Biosynthesis of very long-chain fatty acids (C > 24) in Atlantic salmon: Cloning, functional characterisation, and tissue distribution of an Elovl4 elongase

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Abstract

The elongases of very long-chain fatty acids (Elovl) account for the rate-limiting condensation step of the elongation process in fatty acid (FA) biosynthesis in vertebrates. One member of the Elovl family, Elovl4, has been regarded as a critical enzyme in vertebrates in the production of the so-called very long-chain fatty acids (VLC-FA), a group of compounds that have been scarcely explored in fish. Here we report on the cloning of a novel *elovl4*-like elongase from Atlantic salmon. The salmon *elovl4* cDNA codes for a putative protein containing 306 amino acids. Heterologous expression in yeast demonstrated that salmon Elovl4 efficiently elongated saturated FAs up to 36:0, with 24:0 and 26:0 appearing as preferred substrates. Additionally, salmon Elovl4 effectively converted C20 and C22 polyunsaturated fatty acids to elongated polyenoic products up to C36. Tissue distribution showed that *elovl4* mRNA transcripts are abundant in eye, brain and testes, suggesting that, as described in mammals, these tissues are important metabolic sites for the biosynthesis of VLC-FA. Our results are discussed in comparison with the functional analyses observed in Elovl4 proteins from other vertebrates, and also other Elovl proteins investigated previously in Atlantic salmon.

Keywords

Aquaculture; Atlantic salmon; Elovl4-like elongase; fatty acid biosynthesis; very long-chain fatty acids.

Abbreviations

ARA, arachidonic acid; BHT, butylated hydroxytoluene; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; DTA, docosatetraenoic acid; EI, electron ionization; elf-1α, elongation factor-1α; Elovl (ELOVL), elongase of very long-chain fatty acids; EPA, eicosapentaenoic acid; ER, endoplasmic reticulum; EST, expressed sequence tag; FA, fatty acid; Fad, fatty acyl desaturase; FAME, fatty acid methyl ester; GC-MS, gas chromatography coupled with mass spectrometry; LC-PUFA, long-chain polyunsaturated fatty acids; m/z, mass-to-charge ratio; ORF, open reading frame; PC, phosphatidylcholine; PCR; polymerase chain reaction; PUFA, polyunsaturated fatty acid; qPCR, quantitative real-time PCR; SIM, selected ion monitoring; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase polymerase chain reaction; UTR, untranslated region; VLC-FA, very long-chain fatty acids; VLC-PUFA, very long-chain polyunsaturated fatty acids.

1. Introduction

The very long-chain fatty acids (VLC-FA) are found in most animals and constitute a group of fatty acids (FA) with chain numbers normally ranging from C26 to C40 that can be saturated, monounsaturated or polyunsaturated (Rezanka, 1989; Poulos, 1995). In higher vertebrates, saturated VLC-FA have been found predominantly in tissues such as skin, hair and glands producing wax-like substances, whereas very long-chain polyunsaturated fatty acids (VLC-PUFA) accumulate in retina, brain, testis and spermatozoa (Rezanka and Sigler, 2009). Their relative low abundance compared to shorter chain FA (≤ C24) and the difficulty of their analysis has hindered investigation of the functions and metabolism of VLC-FA. Recent advances in analytical technology will enable more accurate characterisation of VLC-FA that will likely accelerate the understanding of the functions that these compounds play in living organisms (Agbaga et al., 2010b).

The biosynthesis of VLC-FA in mammals has been proposed to proceed through consecutive elongations of appropriate FA substrates catalysed by specific elongases of very long-chain fatty acids (Elovl) (Agbaga et al., 2008). Among them, Elovl4 has been demonstrated to be a critical enzyme in the biosynthesis of both saturated and polyunsaturated VLC-FA. Investigations on genetically-modified mice suggested a role for Elovl4 in the biosynthesis of saturated VLC-FA. Thus, mice lacking a functional ELOVL4 exhibited lower saturated VLC-FA (C > 24) in skin acyl-ceramides, which resulted in severe skin permeability disruptions in the pups that died perinatally (Cameron et al., 2007; Li et al., 2007; Vasireddy et al., 2007). Similarly, mutated *Elovl4*-knockin mice exhibited depleted levels of C32-C36 VLC-PUFA in retinal phosphatidylcholine (PC), indicating that mammalian ELOVL4 is also involved in the biosynthesis of VLC-PUFA (McMahon et al., 2007). The function of Elovl4 was later

confirmed by Agbaga et al. (2008) who demonstrated that the human ELOVL4 efficiently elongated saturated VLC-FA up to C30, and VLC-PUFA from C28 to C38. Very recently, it was suggested that a further Elovl protein, Elovl2, may be involved in the production of n-6 VLC-PUFA that are critical in male fertility (Zadravec et al., 2011).

Recently, we isolated and characterised *elovl4* cDNAs from zebrafish *Danio rerio* (Monroig et al., 2010a) and cobia *Rachycentron canadum* (Monroig et al., 2011). These investigations have demonstrated that, similarly to mammals, teleostei Elovl4 are key enzymes in the biosynthesis of both saturated and polyunsaturated VLC-FAs up to C36. Expression patterns of fish *elovl4* mRNA confirmed that retina, gonads and brain are the major metabolic sites for VLC-FA biosynthesis in teleosts. These findings highlighted the importance that the study of VLC-FA and their biosynthesis have in farmed fish in which fertility issues of broodstock, altered visual acuity, and disruptions of brain function can affect growth performance and the economics of farming.

Atlantic salmon, *Salmo salar* L., is an important species in the fish farming industries of Northern Europe, North America and Chile. It is also an interesting model to investigate FA metabolism as salmon present one of the most highly characterised and complete PUFA biosynthesis pathway, enabling higher replacement of fish oil with vegetable oil in aquafeeds without negative impacts on fish health or growth (Torstensen et al., 2008; Fjelldal et al., 2010). The search for a more sustainable candidate to replace fish oil is currently a major concern in aquaculture due to the dramatic depletion of wild fish stocks, which paradoxically are the source of traditional dietary lipid used in aquaculture. Therefore, it is important to understand the molecular mechanisms underlying the biosynthesis of PUFA and VLC-FA in salmon, and significant advances have been made in recent years with the molecular cloning and

functional characterisation of several genes encoding fatty acyl desaturases (Fad) (Hastings et al., 2005; Zheng et al., 2005; Monroig et al., 2010b), and elongases including Elovl5 and Elovl2 (Hastings et al., 2005; Morais et al., 2009).

The aim of the present study was to further characterise PUFA metabolism in Atlantic salmon, specifically focussing on an area previously unstudied in this important species, VLC-PUFA biosynthesis. Here we report the isolation and molecular cloning of an *elovl4* cDNA, its functional analysis by heterologous expression in baker's yeast, and its mRNA tissue distribution. Results are discussed in relationship with other Elovl proteins previously characterised in Atlantic salmon and other vertebrate species.

2. Materials and Methods

2.1. Molecular cloning of salmon elovl4

The nucleotide sequences of zebrafish *elovl4_b* (gb|NM_199972.1|) and the Japanese medaka EST (gb|DK_113639.1|) were aligned and used to design the primers UNIE4F (forward) and UNIE4R (reverse) that enabled the amplification of a fragment of salmon *elovl4* by PCR performed on eye cDNA (VersoTM cDNA kit, ABgene, Surrey, UK) (see Table 1 for primer details). PCR conditions consisted of a denaturing step at 95°C for 2 min, followed by 33 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s. The PCR product was sequenced (CEQ-8800 Beckman Coulter Inc., Fullerton, USA) and the nucleotide sequence was blasted on GenBank confirming high similarity with other Elovl4 proteins. To obtain the full-length cDNA sequence, specific PCR primers were designed (Table 1), and 3' and 5' rapid amplification of cDNA ends (RACE) PCR was performed on cDNA synthesised from eye total RNA following manufacturer's instructions (FirstChoice® RLM-RACE kit, Ambion, Applied Biosystems, Warrington, UK). RACE PCR products were cloned

into pBluescript KS II vector (Stratagene, La Jolla, USA), sequenced and assembled using Bioedit (BioEdit version 5.0.6, Tom Hall, Department of Microbiology, North Carolina State University, USA) to determine the full-length cDNA sequence.

2.2. Sequence and phylogenetic analysis of Elovl4

The amino acid (AA) sequence deduced from *S. salar elovl4* cDNA was aligned with other orthologues including teleostei cobia *R. canadum* (gb|HM026361|) and zebrafish *D. rerio* Elovl4_a (gb|NP_957090.1|) and Elovl4_b (gb|NP_966266.1|), human ELOVL4 (gb|NP_073563.1|), and bird *Gallus gallus* predicted Elovl4 (gb|XP_419868.2|) using ClustalW2. The AA sequences of salmon Elovl4 and other orthologues from mammals, birds and fish were also compared two by two using the EMBOSS Pairwise Alignment tool (http://www.ebi.ac.uk/Tools/emboss/align/).

A phylogenetic tree was constructed on the basis of the AA sequence similarities between the putative salmon Elovl4, vertebrate Elovl4-, Elovl2- and Elovl5-like proteins using the Neighbour Joining method (Saitou and Nei, 1987). The confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping through 1000 iterations.

2.3. Functional characterisation in yeast

PCR fragments corresponding to the open reading frame (ORF) of the salmon putative *elovl4* were amplified from eye cDNA using the high fidelity Pfu Turbo DNA polymerase (Stratagene, Agilent Technologies, Cheshire, UK). The isolation of the *elovl4* ORF was achieved by performing a two-round PCR. The first PCR was performed with primers designed in the untranslated regions (UTRs), SALE4U5F and SALE4U3R (Table 1). PCR conditions consisted of an initial denaturating step at 95°C

for 1 min, followed by 32 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 2 min, and a final extension at 72°C for 3 min. First round PCR products were used as template for the nested PCR with thermal conditions as described above, and with primers containing restriction sites (underlined in Table 1), SALE4VF (*Hin*dIII) and SALE4VR (*Xho*I). The DNA fragment containing the salmon *elovl4* was digested with the corresponding restriction endonucleases (New England BioLabs, Herts, UK) and cloned into the yeast episomal plasmid pYES2 (Invitrogen, Paisley, UK). The resulting plasmid construct pYES2-*elovl4* was transformed into *Saccharomyces cerevisiae* (strain INVSc1) using the S.C. EasyComp Transformation kit (Invitrogen). A single colony of transgenic yeast was grown in *S. cerevisiae* minimal medium$^{-uracil}$ supplemented with one of the following FA substrates: lignoceric acid (24:0), eicosapentaenoic acid (20:5n-3, EPA), arachidonic acid (20:4n-6, ARA), docosapentaenoic acid (22:5n-3, DPA), docosatetraenoic acid (22:4n-6, DTA) or docosahexaenoic acid (22:6n-3, DHA). DPA and DTA (>98-99% pure) were purchased from Cayman Chemical Co. (Ann Arbor, USA) and the remaining FA substrates (>99 % pure) and chemicals used to prepare the *S. cerevisiae* minimal medium-^{uracil} were from Sigma Chemical Co. Ltd. (Dorset, UK). Substrate FA final concentrations were as previously described (Monroig et al., 2010a). After 2 days, yeast were harvested and washed prior to lipid extraction and FA analyses. Yeast transformed with pYES2 containing no insert were cultured under the same conditions described above and used as control treatment.

2.4. FAME analysis by GC-MS

Yeast samples were homogenised in chloroform/methanol (2:1, v/v) containing 0.01% BHT as antioxidant and total lipid extracted following the Folch method (Folch

et al., 1957). Fatty acid methyl esters (FAME) were subsequently prepared, extracted and purified (Christie, 2003). FAME were identified and quantified using a gas chromatograph (GC8000) coupled to a MD800 mass spectrometer (ThermoFisher Scientific, Hemel Hempstead, UK) as described previously (Monroig et al., 2010a). Elongation activities on PUFA substrates were calculated as the proportion of substrate FA converted to elongated FA product(s) [product area / (product area + substrate area)] x 100. Conversion rates from 24:0 were not calculated as yeast endogenously contains several of the FA involved in the elongation pathway and thus individual saturated FA (≥ C24) contents from *elovl4*-transformed yeast were compared to those of control yeast (Monroig et al., 2010a).

2.5. Tissue distribution of salmon Elovl4

Tissue expression of *elovl4* was measured by quantitative real-time PCR (qPCR). Total RNA was extracted from eye, brain, testes, liver, muscle, heart, gills, kidney, intestine and spleen from salmon parr (40 g, n = 3) maintained at the Institute of Aquaculture Freshwater Research Facility (Buckieburn, Scotland), using Tri Reagent (Sigma, Poole, UK). One μg of DNase (Ambion, UK) treated total RNA was reverse transcribed into cDNA using SuperScript™ II Reverse Transcriptase (Invitrogen) and primed with oligo dT (AB Applied Biosystems, California, USA). qPCR analyses were performed using a Quantica Machine (Techne, Cambridge, UK) in a final volume of 20 μl containing 5 μl diluted (1/10) cDNA, 0.5 μM of each primer, and 10 μl of Absolute™ QPCR Sybr Green® Mix 2x (ABgene, Epsom, UK). Amplifications were carried out with a systematic negative control (NTC, no template control) containing no cDNA. Thermal cycling was initiated at 95°C for 15 min, followed by 40 cycles with a denaturing step at 95°C for 15 s, annealing at 60°C (*elovl4*) or 61°C (*elf-1α*) for 15 s,

and extension at 72°C for 15 s. After the amplification cycle, a melting curve was performed with 0.5°C increments ranging between 75°C and 90°C to ensure the amplification of a single product. In addition, the qPCR product sizes were checked by agarose gel electrophoresis and the identity of random samples confirmed by sequencing. No primer-dimer formation occurred in the NTC. *elf-1α* was used as a reference gene for normalisation of gene expression. Primers used for qPCR analyses are shown in Table 1.

2.6. Statistical analysis

Results were expressed as mean normalised ratios (± SE) between the copy numbers of the putative *elovl4* transcripts and the copy numbers of the reference gene, *elf-1α*. Differences in the expression of *elovl4* among tissues were analysed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test at a significance level of $P \leq 0.05$ (PASWS 18.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Salmon elongase Elovl4 sequence and phylogenetics

A 1,825 bp full-length cDNA sequence (5'UTR 231 bp, ORF 921 bp, 3'UTR 673) was obtained by 5' and 3' RACE PCR and deposited in the GenBank database under the accession number HM208347. The salmon *elovl4* ORF encodes a putative protein of 306 AA that shares 64.1-66.2% AA identity to mammalian orthologues including human, mouse and rat ELOVL4, and 62.6-63.3% identity with predicted Elovl4 sequences from birds. Among fish Elovl4, phylogenetic analysis showed two clusters, one including *Takifugu rubripes* Elovl4 and *Danio rerio* Elovl4_a (70.2 and 73.8% AA identity to salmon Elovl4, respectively), and the other grouping the newly cloned

salmon Elovl4 together with *D. rerio* Elovl4_b (86.0% identity), *Rachycentron canadum* and *Tetraodon nigroviridis* (both presenting 83.4% identity with salmon Elovl4) (Fig.1). All fish Elovl4 elongases grouped together with the mammalian and bird orthologues, and more distantly from other members of the Elovl family such as Elovl2 and Elovl5 from fish and mammals.

The newly cloned Elovl4 contains the three typical features characteristic of Elovl proteins: a single HXXHH histidine box motif, five putative transmembrane-spanning domains containing hydrophobic AA stretches, and a carboxyl-terminal targeting signal believed to be responsible for the retrieval/retention of transmembrane protein to the endoplasmic reticulum (ER) (Fig. 2). The ER retrieval signal from salmon Elovl4 possesses single lysine and arginine residues at the carboxyl terminus, RXKXX. In addition, the 17 AA residues that have been established to be highly conserved across 22 member of the Elovl family were identified (Fig. 2).

3.2. Functional characterisation

The salmon putative Elovl4 was functionally characterised by determining the FA profiles of transformed *S. cerevisiae* containing either the empty pYES2 vector (control) or the vector with the *elovl4* ORF insert and grown in presence of potential FA substrates. To test the ability of salmon Elovl4 to biosynthesise saturated VLC-FA, transgenic yeast were grown in presence of lignoceric acid (24:0) (Table 2, Fig. 3). Control yeast transformed with empty vector contained measurable levels of the substrate 24:0 (7.1% of total saturates ≥ C24), 26:0, the major component among total saturates ≥ C24 representing 84.8%, and 28:0 (7.0% of total saturates ≥ C24). Further saturated VLC-FA up to C34 were also detected in small amount (Table 2). On the other hand, the pYES2-*elovl4* transformed yeast showed a different profile of saturated FAs ≥

C24 compared to the control yeast, with decreased contents of 24:0 and 26:0, and increased levels of 28:0 (3.5-fold), 30:0 (9.5-fold), 32:0 (3.0-fold) and 36:0, the latter being absent in control yeast. These results confirmed that salmon Elovl4 is involved in the biosynthesis of saturated VLC-FA.

The role of salmon Elovl4 in the biosynthesis of VLC-PUFA was investigated by culturing yeast transformed with the *elovl4* ORF in the presence of one of either C20 (20:5n-3 and 20:4n-6) or C22 (22:5n-3, 22:4n-6 and 22:6n-3) PUFA substrates. GC-MS analyses confirmed that control yeast did not have the ability to elongate LC-PUFA consistent with the previously reported lack of a PUFA elongase in *S. saccharomyces* strain INVSc1 (Agaba et al., 2005). However, pYES2-*elovl4* yeast elongated PUFA, and VLC-PUFA with chain lengths up to C36 were produced (Table 3; Fig. 4). Elovl4 showed higher activity towards 20:5n-3 and 20:4n-6 with up to 32.5% and 29.9% of each substrate converted to longer products, respectively, whereas C22 PUFA substrates were elongated to a lower extent (Table 3). It is noteworthy that salmon Elovl4 elongase was able to convert 20:5n-3 and 22:5n-3 to 24:5n-3, an intermediate substrate for DHA biosynthesis via the so-called Sprecher shunt pathway (Sprecher, 2000). In contrast, salmon Elovl4 had only very low activity towards DHA itself, whose conversion rate was only 5.4% (Table 3). This result was consistent with DHA not being a preferred substrate for elongation by Elovl4 (Suh and Clandinin, 2005).

3.3. Tissue expression of elovl4

Tissue distribution of *elovl4* mRNA transcript was measured by real-time qPCR. Results indicate that *elovl4* was expressed in all tissues analysed, with the eye exhibiting significantly higher expression than any other ($P<0.05$) (Fig. 5). Although no significant differences were found in brain and testes they also showed high levels of transcripts,

with 4.9- and 3.8-fold increase, respectively, compared to liver expression signals. Liver exhibited the lowest expression levels, despite being regarded as a major metabolic site of LC-PUFA biosynthesis (Tocher, 2003).

4. Discussion

Human *ELOVL4* was discovered as a gene causing an inherited dominant form of Stargardt-like macular dystrophy (Bernstein et al., 2001; Zhang et al., 2001). Its AA sequence similarities with other Elovl proteins, together with its localisation in the ER (Grayson et al., 2005), the site of long-chain FA synthesis, suggested that Elovl4 had some functional role in FA biosynthesis. The first Elovl4 elongase that was functionally characterised, the human enzyme ELOVL4, confirmed a role in the biosynthesis of VLC-FA including both saturated and polyunsaturated (Agbaga et al., 2008). Despite the attention devoted to investigate the FA composition of fish and fish oil, the difficulties of analyses have meant that VLC-FA have been barely investigated in fish, with GC analysis routinely terminating with 22:6n-3 (DHA) or 24:1 (Ackman, 2000). However, VLC-FA longer than C24 are found in fish, as reported by Aveldaño (1988) who reported the presence of polyene VLC-FA up to C36 in retinal lipids of two fish species. In order to elucidate the molecular mechanisms of VLC-FA biosynthesis in fish, we have focussed on the isolation and characterisation of Elovl4 enzymes from a freshwater model species, zebrafish, the marine teleost cobia, and now the diadromous salmonid, Atlantic salmon.

The salmon Elovl4 possesses conserved regions, which coincide with predicted functional motifs that are common to all Elovl proteins. The histidine box, HXXHH, conserved in all elongases and also characteristic of desaturase and hydrolase enzymes containing a di-iron-oxo cluster (Fe-O-Fe), is involved in the coordination of electron

reception during reduction reactions occurring during FA elongation (Jakobsson et al., 2006). By sequence comparison with vertebrate Elovl4 orthologues, five conserved putative transmembrane-spanning domains containing hydrophobic AA stretches can be predicted (Zhang et al., 2003). In addition the 17-AA motif described by Leonard et al. (2004) to be a common feature of elongases, was also identified in salmon Elovl4. The ER retrieval signal is essential for Elovl4 to integrate into the ER where its primary function occurs. Typically ELOVL4 proteins possess dilysine motifs (KKXX or KXKXX) at the C-terminus as observed in several mammals including human and mouse, as well as birds, and the zebrafish Elovl4_b (Zhang et al., 2003). The ER retrieval signal of salmon Elovl4 contains an arginine residue in position –5 from the C-terminus of the protein, and conserves one lysine residue in the position –3 (RXKXX), the latter regarded as a critical residue for ER retrieval signal function (Jackson et al., 1990). The RXKXX pattern is common to other teleostei Elovl4 including the puffer fish *T. rubripes* and *T. nigroviridis*, the *D. rerio* Elovl4_a, and cobia *R. canadum* (Zhang et al., 2003; Monroig et al., 2010a, 2011).

Elovl4 proteins are highly conserved through evolution (Leonard et al., 2004). Consistent with that, the phylogenetic analysis revealed that the salmon *elovl4* cDNA encodes a protein more similar to other Elovl4 orthologues from mammals, birds and fish, than the other Elovl family proteins characterised previously in Atlantic salmon, the Elovl5_a and Elovl5_b, and Elovl2 (Hastings et al., 2005; Morais et al., 2009). Regarding fish Elovl4 proteins, two groups were identified with *T. rubripes* Elovl4 and *D. rerio* Elovl4_a grouping separately from *D. rerio* Elovl4_b, *R. canadum*, *T. nigroviridis* and Atlantic salmon Elovl4. This clustering pattern is consistent with the differing functional activities between the two fish Elovl4 groups. The two Elovl4 present in zebrafish, Elovl4_a and Elovl4_b, were the first non-human Elovl4 proteins

functionally characterised (Monroig et al., 2010a), and they showed marked differences in their substrate specificity. Whereas zebrafish Elovl4_a was only able to produce saturated VLC-FA, the b isoform was efficient in the synthesis of both saturated VLC-FA and VLC-PUFA up to C36. The other members of the same phylogenetic cluster, cobia (Monroig et al., 2011) and Atlantic salmon, have Elovl4 enzymes confirmed to have similar functions to those described for zebrafish Elovl4_b. Thus, yeast cells expressing the salmon *elovl4* showed decreased percentages of 24:0 and 26:0, and concomitant increased levels of longer FA products. This indicates that fish Elovl4 have both 24:0 and 26:0 as preferred substrates.

In addition to a role in the biosynthesis of saturated VLC-FA, salmon Elovl4, as with zebrafish Elovl4_b (Monroig et al., 2010a) and cobia Elovl4 (Monroig et al., 2011), also appears to be a critical enzyme required for the biosynthesis of VLC-PUFA. Salmon Elovl4 showed high efficiency in the elongation of C20 and C22 PUFA substrates that led to the production of n-3 and n-6 polyenes containing up to C36. The VLC-PUFA are FA uniquely found in specific lipid molecules of retina (Alvedaño, 1987, 1988), brain (Robinson et al., 1990), and testis (Furland et al., 2003, 2007a,b; Zadravec et al., 2011). Although VLC-PUFA contents were not determined in salmon tissues in the present study, the tissue distribution analysis of *elovl4* mRNA transcripts is consistent with eye (probably retina), brain and testis being prominent metabolic sites for the biosynthesis of VLC-PUFA in Atlantic salmon. Moreover, these results suggest that, of the Elovl proteins characterised in Atlantic salmon, only Elovl4 is able to produce PUFA products longer than C24. Previously, Elovl5 and Elovl2 elongases involved in LC-PUFA biosynthesis were functionally characterised with salmon Elovl5_a and Elovl5_b showing high elongation efficiency towards C18 and C20 PUFA, whereas Elovl2 produced C24 PUFA from C20 and C22 PUFA and, in all cases, the production of

longer PUFA was negligible (Hastings et al., 2005; Morais et al., 2009). Interestingly, a recent study has shown that mice ELOVL2 participates in the biosynthesis of n-6 VLC-PUFA that are critical in male fertility, and that 24:5n-6 appears a preferred substrate for murine ELOVL2 (Zadravec et al., 2011). Clearly, future investigations should elucidate the role that Elovl2 can play in the production of such potentially indispensable compounds in farmed fish.

Based on the functional analysis data obtained from yeast expression studies of several genes encoding both desaturases and elongases, we can now predict the LC- and VLC-PUFA biosynthetic pathway from the dietary essential fatty acids α-linolenic (18:3n-3) and linoleic (18:2n-6) in Atlantic salmon (Fig. 6). Together with the conversions previously described for the fatty acyl desaturases (Hastings et al, 2005; Zheng et al., 2005; Monroig et al., 2010b) and the Elovl2 and Elovl5 elongases (Hastings et al., 2005; Morais et al., 2009), the Elovl4 is now expanding the biosynthetic pathway. It is noteworthy that Atlantic salmon Elovl4, as Elovl2, may participate in the biosynthesis of DHA, as it is able to convert 22:5n-3 to 24:5n-3, which can be further Δ6-desaturated to 24:6n-3 that subsequently is chain-shortened to DHA (Sprecher, 2000). These results further confirm that some teleostei Elovl4, in contrast to mammalian orthologues (Agbaga et al., 2010a), can play an active role in the biosynthesis of DHA (Monroig et al., 2010a; 2011).

In conclusion, the cDNA of an Elovl4 elongase of Atlantic salmon was cloned and characterised. The salmon Elovl4 possessed all the features of Elovl proteins and is phylogenetically closer to other Elovl4 orthologues of other animal classes than to other Elovl proteins of Atlantic salmon. The functional analysis demonstrated that Elovl4 is involved in the biosynthesis of both saturated VLC-FA and VLC-PUFA, similar to the pattern of activity described in some other Elovl4 elongases from fish species including

zebrafish (isoform Elovl4_b) and cobia. Our results also suggest that shorter (C20-C22) PUFA are suitable precursors for further elongation to VLC-PUFA by Elovl4. Further studies are investigating the impact that vegetable oil-based diets, devoid of potential Elovl4 C20-22 PUFA precursors, have on *elovl4* transcriptional regulation and activity and on the biosynthesis and metabolism of VLC-PUFA in farmed fish.

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FIGURE CAPTIONS

Fig 1. Phylogenetic tree comparing the salmon Elovl4 with elongase proteins from other organisms. The tree was constructed using the Neighbor Joining method using MEGA4. The horizontal branch length is proportional to amino acid substitution rate per site. The numbers represent the frequencies (%) with which the tree topology presented was replicated after 1000 iterations.

Fig 2. Comparison of the deduced amino acid (AA) sequence of the Atlantic salmon Elovl4 with those of zebrafish *Danio rerio* (isoforms Elovl4_a and Elovl4_b) and cobia *Rachycentron canadum*. The AA sequences were aligned using ClustalX, and identity/similarity shading was based on a 60% identity threshold. Identical residues are shaded black and similar residues are shaded grey. Indicated are the conserved HXXHH histidine box motif, five (I-V) putative membrane-spanning domains predicted by

Zhang et al. (2003), and the ER retrieval signal. An asterisk indicates each of the 17 AA residues conserved across Elovl proteins (Leonard et al., 2004).

Fig 3. Role of Atlantic salmon Elovl4 elongase in the biosynthesis of saturated very long chain fatty acids (VLC-FA). Yeast transformed with pYES2 vector containing the *elovl4* ORF (A) as insert were grown in the presence of α-lignoceric acid (24:0), and fatty acid composition determined. Saturated VLC-FA from control yeast transformed with empty pYES2 are shown in panel B. Substrate 24:0 ("*") and its corresponding elongated products are indicated accordingly. Vertical axis, EI-MS response; horizontal axis, retention time.

Fig 4. Role of Atlantic salmon Elovl4 elongase in the biosynthesis of very long chain polyunsaturated fatty acids (VLC-PUFA). Yeast transformed with pYES2 vector containing the ORF of *elovl4* as insert were grown in the presence of PUFA substrates 20:5n-3 (EPA), and 22:5n-3 (DPA), and fatty acid composition determined. Substrates ("*") and their corresponding elongated products are indicated accordingly in the panels. Vertical axis, EI-MS response; horizontal axis, retention time.

Fig 5. Tissue expression of *elovl4* transcripts (mRNA) in Atlantic salmon. Absolute number of copies were quantified by qPCR and normalised by absolute levels of *elf-1α*. Results are means (±SE) of analysis performed in triplicate. * $P<0.05$ as determined by one-way ANOVA and Tukey's test.

Fig 6. The biosynthesis pathway of long-chain and very long-chain polyunsaturated fatty acids from α-linolenic (18:3n-3) and linoleic (18:2n-6) acids. Enzymatic activities shown in the scheme are predicted from heterologous expression in *S. cerevisiae* of the Δ5 fatty acyl desaturase (Fad), the three Δ6 Fad (Hastings et al., 2005, Zheng et al.,

2005; Monroig et al., 2010b), and Elovl2 and Elovl5 elongases (Hastings et al., 2005; Morais et al., 2009). Steps catalysed by the newly characterised Atlantic salmon Elovl4 are also shown.

Table 1. Details of primer pairs (restriction sites for *Hin*dIII and *Xho*I underlined) used for the cloning of salmon *elovl4* ORF in pYES2, and qPCR analysis of tissue expression.

Aim	Transcript	Primer	Primer sequence	Amplicon size	Ta	Accession no.[a]
5'RACE	*elovl4*	*SALE4R3*	*5'-CCACCAGAGGAATTTCTGGA-3'*	*763 bp*	*57°C*	*HM208347*
	elovl4	*SALE4R2*	*5'-ACTTGATTCCGATCCACCAG-3'*	*875 bp*	*59°C*	*HM208347*
3'RACE	*elovl4*	*SALE4bF4*	*5'-GTTCTGCCCACAAGGTGG-3'*	*837 bp*	*58°C*	*HM208347*
	elovl4	*SALE4bF5*	*5'-GCCCGTCACTAATGGTGTC-3'*	*815 bp*	*59°C*	*HM208347*
ORF cloning	*elovl4*	*UNIELO4F1*	*5'-GTCTACAACTTCAGCATGGTG-3'*	*439 bp*	*55°C*	*HM208347*
		UNIELO4R2	*5'-GGAACTGGATCATCTGAATAAT-3'*			
	elovl4	*SALE4U5F*	*5'-TCTCGAGAAGCATAAGCAGGA-3'*	*1,147 bp*	*58°C*	*HM208347*
		SALE4U3R	*5'-ATGCCAGGTACAATGCATTC-3'*			
	elovl4	*SALE4VF*	*5'-CCCAAGCTTAGGATGGAAGCTGTCACC-3'*	*942 bp*	*60°C*	*HM208347*
		SALE4VR	*5'-CCGCTCGAGTTACTCCCTTTTCGCTCTT-3'*			
	elovl4	*SAL45UF2*	*5'-TCCGCTTGCCTTCTGTAGTT-3'*	*1,653 bp*	*55°C*	*HM208347*
		SAL43UR1	*5'-CCCCTCAGTCCTATTGGTCA-3'*			
qPCR	*elovl4*	*SAL4q3UF6*	*5'-TTGTCAAATTGGTCCTGTGC-3'*	*191 bp*	*61°C*	*HM208347*
		SAL4q3UR6b	*5'-TTAAAAGCCCTTTGGGATGA-3'*			
	elf-1a	*qPCREloAF*	*5'-TCTGGAGACGCTGCTATTGTTG-3'*	*176 bp*	*59°C*	*AF321836*
		qPCREloAR	*5'-GACTTTGTGACCTTGCCGCTTGAG-3'*			

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

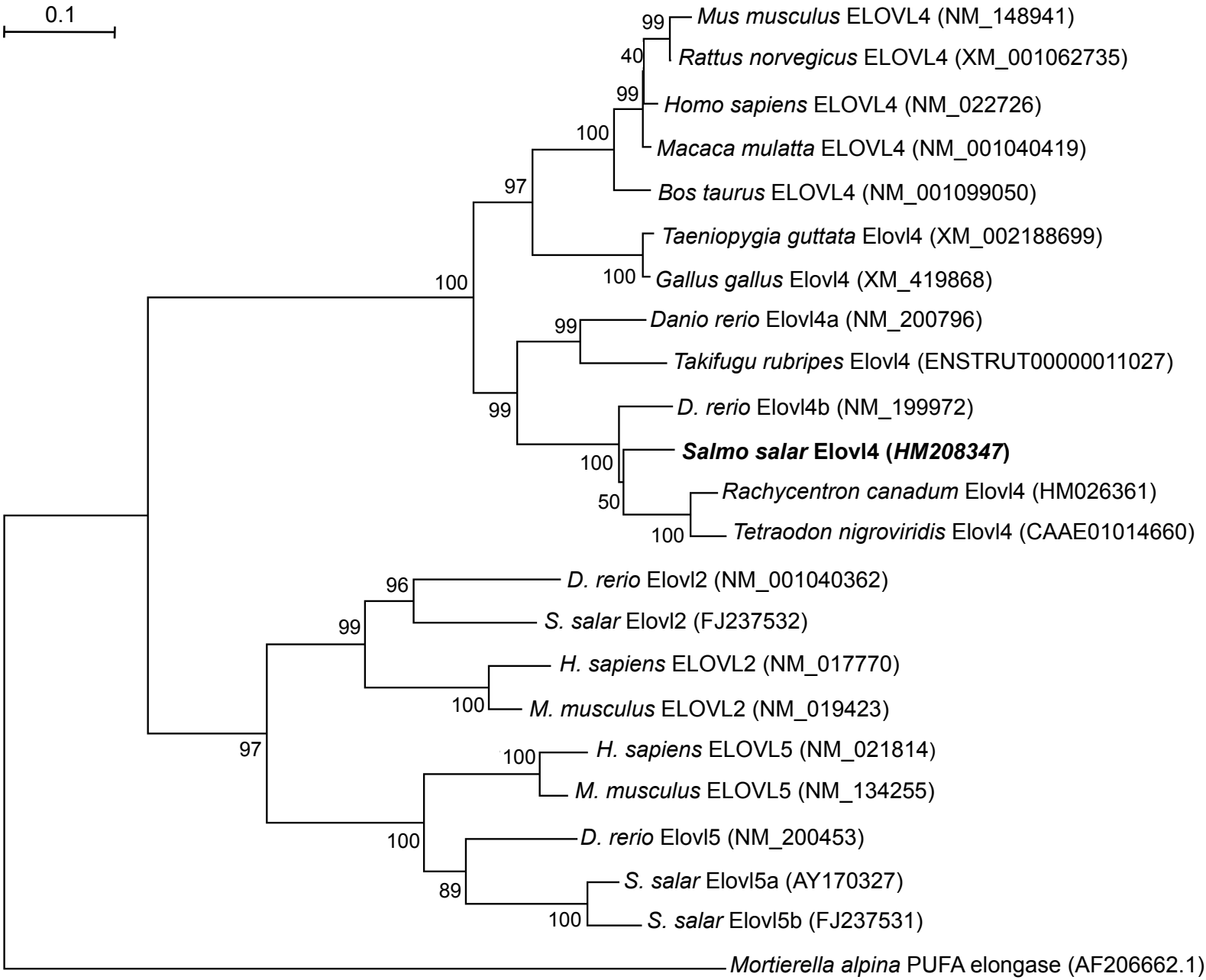
Table 2. Functional characterisation of Atlantic salmon Elovl4 elongase and its role in biosynthesis of very long-chain saturated fatty acids (VLC-FA). Results are expressed as amount of each individual FA as a percentage of total peak area for all saturated VLC-FA (C ≥ 24). The yeast were transformed with either the empty pYES2 vector (control) or the pYES2 vector containing the salmon *elovl4* ORF.

FA	Elovl4	Control
24:0 *	3.6	7.1
26:0	65.5	84.8
28:0	24.6	7.0
30:0	4.8	0.5
32:0	0.6	0.2
34:0	0.1	0.3
36:0	0.8	0.0

* Lignoceric acid used as exogenously added substrate.

Table 3. Functional characterisation of Atlantic salmon Elovl4 elongase and its role in the biosynthesis of very-long-chain polyunsaturated fatty acids (VLC-PUFA) Data show the conversions of PUFA substrates and are expressed as percentage of fatty acid substrate converted to elongated product at each step of the pathway and overall (Total).

PUFA substrate	Product	Conversion (%) Elovl4	Activity
20:5n-3	22:5n-3	15.4	C20 → 22
	24:5n-3	3.3	C22 → 24
	26:5n-3	0.6	C24 → 26
	28:5n-3	0.1	C26 → 28
	30:5n-3	1.4	C28 → 30
	32:5n-3	7.4	C30 → 32
	34:5n-3	4.0	C32 → 34
	36:5n-3	0.3	C34 → 36
	Total	32.5	
20:4n-6	22:4n-6	11.5	C20 → 22
	24:4n-6	3.1	C22 → 24
	26:4n-6	0.9	C24 → 26
	28:4n-6	0.6	C26 → 28
	30:4n-6	5.5	C28 → 30
	32:4n-6	7.2	C30 → 32
	34:4n-6	1.1	C32 → 34
	36:4n-6	0.1	C34 → 36
	Total	29.9	
22:5n-3	24:5n-3	5.7	C22 → 24
	26:5n-3	0.7	C24 → 26
	28:5n-3	0.2	C26 → 28
	30:5n-3	1.9	C28 → 30
	32:5n-3	9.8	C30 → 32
	34:5n-3	3.6	C32 → 34
	36:5n-3	0.1	C34 → 36
	Total	22.1	
22:4n-6	24:4n-6	4.3	C22 → 24
	26:4n-6	1.2	C24 → 26
	28:4n-6	0.8	C26 → 28
	30:4n-6	8.2	C28 → 30
	32:4n-6	11.1	C30 → 32
	34:4n-6	1.5	C32 → 34
	36:4n-6	0.1	C34 → 36
	Total	27.1	
22:6n-3	24:6n-3	3.2	C22 → 24
	26:6n-3	0.3	C24 → 26
	28:6n-3	0.0	C26 → 28
	30:6n-3	0.1	C28 → 30
	32:6n-3	1.3	C30 → 32
	34:6n-3	0.4	C32 → 34
	36:6n-3	0.0	C34 → 36
	Total	5.4	


0.1
99
Mus musculus ELOVL4 (NM_148941)
40
Rattus norvegicus ELOVL4 (XM_001062735)
99
Homo sapiens ELOVL4 (NM_022726)
100
Macaca mulatta ELOVL4 (NM_001040419)
97
Bos taurus ELOVL4 (NM_001099050)
Taeniopygia guttata Elovl4 (XM_002188699)
100
Gallus gallus Elovl4 (XM_419868)
100
99
Danio rerio Elovl4a (NM_200796)
Takifugu rubripes Elovl4 (ENSTRUT00000011027)
99
D. rerio Elovl4b (NM_199972)
100
Salmo salar Elovl4 (HM208347)
50
Rachycentron canadum Elovl4 (HM026361)
100
Tetraodon nigroviridis Elovl4 (CAAE01014660)
96
D. rerio Elovl2 (NM_001040362)
99
S. salar Elovl2 (FJ237532)
H. sapiens ELOVL2 (NM_017770)
100
M. musculus ELOVL2 (NM_019423)
97
100
H. sapiens ELOVL5 (NM_021814)
M. musculus ELOVL5 (NM_134255)
100
D. rerio Elovl5 (NM_200453)
89
S. salar Elovl5a (AY170327)
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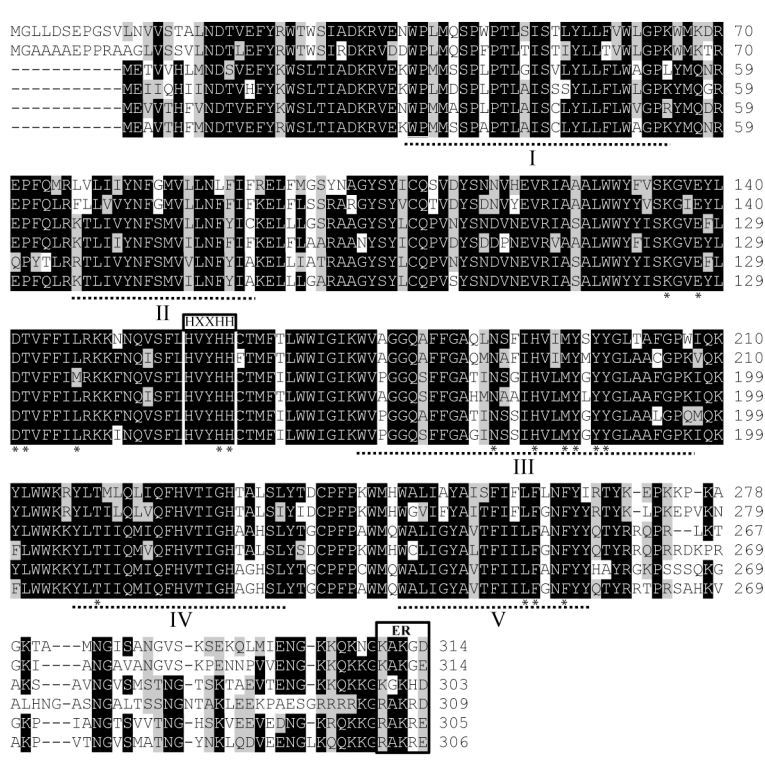
S. salar Elovl5b (FJ237531)
Mortierella alpina PUFA elongase (AF206662.1)


Homo sapiens
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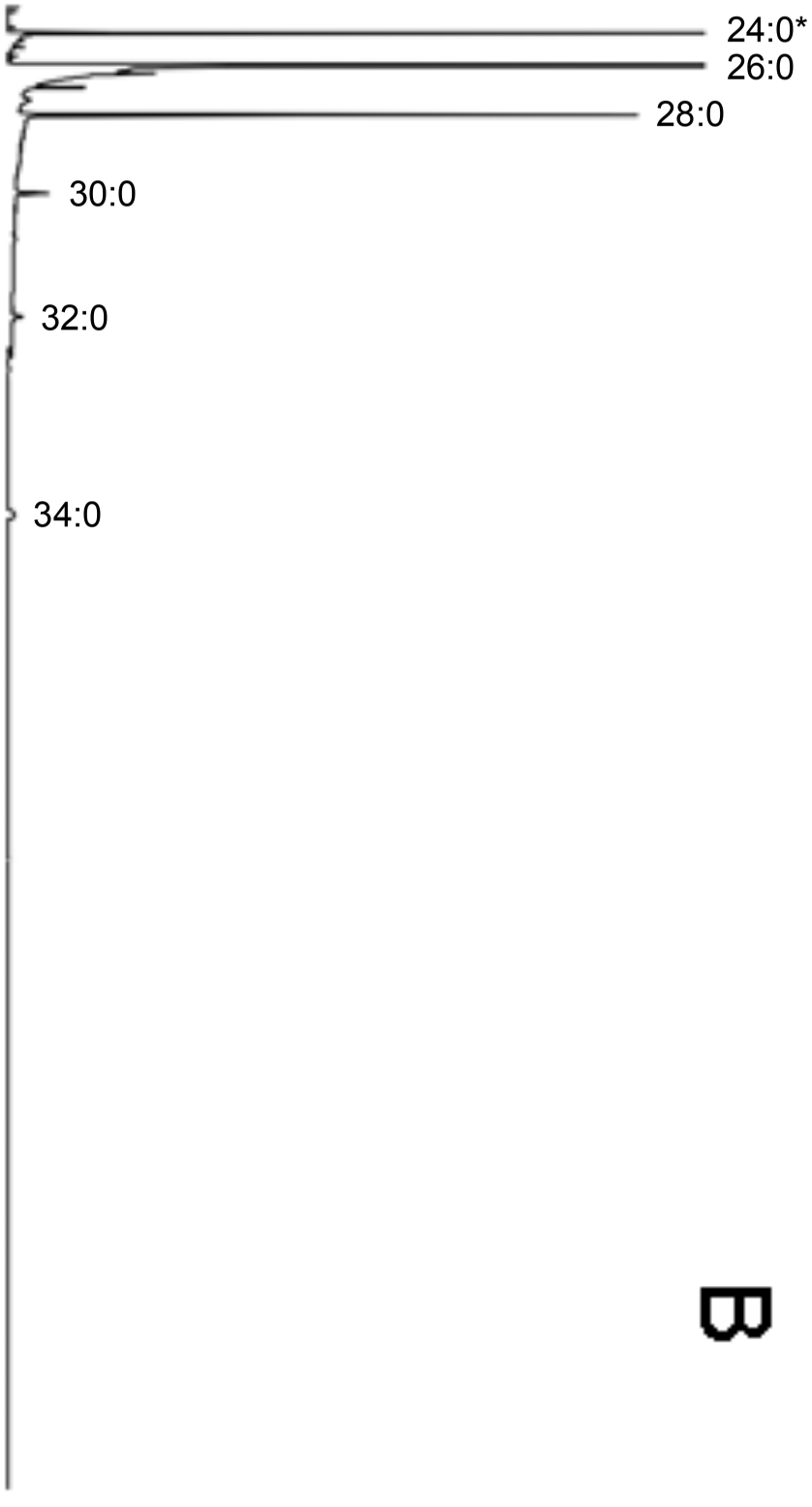
D. rerio a
Rachycentron canadum
Salmo salar
I
II
HXXHH
III
IV
V
ER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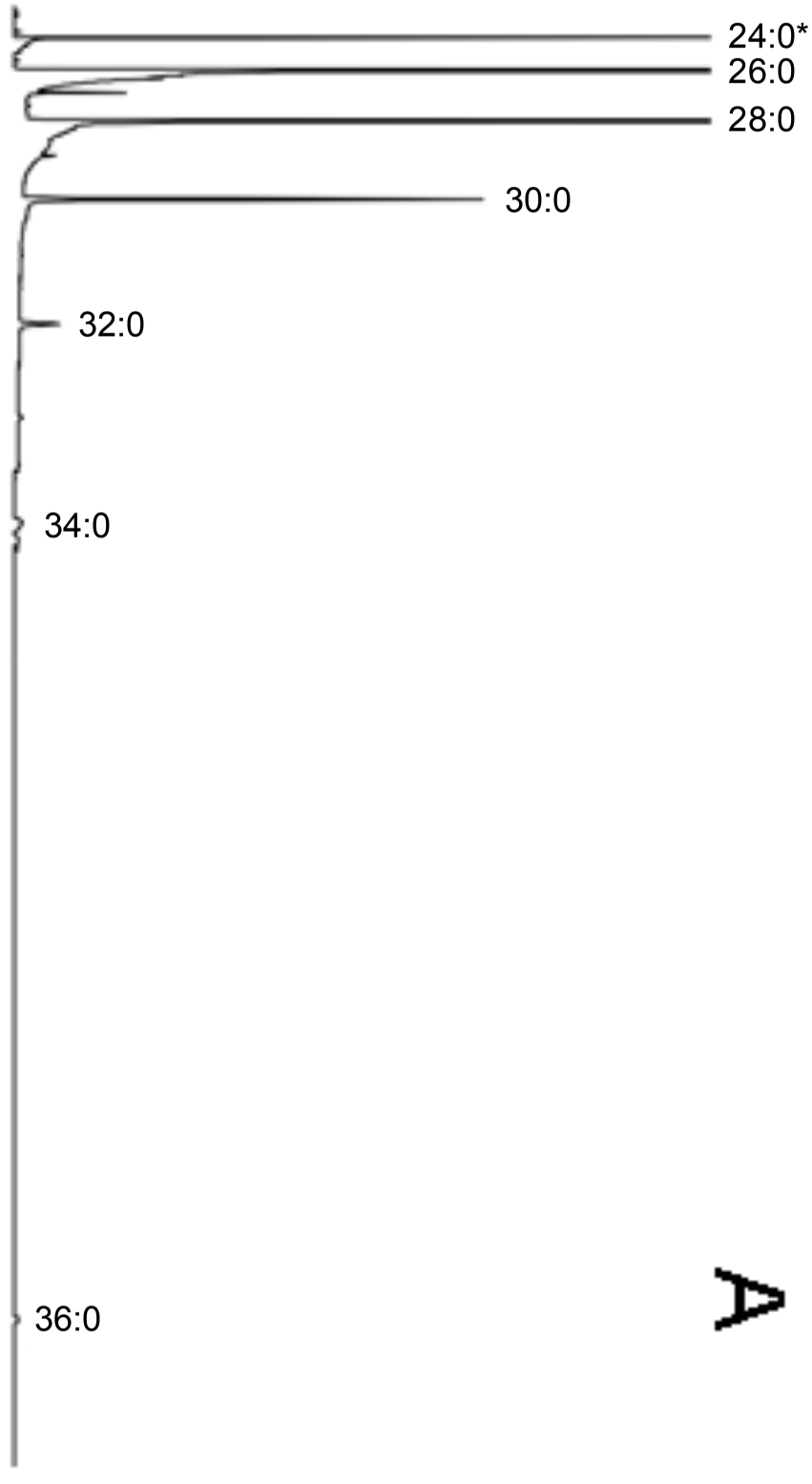
32:0
34:0
B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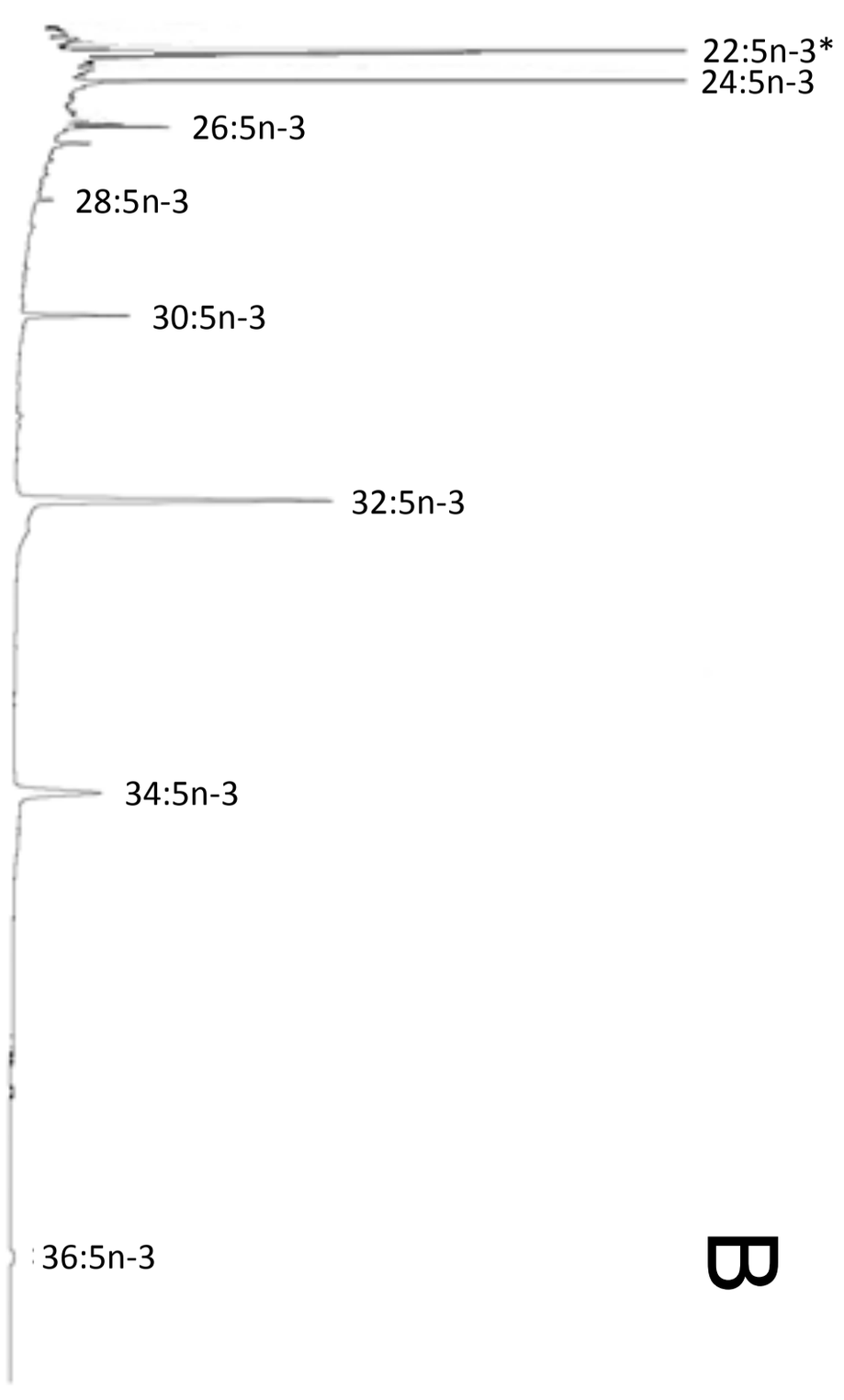
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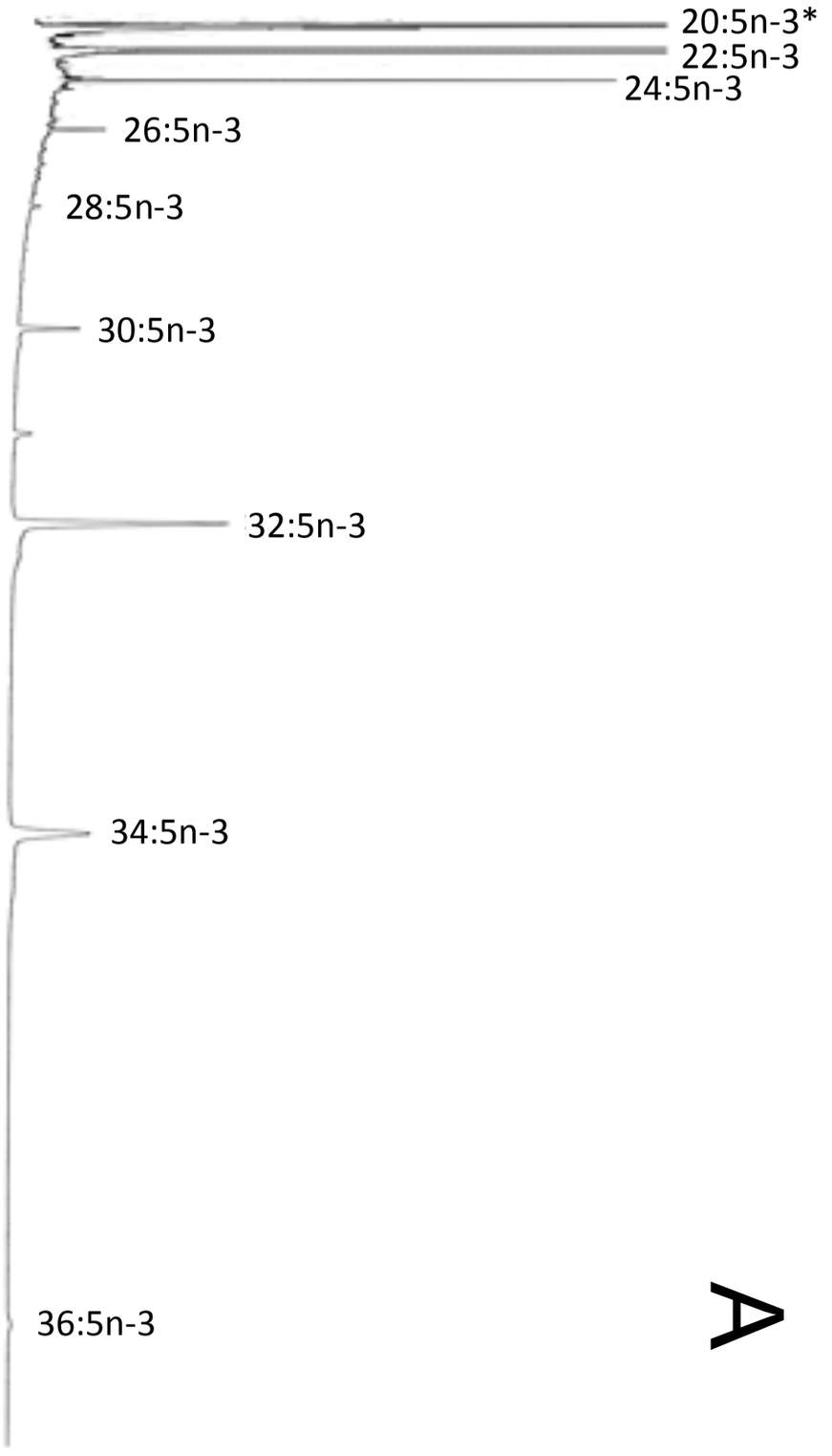
B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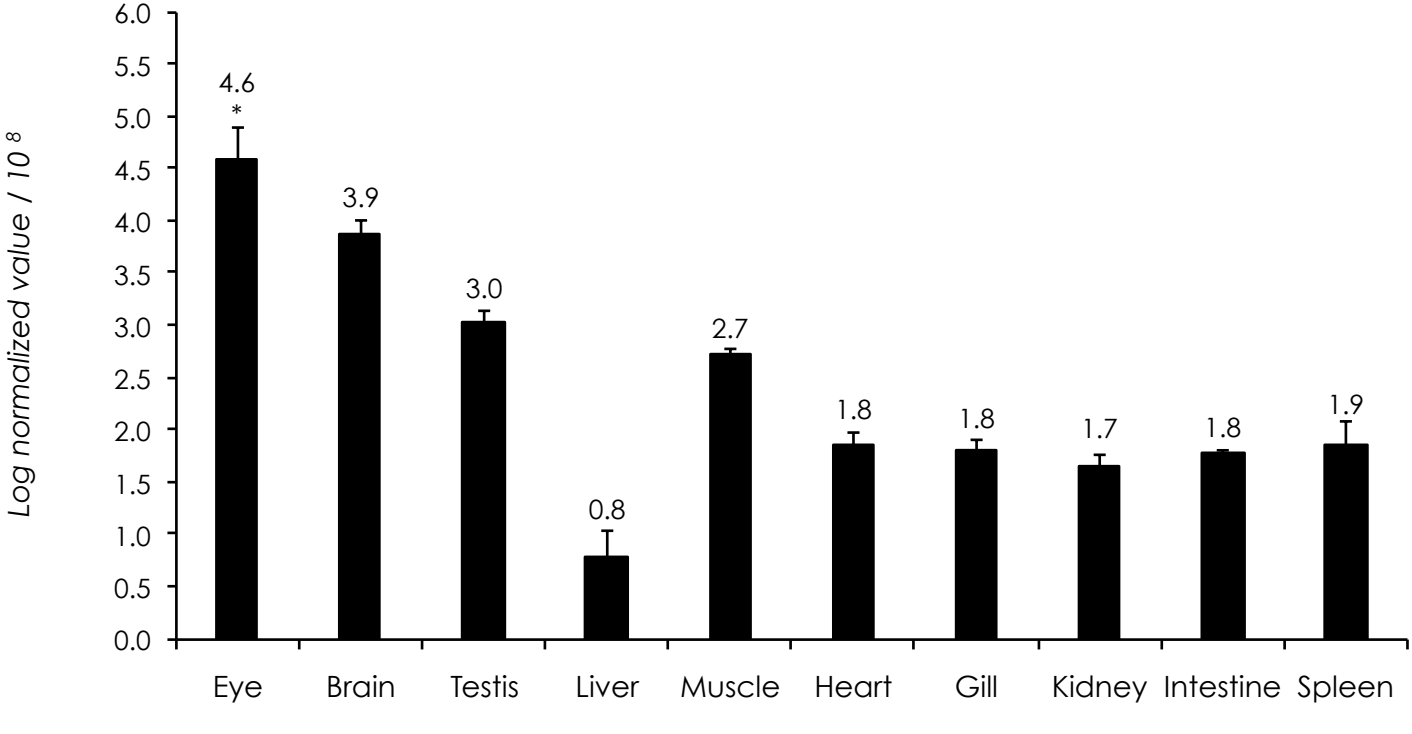
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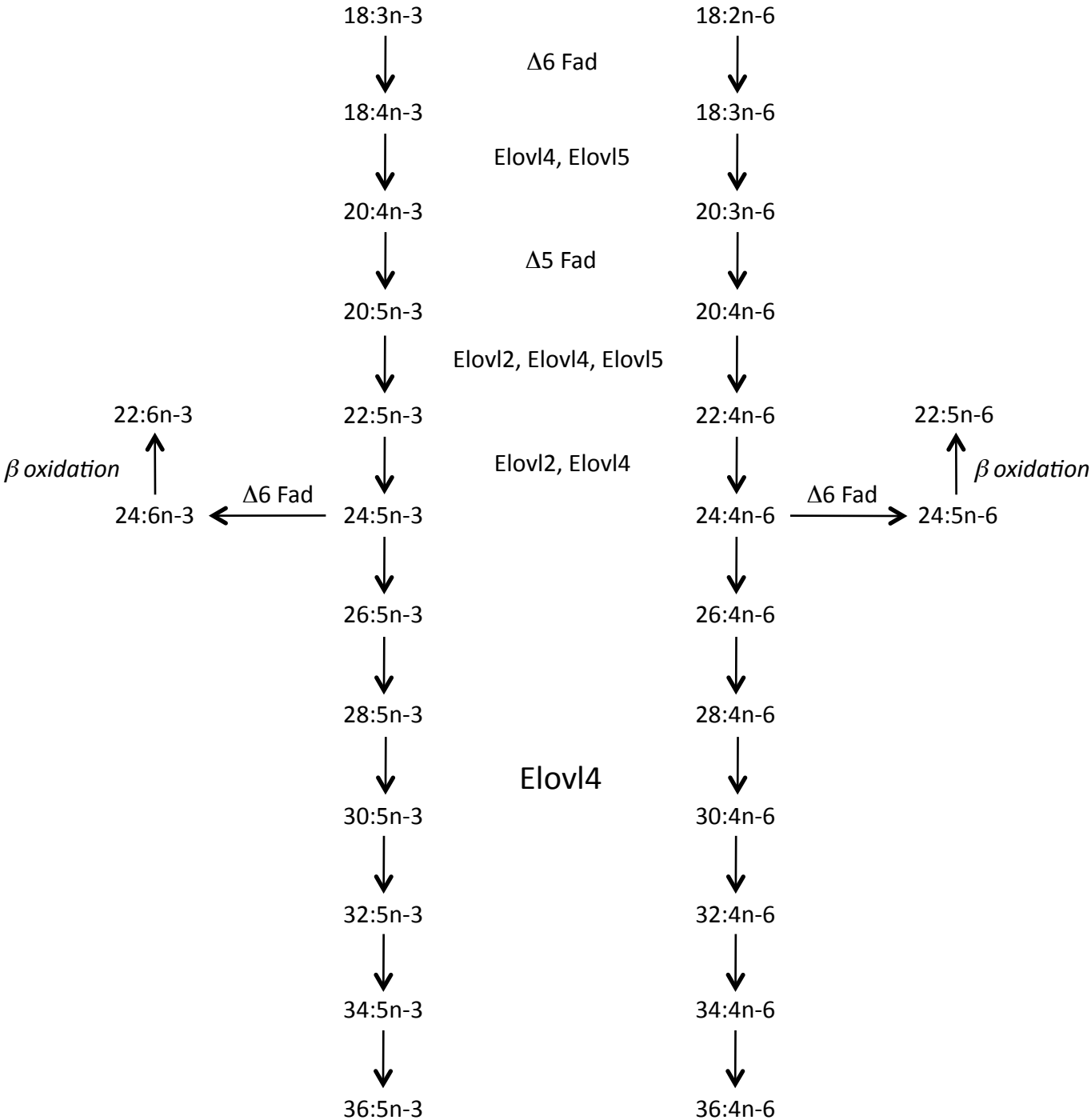
1.0
0.5
0.0
4.6
*
3.9
3.0
0.8
2.7
1.8
1.8
1.7
1.8
1.9
Eye
Brain
Testis
Liver
Muscle
Heart
Gill
Kidney
Intestine
Spleen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Δ6 Fad
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Elovl4, Elovl5
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Δ5 Fad
20:5n-3
20:4n-6
Elovl2, Elovl4, Elovl5
22:6n-3
22:5n-3
22:4n-6
22:5n-6
β oxidation
Elovl2, Elovl4
β oxidation
Δ6 Fad
Δ6 Fad
24:6n-3
24:5n-3
24:4n-6
24:5n-6
26:5n-3
26:4n-6
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28:4n-6
Elovl4
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