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Long-chain polyunsaturated fatty acid biosynthesis in the euryhaline herbivorous teleost *Scatophagus argus*: Functional characterization, tissue expression and nutritional regulation of two fatty acyl elongases

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Abstract:

Both the spotted scat *Scatophagus argus* and rabbitfish *Siganus canaliculatus* belong to the few cultured herbivorous marine teleost, however, their fatty acyl desaturase (Fad) system involved in long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis are different. The *S. argus* has a Δ6 Fad, while the rabbitfish has Δ4 and Δ6/Δ5 Fad, which were the first report in vertebrate and marine teleost, respectively. In order to compare the characteristics of elongases of very long-chain fatty acids (Elovl) between them, two Elovl cDNAs were cloned from *S. argus* in the present study. One has 885 bp of open read fragment (ORF) encoding a protein with 294 amino acid (aa) showing Elovl5 activity functionally characterized by heterologous expression in yeast, which was primarily active for the elongation of C18 and C20 PUFA. The other has 915 bp of ORF coding for a 305 aa protein showing Elovl4 activity, which was more efficient in the elongation of C20 and C22 PUFA. Tissue distribution analyses by RT-PCR showed that *elovl5* was highly expressed in liver compared to other tissues determined, whereas *elovl4* transcripts were only detected in eye. The expression of *elovl5* and *elovl4* were significantly affected by dietary fatty acid composition, with highest expression of mRNA in liver and eye of fish fed a diet with an 18:3n-3/18:2n-6 ratio of 1.7:1. These results indicated that the *S. argus* has a similar Elovl system in the LC-PUFA biosynthetic pathway to that of rabbitfish although their Fad system was different, suggesting that the diversification of fish LC-PUFA biosynthesis specificities is more associated with its Fad system. These new insights expand our knowledge and understanding of the molecular basis and regulation of LC-PUFA biosynthesis in fish.

Key words: Elovl5, Elovl4, LC-PUFA biosynthesis, *Scatophagus argus*

Introduction

The long-chain (C20-24) polyunsaturated fatty acids (LC-PUFA), particularly eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) acids found primarily in fish and seafood, are regarded as beneficial in a series of human pathologies including metabolic disorders, cardiovascular, inflammatory and neurological diseases (Muhlhausler & Ailhaud, 2013; Delgado-Lista et al., 2012; Awada et al., 2013; Campoy et al., 2012). Comparing to freshwater and salmonids species, marine fish are generally thought to have limited capability or inability for *de novo* LC-PUFA biosynthesis (Sargent et al., 2002; Tocher, 2010). As marine fish are a major source of n-3 LC-PUFA for humans, the biosynthesis and metabolic regulation of these key nutrients have become areas of considerable research in recent years (Tocher, 2010; Xie et al., 2014; Zhang et al., 2014; Kabeya et al., 2015).

The biosynthesis of LC-PUFA from the C18 precursors α-linolenic acid (LNA, 18:3n-3) and linoleic acid (LA, 18:2n-6) consists of sequential reactions catalyzed by a series of fatty acyl desaturase (Fads) and elongase of very long-chain fatty acid (Elovl) enzymes such as Δ6 Fad, Δ5 Fad, Δ6/Δ5 Fad, Δ6/Δ8 Fad, Δ4 Fad, Elovl5, Elovl4 and Elovl2 (Torstensen & Tocher, 2010; Li et al., 2010; Monroig et al., 2011a; Fonseca-Madrigal et al., 2014; Castro et al., 2016). Differences or absence in the activity of enzymes in one or more steps of the pathways result in differential LC-PUFA biosynthetic capability in fish (Castro et al., 2016). Currently, existing data show that the capability for LC-PUFA biosynthesis in marine fish is more diverse than that in other vertebrates (Fonseca-Madrigal et al., 2014) and the diversity was primarily associated with differences in the compliment of enzymes/activities in the pathway of LC-PUFA biosynthesis. Among marine fish, Δ6 Fad and Elovl5 cDNAs have been identified in more than a dozen species (Monroig et al., 2011b; Castro et al., 2016). However, Elovl4 has been investigated to a lesser extent but was reported in cobia (*Rachycentron canadum*) (Monroig et al., 2011c), rabbitfish (*Siganus canaliculatus*) (Monroig et al., 2012), Nibe croaker (*Nibea mitsukurii*) (Kabeya et al., 2015) and orange-spotted grouper (*Epinephelus coioides*) (Li et al., 2015). Δ4 Fad has been identified in two marine fish, rabbitfish and Senegalese sole (*Solea senegalensis*, 1858) (Li et al., 2010; Morais et al., 2012), while Δ6/Δ5 Fad was found only in rabbitfish (Li et al., 2010) among the marine teleost. Thus, to date, rabbitfish is the only marine teleost in which Fad and Elovl enzymes that possess all the activities required for the production of LC-PUFA from C18 PUFA have been found.

With respect to the differences in the capability for LC-PUFA biosynthesis among marine fish, Castro et al. (2012) hypothesized that the losses and diversifications of crucially important genes in the LC-PUFA biosynthetic pathway during fish evolution might be linked to habitat-specific food web characteristics, such as LC-PUFA availability, in different environments. More recently, other confounding factors including "trophic ecology" and diadromy have been proposed (Morais et al., 2012; Monroig et al., 2013). Herbivorous rabbitfish has a wide distribution in the coral reefs of the Indo-Pacific region (Woodland, 1983) and can also live in brackish water (Li et al., 2008), and feeds on a range of macroalgae including *Enteromorpha prolifra* and *Gracilaria lemaneiformis* (You et al., 2014).

Both the spotted scat (*Scatophagus argus*) and rabbitfish are economically important cultured teleost. The spotted scat has similar habitat (euryhaline) and feeding (herbivore) habits to rabbitfish, and is distributed widely in freshwater, brackish and marine habitats of the Indo-Pacific, South and South East Asia (Barry & Fast, 1992; Gandhi, 2002; Yoshimura et al., 2003). In order to know whether *S. argus* has an enzymatic complement for LC-PUFA biosynthesis similar to that of rabbitfish, we aimed to clone and functionally characterize all the genes involved in LC-PUFA biosynthesis in this species. Our previous study showed that the Fads2 of *S. argus* was a monofunctional Δ6 desaturase enzyme (Δ6 Fad), which is in contrast to the more diverse enzymatic complement (Δ4 Fad and Δ6/Δ5 Fad) found in rabbitfish (Li et al., 2010), suggesting that the above mentioned diversification also exists within marine herbivorous fish (Xie et al., 2014). Besides, two elongases including Elovl4 and Elovl5 were identified in rabbitfish (Oscar et al., 2012). In order to compare the characteristics of Elovl system between spotted scat and rabbitfish, and provide basis for fully understanding the LC-PUFA biosynthetic capability of *S. argus*, the present study reports the cloning, functional characterization, tissue expression and nutritional regulation of two Elovl cDNAs encoding putative Elovl4 and Elovl5, key enzymes with well-demonstrated roles in LC-PUFA biosynthesis in fish (Castro et al., 2016).

Materials and Methods

Experimental fish and sampling

Juvenile *S. argus* (body mass around 4.3 g) were purchased from a commercial hatchery in Zhuhai (Guangdong, China). Six isoproteic and iso-lipidic experimental diets (D1-D6) were

formulated with 32 % crude protein and 8 % crude lipid (soybean oil, perilla oil or fish oil as lipid source). Diet D2 contained fish oil (FO) as control, and diets D1, D3-D6 contained different proportions of soybean oil and perilla oil, which resulted in LNA: LA ratios of 0.14, 0.57, 0.84, 1.72 and 2.85, respectively. The detailed dietary formulations, proximate and fatty acid compositions were shown in Xie et al. (2014).

All juvenile *S. argus* were reared in floating cages (0.6×0.6×3.0 m) located on the coast near Nan'ao Marine Biological Station (NAMBS), Shantou University, and fed an equal mix of the six experimental diets for two weeks before the start of feeding trial. The feeding trial was conducted in 18 cages at ambient temperature, salinity and photoperiod, with each cage containing 25 fish that were allocated randomly. Fish in triplicate cages were fed one of the experimental diets twice a day (at 9:00 and 16:00 h) for 8 weeks. At the end of the feeding trial, fish were anaesthetized with 0.01% 2-phenoxyethanol (Sigma-Aldrich Inc., USA), and livers of 54 fish (3 fish per replicate cage) were collected, frozen in liquid nitrogen and stored at -80 °C prior to the analysis of *elovl* mRNA expression by quantitative PCR (qPCR). In order to determine the tissue distribution of *elovl5* and *elovl4* transcripts, eye, brain, liver, muscle, heart, gills, spleen, kidney and intestine were collected from wild *S. argus* (50–60 g) captured from the coast near NAMBS, after fish were anaesthetized with 0.01% 2-phenoxyethanol. Tissue samples were frozen in liquid nitrogen immediately after collection and stored at -80 °C until RNA extraction.

Molecular cloning of elovl5 and elovl4 cDNAs

Total RNA was extracted from *S. argus* liver and eye using Trizol reagent (Invitrogen, USA) and reverse transcribed into cDNA using random primers and an appropriate RT-PCR kit (Invitrogen, USA). For *elovl5*, degenerate primers (E5F1 and E5R1, Table1) were designed on the basis of alignment of fish *elovl5* including rabbitfish (GU597350), cobia (FJ440239)，zebrafish (*Danio rerio*) (NM_200453) and rainbow trout (*Oncorhynchus mykiss*) (AY605100), and used for amplifying partial fragments of putative *elovl5* cDNA from *S. argus* by polymerase chain reaction (PCR). For *elovl4*, degenerate primers (E4F1 and E4R1, Table1) were designed on the basis on the alignment of several fish *elovl4* including cobia (HM026361), rabbitfish (JF320823), and zebrafish Elovl4a (NM_200796) and Elovl4b (NM_199972), and used for amplifying partial fragments of putative *elovl4* cDNA fragment by PCR. For both *elovl* cDNAs, PCR (RT-PCR kit, Invitrogen, USA) consisted of an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at

94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. For both genes, the PCR fragments of the expected size were subsequently cloned into the pMD18-T vector (Takara, Dalian, China) and sequenced (Sangon, Shang Hai, China). Gene-specific primers were then designed to produce the full-length cDNA by 5' (primers E5R2/E5R3 and E4R2/E4R3 for *elovl5* and *elovl4*, respectively) and 3' (E5F2 and E5F3, E4F2 and E4F3) rapid amplification of cDNA ends (RACE) PCR (Gene Racer ™ Kit, Invitrogen, USA) (Table1).

Sequence and phylogenetic analysis of Elovl5 and Elovl4

The deduced amino acid (aa) sequences of the newly cloned elongases were aligned with their corresponding orthologues from rabbitfish (Elovl5, ADE34561; Elovl4, ADZ73580), Nibe croaker (Elovl5, ACR47973; Elovl4, AJD80650), cobia (Elovl5, ACJ65150; Elovl4, ADG59898), Atlantic salmon (Elvol5a, AAO13175; Elovl5b, ACI62499; Elovl4, ADJ95235) and zebrafish (Elovl5, NP_956747; Elovl4b, NP956266) using ClustalW2 (Higgins & Sharp, 1989). The aa sequence identities between deduced Elovl proteins from *S. argus* and other vertebrate homologues were compared by the EMBOSS Needle Pairwise Sequence Alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). A phylogenetic tree comparing the aa sequence similarities of different types of elongases (Elovl2, Elovl4 and Elovl5) from a variety of vertebrate lineages was constructed using the neighbor-joining method (Saitou & Nei, 1987). Confidence in the resulting phylogenetic tree branch topology was measured through bootstrapping through 1000 iterations.

Functional characterization of cloned elongase genes in yeast

Functional characterization of the *S. argus* putative elongase genes was conducted by expressing their open reading frame (ORF) in yeast *Saccharomyces cerevisiae*. Expression primers listed in Table 1 (*elovl5*: E5F4 and E5R4, *elovl4*: E4F4 and E4R4) containing restriction sites *Bam*HI and *Xba*I were designed for amplification of the *elovl5* and *elovl4* ORFs from liver and eye cDNA using high-fidelity DNA polymerase (TianGen, Beijing, China) under the following conditions: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s and extension at 72 °C for 3 min, and a final extension at 72 °C for 10 min. The DNA fragments were purified and digested with the corresponding restriction endonucleases (New England Biolabs, UK) and ligated into the yeast episomal plasmid pYES2 (Invitrogen). The recombinant plasmids (pYES2-*elovl5* or pYES2-*elovl4*) were transformed into *S. cerevisiae* (strain

INVSc1, Invitrogen) using the S.C. Easy Comp Transformation kit (Invitrogen).

A single colony expressing either the *elovl5* or *elovl4* ORF was grown on *S. cerevisiae* minimal medium minus uracil (SCMM$^{-uracil}$). Stearidonic acid (18:4n-3), γ-linolenic acid (18:3n-6), EPA (20:5n-3), arachidonic acid (ARA, 20:4n-6), docosapentaenoic acid (DPA, 22:5n-3) or docosatetraenoic acid (DTA, 22:4n-6) were used as substrates for testing the elongase activity of the *S. argus elovl5* and *elovl4*. All the fatty acids were purchased from Cayman Chemicals Co (Ann Arbor, MI, USA). The PUFA substrates were added at final concentrations of 0.5 (C18), 0.75 (C20) and 1.0 (C22) mM (Li et al., 2010). After two days culture, yeast cells were harvested and washed as described previously (Li et al., 2010).

Lipid extraction and fatty acid analysis

Yeast samples were homogenized in chloroform/methanol (2:1, v/v) containing 0.01 % BHT as antioxidant, and total lipid extracted according to the Folch method (Folch et al., 1957). Fatty acid methyl esters (FAME) were prepared by transesterification with boron trifluoride etherate (ca. 48 %, Acros Organics, NJ, USA) as described previously (Li et al., 2008; Xie et al., 2014). FAME were purified by TLC, resuspended in hexane (Berry, 2004), and separated using a gas chromatograph (GC2010-plus, Shimadzu, Japan) as described in detail previously (Li et al., 2010). The activity of elongase was calculated as the proportion of substrate fatty acid converted to elongated FA products as follows: 100 × [individual product area / (all product areas + substrate area)] (Li et al., 2010).

Tissue distribution of elovl5 and elovl4 mRNA

In order to determine the distribution of elongase mRNA in *S. argus*, total RNA (1 μg) from eye, brain, liver, muscle, heart, gills, spleen, kidney and intestine was reverse transcribed into cDNA (Cloned AMV First-Strand cDNA Synthesis Kit, Invitrogen). RT-PCR was carried out with an initial denaturing step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 40 s, and a final extension at 72 °C for 5 min. The expression of the housekeeping gene *18S rRNA* was used as internal control to check the efficiency of cDNA synthesis and cDNA integrity. The primer pairs used for RT-PCR are given in Table 1.

Expression of elovl5 and elovl4 mRNA in liver and eye in response to diets with different 18:3n-3/18:2n-6 ratios

The levels of *elovl5* and *elovl4* mRNA were measured by quantitative real-time PCR (qPCR) in liver and eye, respectively, from fish fed on the experimental diets D1-D6. QPCR primers were

E5qF/E5qR and E4qF/E4qR for *elovl5* and *elovl4*, respectively (Table 1). One μg total RNA from liver and eye was reverse transcribed into cDNA according to manufacturers instruction (Takara). The 20 μL reaction system consisted of 2 μL diluted cDNA, 0.4 μL for each primer (10 μmol), 10 uL SYBR Premix, and 7.2 μL sterile double distilled water. PCR amplifications were carried out using a Lightcycler 480 real-time PCR detection system (Roche, Switzerland) with an initial denaturing step at 95 °C for 30s, followed by 40 cycles of denaturation at 95 °C for 5s, with a final step at 60 °C for 31 s. The mRNA levels of *elovl5* and *elovl4* in the liver and eye of *S. argus* in each dietary groups were normalized relative to the expression of *18S rRNA* calculated by the comparative threshold cycle (Ct) method (Whelan et al., 2003).

Statistics

The *elovl* mRNA expression data were presented as means ± standard error of mean (n = 9). Differences in the expression of *elovl5* and *elovl4* (tissue distribution and nutritional regulations experiments) were analyzed by one-way ANOVA followed by Tukey's multiple comparison. All analyses were conducted using SPSS v17.0 (SPSS Inc., Chicago, IL, USA).

Results

3.1. Sequence and phylogenetic analyses of S. argus elovl5 and elovl4 cDNAs

The newly cloned *S. argus* elongase cDNAs were 1390 bp (*elovl5*) and 1484 bp (*elovl4*) in full-length, and deposited in the GenBank database with the accession numbers KF029625 and KF029624, respectively. The *elovl5*-like cDNA had a 885 bp ORF encoding a peptide of 294 aa, whereas the *elovl4*-like cDNA had a 918 bp ORF encoding a protein of 304 aa. When compared to other teleost Elovl5 and Elovl4 sequences, the *S. argus* Elovl5 was 71-85 % identical to teleost Elovl5 including zebrafish, Atlantic salmon, rabbitfish, Nibe croaker and cobia, while *S. argus* Elovl4 shares aa sequence identities of 84-97 % to Elovl4 from teleosts including zebrafish, Atlantic salmon, Nibe croaker, cobia and rabbitfish.

Similar to other teleost Elovl-like proteins, both *S. argus* Elovl5 and Elovl4 deduced proteins possessed the histidine box motif (HXXHH) conserved in the elongase family (Fig. 1) (Jakobsson et al., 2006). They have lysine or arginine residues at the carboxyl terminus, more specifically KXRXX in Elovl5 and RXKXX in Elovl4, regarded as putative endoplasmic reticulum (ER) retrieval signals. Five putative transmembrane-spanning regions containing hydrophobic aa stretches were predicted by comparison with other vertebrate ELOVL proteins (Fig. 1).

A neighbor-joining phylogenic tree was constructed based on the deduced elongase aa sequences from Elovl2, Elovl4 and Elovl5 retrieved from fish and other vertebrate genomes. Our results showed that sequences from the same type of elongase (Elovl2, Elovl4 and Elovl5) clustered together regardless of the vertebrate lineage considered. Thus, the herein characterized *S. argus* Elovl4 and Elovl5 grouped with other orthologues from other fish and more distantly other vertebrates (Fig. 2).

3.2. Functional characterization

The two putative Elovl isolated from *S. argus* were functionally characterized by heterologous expression in yeast *S. cerevisiae* grown in the presence of the following FA substrates: 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3 or 22:4n-6. The FA composition of control yeast transformed with the empty pYES2 vector was 16:0, 16:1 isomers (16:1n-9 and 16:1n-7), 18:0 and 18:1n-9, as well as any exogenously added PUFA substrate (data not shown). This was consistent with the earlier observations that *S. cerevisiae* lacks PUFA elongase activity (Agaba et al., 2004; Monroig et al., 2012). Interestingly, yeast transformed with pYES2-*elovl5* were able to convert C18 to C22 PUFA substrates to corresponding elongated products (Fig. 3). As shown in Table 2, *S. argus* Elovl5 had an apparent preference for C18 and C20 over C22 FA substrates. Moreover, n-3 PUFA were elongated to a greater extent compared to their corresponding n-6 isomers with, for example, almost 73% of added 18:4n-3 was elongated whereas only 40% of added 18:3n-6 was elongated.

When the *S. argus* Elovl4 cDNA was expressed in the yeast cells, evidence of elongation of all fatty acids was observed (Fig. 4, Table 2). Moreover, *S. argus* Elovl4 was more effectively convert both C20 and C22 PUFA substrates to C24 products with no obvious preference in terms of fatty acyl chain length (C20 vs C22) or FA series (n-3 vs n-6).

3.3. Tissue expression of Elovl4 and Elovl5

RT-PCR was used to analyze the expression of *elovl5* and *elovl4* in *S. argus* tissues (Fig. 5). The transcript of *elovl5* was detected in all tissues, with apparent higher expression levels in liver compared to eye, intestine and brain. However, the expression of *elovl4* was only detected in eye. As expected, the housekeeping gene *18S rRNA* was expressed in all tissues analyzed (Fig. 5).

3.4. Effects of dietary fatty acid composition on the mRNA expression level of elovl4 and elovl5

Nutritional regulation of the newly cloned Elovls was analyzed by qPCR in liver (*elovl5*) and eye (*elovl4*). Compared to fish fed the control diet based on FO (D2), livers from fish fed vegetable oil-based diets showed higher ($P<0.05$) expression of *elovl5* except for fish fed diet D3, which had

with the same LNA/LA ratio as D2 (Fig. 6B). The highest expression level of *elovl5* was detected in fish fed diet D5 with a dietary LNA/LA ratio of 1.72 (Fig. 6A). A similar pattern was observed for the expression of *elovl4* in eye. Thus, fish fed all vegetable oil-based diets, except D3, showed a significantly higher expression of *elovl4* compared to fish fed FO. Like *elovl5* in liver, the highest expression of *elovl4* in eye was observed in fish fed diet D5 (Fig. 6B).

Discussion

Elovl enzymes account for the condensation step of the elongation reaction resulting in the addition of 2 carbon atoms to the pre-existing FA substrate (Jakobsson et al., 2006; Guillou et al., 2010). Investigations in many fish species have demonstrated that Elovl5 preferentially elongates C18 and C20 PUFA, with residual conversion toward C22 substrates (Agaba et al., 2004, 2005; Monroig et al., 2012). On the other hand, Elovl4 has been regarded as participating in the biosynthesis of very long-chain (> C24) PUFA, although studies in fish have revealed a role in the biosynthetic pathways of long-chain (C20-24) PUFA (Castro et al., 2016). To date, *elovl5* cDNAs have been identified in numerous fish species, while Elovl4 has been studied in a lesser number of species (Castro et al., 2016). In the present study, we provide evidence for the existence of both Elovl5 and Elovl4 encoding cDNAs and demonstrate their role in the biosynthesis of long-chain PUFA in the euryhaline teleost *S. argus*.

The deduced aa sequences of *S. argus* Elovl5 and Elovl4, containing all the main structural features common for Elovl protein family members (Jakobsson et al., 2006), shared high similarity to other fish orthologues. Consistent with this, the functional characterization of the newly cloned Elovl-like encoding cDNAs showed similar substrate specificities as those described in rabbitfish. Thus the *S. argus* Elovl5 has high activity towards C18 and C20 PUFA substrates, and relatively low activity towards C22 PUFA. In addition to rabbitfish (Monroig et al., 2012), these results are consistent with previously reported activities in other Elovl5 proteins characterized in cobia (Zheng et al., 2009), southern bluefin tuna (*Thunnus maccoyii*) (Gregory et al., 2010), Asian sea bass (*Lates calcarifer*) (Mohd-Yusof et al., 2010), Atlantic bluefin tuna (*Thunnus thynnus* L.) (Morais et al., 2011), rabbtifish (Monroig et al., 2012), Nibe croaker (Kabeya et al., 2015). In contrast to the Elovl5, *S. argus* Elovl4 may effectively elongate the tested C20 and C22 PUFA, generating products up to C24 in length. However, previously reported Elovl4 from zebrafish, Atlantic salmon, cobia, rabbitfish and orange-spotted grouper Elovl4, have the ability to catalyze the conversion of

C20 and C22 PUFA up to C36 PUFA (Monroig et al., 2010; Carmona-Antoñanzas et al., 2011; Monroig et al., 2011c, 2012; Li et al., 2015). The capability of the *S. argus* Elovl4 for elongation of C22 and C24 PUFA substrates up to C36 products was not found, and a recent investigation on the Nibe croaker Elovl4 (Kabeya et al., 2015) reported similar activities as those obtained for *S. argus* Elovl4, i.e. elongation products up to C24. Although elongation products longer than C24 could have been produced in yeast at amounts below the detection level, this results were similar to those reported in other fish species (Monroig et al., 2011b) and mammals (Agbaga et al., 2008). Taken together, it is still reasonable to believe that the *S. argus* Elovl4 plays a prominent role in the biosynthesis of VLC-PUFA. This is consistent with the mRNA tissue distribution showing that the *S. argus elovl4* was highly expressed in eye, a major metabolic site for VLC-PUFA (Agbaga et al., 2010) where these compounds accumulate in photoreceptor cell phospholipids (Aveldaño, 1988; Agbaga et al., 2010; Harkewicz et al., 2012).

The restricted pattern of *elovl4* mRNA found in *S. argus* tissues is largely consistent with that of rabbitfish, although brain also showed expression of *elovl4* in the latter (Monroig et al., 2012). In contrast, more widespread distribution of *elovl4* mRNA has been observed in the marine teleosts cobia and orange-spotted grouper, where *elovl4* transcripts were detected in eye, brain, testis, liver, kidney, muscle and stomach (Monroig et al., 2011c; Li et al., 2015). Although further studies are required to draw a firm conclusion, the difference in the distribution pattern of *elovl4* mRNAs of *S. argus* and rabbitfish compared to other teleost fish may be linked to their feeding habits. For *S. argus elovl5* mRNA, a wide spread distribution pattern was obtained, with greatest expression level in liver, eye, intestine and brain. This is similar to the tissue expression pattern obtained from rabbitfish, in which the expression of an *elovl5* was greatest in liver, followed by intestine and brain. In contrast, studies on carnivorous marine fish, including cobia, Asian sea bass, Nibe croaker, meagre, Japanese eel and Northern pike, showed that the expression of *elovl5* transcript was substantially higher in brain than other tissues (Zheng et al., 2009; Mohd-Yusof et al., 2010; Yamamoto et al., 2010; Monroig et al., 2013; Carmona-Antoñanzas et al., 2013; Wang et al., 2014).

From the functional and phylogenetic analysis, and tissue distribution of *elovl5* and *elovl4* transcripts, it is possible to conclude that the elongase complement involved in LC-PUFA biosynthesis in *S. argus* is similar to that characterized in rabbitfish (Monroig et al., 2012). While it is unclear if phylogeny and/or feeding habits can partly explain such similarity between *S. argus* and rabbitfish elongation capability, it is clear that such resemblance does not extend to Fads

complement. Thus, the sole Fads2 found in *S. argus* (Xie et al., 2014), as observed in many fish species, such as cobia, Nibe croaker, Japanese eel (*Anguilla japonica*) and common carp (*Cyprinus carpio* var. Jian) (Zheng et al., 2009; Yamamoto et al., 2010; Wang et al., 2014; Kabeya et al., 2015; Ren et al., 2013), was characterized with Δ6-desaturase activity but no Δ5- and Δ4-desaturase (Xie et al., 2014). In contrast, the rabbitfish possess at least two Fads2 desaturases, a dual Δ6/Δ5 desaturase and a Δ4 desaturase, the latter being the first record of a Δ4 desaturation activity in vertebrates (Li et al., 2010). Additionally, the distribution of *S. argus fads2* mRNA, with highest expression in liver, followed a pattern typically found in freshwater/salmonid species in contrast to carnivorous marine species whereby brain has shown the highest levels of *fads2* transcription (Monroig et al., 2011b). These results further confirm the enormous diversification of fish LC-PUFA biosynthesis specificities that has been previously hypothesized to be associated with factors including habitat, trophic level and ecology, as well as species-specific evolutionary history (Fonseca-Madrigal et al., 2014).

The ability of fish to regulate LC-PUFA biosynthesis has been extensively investigated in commercially important species in order to understand the metabolic impact of replacing FO by VO devoid of LC-PUFA in aquafeed (Ling et al., 2006; Jordal et al., 2005; Li et al., 2008; Thanuthong et al., 2011; Navarro-Guillen et al., 2014; Xie et al., 2014, 2015; Kuah et al., 2015). We herein showed that dietary lipid resource also affected the expression of *elovl5* and *elovl4* in *S. argus*. Generally speaking, both *elovl4* and *elovl5* were up-regulated in *S. argus* in response to low dietary LC-PUFA (high VO) input. While nutritional regulation of *elovl5* in liver has often been reported (Ling et al., 2006; Morais et al., 2009; Yamamoto et al., 2010; Thanuthong et al., 2011; Navarro-Guillen et al., 2014; Xie et al., 2015; Kuah et al., 2015), regulatory mechanisms of LC-PUFA in eye have less been investigated despite eye accumulating large amounts of LC-PUFA in some species (Aveldaño, 1988; Agbaga et al., 2010; Harkewicz et al., 2012). Li et al. (2015) recently described an up-regulation of *elovl4* in viscera of orange-spotted grouper larvae. Although the specific mechanism remains to elucidated, it is largely accepted that the increased expression of key enzymes including *elovl* and *fads* involved in LC-PUFA biosynthesis pathway stimulated by dietary VO is a biochemical/molecular mechanism that can at least partially compensate dietary essential fatty acid deficiencies (Tocher et al., 2003).

In addition to the dietary lipid source (FO v. VO), the ratio of dietary LNA and LA also influenced the expression of key enzymes involved in LC-PUFA biosynthesis. Functional

characterization of Fads and Elovl isolated from various teleosts species have often revealed a higher activity towards n-3 compared to n-6 PUFA substrates (Morais et al., 2009; Zheng et al., 2009; Li et al., 2010; Monroig et al., 2012; Monroig et al., 2013; Xie et al., 2014; Kabeya et al., 2015). Previous studies have revealed that Fad gene expression and enzymatic activity varied with dietary LNA/LA ratio. For example, up-regulation of Δ6 *fads2* gene expression was measured in rabbitfish, Murray cod (*Maccullochella peelii peelii*), rainbow trout and *S*. *argus* fed high dietary ratios of LNA/LA (Li et al., 2008; Senadheera et al., 2011; Thanuthong et al., 2011; Xie et al., 2014). An excess of LNA in the diet could also block Δ6 *fads* gene expression (Izquierdo et al., 2008; Xie et al., 2014). Unlike desaturases, few studies have reported the influence of dietary LNA/LA ratio on elongase gene expression. In the present study, the mRNA expression of *elvol5* and *elovl4* was highest in liver and eye of *S*. *argus* fed a diet with an LNA/LA ratio of around 1.72. As prior to the elongase activity, the Δ6 desaturase acting on LNA and LA increased with the elevation of the dietary ALA/LA ratio (Thanuthong et al., 2011; Xie et al., 2014). In our previous study, the highest Δ6 fads mRNA expression was detected in liver of *S*. *argus* fed a diet with an LNA/LA ratio of 1.72 (Xie et al., 2014), which means the high level Elovls substrate are alviable to Elvol5 and Elovl4 in the dietary treatment with an LNA/LA ratio of 1.72. Therefore, it appears that dietary ratio of LNA/LA can influence the expression of fads and elovl and LC-PUFA biosynthesis efficiency could be optimized with particular dietary levels of C18 PUFA.

In summary, the present study showed that *S*. *argus* has at least two Elovl with high sequence, function and distribution homology to Elovl4 and Elovl5 reported previously in another herbivorous species, the rabbitfish. However, the Fads complement of *S*. *argus* and rabbitfish is remarkably different and this suggests that the diversification of fish LC-PUFA biosynthesis specificities is highly varied. Replacing FO with a VO blend with a dietary ratio of LNA/LA of 1.72 resulted in highest expression of *elovl5* and *elovl4* in liver and eye of *S*. *argus*, respectively. These discoveries will expand our knowledge in understanding the molecular basis and regulation of LC-PUFA biosynthesis in fish.

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548 Table 1

549 Primers used for cDNA cloning or determining gene expression of *Scatophagus argus* elongases

Aim	Primer	Primer sequence	Accession No[1]
First fragment cloning	E5F1	5’-GGTACTACTTCTCCAAGCTCAT-3’	KF029625
	E5R1	5’-GTGATGTATCTCTTCCACC-3’	
	E4F1	5’-GTCTACAACTTCAGCATGGTG-3’	KF029624
	E4R1	5’-GGAACTGGATCATCTGAATAA-3’	
3’RACE	E5F2	5’-ACAGCTTCGTCCACGTCGTGATGTA-3’	KF029625
	E5F3	5’-TTCGTTATGAACTGGCAACCCTGTG-3’	
	E4F2	5’-TGGCAGCCTTGGGACCTCAG-3’	KF029624
	E4F3	5’-GTGGATTGGCATCAAATGGGTC-3’	
5’RACE	E5R2	5’-TTCAGCATGGTAGCGTGGTGGTAGA-3’	KF029625
	E5R3	5’-TGTTTATGGCGGCACCGAAGTATGA-3’	
	E4R2	5’-GCGAGGGATGTAAGGGTTTCTTCAGAC-3’	KF029624
	E4R3	5’-GTGGATGGAAGAGTTGATGGTTGC-3’	
ORF cloning	E5F4	5’-CCCAAGCTTCAAATGGAGACCATCAATC-3’	KF029625
	E5R4	5’-CCGCTCGAGTCAATCCATCCTCAGCTT-3’	
	E4F4	5’-CCCAAGCTTGCCATGGAGGTTGTAACAC-3’	KF029624
	E4R4	5’-CCGCTCGAGTTACTCTCTTTTTGCTCT-3’	
RT-PCR and qPCR	E5qF	5’-ATGAACTGGCAACCCTGTGG-3’	KF029625
	E5qR	5’-ATATGGCTGCACACATCGTCTG-3’	
	E4qF	5’-TAGCAGACAAGAGGGTGGAGAA-3’	KF029624
	E4qR	5’-CTATGAGGGTCTTCCTGAGTGTA-3’	
	18SF	5’-CGCCGAGAAGACGATCAAAC-3’	AJ427629
	18SR	5’-TGATCCTTCCGCAGGTTCAC-3’	

550 [1] GenBank(http://www.ncbi.nlm.nih.gov/)

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Table 2

Functional characterization of the *Scatophagu argus* elongases in *Saccharomyces cerevisiae* yeast. Results are expressed as a percentage of total substrate fatty acid converted to elongated products.

Elongase	Substrate fatty acid	Product	%Conversion	Activity
Elovl5	18:3n-6	20:3n-6	43.9	C18→20
		22:3n-6	10.3	C20→22
	18:4n-3	20:4n-3	72.9	C18→20
		22:4n-3	20.8	C20→22
	20:4n-6	22:4n-6	32.0	C20→22
		24:4n-6	6.9	C22→24
	20:5n-3	22:5n-3	35.8	C20→22
		24:5n-3	10.1	C22→24
	22:4n-6	24:4n-6	7.5	C22→24
	22:5n-3	24:5n-3	11.5	C22→24
Elovl4	18:3n-6	20:3n-6	7.6	C18→20
	18:4n-3	20:4n-3	12.3	C18→20
	20:4n-6	22:4n-6	37.3	C20→22
		24:4n-6	19.3	C22→24
	20:5n-3	22:5n-3	35.2	C20→22
		24:5n-3	21.6	C22→24
	22:4n-6	24:4n-6	26.5	C22→24
	22:5n-3	24:5n-3	34.8	C22→24

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581  SaE5     METINLKLNAQLETWIGPR---DQRVRGWLLLDNYPPTFALTVIYLLIVWMGPKYMKYRQPYSCRGLLVFYNLGLTLL 75
582  ScE5     MEDFNRKLNSYFESWIGPR---DQRLQGWLLLDNYPPTFALTVVYLLIVWLGPKYMKNRPAYSCRGLMVIYNLGLTLL 75
583  RcE5     METFNHKLNAYIESWMGPR---DQRVKGWLLLDNYPPTFALTVMYLLIVWMGPKYMKHRQPYSCRGLLVLYNLGLTLL 75
584  NmE5     METFNHKLNTYLESWMGPR---DQRVRGWLLLDNYPPTFALTVMYLVIVWMGPKYMKHRQPYSCRGLLVLYNLGLTLL 75
585  SsE5a    METFNYKLNMYIDSWMGPR---DERVQGWLLLDNYPPTFALTVMYLLIVWLGPKYMRHRQPVSCRGLLLVYNLGLTIL 75
586  SsE5b    MEAFNHKLNTYIDSWMGPR---DERVQGWLLLDNYPPTFALTLMYLLIVWLGPKYMRHRQPVSCQGLLVLYNLALTLL 75
587  DrE5     METFSHRVNSYIDSWMGPR---DLRVTGWFLLDDYIPTFIFTVMYLLIVWMGPKYMKNRQAYSCRALLVPYNLCLTLL 75
588  SaE4     MEVVTHFVNDTVEFYKWSLTIADKRVEKWPMMSSPLPTLAISCLYLLFLWAGPRYMQDRQPCTLRKTLIVYNFSMVVL 78
589  ScE4     MEVVTHFVNDTVEFYKWSLTIADKRVEKWPMMSSPLPTLAISCLYLLFLWAGPRYMQDRQPFTLRKTLIVYNFSMVVL 78
590  RcE4     MEVVTHFVNDTVEFYKWSLTIADKRVENWPMMASPLPTLAISCLYLLFLWVGPRYMQDRQPYTLRRTLIVYNFSMVVL 78
591  NmE4     MEAVTHFVNDTVEFYKWGLTIADKRVENWPMMSSPLPTLAISCLYLLFLWAGPRYMQDRQPFTLRKTLIVYNFSMVVL 78
592  SsE4     MEAVTHFMNDTVEFYRWSLTIADKRVEKWPMMSSPAPTLAISCLYLLFLWAGPKYMQNREPFQLRKTLIVYNFSMVIL 78
593  DrE4b    METVVHLMNDSVEFYKWSLTIADKRVEKWPMMSSPLPTLGISVLYLLFLWAGPLYMQNREPFQLRKTLIVYNFSMVLL 78
594           ** .    :*  .. :.:::  * *:  * :: .  **: ::  **:::* ** **: *.:  :: **: :.:*::.*
595                                                I                      II
596  SaE5     SFYMFYELVTAVWYGGYNFYCQNS-HSAEEADNKIMNVLWWYYFSKLIEFMDTFFFILRKNNHQISFLHVYHHATMLN 150
597  ScE5     SFYMFYELGSAIWFGGYHFYCQNT-HSLPEMDNKVMRALWWYYFSKLIEFMDTFFFILRKNNHQITFLHIYHHASMFN 150
598  RcE5     SFYMFYELVTAVWHGGYNFYCQDT-HSAEEVDNKIINVLWWYYFSKLIEFMDTFFFILRKNNHQITFLHIYHHATMLN 150
599  NmE5     SFYMFYELVTAVWHGGYNFYCQDI-HSAQEVDNKIINVLWWYYFSKLIEFMDTFFFILRKNNHQITFLHIYHHASMLN 150
600  SsE5a    SFYMFYEMVSAVWHGDYNFYCQDT-HSAGETDTKIINVLWWYYFSKLIEFMDTFFFILRKNNHQITFLHIYHHASMLN 150
601  SsE5b    SFYMFYEMVSAVWQGGYNFYCQDT-HSAGETDTKIINVLWWYYFSKVIEFMDTFFFILRKNNHQITFLHIYHHASMLN 150
602  DrE5     SLYMFYELVMSVYQGGYNFFCQNT-HSGGDADNRMMNVLWWYYFSKLIEFMDTFFFILRKNNHQITFLHVYHHATMLN 150
603  SaE4     NFYIAKELLLGSRAAGYSYLCQPVNYSNDVNEVRIASALWWYYISKGVEFLDTVFFILRKKFNQVSFLHVYHHCTMFI 154
604  ScE4     NFYIAKELLLGSRAAGYSYLCQPVNYSNDVNEVRIASALWWYYISKGVEFLDTVFFILRKKFNQVSFLHVYHHCTMFI 154
605  RcE4     NFYIAKELLIATRAAGYSYLCQPVNYSNDVNEVRIASALWWYYISKGVEFLDTVFFILRKKFNQVSFLHVYHHCTMFI 154
606  NmE4     NFYIAKELLLGSRAAGYSYLCQPVNYSNDVNEVRIASALWWYYISKGVEFLDTVFFIMRKKFNQVSFLHVYHHCTMFI 154
607  SsE4     NFYIAKELLLGARAAGYSYLCQPVSYSNDVNEVRIASALWWYYISKGVEYLDTVFFILRKKINQVSFLHVYHHCTMFI 154
608  DrE4b    NFYICKELLLGSRAAGYSYLCQPVNYSNDVNEVRIASALWWYYISKGVEFLDTVFFIMRKKFNQVSFLHVYHHCTMFI 154
609           :::: *:  .   ..* : **   :*    : ::  .**** ::** : *::**.***:**: :*::***:***.:*:
610              II
611  SaE5     IWWFVMNWQPCGHSYFGAAINSFVHVVMYSYYGLSAI-PGIRPYLWWKKYITQLQMIQFFLTMCQTMCAAIWPCGVPV 225
612  ScE5     IWWFVMNWIPCGHSYFGASLNSFVHVVMYSYYGLSAV-PSLRPYLWWKKYITQLQLVQFFLTMFQTYCAVLWPCGFPI 225
613  RcE5     IWWFVMNWIPCGHSYFGASLNSFVHVVMYSYYGLSAI-PAMRPYLWWKKYITQLQLIQFFLTMSQTMCAVIWPCDFPR 225
614  NmE5     IWWFVMNWVPCGHSYFGASLNSFVHVVMYSYYGLSAI-PAMRPYLWWKRYITQLQLVQFFLTMSQTMCAVVWPCGFPM 225
615  SsE5a    IWWFVMNWVPCGHSYFGASLNSFIHVLMYSYYGLSAV-PALRPYLWWKKYITQGQLIQFFLTMSQTICAVIWPCGFPR 225
616  SsE5b    IWWFVMNWVPCGHSYFGASLNSFVHVLMYSYYGLSAV-PAIRPYLWWKKYITQGQLIQFFLTMSQTICAVIWPCGFPR 225
617  DrE5     IWWFVMNWVPCGHSYFGATFNSFIHVLMYSYYGLSAV-PALRPYLWWKKYITQGQLVQFVLTMFQTSCAVVWPCGFPM 225
618  SaE4     LWWIGIKWVPGGQSFFGATINSSIHVLMYGYYGLAALGPQMQKYLWWKKYLTIIQMIQFHVTIGHAGHSLYTGCPFPA 229
619  ScE4     LWWIGIKWVPGGQSFFGATINSSIHVLMYGYYGLAALGPQMHKYLWWKKYLTIIQMIQFHVTIGHAGHSLYTGCPFPA 229
620  RcE4     LWWIGIKWVPGGQAFFGATINSSIHVLMYGYYGLAALGPQMQKYLWWKKYLTIIQMIQFHVTIGHAGHSLYTGCPFPC 229
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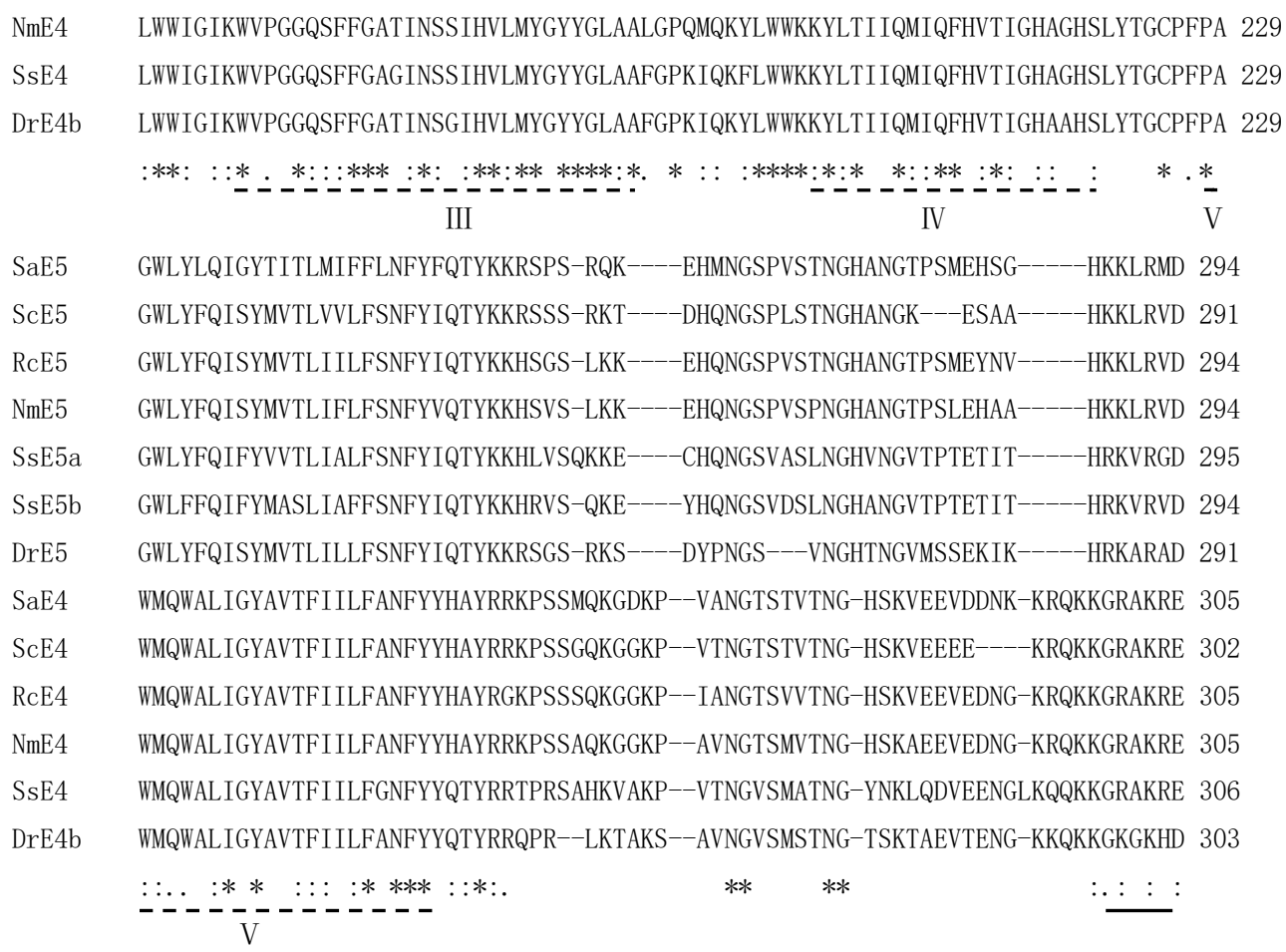


Figure 1. Alignment of the deduced amino acid (aa) sequences of elongases Elovl5 (E5) and Elovl4 (E4) isolated from *Scatophagus argus* (Sa) with their corresponding orthologues, including rabbitfish (*Siganus canaliculatus*, Sc) Elovl5 (ADE34561) and Elovl4 (ADZ73580), cobia (*Rachycentron canadum*, Rc) Elovl5 (ACJ65150) and Elovl4 (ADG59898)，Nibe croaker (*Nibea mitsukurii*, Nm) Elovl5 (ACR47973) and Elovl4 (AJD80650), Atlantic salmon (*Salmo salar*, Ss) Elvol5a (AAO13175), Elovl5b (ACI62499) and Elovl4 (ADJ95235), zebrafish (*Danio rerio*, Dr) Elovl5 (NP_956747) and Elovl4b (NP956266). Deduced aa sequences were aligned using ClustalW2. Identical and similar residues are marked with '*' and ':', respectively. The conserved histidine box HXXHH is shaded grey, five putative transmembrane domains are dash-underlined, and the putative ER retrieval signal is solid underlined.

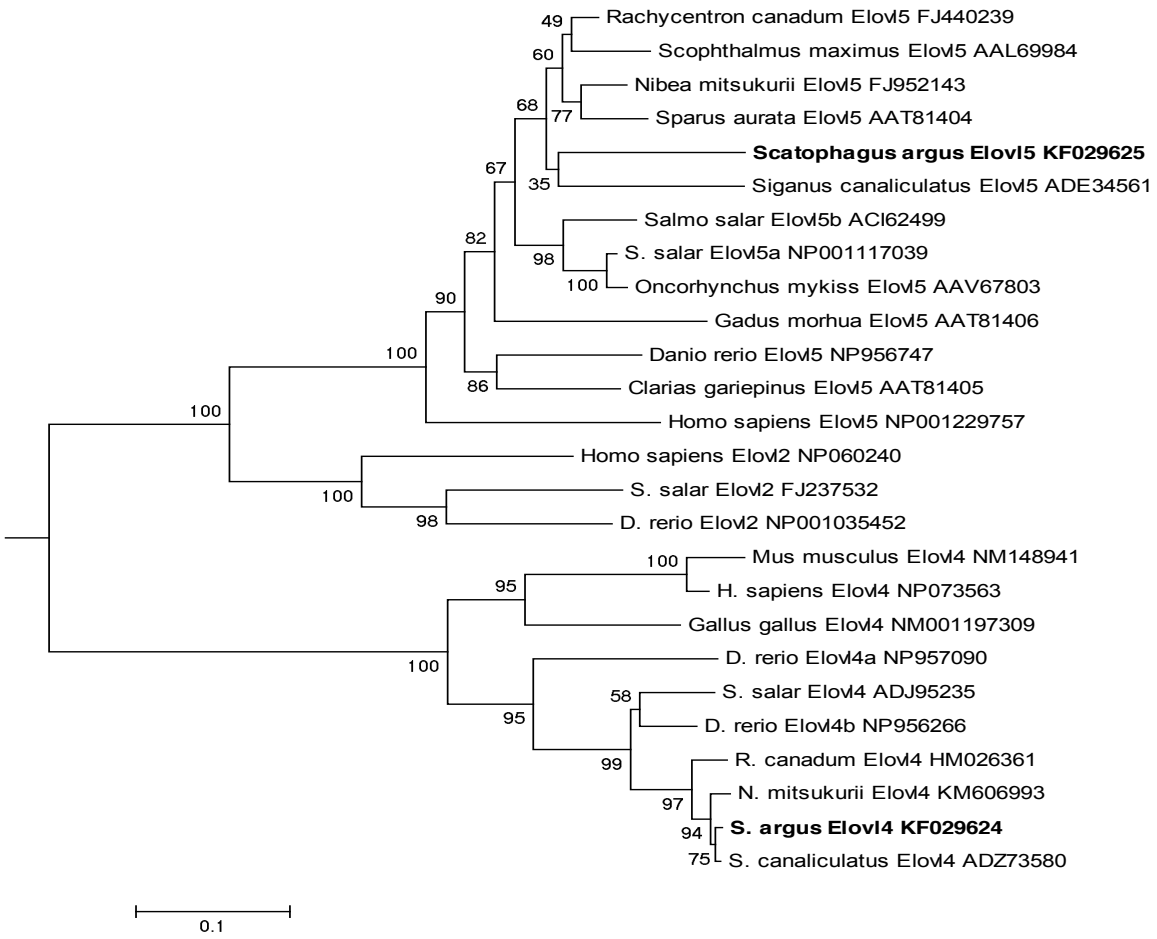


Figure 2. Phylogenetic tree comparing the deduced amino acids of *Scatophagus argus* Elovl4 and Elovl5 with other Elovl members from fish and mammals. The tree was constructed using the neighbor-joining method (Saitou and Nei,1987) with MEGA6. The horizontal branch length is proportional to the substitution rate per site. Numbers represent the frequencies with which the tree topology presented was replicated after 1000 bootstrap iterations.

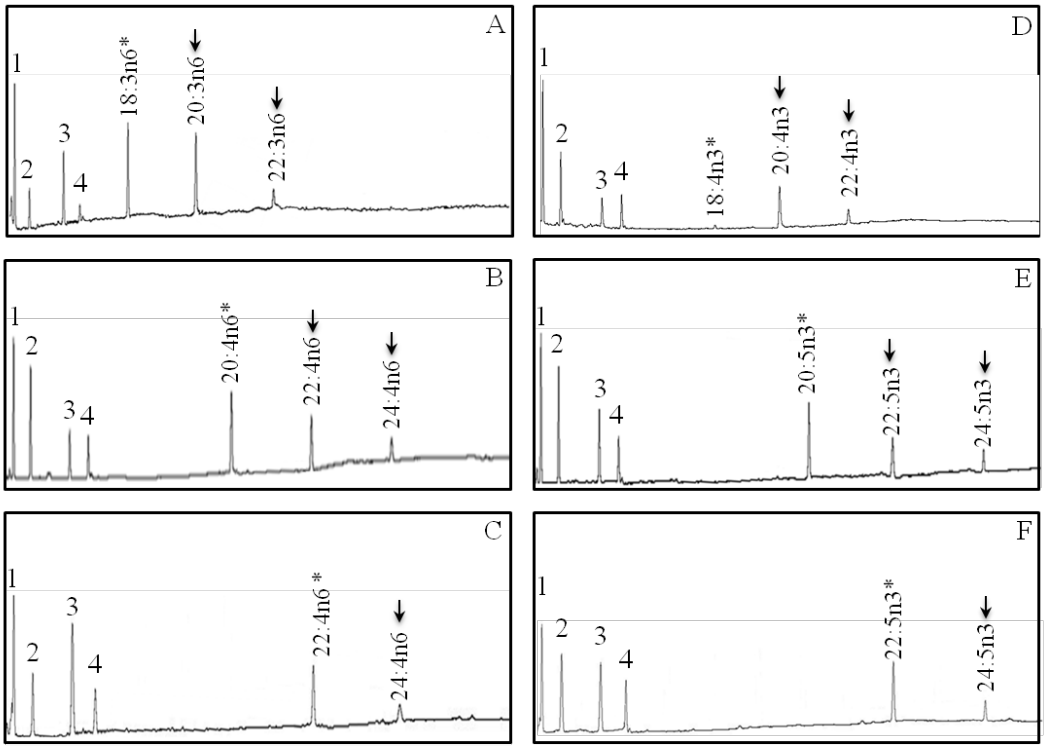


Figure 3 Functional characterization of *Scatophagus argus* putative Elovl5 in yeast *Saccharomyces cerevisiae*. FAMEs were extracted from yeast transformed with the pYES2-*elovl5*, and grown in the presence of PUFA substrates18:3n-6 (A), 18:4n-3 (B), 20:4n-6(C), 20:5n-3 (D), 22:4n-6 (E) and 22:5n-3 (F). Based on retention times, substrates (*) and their corresponding elongated products (↓) are indicated accordingly. Peaks 1–4 represent the main endogenous FAs of *S*. *cerevisiae*, namely 16:0, 16:1 isomers, 18:0 and 18:1n-9, respectively. Vertical axis, FID response; horizontal axis, retention time.

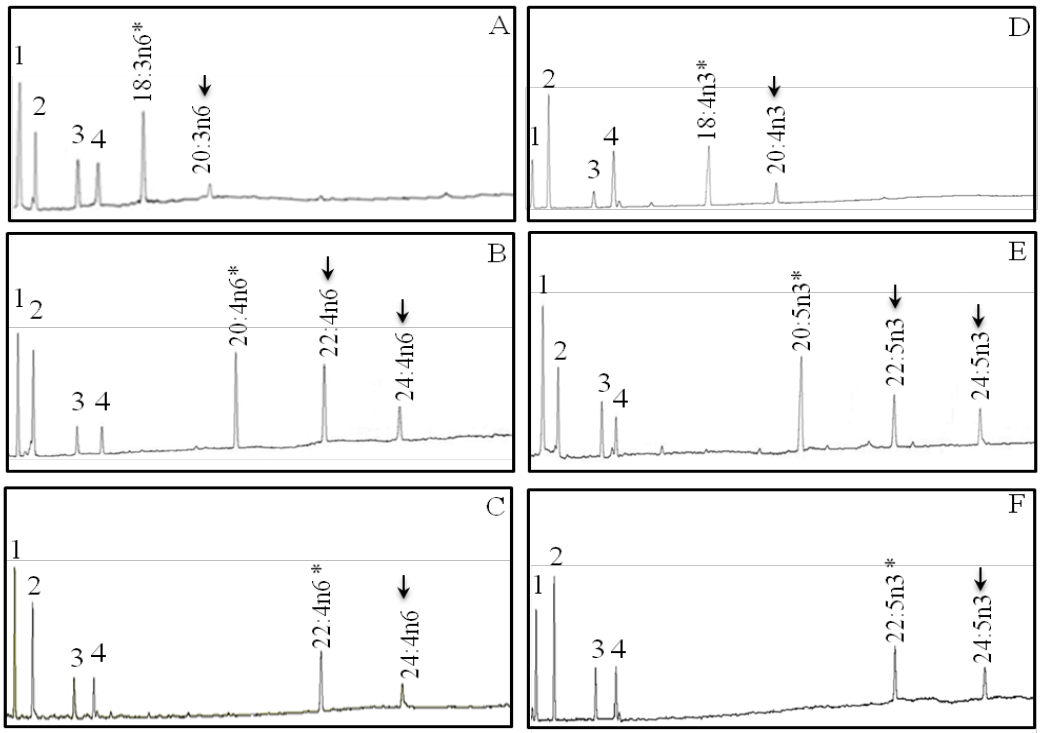
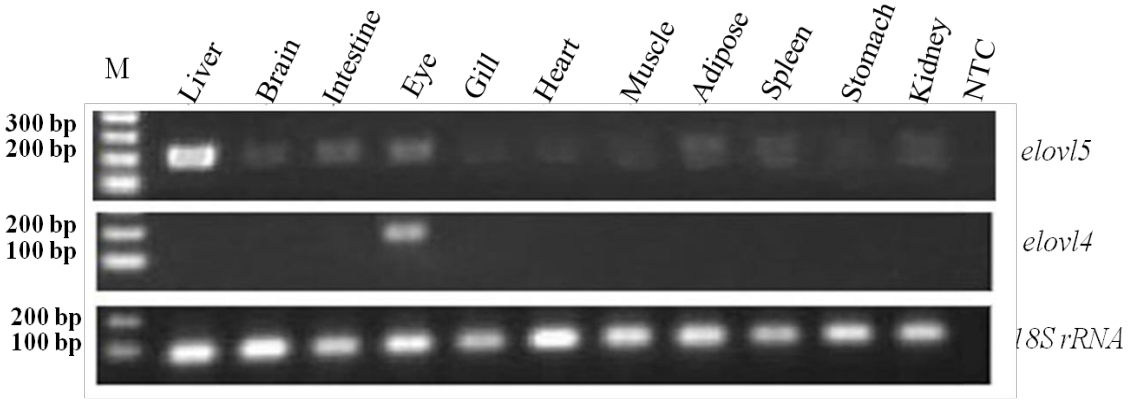


Figure 4 Functional characterization of *Scatophagus argus* putative Elovl4 in yeast *Saccharomyces cerevisiae*. FAME were extracted from yeast transformed with the pYES2-*elovl4*, and grown in the presence of PUFA substrates 18:3n-6 (A), 18:4n-3 (B), 20:4n-6(C), 20:5n-3 (D), 22:4n-6 (E) and 22:5n-3 (F). Based on retention times, substrates (*) and their corresponding elongated products (↓) are indicated accordingly. Peaks 1–4 represent the main endogenous FAs of *S*. *cerevisiae*, namely 16:0, 16:1 isomers, 18:0 and 18:1n-9, respectively. Vertical axis, FID response; horizontal axis, retention time.



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712 Figure 5. Tissue-specific expression of *elovl5* and *elovl4* from *S. argus*. Expression of the housekeeping gene *18S*

713 *rRNA* is also shown. NTC: no template control.

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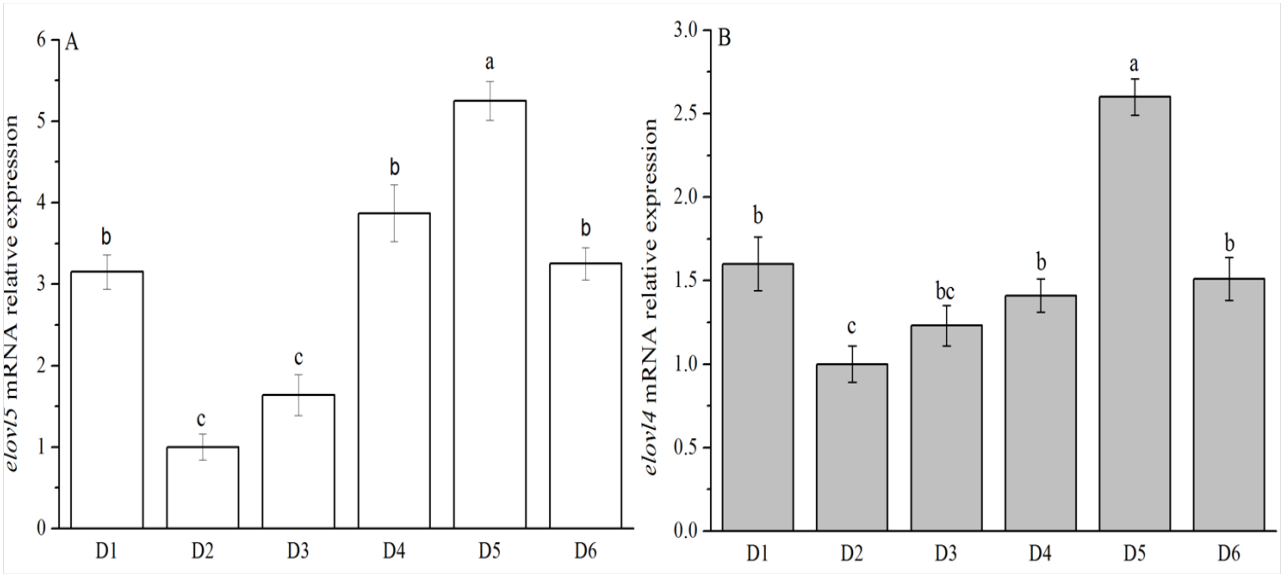

Figure 6. Relative expression levels of *elovl5* (A) and *elovl4* (B) in livers and eyes collected from *S. argus* fed six experimental diets. Expression values were normalized to those of *18S rRNA*. Data are means ± SEM (n = 6). Bars with different superscripts are significantly different ($P<0.05$, one-way ANOVA and Tukey's tests). D2: control diet with fish oil as lipid source; D1, D3–D6: diets with blended vegetable oils as lipid source with dietary LNA/LA ratios of 0.14, 0.57, 0.84, 1.72, and 2.85, respectively.