*, whole-mount *in situ* hybridization (WISH) was performed on 24 hpf zebrafish embryos using digoxygenin (DIG)-labelled antisense riboprobes as previously described [49].

Antisense riboprobes were made from linerarised full length *Danio rerio fad*, *elovl5* and *elovl2* cDNAs.

Tissue distribution of fad, elovl5 and elovl2 mRNA transcripts in zebrafish adults

Expression of the target genes was also measured in adult tissues by quantitative real-time PCR (qPCR). Total RNA from eye, gill, liver, brain, ovary, testis, kidney, muscle, intestine and adipose tissue was extracted as described above, and 1 µg of total RNA reverse transcribed into cDNA (M-MLV reverse transcriptase, Promega). The qPCR was performed using primers shown in Table 1. Copy numbers of target genes were normalised with copy number of the reference gene *18s* rRNA [48]. PCR amplicons of each gene were cloned into pBluescript II KS (Stratagene) that was then linearised, quantified spectrophotometrically (NanoDrop ND-1000, Thermo Scientific, Wilmington, USA), and serial-diluted to generate a standard curve of known copy numbers. The qPCR amplifications were carried out in triplicate using a Quantica machine (Techne, Cambridge, UK) in a final volume of 20 µl containing 5 µl diluted (1/10) cDNA, 0.5 µM of each primer and 10 µl AbsoluteTM QPCR SYBR® Green mix (ABgene, Epsom, UK). Amplifications were carried out with a systematic negative control (NTC - no template control, containing no cDNA). The qPCR profiles contained an initial activation step at 95 °C for 15 min, followed by 40 cycles: 15 s at 95 °C, 15 s at the specific primer pair annealing Tm (Table 1) and 10-15 s at 72 °C. After the amplification phase, a dissociation curve of 0.5 ºC increments from 75 ºC to 90 °C was performed, enabling confirmation of the amplification of a single product in each reaction. The qPCR product sizes were checked by agarose gel electrophoresis and their identity confirmed by sequencing. No primer-dimer formation occurred in the NTC. All reactions were carried out in triplicate and a linear

standard curve was drawn, and absolute copy number of the targeted gene in each sample was calculated.

Fatty acid analyses of zebrafish embryos

In order to monitor the FA changes during embryogenesis, pools of 150-200 embryos were sampled at different stages (0, 9, 24, 48 and 72 hpf) and total lipid extracted, FA methyl esters prepared and analysed as described above.

Statistics

For tissue expression profiles, results expressed as mean normalised values (± SE) corresponding to the ratio between the copy numbers of *fad*, *elovl5* and *elovl2* transcripts and the copy numbers of the reference gene, *18s* rRNA. A one-way analysis of variance (ANOVA) followed by Tukey HSD test ($P<0.05$) was performed to compare the expression level among tissue samples (SPSS, Chicago, USA).

Results

Zebrafish elongase (Elovl2) sequence and phylogenetics

The new zebrafish elongase ORF encodes a protein of 295 AA, sharing 73.6 % identity in AA sequence to the salmon Elovl2, 65.8 - 68.1 % AA identity to mammalian homologues, and 66.9 - 68.4 % identity with predicted Elovl2 sequences from amphibians and birds. The phylogenetic tree (Fig. 2) shows that zebrafish Elovl2 elongase clusters most closely with salmon Elovl2, the only Elovl2 elongase cloned and characterised in fish so far. The fish Elovl2 elongases cluster with the

mammalian, amphibian and bird Elovl2-like elongases, and more distantly from Elovl5-like elongases from mammals and fish.

Functional characterisation

The zebrafish putative Elovl2 elongase was functionally characterised by determining the FA profiles of *S. cerevisiae* transformed with pYES2 containing *elovl2* cDNA ORF insert and grown in the presence of potential FA substrates. The FA composition of the wild yeast consists essentially of 16:0, 16:1n-7, 18:0 and 18:1n-9 [43]. Control treatments consisting of yeast transformed with pYES2 vector without elongase insert contained these FA together with whichever exogenous FA was added as substrate (data not shown), this result being consistent with the well established lack of PUFA elongase activity in *S. cerevisiae* [30,32]. Zebrafish Elovl2 shows activity towards FA substrates from 18 to 22 carbons, with the highest specificity on C20 and C22 substrates (Table 2). The traces show the major endogenous FA (16:0. 16:1n-7, 18:0 and 18:1n-9) and additional peaks corresponding to the substrate and elongation products (Fig. 3). Thus exogenously added 18:4n-3 (Fig. 3A) and 18:3n-6 (Fig. 3B) were elongated to their corresponding C20, C22 and C24 elongation products 20:4n-3, 22:4n-3 and 24:4n-3 (from 18:4n-3) and 20:3n-6, 22:3n-6 and 24:3n-6 (from 18:3n-6). Total conversion of C18 substrates ranged from 20.1 - 23.0 % (Table 2). Higher elongation rates were observed for C20 substrates 20:5n-3 (78.4 %) and 20:4n-6 (65.3 %), being elongated to C22, C24 and C26 products (Fig. 3C-D). Elovl2 also elongated C22 FA substrates to C24 and C26 elongation products. Thus, yeast transformed with pYES2-*elovl2* converted 22:5n-3 to 24:5n-3 and 26:5n-3 (Fig. 3E), and 22:4n-6 was elongated to 24:4n-6 and 26:4n-6 (Fig. 3F). Comparison of peak areas of the endogenous fatty acids in yeast indicates Elovl2 shows some capability to elongate

monounsaturated fatty acids such as 16:1n-7 to 18:1n-7 (5.2 - 7.0 %) and 18:1n-9 to 20:1n-9 (1.5 - 3.1 %). No evidence for elongation of saturated FAs was observed with the zebrafish Elovl2.

Spatial-temporal expression of fad, elovl5 and elovl2 in zebrafish

Temporal expression of *fad*, *elvol5* and *elovl2* was studied by RT-PCR on cDNA samples obtained from embryos at different developmental stages from 0 to 72 hpf (Fig. 4). Results reveal that all three genes are expressed from the zygote stage (0 hpf), with transcripts detected throughout embryonic development. Although comparisons of transcript levels from RT-PCR analyses have to be made cautiously, some temporal patterns can be observed in the expression of *fad*, with a noticeable increasing expression from 12 hpf onwards. Also obvious was the pattern shown by *elovl2*, which showed low expression until 9 hpf, with evident increased expression during 12 to 72 hpf. Changes in expression of *elovl5* with development were less obvious, and *β-actin* reference gene expression was constant during development of zebrafish embryos.

To examine the spatial expression of zebrafish *fad*, *elovl5* and *elovl2*, WISH was performed on 24 hpf zebrafish embryos (Fig. 5). Zebrafish *fad* (Fig. 5B) and *elovl2* transcripts (Fig. 5F) were widely distributed in the head region and specifically in the yolk syncytial layer (YSL) (Fig. 5B, F insets). Similar to the expression patterns of zebrafish *fad* and *elovl2*, zebrafish *elovl5* was also uniformly expressed in the YSL (Fig. 5D inset). However, unlike *fad* and *elovl2*, *elovl5* was specifically expressed in the pronephric ducts of 24 hpf embryos (Fig. 6D). Embryos treated with control sense probes did not show any signal (Fig. 5A, C, E).

Adult tissue distribution of *fad*, *elvol5* and *elovl2* mRNA transcripts was analysed by qPCR (Fig. 6). Results indicate that these genes are expressed in all tissues analysed, with significantly higher levels of these transcripts found in liver than any other tissue. Although no significant differences were found, intestine and brain also showed high levels of transcripts, especially *fad* and *elovl2*. Muscle and gill appear to be tissues with very low expression of the three genes. Generally speaking, expression of zebrafish *fad* gene was higher than those of elongase genes.

Fatty acid composition of zebrafish embryos

Activity of the enzymes involved in LC-PUFA biosynthesis during zebrafish embryogenesis was estimated by comparing levels of C18 substrates (18:3n-3 and 18:2n-6) with levels of all potential desaturation/elongation products (Fig. 7). Total amount of C18 precursors decreased by around 50% over the time-course of embryogenesis, and the levels of products of the biosynthetic pathway showed a steady increase as development proceeded (Fig. 7). Contents of DHA, the most abundant PUFA in zebrafish embryos, initially decreased until 9 hpf, and then increased until the end of embryonic development. The fatty acid profiles (μg of fatty acid per mg of total lipid) of zebrafish embryos at different stages of development are shown in Table 3.

Discussion

Our overall objective is to elucidate the molecular mechanisms controlling LC-PUFA synthesis in vertebrates. Using zebrafish as a model species, the specific aim of the present study was to determine the ontogenic changes in expression of genes of the LC-PUFA synthesis pathway during development. In order to do this, we examined

all the key genes of LC-PUFA synthesis pathway. Previously, we cloned a Fad cDNA from zebrafish that was unique among vertebrate Fads in showing dual Δ6/Δ5 activity [43]. The enzyme product displayed all the fatty acyl desaturation activities required for the synthesis of EPA and DHA [50]. Subsequently, a PUFA elongase cDNA was also isolated from zebrafish [30]. In mammals, *ELOVL2* and *ELOVL5* have been shown to participate in LC-PUFA biosynthesis [8,9,51,52]. Mammalian *ELOVL5* is predominantly involved in the elongation of C18 and C20 PUFA, whereas *ELOVL2* has greatest activity in the elongation of C20 and C22 PUFA and, therefore, appears to be a critical enzyme for the synthesis of C22 and C24 LC-PUFAs [6,8, 51,52]. Functional characterisation showed the first cloned zebrafish PUFA elongase [28] to be similar to elongases found in several other fish species [31,32,38], now all designated as Elovl5 [33]. In contrast to mammalian Elovl5s, fish Elovl5s displayed C22 elongation activity, albeit low, and so it was speculated that Δ6/Δ5 Fad and Elovl5 were the only desaturase and elongase necessary for LC-PUFA synthesis in zebrafish [50]. However, whereas sequence similarity searches against the zebrafish draft genome assembly (Zv7) revealed no further Fad genes, a further elongase-like gene was present in chromosome 24 that, if expressed, could potentially participate in LC-PUFA production. We now report the cDNA cloning and functional characterisation of this second zebrafish elongase (gb|NP_001035452|).

The AA sequence of the newly cloned zebrafish elongase shows high identity to the recently cloned salmon elongase cDNA, which has been shown to be an Elovl2 orthologue [33], and relatively high identity to mammalian ELOVL2s. Phylogenetic analysis groups the zebrafish elongase into a cluster with greatest similarity to salmon Elovl2 and other Elovl2-like genes from mammals, amphibians and birds, and more distantly from Elovl5 elongases. Functional characterisation of the zebrafish cDNA

confirms that the encoded protein elongated C20 and C22 PUFA and so the elongase is designated as an Elovl2. Recombinant yeast containing zebrafish Elovl2 cDNA also produced C26 PUFA from their corresponding C20 and C22 substrates, although these conversions are unlikely to occur *in vivo* because of competition with Δ6 Fad for intermediate C24 PUFAs [6]. As described for mouse and salmon, zebrafish *elovl2* cDNA encodes an enzyme that also has C18-20 elongase activity [8,33]. This is in contrast to human ELOVL2, which is only active towards C20 and C22 substrates [8]. Importantly, the major difference in comparison to zebrafish Elovl5 [30] and other fish Elovl5s, is the high activity towards C22 PUFA shown by zebrafish Elovl2. Therefore, Elovl2 is a key component in the biosynthesis of DHA, where two consecutive elongation steps from 20:5n-3 to 22:5n-3 and 22:5n-3 to 24:5n-3 are required, followed by Δ6 desaturation and chain-shortening [6,53]. These results prove that zebrafish possess all the enzymatic activities required for LC-PUFA synthesis [6], with Δ6 and Δ5 desaturation performed by a single protein [43], and elongation of PUFAs ranging from C18 to 22 catalysed by Elovl5 [30] and the herein characterised Elovl2. The capability of zebrafish for LC-PUFA biosynthesis was previously assessed in isotopic studies with primary hepatocytes showing that the pathway for EPA and DHA synthesis was fully functional [54]. This conclusion is supported by the molecular cloning of the Δ6/Δ5 Fad [43], Elovl5 [30], and the newly characterised Elovl2.

Expression of all Fad and Elovl activities required for LC-PUFA biosynthesis, presents zebrafish as an excellent model to study relationships between expression of these genes and important developmental events where high demands for LC-PUFA are required, especially the formation of neuronal tissues critical for the viability of the embryo [10,16]. In humans, such high requirements for LC-PUFAs are mostly

delivered to the fetus by transfer across the placenta, since fetus LC-PUFA biosynthesis capability has been suggested to be insufficient [17]. Similar to avians, where embryos have been demonstrated to biosynthesise LC-PUFA [55], our results suggest that LC-PUFA biosynthesis occurs in zebrafish embryos, as supported by the presence of *fad*, *elovl5* and *elovl2* transcripts during embryogenesis, and the dynamic FA composition of embryos denoting endogenous production of LC-PUFA.

Temporal expression patterns show that genes of LC-PUFA biosynthesis enzymes in zebrafish are detected at the zygote stage (0 hpf). The only explanation for this is that maternal transfer of the target gene mRNA takes place in zebrafish, since zygotic gene activation is delayed until midblastula transition, which begins at the 512 cell stage at 2.75 hpf [45]. This highlights that the maternal role in LC-PUFA supply to fish embryos is not only transfer of preformed LC-PUFA [21,22], but also transfer of mRNA transcripts that can potentially be translated to active proteins. Expression of *fad*, *elovl5* and *elovl2* genes continues to the end of embryogenesis (72 hpf), and so the pathway could be active throughout to assure the high demands of forming tissues such as brain and retina for LC-PUFAs.

Beyond maternal mRNA transfer and its potential role in LC-PUFA biosynthesis in early stage embryos, the results raise the question of when the embryo itself begins to activate the pathway. Despite the steady increase in total LC-PUFA content during embryogenesis, DHA initially decreases from 0 to 9 hpf. This could indicate that, although mRNA transcripts of *fad*, *elovl5* and *elovl2* were detected during the early developmental stages (0-9 hpf), the biosynthesis pathway is not fully active, at least for producing C22 PUFAs. Supporting this idea is the fact that *elovl2* mRNA transcripts are very low until 9 hpf, possibly limiting biosynthesis of specifically DHA during early embryogenesis [8]. From 9 hpf onwards *de novo* transcription of

embryonic genes likely occurs as indicated by increased levels of *fad* and *elovl2* transcripts from 12 hpf. We may speculate that the increase in expression of *fad* and *elovl2* is due to the development of the central nervous system and retina, occurring in zebrafish at gastrula:bud (10.0 - 10.33 hpf) and 5-9 somites (11.66 - 14.0 hpf), respectively [45]. The spatial expression of *fad* and *elovl2* in zebrafish embryos supports this hypothesis.

Spatial expression patterns of FA metabolism enzymes in zebrafish was first studied by Hsieh and co-workers [56], who determined that stearoyl-CoA desaturase, the enzyme responsible for the synthesis of 18:1n-9 from 18:0, is evenly expressed in all embryo tissues. A more specific expression has now been observed for genes encoding enzymes of the LC-PUFA biosynthesis pathway, with *fad* and *elovl2* genes highly expressed in the head area of zebrafish embryos, probably related to the requirement for ARA and DHA in developing neuronal tissues [10-17]. Interestingly, the Elovl5 elongase was specifically expressed in the pronephric ducts of 24 hpf embryos. Although Elovl5 elongase has been reported to be expressed in kidney of adult fish [33,36,46], there is no obvious explanation for such a specific expression in the pronephric ducts of the embryonic kidney, and further investigations are required to elucidate these findings.

The spatial gene expression data also reveals that the yolk syncytial layer (YSL) may also be an important tissue for embryonic LC-PUFA biosynthesis in zebrafish. The YSL, a structure unique to teleosts, forms a boundary layer between the embryo and the yolk mass. Consequently, all nutrients contained in the yolk must pass through the YSL before being utilised by the developing tissues in the embryo [57]. Indeed the presence of proteolytic enzyme activities in teleost YSL has been reported previously, in agreement with an active role in resorption of yolk lipoproteins [58,59].

Our results show that YSL is likely also to be active in remodelling PUFA during zebrafish embryogenesis. Thus, in addition to hydrolysis of the abundant lipids contained in the yolk [60], the YSL may also influence the composition of the hydrolysed and absorbed FA in a number of ways including conversion of C18 FA and alteration of EPA/DHA ratio prior to transfer to the developing embryonic tissues. As aforementioned, retinal membranes are composed by DHA-rich phospholipids [61,62], and therefore LC-PUFA biosynthetic activity could be expected in developing eye. However, no clear expression of *fad*, *elvol5* and *elovl2* genes in retina was detected in the present study. Previously, zebrafish embryo retina/eye tissue was found to express Elovl4 elongase [63], speculated to be a photoreceptor-specific component of the LC-PUFA biosynthesis pathway [9]. Recently it was shown that Elovl4 was required for the production of C28-C38 very long chain PUFA in retina, brain and sperm [64], and is implicated in the synthesis of very long chain omega-hydroxylated fatty acids present in ceramides of the epidermal permeability barrier in mammals [65].

The present study also demonstrates that adult zebrafish expressed Δ6/Δ5 *fad*, *elovl5* and *elovl2* genes in all tissues analysed. In agreement with previous studies on freshwater fish, our results show that the genes in zebrafish are predominantly expressed in liver, intestine and brain implicating these tissues as the most active in LC-PUFA biosynthesis [33,46]. This is consistent with liver and intestine being the major sites of lipid synthesis and distribution. Furthermore, liver and intestine have been described to be the primary tissues for LC-PUFA synthesis in salmonids [66,67]. Comparison of transcript levels indicates that *fad* expression is consistently higher than that of both elongases. This could be related to the fact that zebrafish Fad, having

dual Δ6/Δ5 activity, is required for all desaturation steps necessary in LC-PUFA biosynthesis [43].

In conclusion the present study demonstrates that zebrafish Elovl2 shows substrate specificity towards C20- and C22-PUFA, indicating its important role in synthesis of LC-PUFA, particularly DHA. All three genes, *fad*, *elovl5* and *elovl2*, are ubiquitously expressed in adult zebrafish tissues with highest expression levels in liver, intestine and brain. Our results demonstrate the presence of *fad*, *elovl5* and *elovl2* transcripts from the zygote stage indicating that maternal transfer of mRNA occurs in zebrafish. Subsequent increases of *fad* and *elovl2* transcript levels however, suggest endogenous embryonic expression is activated at later stages when required for neuronal tissues development. DHA levels during zebrafish embryogenesis and spatial expression of *fad* and *elovl2* support this hypothesis. The WISH data also indicated that other tissues such as YSL and the pronephric ducts have roles in LC-PUFA metabolism in early embryogenesis in *D. rerio*. Whereas the role of YSL appears obvious in remodelling of yolk FA, the role of the pronephric ducts is both intriguing and obscure and requires further investigation.

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Legends to Figures

Fig. 1. Biosynthesis pathways of long-chain polyunsaturated fatty acids from C18 precursors, 18:3n-3 and 18:2n-6 [6].

Fig. 2. Phylogenetic tree comparing the putative zebrafish Elovl2, Elovl2 orthologs and Elvol5 proteins The tree was constructed using the Neighbour Joining method [47] using MEGA4. The horizontal branch length is proportional to amino acid substitution rate per site. The numbers represent the frequencies (%) with which the tree topology presented was replicated after 1000 iterations.

*Predicted proteins (GenBank).

Fig. 3. Functional characterisation of the zebrafish putative elongase Elovl2 in transgenic yeast (*Saccharomyces cerevisiae*) grown in the presence of fatty acid substrates 18:4n-3 (A), 18:3n-6 (B), 20:5n-3 (C), 20:4n-6 (D), 22:5n-3 (E) and 22:4n-6 (F). Fatty acids were extracted from yeast transformed with pYES2 vector containing the ORF of the putative elongase cDNA as an insert. Peaks 1-4 represent the main endogenous FAs of *S. cerevisiae*, namely 16:0 (1), 16:1n-7 (2), 18:0 (3) and 18:1n-9 (4). Substrates ("*") and their corresponding elongated products are indicated accordingly in panels A-F. Vertical axis, FID response; horizontal axis, retention time.

Fig. 4. RT-PCR analyses of the temporal expression patterns of *fad*, *elvol5*, and *elovl2* during zebrafish *Danio rerio* embryogenesis (0 to 72 hpf at 28.5 ºC). Expression of the housekeeping gene *β-actin* is also shown. hpf, hours post-fertilization; NTC, no template control.

Fig. 5. Whole mount *in situ* hybridization showing the expression of *fad* (A, B), *elovl5* (C, D), and *elovl2* (E, F) in 24 hpf embryos. Embryos were hybridised with either sense (A, C, D) or antisense probes (B, D, F). Strong signal was observed in the head region and yolk syncytial layer (B, F inset) of 24-hpf embryos when antisense *fad* and *elovl2* probes were used (A), but no signal was observed for sense probe (E). Similar results were observed for *elovl5* (C, D), however, its expression was specifically localised in the pronephric ducts (D) and the yolk syncytial layer (D inset). Lateral views, dorsal upward, anterior to the left (A-F). YSL, yolk syncytial layer; PD, pronephric ducts; H, head; e, eye. Scale bars: 100 μm.

Fig. 6. Tissue distribution of the *fad*, *elovl5* and *elovl2* transcripts (mRNA) in zebrafish adults. Absolute copy numbers were quantified for each transcript and were normalised by absolute levels of 18s RNA. Results are means ± S.E. (n = 3). L, liver; I, intestine; B, brain; E, eye; K, kidney; A, adipose; M, muscle; O, ovary; T, testis; G, gill. * $P < 0.05$ as determined by one-way ANOVA and Tukey's test.

Fig. 7. Fatty acid contents during zebrafish embryogenesis. Contents (μg of fatty acid per mg of total lipid) of substrates (sum of 18:3n-3 and 18:2n-6) and potential products (sum of 18:4n-3, 18:3n-6, 20:3n-3, 20:4n-3, 20:2n-6, 20:3n-6, 20:5n-3, 20:4n-6, 22:4n-3, 22:5n-3, 22:6n-3, 22:4n-6, 24:5n-3, 24:4n-6, 24:6n-3 and 24:5n-6) of long-chain polyunsaturated fatty acid biosynthesis enzymes Fad, Elovl5 and Elovl2. Levels of docosahexaenoic acid (DHA; 22:6n-3) are also shown.

Tables

Table 1. Sequence and annealing temperature (Tm) of the primer pairs used, size of the fragment produced and accession number of the sequence used as reference for primer design, for Elovl2 ORF cloning, reverse transcriptase PCR (RT-PCR) performed in embryo samples, and quantitative real time PCR (qPCR) determinations of transcripts in adult tissues.

Aim	Transcript	Primer	Primer sequence	Fragment	Tm	Accession No[1].
ORF cloning	*elovl2*	Elovl2VF	5'-CCCAAGCTTAGGATGGAATCATATGAAAAAATTGATAAG-3'	184 bp	60ºC	NM_001040362
		Elovl2VR	5'-CCGCTCGAGTCACTGTAGCTTCTGTTTGGAG-3'			
RT-PCR	*fad*	FadF1	5'-AGGAGGTGCAGAAACACACC-3'	1264 bp	60ºC	AF309556
		FadR1	5'-CTCGCCAGATTTCTCCAAAG -3'			
	elovl5	Elovl5F1	5'-CTCAGGGTCACAGGATGGTT-3'	768 bp	60ºC	NM_200453
		Elovl5R1	5'-CTCCATTAGTGTGGCCGTTT-3'			
	elovl2	Elovl2F1	5'-AAAGAGATACCCGCGTGAGA-3'	810 bp	60ºC	NM_001040362
		Elovl2R1	5'-TTGGAGTTGGCTCCGTTTAG-3'			
	β-actin	β-ActinF1	5'-CTCTTCCAGCCTTCCTTCCT-3'	246 bp	60ºC	NM_131031
		β-ActinR1	5'-CACCGATCCAGACGGAGTAT-3'			
qPCR	*fad*	FadF2	5'-CATCACGCTAAACCCAACA-3'	158 bp	60ºC	AF309556
		FadR2	5'-GGGAGGACCAATGAAGAAGA-3'			
	elovl5	Elovl5F2	5'-TGGATGGGACCGAAATACAT-3'	173 bp	60ºC	NM_200453
		Elovl5FR2	5'-GTCTCCTCCACTGTGGGTGT-3'			
	elovl2	Elovl2F2	5'-CACTGGACGAAGTTGGTGAA-3'	184 bp	60ºC	NM_001040362
		Elovl2R2	5'-GTTGAGGACACACCACCAGA-3'			
	18s	18sF1	5'-CCGCTATTAAGGGTGTTGGA-3'	134 bp	62ºC	NM_173234
		18sR1	5'- GGCGAGGGTTCTGCATAATA-3'			

[1] GenBank (http://www.ncbi.nlm.nih.gov/)

Table 2. Functional characterisation of the newly characterised Elovl2 elongase. Results are expressed as a percentage of total fatty acid (FA) substrate converted to elongated product. Percentage of stepwise conversion into intermediary products of the elongation pathway is also shown.

FA Substrate	Product	% Conversion	Activity
18:4n-3	20:4n-3	6.0	C18→20
	22:4n-3	7.0	C20→22
	24:4n-3	10.0	C22→24
	26:4n-3	0.0	C24→26
		Total: 23.0	
18:3n-6	20:3n-6	7.1	C18→20
	22:3n-6	4.2	C20→22
	24:3n-6	8.8	C22→24
	26:3n-6	0.0	C24→26
		Total: 20.1	
20:5n-3	22:5n-3	7.7	C20→22
	24:5n-3	63.1	C22→24
	26:5n-3	7.6	C24→26
		Total: 78.4	
20:4n-6	22:4n-6	3.9	C20→22
	24:4n-6	52.2	C22→24
	26:4n-6	9.2	C24→26
		Total: 65.3	
22:5n-3	24:5n-3	43.2	C22→24
	26:5n-3	11.0	C24→26
		Total: 54.2	
22:4n-6	24:4n-6	34.1	C22→24
	26:4n-6	9.3	C24→26
		Total: 43.4	

Table 3. Fatty acid composition of zebrafish embryos at different stages of development. Results are expressed in µg of fatty acid per mg of total lipid.

Fatty acid	*0 hpf*	*9 hpf*	*24 hpf*	*48 hpf*	*72 hpf*
14:0	2.3	5.1	5.1	4.4	3.2
15:0	1.2	2.0	2.1	1.4	1.7
16:0	141.2	130.8	120.7	121.4	122.8
18:0	53.6	47.3	42.5	41.4	42.2
20:0	0.0	0.5	0.6	1.0	1.2
Total saturated	198.4	185.6	171.1	169.6	171.0
16:1n-9	3.5	3.4	3.4	3.3	3.7
16:1n-7	8.2	18.6	18.0	14.1	12.2
18:1n-9	87.6	93.1	85.5	80.1	81.8
18:1n-7	18.9	24.8	24.3	21.6	19.6
20:1[1]	3.0	5.3	6.8	4.3	3.2
22:1[2]	0.0	3.1	3.8	0.0	0.0
24:1n-9	0.0	0.4	0.5	0.3	0.3
Total monounsaturated	121.3	148.7	142.4	123.8	120.8
18:2n-6	41.5	21.8	22.7	23.5	17.6
18:3n-6	0.0	0.7	0.8	0.8	0.6
20:2n-6	2.5	1.5	1.8	1.7	1.9
20:3n-6	4.9	3.5	3.9	4.1	4.8
20:4n-6	11.7	14.2	15.6	16.3	16.3
22:4n-6	1.5	0.9	0.9	1.0	1.3
22:5n-6	1.0	4.1	4.2	5.1	5.1
Total n-6 PUFA	63.0	46.7	50.0	52.4	47.6
18:3n-3	3.2	4.5	3.4	3.1	2.6
18:4n-3	0.0	0.8	1.0	0.0	0.6
20:3n-3	0.0	1.0	0.9	0.8	0.9
20:4n-3	1.1	3.1	2.5	2.5	2.1
20:5n-3	19.3	44.4	43.4	41.3	42.9
22:5n-3	5.9	13.0	15.9	11.9	13.3
22:6n-3	91.5	63.1	74.4	86.1	89.7
Total n-3 PUFA	121.0	129.9	141.4	145.7	152.1
C16 PUFA	0.0	4.2	3.0	3.8	3.6
Total PUFA	184.1	180.8	194.4	201.9	203.3

[1] predominantly n-9 isomer; [2] predominantly n-11 isomer;
PUFA, polyunsaturated fatty acid; hpf, hours post-fertilization

Figures

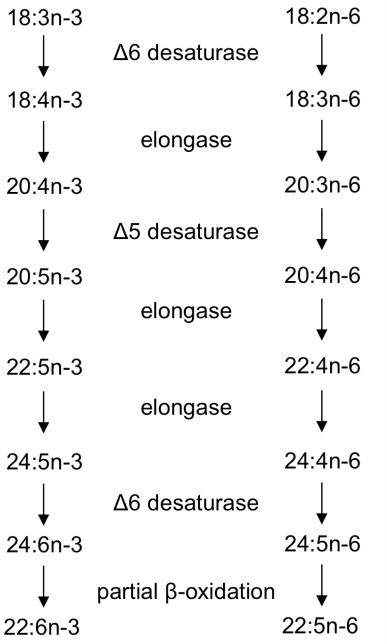

Fig. 1

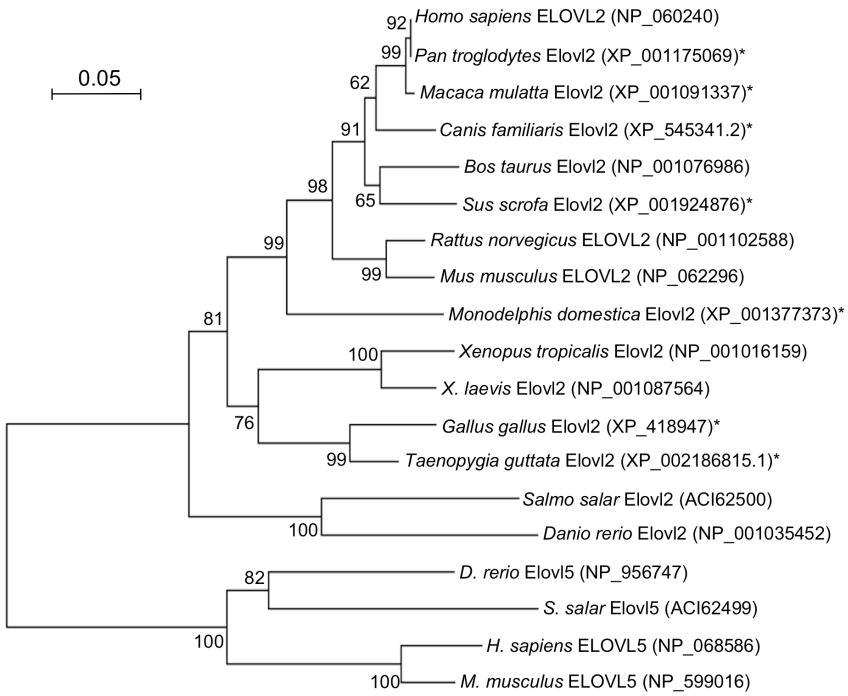

Fig. 2

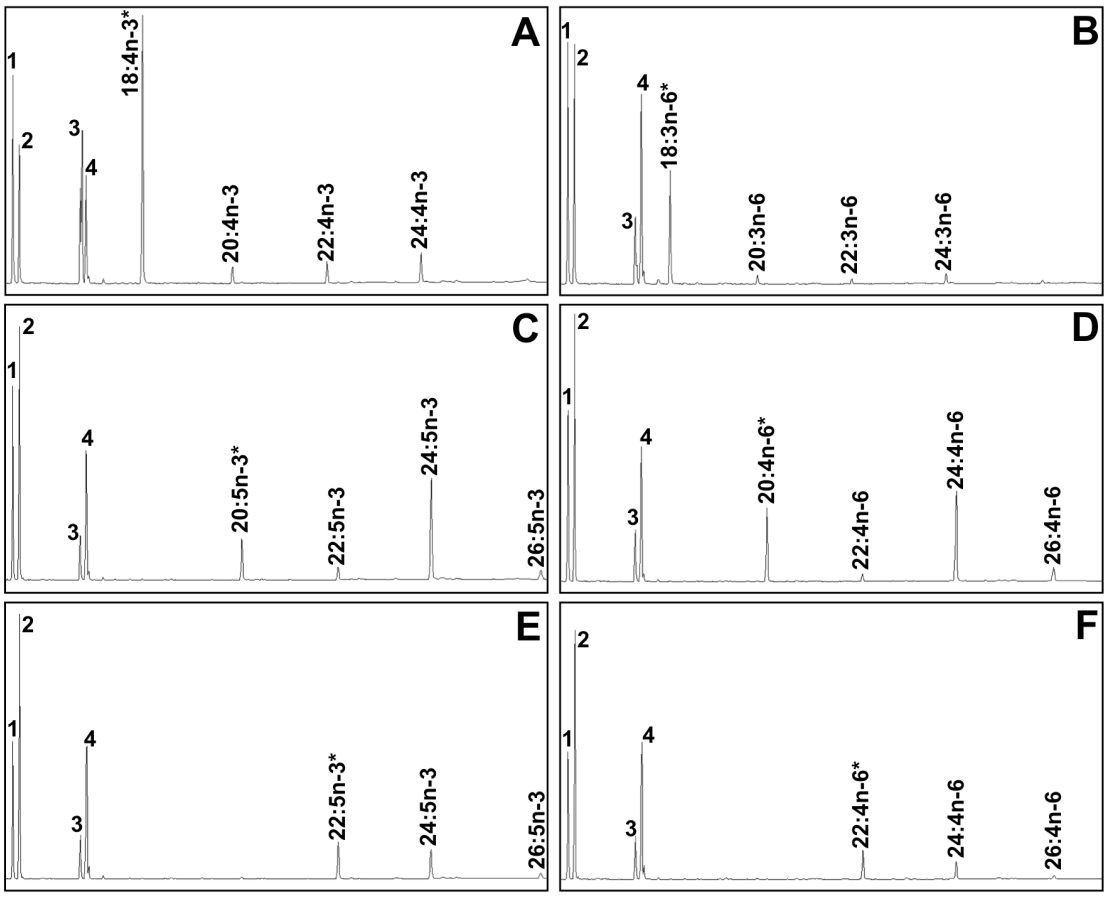
A
1
2
3
4
18:4n-3*
20:4n-3
22:4n-3
24:4n-3
B
1
2
3
4
18:3n-6*
20:3n-6
22:3n-6
24:3n-6
C
1
2
3
4
20:5n-3*
22:5n-3
24:5n-3
26:5n-3
D
1
2
3
4
20:4n-6*
22:4n-6
24:4n-6
26:4n-6
E
1
2
3
4
22:5n-3*
24:5n-3
26:5n-3
F
1
2
3
4
22:4n-6*
24:4n-6
26:4n-6

Fig. 3

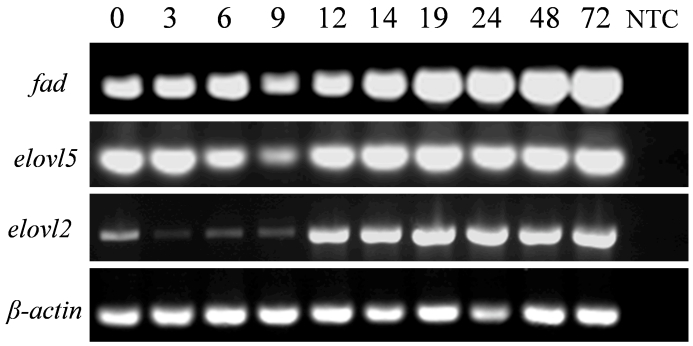
0 3 6 9 12 14 19 24 48 72 NTC
fad
elovl5
elovl2
β-actin

Fig. 4

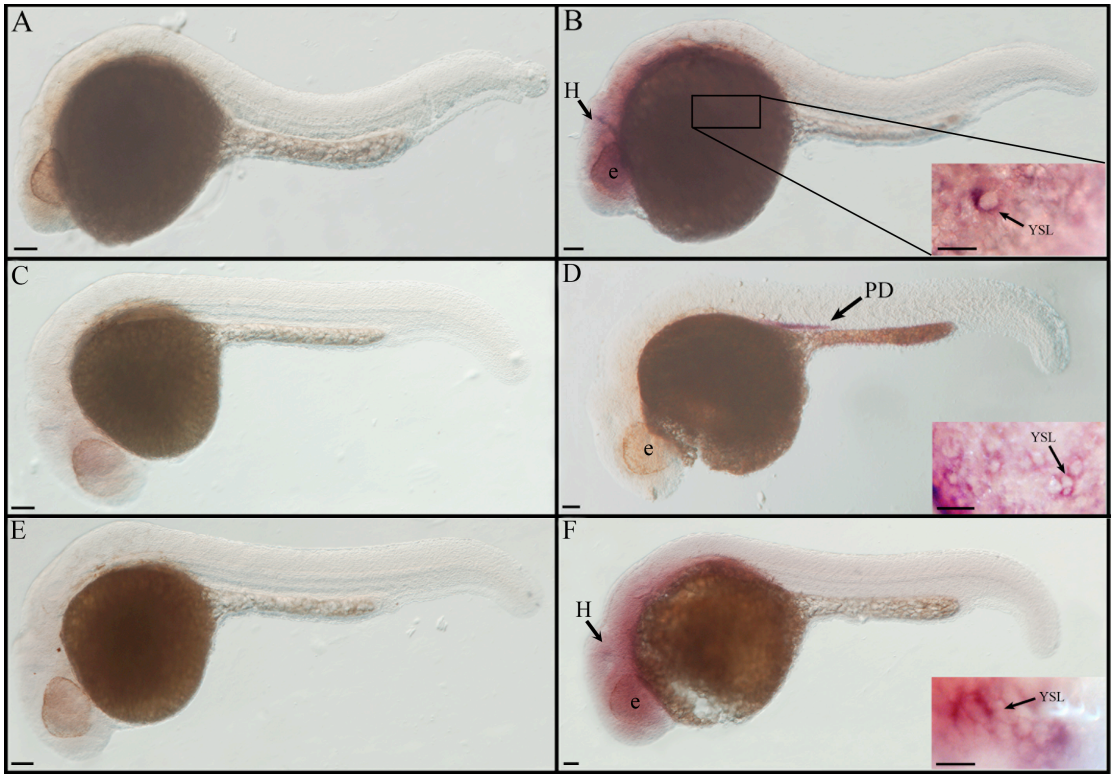
A
B
H
e
YSL
C
D
PD
e
YSL
E
F
H
e
YSL

Fig. 5

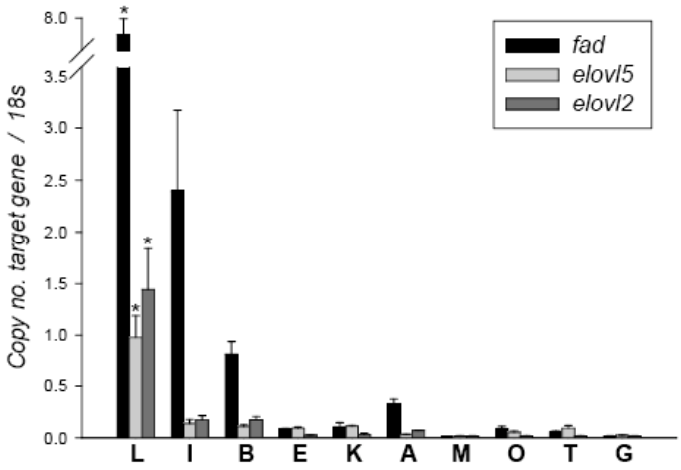
fad
elovl5
elovl2
Copy no. target gene / 18s
8.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
L
I
B
E
K
A
M
O
T
G

Fig. 6

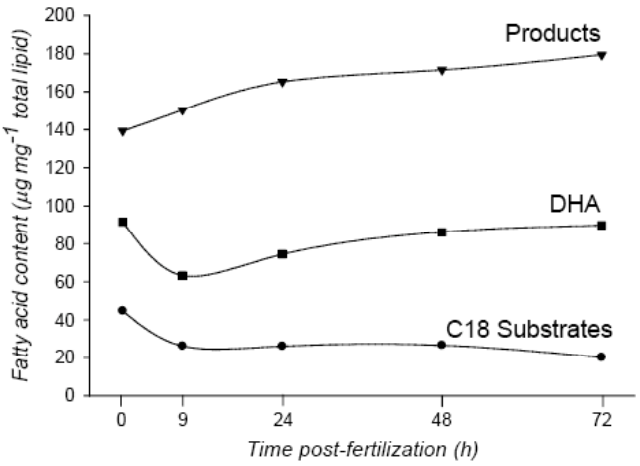
Products
DHA
C18 Substrates
Fatty acid content (μg mg-1 total lipid)
Time post-fertilization (h)
0
9
24
48
72
200
180
160
140
120
100
80
60
40
20
0

Fig. 7