Molecular and functional characterization of a putative *elovl4* gene and its expression in response to dietary fatty acid profile in Atlantic bluefin tuna (*Thunnus thynnus*)

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Abstract

Elongation of very long-chain fatty acid 4 (Elovl4) proteins are involved in the biosynthesis of very long-chain (>C_{24}) fatty acids and in many teleost fish species they are key enzymes in the pathway for the production of docosahexaenoic acid (DHA; 22:6n-3) from eicosapentaenoic acid (EPA; 20:5n-3). Therefore, Elovl4 may be particularly important in Atlantic bluefin tuna (ABT; *Thunnus thynnus*) characterised by having high DHA to EPA ratios. The present study cloned and characterised both the function and expression of an *elovl4* cDNA from ABT. The Elovl4 had an open reading frame of 915 base pairs encoding a putative protein of 304 amino acids. Functional characterisation demonstrated that the Elovl4 enzyme had elongase activity towards all the polyunsaturated fatty acid (PUFA) substrates assayed. The ABT Elovl4 contributed to DHA biosynthesis by elongation of EPA and DPA to 24:5n-3, the latter being desaturated to 24:6n-3 by the action of *fads2* (Δ6 desaturase). Additionally, the ABT Elovl4 has a role in the biosynthesis of very long-chain PUFA up to C_{34}, compounds of key structural roles in neural tissues such as eye and brain, which had high levels of *elovl4* transcripts. Surprisingly, while the relative expression of *fads2*, required for the production of DHA from EPA, was increased in liver of ABT fed a diet with reduced levels of EPA and DHA, expression of *elovl4* was reduced. Results indicated that ABT has enzymes necessary for endogenous production of DHA from EPA and demonstrate that Elovl4b can effectively compensate for absence of Elovl2.

Keywords: Biosynthesis; docosahexaneonic acid; eicosapentaenoic acid; polyunsaturated fatty acids; Sprecher pathway; very long-chain fatty acids.

1. Introduction

Atlantic bluefin tuna (ABT; *Thunnus thynnus*) is a large pelagic migratory fish species that plays an important role as a top predator, influencing Atlantic and Mediterranean marine communities (Shimose and Wells, 2015). Traditionally, ABT fisheries have been supplemented by farming although this has actually relied on the capture of juveniles in the wild, to be fattened in so-called tuna ranches (Benetti et al., 2016). In recent years, considerable efforts have been made to close the life cycle of ABT (van Beijnen, 2017), and success in the production of larvae and juveniles has provided the animals to enable studies into the elucidation of ideal compositions of both live feeds (Betancor et al., 2017a,b) and inert weaning diets (Betancor et al., 2019). Compared to most teleost fish species, tissues of ABT have high levels of the health-beneficial omega-3 long-chain (C_{20-24}) polyunsaturated fatty acid (n-3 LC-PUFA), docosahexaenoic acid (DHA; 22:6n-3) and very high DHA: eicosapentaenoic acid (EPA; 20:5n-3) ratios (Mourente and Tocher, 2003, 2009). This may indicate a high dietary requirement for DHA, as the LC-PUFA profile of teleosts often reflects dietary intake, but also may suggest preferential retention and accumulation and/or biosynthesis of DHA from EPA, as tissue fatty acid compositions also reflect endogenous metabolism to some extent (Tocher, 2003, 2010; Monroig et al., 2018). However, dietary DHA and EPA can currently only be supplied economically by marine raw materials (fish oil and fishmeal) and, with stagnating supply and increasing demand, the trend nowadays in aquafeed formulation is for the use of high levels of terrestrial vegetable oils, naturally devoid of LC-PUFA, as primary lipid sources (Tocher, 2015). This translates into a low dietary intake of n-3 LC-PUFA, which consequently reduces the contents of the beneficial DHA and EPA in farmed fish (Sprague et al., 2016). The impacts of dietary vegetable oil and low levels of dietary n-3 LC-PUFA, especially DHA, on ABT are as yet unknown (Mourente and Tocher, 2009).

LC-PUFA can be biosynthesised from the C_{18} PUFA, α-linolenic acid (18:3n-3) and linoleic acid (18:2n-6), through enzymatic reactions mediated by fatty acyl desaturases (Fads) and elongation of very long-chain fatty acid (Elovl) proteins (Castro et al., 2016; Monroig et al., 2018). Elovl have been considered as rate-limiting enzymes in fatty acid synthesis, with three members identified as being capable of elongating PUFA, namely Elovl2, Elovl4 and Elovl5 (Guillou et al., 2010; Jakobsson et al., 2006). Most studies on teleost Elovl proteins have focussed on the characterisation of Elovl2 and Elovl5 from farmed species (Monroig et al., 2016, 2018). These studies have shown that Elovl5 proteins are found in most teleost species and primarily elongate C_{18} and C_{20} PUFA, whereas Elovl2 acts mainly on C_{20} and C_{22} PUFA (Castro et al., 2016) and is absent in most ray-finned fish (Monroig et al., 2016). In recent years, considerable attention has been given to the presence of Elovl4 proteins in teleosts, with several farmed species shown to possess these elongases (Carmona-Antoñanzas et al., 2011; Monroig et al., 2012; Kabeya et al., 2015; Jin et al., 2017; Li et al., 2017a,b; Oboh et al.,

2017a; Zhao et al., 2019). In mammals, Elovl4 has been demonstrated to be a critical enzyme in the biosynthesis of both very long-chain (>C_{24}) saturated (VLC-SFA) and polyunsaturated fatty acids (VLC-PUFA) (McMahon et al., 2007; Agbaga et al., 2008). However, in zebrafish (*Danio rerio*), the first fish species in which Elovl4 was studied, two genes *elovl4a* and *elovl4b* were identified with both proteins able to elongate saturated fatty acids, but only Elovl4b able to elongate PUFA (Monroig et al., 2010). *In silico* searches have suggested that all teleost species likely have at least one copy of both *elovl4a* and *elovl4b* (Castro et al., 2016). Importantly, teleost Elovl4b are generally able to elongate 20:5n-3 (EPA) and 22:5n-3 to 24:5n-3 (Castro et al., 2016; Monroig et al., 2016), and thus have the capability to play a role in the biosynthesis of DHA via the Sprecher pathway (Sprecher, 2000). Indeed, it has been suggested that the acquisition/retention of this ability by teleost Elovl4b might compensate for the loss of Elovl2 during the evolution history of teleosts (Monroig et al., 2010; 2016). An early study demonstrated that ABT possessed a Fads2 with Δ6 desaturase activity, as well as an Elovl5 with the ability to elongate mainly C_{18} and C_{20} PUFA (Morais et al., 2011). Interestingly, the ABT Elovl5 showed relatively high elongation activity towards 22:5n-3 compared to most teleost Elovl5 (Morais et al., 2011), suggesting that this enzyme has some potential to contribute to the Sprecher pathway as described above for Elovl4b-like proteins, in a species like ABT that lacks Elovl2. Moreover, the presence or otherwise of Elovl4 in ABT was not investigated in the earlier study.

It is known that several factors can regulate the enzymatic machinery involved in LC-PUFA biosynthesis (Monroig et al., 2018). Both environmental and nutritional (diet) factors can influence the expression and activity of the LC-PUFA biosynthetic enzymes (Zheng et al., 2005; Morais et al., 2011; Monroig et al., 2018). Considerable research has demonstrated how dietary fatty acid profile can impact the expression levels of *fads* and *elovl* genes in fish. In salmonids, an up-regulation in *fads2* occurs in fish fed a diet with low levels of LC-PUFA, especially DHA (Betancor et al., 2014, 2015b, 2016), whereas this response is not as pronounced in carnivorous marine species (Torstensen and Tocher, 2011). Few studies have evaluated the regulation of teleost *elovl4* in response to dietary LC-PUFA levels (Li et al., 2017a,b; Zhao et al., 2019). Furthermore, the interrelationship between the expression levels of *elovl4* with those of the different biosynthetic enzymes in the LC-PUFA pathway has not been extensively studied.

The overarching aim of the present study is to further elucidate the biochemical mechanisms underpinning the high DHA:EPA ratio in ABT, specifically investigating LC-PUFA biosynthetic pathways and the production of DHA from EPA. To this end, the cDNA of an *elovl4* was cloned from ABT and its tissue transcript distribution determined. We further established the function of the Elovl4 in VLC-PUFA biosynthesis, and investigated the potential contribution of ABT Elovl4 and Elovl5 to DHA biosynthesis *via* the Sprecher pathway. In addition, ABT juveniles were fed diets with

varying n-3 LC-PUFA levels to investigate the effect of dietary fatty acid composition on the expression levels of the newly characterised *elovl4* as well as other genes of LC-PUFA biosynthesis in ABT, namely *elovl5* and *fads2*. Taking all the data into account, the capability of ABT for the biosyntheis of DHA and their potential to utilise modern, sustainable feeds rich in ingredients of terrestrial origin is discussed.

2. Materials and methods

2.1. Experimental animals

All procedures were conducted in accordance with the regulations set forward by the Spanish RD 53/2013 (BOE 8th February 2013) and Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes. Additionally, all experimental procedures were reviewed and approved by the Animal Welfare and Ethical Review Board (AWERB) of the University of Stirling, Scotland, UK.

Juveniles for the nutritional trial were produced from eggs spawned in summer 2017 from captive wild ABT broodstock fish maintained in a floating net cage located at El Gorguel, off the Cartagena coast, SE Spain. The eggs were transferred to the Planta Experimental de Cultivos Marinos, Instituto Español de Oceanografía (IEO), Puerto de Mazarrón (Murcia), Spain for hatching and initial larviculture (Ortega, 2015; de la Gándara et al., 2016). Fish were weaned from the live feed stage, fed gilthead sea bream (*Sparus aurata* L.) yolk sac larvae as prey, to formulated feed at 27 days after hatch (dah) using a commercial diet (Magokoro®; Marubeni Nisshin Feed Co., Japan; Okada et al., 2014; Kurata et al., 2015; Honryo et al., 2018) and were completely weaned by 32 dah.

Samples of tissues including brain, gill, heart, kidney, spleen, liver, intestine, red and white muscle, adipose tissue, ovary, testis and eye used for cloning and tissue distribution of *elovl4* expression were obtained from eight wild broodstock ABT (4 males and 4 females) allocated to a floating cage located at El Gorguel Bay (as above) and culled for reproductive stage assessment.

2.2. Nutritional trial

A total of 184 ABT juveniles (41 dah; initial weight 3.3 ± 0.6 g) were distributed into four experimental tanks (water volume 5 m^3) at a stocking density of 46 individuals per tank. The fish were fed *ad libitum* two diets using krill oil as the single lipid source (KO) or a blend of krill oil and rapeseed oil (50:50; KORO) for 10 days. The diets were isoproteic (56 %) and isolipidic (15 %) but supplied differing levels of n-3 LC-PUFA (38.4 % versus 25.5 %; Table 1). At the end of the experimental trial, approximately 100 - 150 mg of liver tissue (samples of individual livers from three fish per tank; six per dietary treatment) were placed in 1 ml RNAlater® (Sigma-Aldrich, Dorset, UK)

and processed according to manufacturer's instructions (4 °C for 24 h) before storage at −80 °C prior to RNA extraction and subsequent analysis.

2.3. Tissue RNA extraction and cDNA synthesis

Adult ABT tissue and juvenile liver samples were homogenised in 1 ml of TriReagent® (Sigma-Aldrich) RNA extraction buffer using a bead tissue disruptor (Bio Spec, Bartlesville, Oklahoma, USA). Total RNA was isolated following manufacturer's instructions and quantity and quality determined by spectrophotometry using a Nanodrop ND-1000 (Labtech Int., East Sussex, UK), and electrophoresis using 200 ng of total RNA in a 1 % agarose gel. cDNA was synthesised using 2 µg of total RNA and random primers in 20 µl reactions and the high capacity reverse transcription kit without RNase inhibitor according to the manufacturer's protocol (Applied Biosystems, Warrington, UK).

2.4. Molecular cloning of elovl4

Primers for cloning the cDNA open reading frame (ORF) sequence of *elovl4* were designed on several ABT sequence read archive (SRA) by identifying and assembling the sequences using CAP3 (Huang and Madan, 1999). This was achieved by blasting the black seabream *elovl4b* sequence against the available ABT transcriptomic data from SRA SRX2255758, ERX555873 and ERX555874. Amplification of the first fragment of the gene, which included the ORF and parts of the 5' and 3' regions was achieved by polymerase chain reaction (PCR) using cDNA synthesised from adult ABT brain total RNA as template and primers designed on the 5' (UniE4F, 5'-GCAGTGGTATCAACGCAGAG-3') and 3' (UniE4R, 5'-TCTCTATCCCTTCCCTCCCC-3') regions of the ABT sequences obtained from SRA. PCR conditions consisted of an initial denaturation step at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 80 s, followed by a final extension at 72 °C for 7 min. PCR fragments were purified using the Illustra GFX PCR DNA/gel band purification kit (GE Healthcare, Little Chalfont, Bucks., UK), and sequenced at GATC Biotech Ltd. (Konstanz, Germany).

2.5. Sequence and phylogenetic analysis

The deduced amino acid (aa) sequence of the newly cloned putative ABT *elovl4* was aligned with *elovl4* orthologues from a range of fish species and other vertebrates with the ClustalW tool (BioEdit v7.0.9, Tom Hall, Department of Microbiology, North Carolina State University, USA). A phylogenetic tree was constructed on the basis of the deduced aa sequence of ABT Elovl4 and other fish and vertebrate Elovl4, Elovl2 and Elovl5 sequences using the maximum likelihood method

(Jones et al., 1992) with MEGA 6.0 software (http://www.megasoftware.net/). Confidence in the resulting tree branch topology was measured using bootstrapping through 1,000 replications.

2.6. Functional characterisation of ABT elovl4

PCR fragments corresponding to the ORF of the newly cloned ABT *elovl4* cDNA were amplified from cDNA synthesised from brain RNA, using the high fidelity *Pfu* DNA polymerase (Promega, USA) with primers containing *Hin*dIII (forward) and *Xho*I (reverse) restriction sites (Table 2). PCR conditions consisted of an initial denaturation at 95 °C for 2 min, followed by 32 cycles of denaturation at 95 °C for 30 s, annealing at 66 °C for 30 s, extension at 72 °C for 2 min followed by a final extension at 72 °C for 7 min. The DNA fragments obtained were purified as described above, digested with the appropriate restriction enzymes (New England Biolabs, UK), and ligated into similarly digested pYES2 expression vector (Invitrogen, UK) to produce the plasmid construct pYES2-*elovl4*.

Yeast competent cells InvSc1 (Invitrogen) were transformed with pYES2-elovl4 using the S.c. EasyCompTM Transformation Kit (Invitrogen). Selection of yeast containing the pYES2 constructs was done on *S. cerevisiae* minimal medium minus uracil (SCMM-ura) plates. One single yeast colony was grown in SCMM-ura broth for 2 days at 30 °C, and subsequently subcultured in individual Erlenmeyer flasks until optical density measured at a wavelength of 600 nm (OD600) reached 1, after which galactose (2 %, w/v) and a PUFA substrate at a final concentration of 0.50 mM (C_{18}), 0.75 mM (C_{20}) and 1.0 mM (C_{22}) were added. The fatty acid substrates included γ-linolenic acid (18:3n-6), EPA (20:5n-3), arachidonic acid (20:4n-6), docosapentaenoic acid (22:5n-3), docosatetraenoic acid (22:4n-6) and DHA (22:6n-3). In addition to exogenously added PUFA substrates, some Elovl4 have been shown to elongate saturated FA (Monroig et al., 2018). Consequently, the ability of ABT Elovl4 to elongate yeast endogenous saturated fatty acids was investigated. For that purpose, the saturated fatty acid profiles of yeast transformed with empty pYES2 vector (control) and those of yeast transformed with pYES2-elovl4 were compared after growing the yeast without addition of any substrate. After 2 days, yeast were harvested, washed twice with doubled distilled water and freeze-dried until further analysis. All fatty acid substrates (> 98-99 % pure) used for the functional characterisation assays were obtained from Nu-Chek Prep, Inc (Elysian, MN, USA). Yeast culture reagents including galactose, nitrogen base, raffinose, tergitol NP-40 and uracil dropout medium were obtained from Sigma-Aldrich (Poole, UK).

2.7. Roles of ABT elongase and desaturase enzymes in DHA biosynthesis via the Sprecher pathway

Yeast competent cells InvSc1 (Invitrogen) were co-transformed with two different plasmid constructs prepared as described below. First, the herein cloned ABT *elovl4* ORF and the previously

cloned ABT *elovl5* ORF were ligated into the yeast expression vector p415TEF (a centromeric plasmid with a LEU2 selectable marker) to produce the constructs p415TEF-*elovl4 and* p415TEF-*elovl5* respectively, in which the expression of the ABT *elovl* was controlled under the yeast TEF1 promoter (constitutive expression). Second, the ORF of the ABT *fads2* was cloned into the episomal yeast vector pYES2 to produce the constructs pYES2-*fads2*, in which the Fads expression was under the control of the GAL1 promoter (inducible expression). Selection of transformant yeast containing simultaneously p415TEF-*elovl* (either *elovl4* or *elovl5*) and pYES2-*fads2* was performed by growing the co-transformed yeast on *S. cerevisiae* minimal medium minus uracil minus leucine (SCMM−ura−leu) plates. One single colony was grown in SCMM−ura−leu broth for 24 h at 30 °C, and subsequently subcultured in individual Erlenmeyer flasks at 0.1 OD600 (t_0) and supplemented with either 0.5 mM Na salt of 18:3n-3 (Δ6 desaturation control) or 0.75 mM Na salt of 22:5n-3 (DPA). Co-transformed yeast were then grown for 24 h (t_0 + 24 h) allowing the ABT Elovl (Elovl4 or Elovl5) to convert the exogenously added C_{22} substrate 22:5n-3 into its corresponding C_{24} elongation product 24:5n-3. In order to test the ability of the ABT Fads2 to Δ6 desaturate 24:5n−3 synthesised by yeast, expression of the ABT *fads2* was then induced (t_0 + 24 h) by addition of 2% galactose, after which the recombinant yeast were further grown for 48 h (t_0 + 72 h) before collection. As positive control, yeast co-transformed with *D. rerio* p415TEF-*elovl*2 and ABT pYES2-*fads2* vectors were also grown in SCMM−ura−leu broth as described above.

2.8. Fatty acid analysis

Total lipids were extracted from freeze-dried samples of yeast (Folch et al., 1957) and fatty acid methyl esters (FAME) prepared as described in detail previously (Oboh et al., 2016). Preparation of FAME and peak identification using Gas Chromatograph (GC) coupled with Mass Spectrometry (MS) detector were performed as described by Monroig et al. (2010).

Briefly, the elongation of endogenous saturated fatty acids was assessed by comparison of the areas of the fatty acid of control yeast with those of yeast transformed with pYES2-elovl4. The GC-MS was operated in the electron ionisation (EI) single ion monitoring (SIM) mode. The 24:0, 26:0, 28:0, 30:0, 32:0, 34:0 and 36:0 response values were obtained by using the m/z ratios 382.4, 410.4, 438.4, 466.5, 494.5, 522.5 and 550.5, respectively. For VLC-PUFA analysis, the response values were obtained by using the m/z ratios 79.1, 108.1 and 150.1 in SIM mode. As described in detail by Li et al. (2017), the elongation conversions of exogenously added PUFA were calculated as [areas of first product and longer chain products/(areas of all products with longer chain than substrate + substrate area)] × 100. Moreover, the ability of the ABT Δ6 Fads2 to convert 24:5n-3 to 24:6n-3 was calculated as [area of 24:6n-3 / (area of 24:6n-3 + area of 24:5n-3)] × 100, considering the area of 24:5n-3 as

that generated from exogenously added 22:5n-3 by either the ABT Elovl5 or Elovl4b in the co-transformation assays (Oboh et al., 2017b).

2.9. qPCR analysis

Transcript abundance was determined by quantitative RT-PCR (qPCR) of fatty acyl elongases *elovl4* and *elovl5*, and fatty acyl desaturase Δ6 *fads2*, key genes involved in the pathway for the biosynthesis of LC-PUFA, particularly the production of DHA from EPA in liver, given its active role in lipid metabolism. Elongation factor-1α (*elf1α*) and β-actin (*bactin*) were used as suitable reference genes as they had been determined previously to be stable (Betancor et al., 2017a,b; 2019). The cDNA was diluted 20-fold with milliQ water. The efficiency of the primers for each gene was previously evaluated by serial dilutions of cDNA pooled from the samples to guarantee it was > 85 % for all primer pairs. Analyses by qPCR were performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate 20 µl reaction volumes containing 10 µl of Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific, Hemel Hempstead, UK), 1 µl of the primer corresponding to the analysed gene (10 pmol), 3 µl of molecular biology grade water and 5 µl of cDNA (1/20 diluted). In addition, amplifications were carried out with a systematic negative control (NTC, no template control) containing no cDNA. Standard amplification parameters included a UDG (Uracil-DNA glycosylase) pre-treatment at 50 °C for 2 min, an initial denaturation step at 95 °C for 10 min, followed by 35 cycles: 15 s at 95 °C, 30 s at the annealing Tm and 30 s at 72 °C. Primer sequences for genes are given in Table 2.

2.10. Statistical analysis

Roles of the ABT Elovl4 in the elongation of saturated fatty acids were presented as mean ± SD (n = 3). Comparison of fatty acid profiles from control and yeast expressing the ABT *elovl4* were compared with a Student's t-test. Tissue expression (qPCR) results were expressed as the logarithm of arbitrary units after normalisation against the expression level of the housekeeping gene *ef1α*. One arbitrary unit was set at the lowest expression of the gene per each set of genes. Differences in gene expression among tissues were analysed by one-way ANOVA, data not requiring any transformation. Gene expression in ABT juveniles fed the two dietary treatments was compared by a Student's t-test. Differences were regarded as statistically significant when $P < 0.05$ (Zar, 1999).

3. Results

3.1. Elovl4 sequence and phylogenetic analysis

The ORF of the putative ABT *elovl4* cDNA consisted of 915 bp, encoding a protein of 304 aa. Sequence analysis of the putative Elovl4 protein showed that it contained the conserved histidine dideoxy binding motif (HXXHH), the predicted endoplasmic reticulum (ER) retention signal with an

arginine (R) and lysine (K) at the carboxyl end (RXKXX), as well as several regions containing similar motifs (Fig. 1).

We compared the deduced aa sequence of the ABT Elovl4 with other fish Elovl sequences via BLASTp searches. Our results revealed that the deduced aa sequence of the ABT Elovl showed only 43 % similarity with the previously described ABT Elovl5 sequence (gb|ADX62355.1|). In contrast, phylogenetic analysis showed that the Elovl4 protein of ABT clustered with several other Elovl4-like sequences from teleosts, whereas a separate cluster contained those proteins from mammals and cartilagenous fish (Fig. 2). It is interesting to note that, within teleosts, the herein characterised ABT Elovl4 grouped more closely with Elovl4b-like sequences from orange spotted grouper *E. coioides* (gb|AHI17192.1|; 95 %) and Nibe croaker *Nibea mitsukurii* (gb|AJD80650.1|; 94 %) (Fig. 2). These results strongly suggested that the ABT *elovl4* cDNA characterised here encoded an Elovl4b enzyme, which has been deposited in GenBank under the accession number MN171375.

3.2. Functional characterisation of ABT Elovl4b

Functional characterisation of the ABT Elovl4b protein was carried out in *S. cerevisiae* yeast cells expressing the *elovl4b* ORF and grown in the presence of potential fatty acid substrates. However, the potential activity of the ABT Elovl4b protein for the elongation of saturated fatty acids was first evaluated by comparing the saturated fatty acid profiles of yeast transformed either with an empty pYES2 plasmid (control) or transformed with pYES2-*elovl4b* and grown in the absence of exogenous fatty acid (Table 3). The results showed that pYES2-*elovl4b* transformed yeast contained proportions of 16:0 and 26:0 that were numerically lower (not statistically significant), and those of 28:0 and 30:0 that were significantly higher, than yeast transformed with empty PYES2 plasmid.

To determine the ability of ABT Elovl4b to elongate PUFA, *S. cerevisae* transformed with pYES2-*elovl4b* were grown in the presence of potential PUFA substrates (Table 4). Transgenic yeast containing the *elovl4b* ORF were capable of elongating exogenously added PUFA from C_{18} to C_{22} (Table 4). Thus, tetracosapentaenoic acid (24:5n-3), key intermediate in DHA biosynthesis via the Sprecher pathway, can be produced from both 20:5n-3 and 22:5n-3 by Elovl4b in ABT. However, GC-MS analyses confirmed that even higher conversions were found for n-3 PUFA with chain lengths of C_{24} up to C_{30} before activity declined with longer chain lengths. With n-6 PUFA, highest conversions peaked at C_{28} and declined with longer chain length. The ABT Elovl4b had no activity towards C_{34} PUFA, irrespective of whether of the n-3 or n-6 series (Table 4). Additionally, yeast containing empty vector and grown in the presence of the same PUFA substrates as those transformed with pYES2-*elovl4b* did not show any elongation activity (data not shown), in agreement with yeast endogenous elongases not being able to elongate PUFA (Agaba et al., 2004).

3.3. Tissue distribution of ABT elovl4b

The transcripts for *elovl4b* were found in all of the analysed ABT tissues except the ovaries (Fig. 3). The highest number of mRNA copies were found in eyes, followed by brain and gills. In contrast, the lowest levels of *elovl4b* expression were observed in heart and kidney.

3.4. Roles of ABT elongase and desaturase enzymes in DHA biosynthesis via the Sprecher pathway

The ability of ABT desaturase (Fads2) and elongase (Elovl4b and Elovl5) enzymes to operate the Sprecher pathway was determined by co-transforming yeast with the ORF sequences of either *elovl4b* or *elovl5* and *fads2* (Δ6 desaturase). First, yeast co-transformed the ABT *elovl5* and Δ6 *fads2* did not contain any detectable 24:5n-3 when grown in the presence of 22:5n-3 (Table 5). This result indicated that the ABT Elovl5 does not play a role in DHA biosynthesis via the Sprecher pathway. Yeast co-transformed with the ABT *elovl4b* and Δ6 *fads2* were able to elongate the exogenously added 22:5n-3 to 24:5n-3, confirming the activity of the ABT *elovl4b* in the constitutive expression vector p415TEF (data not shown). Importantly, an additional peak corresponding to 24:6n-3 denoted a Δ6 desaturation of 24:5n-3 by the ABT Fads2 (3.4 % conversion). Such Δ6 desaturation capacity was also observed when ABT Elovl4b/ABT Fads2 co-transformed yeast were supplied with 18:3n-3, which was converted to 18:4n-3 (8.7 % conversion). Overall, this demonstrates that ABT has the potential to operate the Sprecher pathway by simultaneously activating the herein studied Elovl4b to produce 24:5n-3, which is subsequently converted to 24:6n-3 via a Δ6 desaturation catalysed by Fads2. Similar results to those described above for yeast co-transformed with ABT Elovl4b/ABT Fads2 were obtained for the elongation control yeast co-transformed with *D. rerio* Elovl2/*T. thynnus* Fads2, which exhibited a 6.9 % conversion of 24:5n-3 to 24:6n-3. These results suggested that both the ABT Elovl4b and the zebrafish Elovl2 are efficient in providing 24:5n-3 from the shorter-chain precursor 22:5n-3.

3.5. Nutritional regulation of elovl4b expression: an in vivo trial

A trial was conducted to elucidate the nutritional regulation of *elovl4b* in juvenile ABT when different levels of n-3 LC-PUFA were supplied in the diet. The experimental fish were fed either a feed high (KO; 38.4 %) or low (KORO; 25.5 %) in n-3 LC-PUFA. Liver of juvenile ABT fed diet KORO showed lower mRNA copy number of *elovl4b* compared to liver of fish fed diet KO ($p = 0.022$; Fig. 4). Additionally, expression of the □6 *fads2* fatty acyl desaturase and *elovl5* elongase genes so far characterised from ABT and with confirmed roles in biosynthesis of LC-PUFA (Morais et al., 2011), were analysed. Low dietary n-3 LC-PUFA also led to a down-regulation in transcript level of *elovl5* ($p = 0.043$), whereas the expression level of *fads2* was up-regulated in fish fed KORO ($p = 0.044$).

4. Discussion

In the present study, the full length cDNA sequence of a putative *elovl4b* was cloned from ABT. The obtained sequence contained typical domains of Elovl4 family members such as an endoplasmatic reticulum retrieval signal (RXKXX) and a histidine box (HXXHH), similar to those described for other teleosts Elovl4 proteins (Monroig et al., 2010, 2011, 2012; Carmona-Antoñanzas et al., 2011; Kabeya et al., 2015; Li et al., 2017a,b; Jin et al., 2017; Oboh et al., 2017a). The histidine box is a common feature of desaturase and hydrolase enzymes in general, and in elongases is involved in the coordination of electron transfer during elongation of fatty acids (Jakobsson et al., 2006). Furthermore, ABT Elovl4b exhibited other characterisitics of microsomal membrane-bound enzymes, such as multiple transmembrane regions (Jakobsson et al., 2006). Specifically, seven transmembrane regions were predicted, according to the hydropathy analysis of the ABT deduced Elovl4 sequence. The specific number of transmembrane regions can be variable in teleost Elovl4, ranging from five in *E. coioides* (Li et al., 2017a), Atlantic salmon *Salmo salar* (Carmona-Antoñanzas et al., 2011) and *L. crocea* (Li et al., 2017b), six in *N. mitsukurii* (Kabeya et al., 2015), to seven in both African catfish *Clarius gariepinus* (Oboh et al., 2017a) and black seabream *Acanthopagrus schlegelii* (Jin et al., 2017). Similarly, a variable number of transmembrane regions was observed among Elovl4 proteins in a range of other vertebrates and invertebrates in a previous study, although this did not impact the strong sequence homology that, in turn, indicates substantial functional conservation (Zhang et al., 2003).

Phylogenetic analysis showed that the newly obtained ABT Elovl4b aa sequence clustered together with *D. rerio* and *A. schelegelii* Elovl4b sequences, and in a different branch from teleost Elovl4a. An *in silico* study indicated previously that most teleosts likely possess both Elovl4a and Elovl4b (Castro et al., 2016) and, indeed, recent studies have demonstrated both isoforms in several marine and freshwater teleost species (Kabeya et al., 2015; Oboh et al., 2017a; Jin et al., 2017; Yan et al., 2018) as well as zebrafish (Monroig et al., 2010). A common feature of both the a and b forms of Elovl4 in zebrafish was the capacity to biosynthesise VLC-SFA (Monroig et al., 2010). In the present study, the saturated fatty acid (i.e., precursors of VLC-SFA) profile of yeast transformed with ABT *elovl4b* showed significant differences to yeast transformed with empty vector. Thus, higher percentages of 28:0 and 30:0 were observed in yeast transformed with ABT *elovl4b*, which was consistent with data reported for Elovl4 from several other fish species including zebrafish, Atlantic salmon, cobia (*Rachycentron canadum*), African catfish and orange-spotted grouper that all showed 28:0 as a major product of saturated fatty acid elongation by Elovl4 (Monroig et al., 2010, 2011; Carmona-Antoñanzas et al., 2011; Oboh et al., 2017a, Li et al., 2017a, b). This suggests that ABT Elovl4b have some capacity for the production of VLC-SFA.

Heterologous expression in yeast demonstrated that the ABT Elovl4b exhibited high elongation efficiencies towards exogenously added C_{18}, C_{20} and C_{22} PUFA substrates. Most importantly, the key intermediate in the Sprecher pathway for the synthesis of DHA from EPA, 24:5n-3, was biosynthesised by ABT Elovl4b from both EPA and DPA. Subsequently, 24:5n-3 synthesised by the action of Elovl4b can be further converted to 24:6n-3 by the ABT Fads2 confirming that this enzyme not only operates on C_{18} PUFA precursors as described previously (Morais et al., 2011), but also on C_{24} substrates like 24:5n-3. Such desaturase capacity appears to be common among teleost Fads2 with substrate specificities other than Δ4 desaturase (Oboh et al., 2017b). Our study enables us to confirm that ABT has the enzyme machinery necessary for the endogenous production of DHA from EPA and, for first time, provides molecular evidence demonstrating that Elovl4b contributes to this pathway, thus efficiently compensating for lack of Elovl2 in most marine teleosts (Monroig et al., 2010, 2011, 2012; Carmona-Antoñanzas et al., 2011; Kabeya et al., 2015; Oboh et al., 2017a; Jin et al., 2017; Li et al., 2017a,b; Yan et al., 2018; Zhao et al., 2019). While endogenous production of DHA is important to guarantee supply of such a physiolgical important compound for vertebrates, this pathway may be particularly relevant in species such as ABT whose lipids are characterised by having a fatty acid composition with a very high DHA:EPA ratio (Mourente and Tocher, 2003, 2009).

In addition to the role of ABT Elovl4b in DHA biosynthesis, it is important to note that this enzyme also participates in the biosynthesis of VLC-PUFA, since it was able to produce a range of polyenes with chain lengths up to 34 carbons in the yeast expression system. This is largely in agreement with previous studies on teleost Elovl4 proteins although PUFA of up to 36 carbons have often been reported (Monroig et al., 2010, 2011, 2012; Carmona-Antoñanzas et al., 2011; Kabeya et al., 2015; Oboh et al., 2017a; Jin et al., 2017; Li et al., 2017a,b; Yan et al., 2018; Zhao et al., 2019). Importantly, some of the VLC-PUFA detected in yeast in the present study, namely 26:6n-3, 28:6n-3, 30:6n-3, 32:6n-3 and 34:6n-3, were identified in retinal phosphatidylcholine (PC) of European seabass, gilthead seabream, Senegalese sole and Atlantic salmon in previous studies (Garlito et al., 2019). Overall, the results demonstrated the key role of teleost Elovl4b in VLC-PUFA biosynthesis, a metabolic pathway that is particularly active in retina, consistent with tissue expression data.

The mRNA copy number for *elovl4b* in ABT was highest in eye, which reflected the fact that retina is known to contain VLC-PUFA, primarily within PC, suggesting a very specific structural or functional role for these fatty acids and, consequently, Elovl4 in retinal tissue (Aveldaño, 1987). Moreover, the ABT *elovl4b* mRNA tissue distribution was also consistent with data obtained in other teleost fish species, where photoreception/neural tissues (e.g., retina, pineal gland, brain) are generally sites of high expression of *elovl4b* (Monroig et al., 2010, 2011, 2012; Carmona-Antoñanzas et al., 2011; Oboh et al., 2017a; Jin et al., 2017; Li et al., 2017a,b; Yan et al., 2018). However, transcripts of *elovl4b* were found in almost all tissues of ABT, which was similar to the expression

of *elovl4a*, but not *elovl4b* in zebrafish (Monroig et al., 2010). In contrast both *elovl4a* and *elovl4b* were expressed in almost all tissue in African catfish, black seabream and loach (Oboh et al., 2017a; Jin et al, 2017; Yan et al., 2018). Expression levels of *elovl4a* exceeded those of *elovl4b* in most tissues in catfish and, to a lesser extent, in black sea bream (Oboh et al., 2017a; Jin et al, 2017). It is also worth noting that *elovl4b* expression was generally low in liver of ABT, which is also a characteristic shared with *elovl4b* expression in zebrafish (Monroig et al., 2010), and *elovl4b* expression in several other fish species including Atlantic salmon, cobia, African catfish and black seabream (Carmona-Antoñanzas et al., 2011; Monroig et al., 2011; Oboh et al., 2017a; Jin et al., 2017). While the presence of *elovl4b* transcripts in tissues such as retina, pineal and testis appears related to its role in VLC-PUFA biosynthesis, activity of Elovl4b in other tissues might be related with its contribution to biosynthesis of LC-PUFA like DHA as described above.

With the continued expansion of aquaculture production, the development of more sustainable feeds has become increasingly essential (Ytrestøyl et al., 2015; Shepherd et al., 2017; Tocher et al., 2019). This, in turn, reduces the content of the n-3 LC-PUFA, EPA and DHA, in the feeds and the resultant farmed fish products (Henriques et al., 2014; Tocher, 2015; Sprague et al., 2016). This is likely to be a particular issue in a species like ABT that have high EPA and, especially, DHA contents and whose nutritional quality is dependent upon high levels of these fatty acids (Mourente and Tocher, 2003, 2009). In general, low dietary levels of LC-PUFA have been shown to up-regulate the expression of *fads2* desaturases in teleosts as a mechanism to enhance the biosynthesis of EPA and DHA when fed diets with low levels of LC-PUFA (Leaver et al., 2008; Torstensen and Tocher, 2011). Consistent with this, the transcript level of ABT *fads2* (Δ6 desaturase) was up-regulated in ABT fed the diet with lower content of n-3 LC-PUFA (diet KORO) in the present study. Perhaps surprisingly, higher dietary levels of n-3 LC-PUFA (diet KO) tended to increase the transcript copy numbers of both *elovl4b* and *elovl5* elongases in liver of ABT. In contrast, relative expression of *elovl4b* mRNA in visceral mass was reduced in orange-spotted grouper fed diets with graded increased levels of n-3 LC-PUFA (Li et al., 2017a), in liver of large yellow croaker fed high dietary n-3 LC-PUFA (Li et al., 2017b), and in liver of rainbow trout fed a diet high in soybean oil but not linseed oil (Zhao et al., 2019). Similarly, in the study in orange-spotted grouper, relative expression of *elovl4* tended to decrease as dietary DHA:EPA ratio increased (Li et al., 2017a). While the above three studies investigated the nutritional regulation of *elovl4b*-like genes, regulation of *elovl4a* by dietary PUFA has been only reported in loach *M. anguillicaudatus*, where *elovl4a* was up-regulated in fin cell cultures supplemented with 18:2n-6 and 18:3n-3 (Yan et al., 2018). Overall, studies reporting the expression of fatty acid elongases in fish species in response to dietary levels of LC-PUFA have yielded inconsistent results (Monroig et al., 2018) and, while some studies have shown nutritional regulation, many others have not (Leaver et al., 2008; Tocher, 2010; Torstensen and Tocher, 2011).

In conclusion, the present study demonstrated that ABT, *T. thynnus*, possess an Elovl4b with roles in the biosynthesis of VLC-PUFA up to 34 carbons, compounds of key structural roles in neural tissues such as eye (retina) with high presence of *elovl4b* transcripts. Moreover, the ABT Elovl4b contributes to the DHA biosynthesis by elongation of EPA and DPA to 24:5n-3, the latter being desaturated to 24:6n-3 by the action of the ABT Δ6 Fads2. These results confirm that ABT has the enzyme machinery necessary for the endogenous production of DHA from EPA and demonstrate that Elovl4b can effectively compensate for absence of Elovl2 in many teleost species.

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649

650 **Table 1.** Total lipid fatty acid composition (percentage of total fatty acids) of the experimental diets
651 with higher (KO) and lower (KORO) levels of n-3 long-chain polyunsaturated fatty acids used in the
652 feeding trial with juvenile Atlantic bluefin tuna.

	KO	KORO
14:0	6.8	3.6
16:0	16.5	12.1
18:0	4.3	4.2
Total SFA[1]	28.3	20.6
16:1n-7	4.9	3.0
18:1n-9	10.9	27.0
18:1n-7	4.6	3.9
20:1n-9	2.2	2.5
Total MUFA[2]	26.0	39.7
18:2n-6	2.2	7.8
20:4n-6	0.6	0.5
Total n-6 PUFA[3]	3.4	8.7
18:3n-3	0.9	3.9
18:4n-3	2.0	1.1
20:4n-3	0.4	0.3
20:5n-3	13.3	7.7
22:5n-3	2.7	2.0
22:6n-3	22.0	15.5
Total n-3 PUFA[4]	41.5	30.5
Total PUFA	45.8	39.7
Total n-3 LC-PUFA	38.4	25.5
n-3/n-6	12.2	3.5
DHA/EPA	1.6	2.0

653 Results are means of duplicate analyses. [1]Totals include 15:0, 20:0, 22:0 and 24:0; [2]Totals include
654 16:1n-9, 18:1n-11, 20:1n-7, 22:1 isomers and 24:1; [3]Totals include 18:3n-6, 20:2n-6, 22:4n-6 and
655 22:5n-6; [4]Totals include 20:3n-3 and 22:3n-3; DHA, docosahexaenoic acid; EPA, eicosapentaenoic
656 acid; KO, diet formulated with 15 % lipid as krill oil; KORO, diet formulated with 15 % lipid
657 formulated with krill oil and rapeseed oil (1:1, v/v); MUFA, monounsaturated fatty acid; PUFA,
658 polyunsaturated fatty acid; SFA, saturated fatty acid.
659

Table 2. Sequences of primers used for cloning, functional characterisation and quantitative RT-PCR (qPCR).

Primer	Sequences (5'-3')	Purpose
UniE4b-F	GCAGTGGTATCAACGCAGAG	First fragment isolation
UniE4b-R	TCTCTATCCCTTCCCTCCCC	
Ttelovl4bv-F	CCCAAGCTTAAGATGGAGGCTGTAACACA	Functional characterisation
Ttelovl4bv-R	CCGCTCGAGTTACTCCTTTTTCGCTCTTC	
Ttelovl5v-F	CCCAAGCTTAAAATGGAGACTTTCAATTATAAACTGAACA	
Ttelovl5v-R	CCGCTCGAGTCAATCCACCCGCAGTTTCT	
Ttfadsv-F	CCCGAGCTCAATATGGGTGGTGGAGGCCAGC	
Ttfadsv-R	CCGCTCGAGTCATTTATGAAGATATGCATC	
elovl4-F	ATCCAGTTCCACGTGACCAT	Gene expression (qPCR)
elovl4-R	CCATAGAGGTGCCGTTTGTG	
elovl5-F	CCACGCTAGCATGCTGAATA	
elovl5-R	ATGGCCATATGACTGCACAC	
fads2d6-F	CCGTGCACTGTGTGAGAAAC	
fads2d6-R	CAGTGTAAGCGATAAAATCAGCTG	
ef1a-F	CCCCTGGACACAGAGACTTC	
ef1a-R	GCCGTTCTTGGAGATACCAG	
βactin-F	ACCCACACAGTGCCCATCTA	
βactin-R	TCACGCACGATTTCCCTCT	

Table 3. Saturated fatty acid profiles (percentage of total fatty acids) of yeast *Saccharomyces cerevisiae* transformed with either the empty pYES2 vector (Control) or the Atlantic bluefin tuna *elovl4* ORF (Elovl4b). Results are means ± SD (n = 3). Statistical differences observed between treatments (Student t-test, $P < 0.05$) are indicated with an asterisk.

	Control	**Elovl4b**
14:0	1.2 ± 0.7	1.4 ± 1.3
15:0	0.7 ± 0.1	0.6 ± 0.2
16:0	48.0 ± 5.2	45.8 ± 3.4
18:0	34.5 ± 3.6	34.2 ± 3.2
20:0	0.6 ± 0.1	0.7 ± 0.1
22:0	0.8 ± 0.2	0.9 ± 0.1
24:0	0.9 ± 0.1	0.9 ± 0.1
26:0	12.6 ± 2.6	12.3 ± 2.9
28:0	0.5 ± 0.1	2.7* ± 1.0
30:0	0.1 ± 0.0	0.3* ± 0.1

Table 4. Functional characterisation of Elovl4b elongase of Atlantic bluefin tuna by heterologous expression in the yeast *Saccharomyces cerevisiae*. Data are presented as the percentage conversions of polyunsaturated fatty acid (FA) substrates. Individual conversions were calculated according to the formula [areas of first product and longer chain products / (areas of all products with longer chain than substrate + substrate area)] × 100.

FA substrate	Product	% Conversion	Elongation
18:3n-6	20:3n-6	9.5	C18→36
	22:3n-6	24.9	C20→36
	24:3n-6	43.3	C22→36
	26:3n-6	100	C24→36
	28:3n-6	100	C26→36
	30:3n-6	77.0	C28→36
	32:3n-6	20.5	C30→36
	34:3n-6	n.d.	C32→36
	36:3n-6	n.d.	C34→36
20:5n-3	22:5n-3	18.2	C20→36
	24:5n-3	49.1	C22→36
	26:5n-3	62.7	C24→36
	28:5n-3	93.4	C26→36
	30:5n-3	99.4	C28→36
	32:5n-3	92.0	C30→36
	34:5n-3	18.5	C32→36
	36:5n-3	n.d.	C34→36
20:4n-6	22:4n-6	22.5	C20→36
	24:4n-6	56.0	C22→36
	26:4n-6	65.7	C24→36
	28:4n-6	91.1	C26→36
	30:4n-6	97.0	C28→36
	32:4n-6	66.2	C30→36
	34:4n-6	4.6	C32→36
	36:4n-6	n.d.	C34→36
22:5n-3	24:5n-3	24.1	C22→36
	26:5n-3	100	C24→36
	28:5n-3	66.1	C26→36
	30:5n-3	99.3	C28→36
	32:5n-3	88.6	C30→36
	34:5n-3	16.6	C32→36
	36:5n-3	n.d.	C34→36
22:4n-6	24:4n-6	12.4	C22→36
	26:4n-6	54.4	C24→36
	28:4n-6	85.8	C26→36
	30:4n-6	96.5	C28→36
	32:4n-6	56.2	C30→36
	34:4n-6	3.5	C32→36
	36:4n-6	n.d.	C34→36
22:6n-3	24:6n-3	0.7	C22→36
	26:6n-3	100	C24→36
	28:6n-3	100	C26→36
	30:6n-3	100	C28→36
	32:6n-3	25.6	C30→36
	34:6n-3	6.2	C32→36

Table 5. Roles of the Atlantic bluefin tuna (ABT) Elovl5 and Elovl4b elongases and Fads2 fatty acyl desaturase in DHA biosynthesis via the Sprecher pathway. Fatty acid conversions were calculated as the percentage of 24:5n−3 desaturated to 24:6n−3 as [area of 24:6n-3 / (area of 24:6n-3 + area of 24:5n-3)] × 100. Conversions of 18:3n−3 to 18:4n−3 (control for Δ6 desaturation) are also indicated. In order to normalise the percentage conversions, ratios between the activities on 24:5n−3 and those on 18:3n-3 ("$\Delta_{24:5n-3}$ / $\Delta_{18:3n-3}$") are also presented for each co-transformation assay. Conversions detected for the elongation control consisting of the *Danio rerio* Elovl2 (ZF Elovl2) co-expressed with the ABT Fads2 are also indicated.

	% Conversion		
	18:3n-3 → 18:4n-3	**24:5n-3 → 24:6n-3**	**$\Delta_{24:5n-3}$ / $\Delta_{18:3n-3}$**
ABT Elovl5 / ABT Fads2	12.7	n.d.	0.00
ABT Elovl4b / ABT Fads2	8.7	3.4	0.39
ZF Elovl2 / ABT Fads2	9.0	6.9	0.77

n.d., Not detected

Legends to Figures

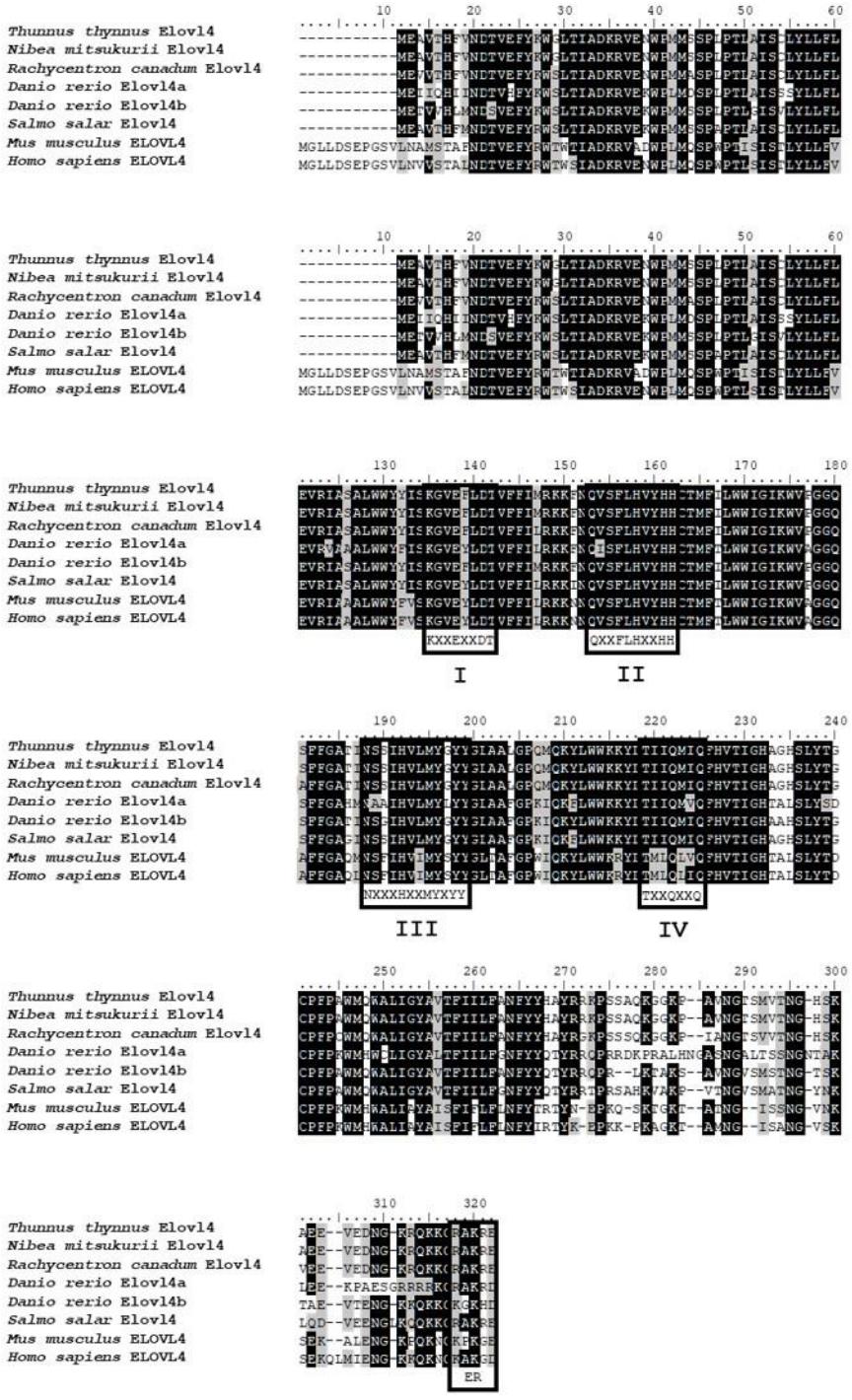
Fig. 1. ClustalW amino acid alignment of the deduced Elovl4 protein of Atlantic bluefin tuna (*Thunnus thynnus*) with Elovl4 proteins from other fish species including *Danio rerio* (Elovl4a, gb|NP_957090.1|; Elovl4b, gb |NP_956266.1|), *Rachycentron canadum* Elovl4b (gb|HM026361|), *Nibea mitsukurii* (gb|AJD80650.1|) and *Salmo salar* Elovl4b (gb |HM208347|), as well as those of mammals, *Mus musculus* (gb |AAG47667.1|) and *Homo sapiens* (gb|NP_073563.1|). Identical residues are shaded black and similar residues (based on the Blosum62 matrix, using ClustalW default parameters) are shaded grey. Indicated are four (i–iv) conserved motifs of Elovl enzymes: (i) KXXEXXDT, (ii) QXXFLHXXHH, (iii) NXXXHXXMYXYY and (iv) TXXQXXQ, as well as the putative endoplasmic reticulum (ER) retrieval signal RXKXX at the C-terminus (Zhang et al., 2003).

Fig. 2. Phylogenetic tree comparing the deduced amino acid sequence of Elovl4 of Atlantic bluefin tuna (*Thunnus thynnus*; highlighted in bold) with Elovl2, Elovl4 and Elovl5 sequences from a range of vertebrates. The tree was constructed using the Maximum Likelihood method and JTT matrix-based model (Jones et al., 1992) using MEGA 6.0 software with a Kimura 2-parameter substitution model. The tree is drawn to scale, with branch lengths measured in the number of is proportional to amino acid substitution rate per site. The *Mortierella alpina* PUFA elongase was included in the analysis as outgroup sequence to construct the rooted tree.

Fig. 3. Distribution of elovl4 transcript in tissues of Atlantic bluefin tuna as determined by qPCR. Values correspond to the log-normalised (*ef1α*) relative expression (RE) of the target gene in each tissue. For comparison, the expression level in ovary, which was the lowest, was defined as 1 before the expression values were then log transformed. The results represent the average of eight individuals (n = 8; 4 males and 4 females; between 200 – 250 kg total weight and 10 to 15 years old) with standard error (SEM), other than for ovary and testis (n = 4). Values with different superscript letters are significantly different (ANOVA; $P < 0.05$). A, adipose tissue; B, brain; E, eye; G, gills; H, heart; I, intestine; K, kidney; L, liver; O, ovary; R, red muscle; S, spleen; T, testis; W, white muscle.

Fig. 4. Effect of diet on the expression of fatty acyl elongases *elovl4* and *elovl5*, and fatty acyl desaturase □6 *fads2* in liver of Atlantic bluefin tuna (*Thunnus thynnus* L.) juveniles fed diets with higher (KO) or lower (KORO) levels of n-3 long-chain polyunsaturated fatty acids. Values are normalised expression ratios with the expression level in fish fed KO set to 1 and are means ± SD of six individuals (n = 6). Values with different superscript letters are significantly different (Student t-

test; $P < 0.05$). KO, diet with 15 % lipid supplied by krill oil; KORO, diet with 15 % lipid supplied by krill oil and rapeseed oil (1:1; v/v).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Mus musculus ELOVL4
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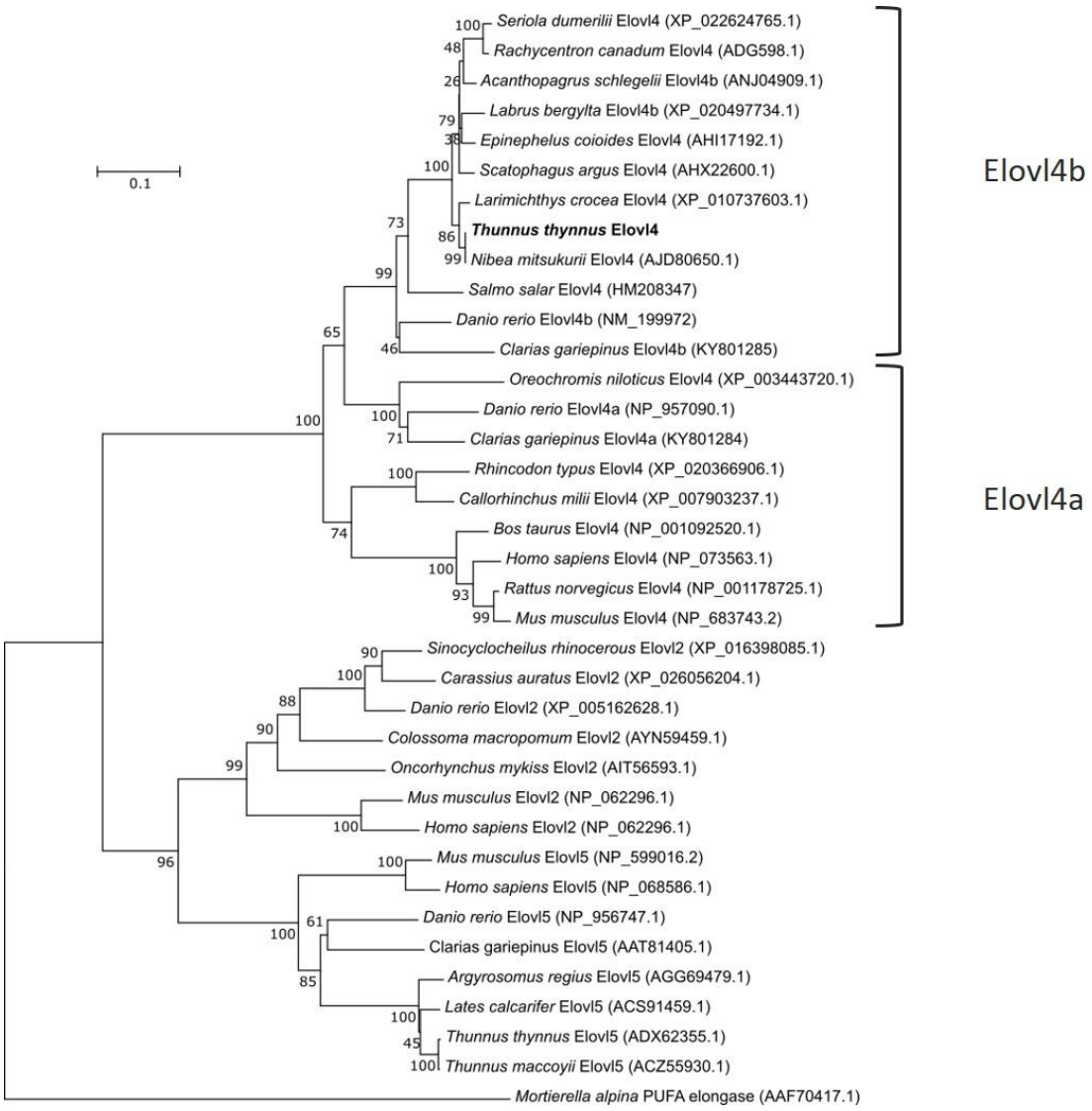
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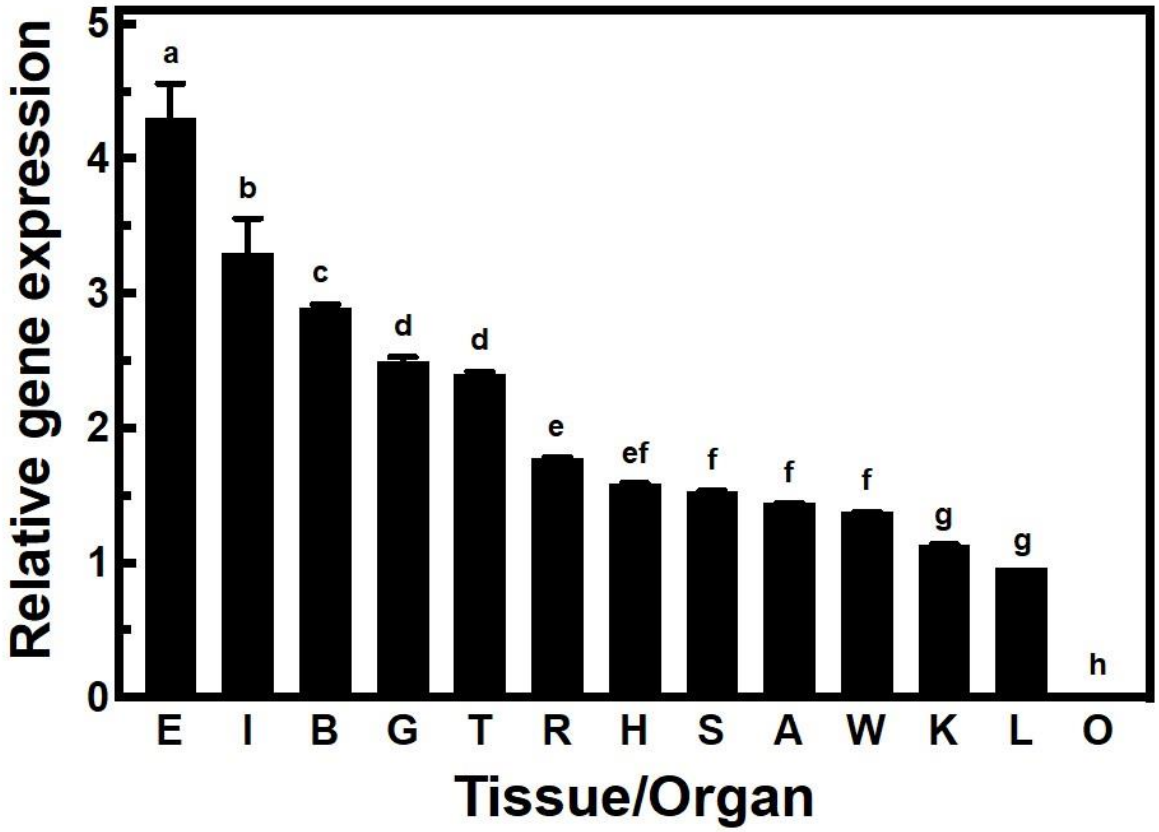
Elovl4b
Elovl4a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S
A
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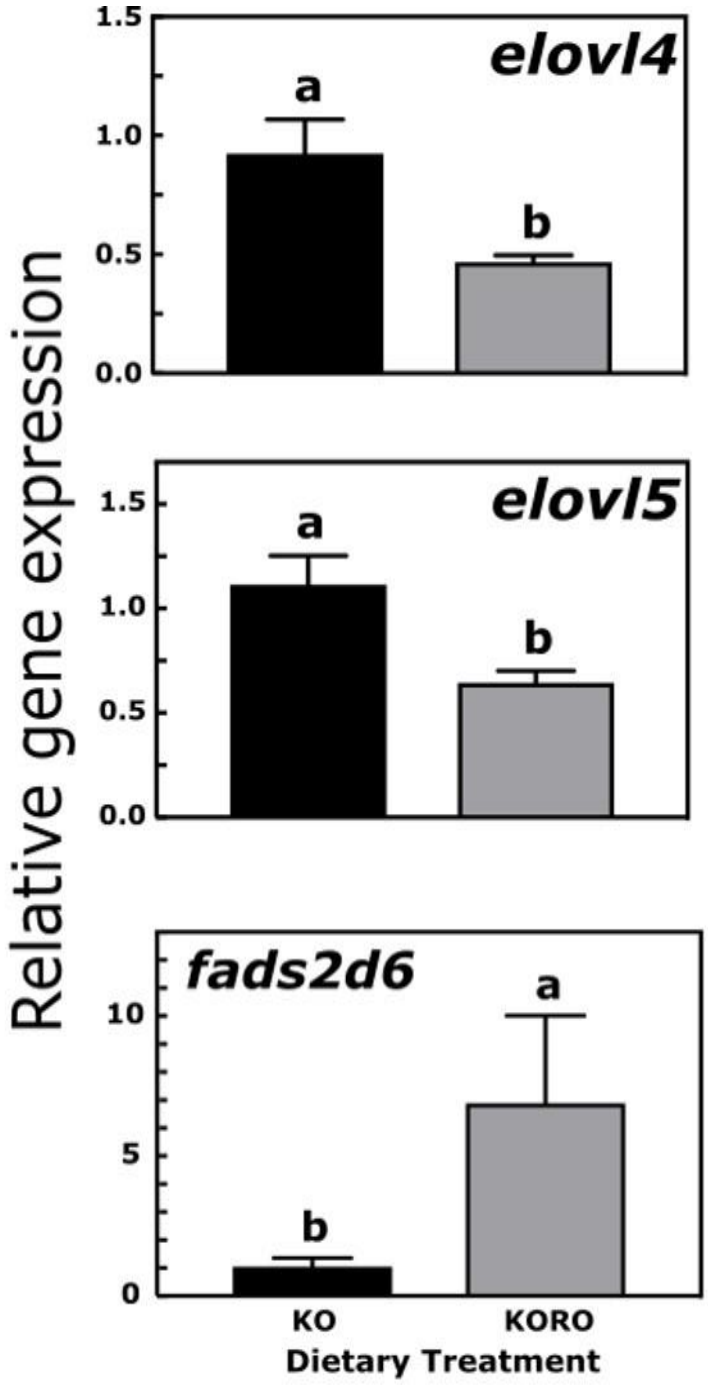
K
L
O
Tissue/Organ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0.0
fads2d6
a
b
10
5
0
KO
KORO
Dietary Treatment