Title

Biosynthesis of long-chain polyunsaturated fatty acids in marine fish: Characterization of an Elovl4-like elongase from cobia *Rachycentron canadum* and activation of the pathway during early life stages

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Summary

Marine fish, unlike freshwater species, have been generally considered to have a limited ability to biosynthesize long-chain polyunsaturated fatty acids (LC-PUFA) from C_{18} precursors due to apparent limited enzymatic activities involved in the pathway. Although LC-PUFA play important physiological roles throughout the entire life cycle, requirements for early life stages are especially high and provision of preformed LC-PUFA in egg lipids appears critical to support the formation of developing tissues where these compounds accumulate. No studies, however, have been conducted to explore the capability of marine fish embryos (here referring to life stages from zygote to the oesophagus opening) for *de novo* synthesis of the LC-PUFA required for normal growth and development. The present study aimed to investigate the activation of the LC-PUFA biosynthetic pathway during embryogenesis of the marine teleost cobia (*Rachycentron canadum*). First, a fatty acyl elongase with sequence similarity to mammalian elongase of very long-chain fatty acids 4 (Elovl4) was isolated, and its biochemical function characterized showing that it catalyzed the production of very long-chain fatty acids (VLC-FA) including both saturated and polyunsaturated fatty acids with chain lenghts ≥ 24 carbons. Notably, cobia Elovl4 was able to elongate 22:5n-3 to 24:5n-3 and thus could play a key role in the biosynthesis of docosahexaenoic acid (22:6n-3), a critical fatty acid in neural tissues. Subsequently, the fatty acid dynamics of embryos at different developmental stages and the temporal expression patterns of target genes including *elovl4*, and the formerly characterized *elovl5* elongase and Δ6 fatty acyl desaturase, were analyzed in order to elucidate the overall activation of the LC-PUFA biosynthetic pathway in cobia embryos. Our results indicated that expression of the LC-PUFA biosynthetic pathway in cobia embryos is initiated at 12-18 hours post-fertilization.

1. Introduction

Fish are the primary source in the human diet of n-3 long-chain polyunsaturated fatty acids (LC-PUFA), which have been demonstrated to promote cardiovascular health and immune function, and protect against neurological and inflammatory conditions (Calder, 2007; Calon and Cole, 2007; Torrejon et al., 2007). With aquaculture increasing its contribution to the overall supply of fish in the human food basket (FAO, 2009), considerable efforts have been made to elucidate the biochemical and molecular mechanisms controlling the biosynthesis of LC-PUFA in fish (Tocher, 2003). These investigations have allowed us to determine that the capacity of fish to biosynthesize LC-PUFA varies with species, and ultimately depends on the enzymatic complement required in the metabolic process.

The accepted biosynthetic pathway in fish consists of consecutive enzymatic reactions that convert C_{18} PUFA 18:3n-3 (α-linolenic acid) and 18:2n-6 (linoleic acid) to LC-PUFA (Fig. 1). Two types of enzymes are responsible for these conversions (Sargent et al., 2002). Fatty acyl desaturases (Fad) introduce double bonds into fatty acyl chains, and elongases of very long-chain fatty acids (Elovl) are responsible for a condensation reaction resulting in a 2-carbon elongation of the pre-existing chain (Jakobsson et al., 2006). Thus for synthesis of arachidonic acid (20:4n-6, ARA), 18:2n-6 is desaturated by Δ6 Fad to 18:3n-6, which is elongated to 20:3n-6 and then desaturated by Δ5 Fad to ARA. Synthesis of eicosapentaenoic acid (20:5n-3, EPA) from 18:3n-3 requires the same enzymes and pathway as for ARA, but synthesis of docosahexaenoic acid (22:6n-3, DHA) reportedly requires two further elongation steps, a further Δ6 desaturation and a peroxisomal chain shortening step (Sprecher, 2000). An alternative more direct way for DHA biosynthesis has been recently described for the

74 first time in vertebrates in the herbivorous marine fish, *Siganus canaliculatus*, which
75 expresses a Δ4 Fad capable of desaturating 22:5n-3 to DHA (Li et al., 2010) (Fig. 1).
76 Most marine fish species have low LC-PUFA biosynthetic capacity due in part to the
77 apparent lack of specific enzymatic activities required in the pathway. Other than a
78 bifunctional Δ6/Δ5 Fad found in *S. canaliculatus* (Li et al., 2010), no Δ5 Fad has been
79 reported in marine species, and all *fad* genes have been characterized as monofunctional
80 Δ6 Fads (Zheng et al., 2004, 2009; Tocher et al., 2006; González-Rovira et al., 2009;
81 Mohd-Yusof et al., 2010). Additionally, marine teleosts appear to lack Elovl2, an
82 enzyme that elongates C_{20} and C_{22} LC-PUFA including 22:5n-3 to 24:5n-3 (Fig. 1) and
83 is thus regarded as an essential enzyme for DHA biosynthesis (Monroig et al., 2009;
84 Morais et al., 2009). Elovl2 functions differ from those of Elovl5, an elongase isolated
85 from a number of marine fish species (Agaba et al., 2005; Zheng et al., 2009; Gregory
86 et al., 2010; Mohd-Yusof et al., 2010), but which has virtually no activity towards C_{22}
87 LC-PUFA. However, marine fish species may have other Elovl enzymes whose
88 functions may partially compensate for the above mentioned incapacity to perform the
89 last elongation steps in the biosynthetic pathway of LC-PUFA.
90 Elovl4 is the most recent member of the Elovl family to be investigated in fish
91 (Monroig et al., 2010). Zebrafish possesses two Elovl4 enzymes that are responsible for
92 the biosynthesis of very long-chain fatty acids (VLC-FAs), including saturated and
93 polyunsaturated FA with chain-lengths $\geq C_{24}$. Studies in mammals have shown that
94 VLC-FA play pivotal functions in phototransduction, fertility and skin permeability
95 (Cameron et al., 2007; Agbaga et al., 2010; Zadravec, 2010), although they have been
96 barely investigated in fish (Alvedaño, 1987). Particularly interesting though, some fish
97 Elovl4 have, in contrast to mammalian Elovl4, the ability to facilitate the synthesis of
98 DHA by possessing Elovl2-like activity. Thus, whereas one zebrafish isoform, Elovl4a,

99 did not show relevant activity towards 22:5n-3, the other isoform, Elovl4b,
100 demonstrated the ability to elongate 22:5n-3 to 24:5n-3 when expressed in yeast
101 (Monroig et al., 2010). These results prompt the question whether marine species have
102 Elovl4 enzymes whose functions resemble those of zebrafish Elovl4a, or contrarily,
103 those of Elovl4b, the latter having important consequences for the production of
104 physiologically essential LC-PUFA including DHA, to compensate for the apparent
105 absence of Elovl2 in marine fish genomes.

106 The insufficiency in LC-PUFA biosynthesis in marine fish may be particularly
107 critical in early developmental stages, where physiological requirements for LC-PUFA,
108 especially DHA, are high due to the rapid formation and development of neural tissues
109 (Bell et al., 1995; Navarro et al., 1997; Benítez-Santana et al., 2007). Whereas dietary
110 LC-PUFA enhancement of broodstock has been shown to improve offspring viability
111 (Rodríguez et al., 1998; Mazorra et al., 2003), it is important to elucidate if early life-
112 stages of marine fish are capable of endogenous biosynthesis to supplement preformed
113 LC-PUFA deposited in the egg. The present study investigated the activation of the LC-
114 PUFA biosynthetic pathway during embryogenesis of the marine teleost, cobia
115 (*Rachycentron canadum*). Cobia is a rapidly emerging aquaculture species with
116 impressive growth performance, excellent flesh quality and many other favourable
117 production-related characteristics (Holt et al., 2007), and some pre-existing knowledge
118 of larval lipid nutrition and LC-PUFA synthesis (Faulk and Holt, 2003; Zheng et al.,
119 2009). Thus, an *elovl4*-like cDNA was isolated from cobia, and its function determined
120 in the yeast expression system, confirming its involvement in the biosynthesis of LC-
121 PUFA. The expression patterns of genes shown to participate in the LC-PUFA synthesis
122 pathway of cobia, including *elovl4* and the formerly characterized *elovl5* and Δ6*fad*

(Zheng et al., 2009), were then determined along with the fatty acid dynamics in embryos collected at different stages of development.

2. Materials and methods

2.1. Fish maintenance

Fertilized eggs of cobia were obtained via photo-thermal conditioning of broodstock maintained in a 42 m^3 recirculating aquaculture system (Holt et al., 2007) at the Fisheries and Mariculture Laboratory of the University of Texas at Austin Marine Science Institute in Port Aransas, Texas. The eggs were transferred to a 500 l tank and incubated at 26 – 30 °C and salinity from 25.0 –33.0 ‰. Samples were collected at the indicated sample points (see sections 2.6 and 2.7), rinsed with distilled water and immediately frozen at -80 ºC until further analyses. Additionally, tissue samples were collected from juvenile cobia (~250 g) reared in 8000 l tanks at the facilities.

2.2. Cobia elovl4 cloning

Total RNA was extracted from brain using TRIzol ® reagent (Gibco BRL, Grand Island, NY, USA). First strand cDNA was synthesized using a Verso™ cDNA kit (ABgene, Rockford, IL, USA) primed with random hexamers. The sequence of the zebrafish *elovl4* (gb|NM_199972|) and the medaka EST (gb|DK113639.1|) were aligned in order to design primers UNIE4F (5'- GTCTACAACTTCAGCATGGTG-3') and UNIE4R (5'- GGAACTGGATCATCTGAATAAT-3') that were used for polymerase chain reaction (PCR) using GoTaq® Colorless Master Mix (Promega, Southampton, UK) on brain cDNA as template. The PCR included an initial denaturing step at 95 ºC for 2 min, followed by 33 cycles of denaturation at 95 ºC for 30 s, annealing at 55 ºC for 30 s, extension at 72 ºC for 40 s, followed by a final extension at

72 ºC for 5 min. The PCR fragment was sequenced (CEQ-8800 Beckman Coulter Inc., Fullerton, USA) and specific primers were designed to produce the full-length cDNA by 5' and 3' rapid amplification of cDNA ends (RACE) PCR (FirstChoice® RLM-RACE kit, Ambion, Applied Biosystems, Warrington, UK) to produce full-length cDNA.

2.3. Sequence and phylogenetic analyses

The deduced amino acid (AA) sequence of the newly cloned cobia *elovl4* cDNA was aligned with human ELOVL4 (NM_022726) and other fish orthologues including zebrafish Elovl4a (gb|NM_200796|) and Elovl4b (gb|NM_199972|), pufferfish *Takifugu rubripes* Elovl4 (derived from EST emb|ENSTRUT00000011027|) and *Tetraodon nigroviridis* Elovl4 (emb|CAG01780|) using ClustalW2. AA sequence identity between Elovl4-like proteins was compared by the EMBOSS Pairwise Alignment Algorithms tool (http://www.ebi.ac.uk/Tools/emboss/align/). Phylogenetic analysis of the AA sequences of Elovl4 from cobia and other vertebrates including fish, birds and mammals was performed by constructing a tree using the Neighbor Joining method (Saitou and Nei, 1987), with confidence in the resulting tree branch topology measured by bootstrapping through 1000 iterations. The AA sequences of Elovl2 and Elovl5, both proteins previously reported in teleosts, were also included in the phylogenetic analysis.

2.4. Functional characterization of cobia Elovl4 by heterologous expression in Saccharomyces cerevisiae

PCR fragments corresponding to the open reading frame (ORF) of the putative *elovl4* elongase were amplified from cobia brain cDNA using the high fidelity Pfu Turbo DNA polymerase (Stratagene, Agilent Technologies, Cheshire, UK). A two-round PCR approach was used with the first round performed with specific primers COBE4U5F

and COBE4U3R (Table 1). PCR conditions consisted of an initial denaturing step at 95 ºC for 2 min, followed by 32 cycles of denaturation at 95ºC for 30 s, annealing at 57 ºC for 30 s, extension at 72 ºC for 1 min 45 s, followed by a final extension at 72 ºC for 5 min. First round PCR products were used as template for the nested PCR with thermal conditions described above, and with primers containing restriction sites (underlined in Table 1) COBE4VF (*Hin*dIII) and COBE4VR (*Xho*I). The DNA fragments were then digested with the corresponding restriction endonucleases (New England BioLabs, Herts, UK) and ligated into a similarly restricted pYES2 yeast expression vector (Invitrogen, Paisley, UK). The purified plasmids (GenElute™ Plasmid Miniprep Kit, Sigma) containing the putative *elovl4* ORF were then used to transform *S. cerevisiae* competent cells (S.c. EasyComp Transformation Kit, Invitrogen). Transformation and selection of yeast with recombinant pYES2-*elovl4* plasmids and yeast culture were performed as described in detail previously (Agaba et al., 2004). Briefly, cultures of recombinant yeast were grown in *S. cerevisiae* minimal medium$^{-uracil}$ supplemented with one of the following fatty acid (FA) substrates: lignoceric acid (24:0), eicosapentaenoic acid (20:5n-3), arachidonic acid (20:4n-6), docosapentaenoic acid (22:5n-3), docosatetraenoic acid (22:4n-6) or docosahexaenoic acid (22:6n-3). Docosapentaenoic and docosatetraenoic acids (> 98 – 99 % pure) were purchased from Cayman Chemical Co. (Ann Arbor, USA) and the remaining FA substrates (> 99 % pure) and chemicals used to prepare the *S. cerevisiae* minimal medium-uracil were from Sigma Chemical Co. Ltd. (Dorset, UK). Lignoceric acid was dissolved in α-cyclodextrin (Singh and Kishimoto, 1983) at 5 μM and added to the yeast cultures at a final concentration of 0.6 μM, whereas PUFA substrates were added at final concentrations of 0.75 (C_{20}) and 1.0 (C_{22}) mM. After 2 days, yeast were harvested and washed for further analyses. Yeast

197 transformed with pYES2 containing no insert were cultured under the same conditions
198 as a control treatment.
199
200 *2.5. Yeast FAME analysis by GC-MS*
201 Total lipids were extracted by homogenization of yeast samples in
202 chloroform/methanol (2:1, v/v) containing 0.01% BHT as antioxidant (Folch et al.,
203 1957). Fatty acid methyl esters (FAME) were subsequently prepared, extracted and
204 purified (Christie, 2003), and identified and quantified using a gas chromatograph
205 (GC8000) coupled to an MD800 mass spectrometer (ThermoFisher Scientific, Hemel
206 Hempstead, UK), and using the methodology described by Monroig et al. (2010).
207 Elongation rates from PUFA substrates were calculated by the proportion of substrate
208 FA converted to elongated FA product as [product area/(product area + substrate area)]
209 x 100. Conversion rates from 24:0 were not calculated as yeast endogenously contains
210 several of the FA involved in the elongation pathway. Instead, contents of individual
211 saturated FA $\geq C_{24}$ from *elovl4*-transformed yeast were calculated and compared to
212 control yeast.
213
214 *2.6. Expression of fad, elovl5 and elovl4 genes during cobia early development and*
215 *elovl4 tissue distribution*
216 To study the expression of the target genes during embryonic development of cobia,
217 pools of ~50 embryos were collected from a single spawn at 0, 3, 6, 12, 18, 24, 36, 48,
218 60, 72 and 84 hours post-fertilization (hpf). This time window encompasses the entire
219 embryogenesis of cobia (Faulk et al., 2007), herein referred to as the period between the
220 zygote stage and the oesophagus opening (Gatesoupe et al., 2001). Total RNA was
221 extracted using Tri Reagent (Sigma) according to the manufacturer's protocol, and 1 µg

of total RNA reverse transcribed into cDNA (Verso™ cDNA kit, ABgene) primed with random hexamers. Expression of *fad*, *elovl5* and *elovl4* transcripts during embryonic development was determined by reverse transcriptase PCR (RT-PCR) with an initial denaturing step at 95 ºC for 2 min, followed by 35 cycles of denaturation at 95 ºC for 30 s, annealing at 56 ºC for 30 s, extension at 72 ºC for 40 s, and a final extension at 72 ºC for 5 min using primers shown in Table 1. Expression of the housekeeping gene *β-actin* was determined to check the efficiency of cDNA synthesis and the cDNA integrity.

Expression of the cobia putative *elovl4* was measured in adult tissues by RT-PCR. Total RNA from pituitary gland, brain, liver, anterior intestine, eye, kidney, red and white muscle, ovary, testis, gill, spleen, skin, stomach, pyloric caeca and spinal cord was extracted as described above, and 1 μg of total RNA reverse transcribed into cDNA. Primers used for expression of *elovl4* and *β-actin* were as described for the embryo samples.

2.7. Fatty acid analyses of cobia embryos

In order to monitor the changes in FA composition during early development, pools of ~200 embryos were sampled at different stages (0, 24, 48 and 72 hpf). Total lipid extraction and FAME preparation were performed as described above for yeast samples. FAME were analysed by gas chromatography and flame ionization detection as described previously (Tocher et al., 2010).

3. Results

3.1. Cobia Elovl4 sequence and phylogenetics

A 2290-bp (excluding polyA tail) full-length cDNA sequence was obtained by 5’ and 3’ RACE PCR and deposited in the GenBank database under the accession number

HM026361. It contains an ORF of 918 bp encoding a putative protein of 305 AA, sharing 42.1 % AA sequence identity with the previously described cobia Elovl5-like elongase (Zheng et al., 2009). Cobia putative Elovl4 possesses the histidine dideoxy binding motif HXXHH, and the putative endoplasmic reticulum (ER) retrieval signal with an arginine (R) and a lysine (K) residues at the carboxyl terminus, RXKXX (Fig. 2) (Jakobsson et al., 2006). By sequence comparison with a mouse ELOVL4 (Zhang et al., 2003), five putative transmembrane-spanning domains, containing hydrophobic AA stretches, can be predicted (Fig. 2).

The deduced AA sequence from the Elovl4 cDNA predicts a protein that is 62.7 - 63.2 % identical to several mammalian ELOVL4-like elongases including human, mouse and rat, and 62.3 - 63.2 % identical to predicted Elovl4 proteins from birds. When the AA sequence of cobia Elovl4 was compared to fish Elovl4s, high identity scores were found with *Tetraodon nigroviridis* Elovl4 (91.5 % identical) and zebrafish Elovl4b (82.0 % identical), whereas lower identity scores were observed when compared with zebrafish Elovl4a (70 % identity) and *Takifugu rubripes* (66.4 % identity). Differentiation among fish Elovl4 proteins is also reflected in the phylogenetic analysis. Although all fish Elovl4 proteins grouped with the mammalian and bird orthologues, and separately from other members of the Elovl family such as Elovl2 and Elovl5, two clusters appeared to exist, with cobia Elovl4 grouping with zebrafish Elovl4b and pufferfish *Tetraodon nigroviridis* predicted Elovl4, and more distantly a group including zebrafish Elovl4a and *Takifugu rubripes* Elovl4 (Fig.3).

3.2. Functional characterization

The cobia putative Elovl4 elongase was functionally characterized by determining the FA profiles of transgenic *S. cerevisiae* containing the cobia *elovl4* cDNA ORF and

grown in the presence of potential FA substrates. In order to test the ability of cobia Elovl4 to elongate saturated VLC-FA, yeast transformed with pYES2 containing the putative *elovl4* ORF or no insert (control) were incubated with lignoceric acid (24:0) (Table 2; Fig. 4). The results confirm that cobia Elovl4 is involved in the biosynthesis of saturated VLC-FA. Thus, control yeast transformed with empty vector and incubated in the presence of 24:0 contained measurable amounts of 24:0 (10.2 % of total saturates ≥ C_{24}), 26:0 (79.7 %) and 28:0 (7.9 %), with traces of 30:0 and 32:0 (Table 2). In contrast, *elovl4*-transformed yeast showed a different profile of saturated VLC-FAs ≥ C_{24} compared to control yeast, with decreased contents of 24:0 and 26:0, and concomitant increased levels of 28:0 (4.3-fold), 30:0 (9.7-fold) and 32:0 (4.1-fold) (Table 2). These results suggest that at least 24:0, 26:0 and 28:0 are good substrates for cobia Elovl4.

In order to test the role of cobia Elovl4 in the biosynthesis of VLC-PUFA, transgenic yeast transformed with Elovl4 ORF were incubated with C_{20} (20:5n-3 and 20:4n-6) and C_{22} (22:5n-3, 22:4n-6 and 22:6n-3) PUFA substrates (Table 3; Fig. 5). The FA composition of the yeast transformed with pYES2 vector containing no insert (control) is characterized by having only 16:0, 16:1n-7, 18:0 and 18:1n-9, together with whichever exogenous FA was added, consistent with *S. cerevisiae* possessing no PUFA elongase activity (Agaba et al., 2004). GC-MS analyses revealed that cobia Elovl4 elongated 20:5n-3 and 20:4n-6 with conversions of 33 % and almost 55 %, respectively (Table 3). Cobia Elovl4 also showed high activity towards the C_{22} substrates, 22:5n-3 and 22:4n-6, with conversions of 34 % and 41 %, respectively (Table 3). Fatty acids produced by *elovl4*-transformed yeast incubated with PUFA included polyenes up to C_{36}, with C_{32} PUFA consistently being the most abundant products (Table 3; Fig. 5). It is noteworthy that cobia Elovl4 was able to convert both 20:5n-3 and 22:5n-3 to 24:5n-3, the C_{24} substrate for Δ6 Fad in DHA (22:6n-3) synthesis. However, in contrast cobia

297 Elovl4 showed very little activity towards DHA itself, which was only marginally
298 converted to longer products (Table 3).
299
300 *3.3. Temporal expression patterns of fad, elovl5 and elovl4*
301 Temporal expression of *fad*, *elovl5* and *elovl4* was studied by RT-PCR using cDNA
302 samples obtained from embryos at different developmental stages from 0 to 84 hpf (Fig.
303 6). Transcripts of the three target genes were detected from the zygote stage (0 hpf),
304 indicating that mRNA transcripts of these genes are transferred maternally (Monroig et
305 al., 2009, 2010). Although comparisons of transcript levels from RT-PCR analyses have
306 to be made with caution, some temporal patterns can be predicted in the expression of
307 the target genes. The three target genes showed low expression at the beginning of the
308 experimental period, with a noticeable signal increase from 18 hpf (*elovl4*) and 36 hpf
309 (Δ6*fad* and *elovl5*) onwards. The expression of the housekeeping gene *β-actin* remained
310 constant during early development of cobia.
311 Adult tissue distribution of *elovl4* mRNA transcript was determined by RT-PCR (Fig.
312 7). The results revealed that cobia *elovl4* was expressed in most of the tissues analyzed,
313 with eye (probably retina), brain and pituitary gland showing high expression signals.
314 Only low expression of *elovl4* was detected in liver and no expression was detected in
315 pyloric caeca, two major metabolic sites in the biosynthesis of C_{18-22} LC-PUFA in fish
316 (Fig. 7).
317
318 *3.4. Fatty acid composition of cobia embryos*
319 Overall activity of the LC-PUFA synthesis pathway during cobia embryogenesis was
320 estimated by analyzing the FA composition (% of total FA) of embryos collected at
321 different developmental stages (Table 4). The percentages of C_{18} PUFA precursors,

18:3n-3 and 18:2n-6, were generally constant over the time-course of cobia embryogenesis. The effects of embryogenic development on LC-PUFA levels were variable depending upon the actual fatty acid. For instance, the proportion of DHA, the most abundant LC-PUFA in cobia embryos, appeared to initially decrease and then increase in later development without any clear trend or obvious pattern. Note that, unlike transgenic yeast samples from Elovl4 functional characterization (see above), VLC-FA could not be determined in the embryo samples. Whereas FA up to C_{22} are present in measurable amounts in lipids, VLC-FAs selectively accumulate in specific lipid classes of certain tissues such as retina, brain and gonads (Poulos, 1995), which are not fully developed in embryonic stages. Therefore, the analysis of these compounds requires large samples of specific tissues and thus is impractical in fish embryos.

4. Discussion

Elovl cDNAs including Elovl5- and Elovl2-like elongases have been cloned and functionally characterized from several fish including freshwater, salmonid and marine species (Agaba et al., 2004, 2005; Zheng et al., 2005; Monroig et al., 2009; Morais et al., 2009; Gregory et al., 2010; Mohd-Yusof et al., 2010). More recently Elovl4 proteins from zebrafish have been investigated, representing the first non-human Elovl4-like proteins that have been functionally characterized (Monroig et al., 2010). *ELOVL4* was first identified as a gene causing a dominant form of Stargardt-like macular dystrophy in humans (Bernstein et al., 2001; Zhang et al., 2001). Its localization in the ER (Grayson et al., 2005), the site of long-chain FA synthesis, its AA sequence similarities with other elongase family proteins and its high expression levels in tissues having high requirements for VLC-FAs, suggested a role for ELOVL4 in FA biosynthesis. The actual function of ELOVL4, however, was recently confirmed by Agbaga et al. (2008)

347 who demonstrated that the human enzyme participates in the biosynthesis of VLC-FAs
348 including both saturated and polyunsaturated FAs. Whereas saturated VLC-FAs play
349 essential structural functions in the maintenance of skin permeability in mammals
350 (Uchida et al., 2008), the functions of the very long-chain polyunsaturated fatty acids
351 (VLC-PUFAs) appear to be related to their unusually long aliphatic chains (C_{24}-C_{38})
352 and the consequent characteristic that some VLC-PUFA possess by combining the
353 properties of saturated fatty acid in the proximal end with those of PUFA in the distal
354 end (Agbaga et al., 2008). Thus, VLC-PUFA are compounds uniquely found in specific
355 lipid molecules of retina (Alvedaño, 1987, 1988), brain (Robinson et al., 1990), and
356 testis (Furland et al., 2003, 2007a,b).

357 Cobia Elovl4 exhibits characteristic features of microsomal-bound enzymes including
358 a single histidine box redox centre motif, a canonical ER retention signal and multiple
359 transmembrane regions (Jakobsson et al., 2006; Molday et al., 2010). Phylogenetic
360 analysis suggested that the newly cloned *elovl4* cDNA encodes a protein more similar to
361 other Elovl4 proteins from mammals, birds and fish, in comparison to other fish Elovl
362 proteins including the previously characterized cobia Elovl5 (Zheng et al., 2009). This
363 is in agreement with Elovl4 proteins being highly conserved through evolution (Lagali
364 et al., 2003). Interestingly, fish Elovl4s themselves clustered in two separate groups
365 with zebrafish Elovl4a and *Takifugu rubripes* Elovl4 in one group, and zebrafish
366 Elovl4b, *Tetraodon nigroviridis* Elovl4 and cobia Elovl4, in the other. The clustering
367 pattern observed for fish Elovl4s is consistent with members of the two groups having
368 different functions.

369 The functional analysis of cobia Elovl4 revealed great similarities with the formerly
370 characterized zebrafish Elovl4b (Monroig et al., 2010). Whereas zebrafish Elovl4a was
371 only able to produce saturated VLC-FA, zebrafish Elovl4b, and now also cobia Elovl4,

372 are efficient in the synthesis of both saturated VLC-FA and VLC-PUFA up to C_{36}.
373 Decreased proportions of 26:0 and concomitant increased levels of 28:0 and 30:0 in
374 transgenic yeast expressing cobia *elovl4* indicate its involvement in the biosynthesis of
375 28:0 and 30:0 from 26:0. This is in agreement with the conversions shown by
376 mammalian ELOVL4 using genetically engineered mice (Cameron et al., 2007; Li et
377 al., 2007a,b; Vasireddy et al., 2007) and human cell lines not naturally expressing
378 *ELOVL4* (Agbaga et al., 2008). Additionally, the cobia Elovl4 elongated C_{20} and C_{22}
379 PUFA substrates that were converted to polyenes up to C_{36} of the n-3 and n-6 series.
380 These compounds are relatively abundant in specific lipid classes of tissues including
381 retina, testis and brain of vertebrates including fish (Poulos, 1995). Although VLC-FA
382 were not measured in cobia tissues for this study, the presence of *elovl4* mRNA
383 transcripts in some of those tissues including eye and brain suggests that these are also
384 metabolic sites for VLC-FA biosynthesis in fish. These findings highlight the
385 importance that the study of VLC-FA and their biosynthesis might have in farmed fish
386 in which altered visual acuity (critical in visual predators such as most cultured fish
387 species) and disruptions of brain functioning can jeopardize normal development of
388 fish. Also interesting is the fact that cobia Elovl4 appears to be highly expressed in
389 pituitary gland (hypophysis). Although it is well known that vertebrate brain regions
390 including pituitary gland accumulate LC-PUFA (Carrié et al., 2000), the presence of
391 *elovl4* mRNA indicates that an active biosynthesis of VLC-FAs, probably
392 polyunsaturated acyl chains, may occur in fish hypophysis.

393 DHA is one of the most abundant LC-PUFAs in tissues such as eye, brain and
394 gonads, the likely reason why Elovl4, highly expressed in these tissues, was initially
395 believed to be involved in the biosynthesis of DHA in mammals. Several studies,
396 however, have shown that mammalian Elovl4 does not directly participate in DHA

biosynthesis, but acts on longer (> C_{26}) polyunsaturated substrates (Molday et al., 2010). The efficiency of cobia Elovl4 for the conversion of 22:5n-3 to 24:5n-3 suggests that, in contrast to mammalian orthologues, some fish Elovl4s have a potential role in the biosynthesis of DHA via the so-called Sprecher Pathway, in which 24:5n-3 is the substrate for Δ6 Fad producing 24:6n-3, which is subsequently chain-shortened to DHA (Sprecher, 2000). Whereas Elovl4 encountered in other marine fish genomes including fugu *Takifugu rubripes* and stickleback *Gasterosteus aculeatus* have not been functionally characterized, our results on cobia Elovl4 confirm that marine fish possess Elovl4 involved in DHA biosynthesis that may act to compensate for the apparent lack of Elovl2-like proteins. Interestingly such a role in DHA production predicted for cobia Elovl4 is in contrast to the elongation activity shown by this protein on DHA itself. Despite its activity towards similar substrates such as 22:5n-3, Elovl4 did not show much activity towards DHA which was only marginally elongated. This is in agreement with functional analysis of zebrafish Elovl4s (Monroig et al., 2010), and studies in mammals where retina preparations showed active elongation of radiolabeled 22:5n-3, whereas DHA remained virtually unmodified and was, in contrast, directly esterified into phospholipids without further metabolism (Rotstein et al., 1996; Suh and Clandinin, 2005).

Early developmental stages of organisms including fish show high requirements for LC-PUFA to support the formation of specific tissues where they are selectively accumulated in particular lipid classes (Tocher, 2003). Whereas deposition of preformed essential LC-PUFA in the embryo has been proven to depend on broodstock diet (Rodríguez et al., 1998; Mazorra et al., 2003; Izquierdo et al., 2001) and genetic makeup (Pickova et al., 1997), the ability of fish embryonic stages to endogenously biosynthesize essential LC-PUFA has remained unexplored. Based on the key elongase

and desaturase mRNA levels and the dynamics of FA biosynthesis products investigated in a recent study (Monroig et al., 2009), we predicted that zebrafish embryos are capable of LC-PUFA biosynthesis during early developmental stages. Similarly, cobia embryos also express a Δ6*fad* and the elongases *elovl4* and *elovl5*, all proved to participate in the biosynthesis pathway of LC-PUFA (Zheng et al., 2009). However, endogenous biosynthesis does not appear to be functional during the initial 24 h for cobia embryogenesis, with very low expression signals of Δ6*fad* and *elovl5* during this period. This argument is in part supported by the depletion of LC-PUFA, especially DHA and to a lesser extent EPA, during very early embryogenesis, when the expected utilization of saturated and monounsaturated FAs for energy supply would perhaps produce an increase in the relative amounts of DHA, which is preferentially retained in lipid cell membranes (Tocher et al., 1985; Fraser et al., 1988). The increased expression signals for *elovl4* (18 hpf) and Δ6*fad* and *elovl5* (36 hpf) suggest a potential activation of parts of the LC-PUFA biosynthetic pathway at later stages of cobia embryogenesis, possibly to fulfil the requirements of DHA necessary for the developing neuronal tissues. However, this was not clearly reflected in embryonic DHA levels and further experiments using embryonic cell culture preparations incubated with radiolabeled substrates are required to clarify how the increased expression of LC-PUFA biosynthetic genes affects enzymatic activities in the LC-PUFA biosynthetic pathway.

In conclusion, the present investigation demonstrates that cobia express an Elovl4-like protein with high similarity to other Elovl4 orthologues from vertebrates and whose function differs from that of the previously characterized Elovl5 in this species. Cobia Elovl4 is able to elongate both saturated and polyunsaturated substrates to products up to C_{36}. Notably, cobia Elovl4 can participate in the biosynthesis of DHA. Our results also demonstrate the presence of *elovl4*, *elovl5* and Δ6*fad* transcripts during

447 embryogenesis suggesting that parts of the LC-PUFA synthesis pathway may be
448 activated during development of embryos of marine fish species.
449
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457
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657

658 **Figure captions**

659 Fig. 1. Biosynthesis pathways of long-chain polyunsaturated fatty acids from C_{18}
660 precursors 18:3n-3 and 18:2n-6. Enzymatic activities shown in the scheme are predicted
661 from heterologous expression in *Saccharomyces cerevisiae* of genes isolated from fish
662 species. Dotted arrows indicate conversions only reported by a Δ4 desaturase from
663 rabbitfish *Siganus canaliculatus*.
664 *Conversion only reported for zebrafish Elovl4b (Monroig et al., 2010).
665

Fig. 2. ClustalW2 amino acid alignment of cobia Elovl4 with human ELOVL4 (gb|NP_073563.1|) and fish Elovl4-like proteins including *T. rubripes* (emb|ENSTRUT00000011027|), zebrafish (gb|NM_200796| and gb|NM_199972|), and *T. nigrov*iridis (emb|CAG01780|). Identical residues are shaded black and similar residues (based on the Gonnet matrix, using ClustalW2 default parameters) are shaded grey. Indicated are the conserved histidine box motif HXXHH, five (I-V) putative membrane-spanning domains, and the putative endoplasmic reticulum (ER) retrieval signal (Zhang et al., 2003).

Fig. 3. Phylogenetic tree comparing the putative cobia Elovl4, with other Elovl4 orthologues and Elovl2- and Elovl5-like elongases. The tree was constructed using the Neighbour Joining method (Saitou and Nei, 1987) 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. 4. Role of cobia Elovl4 in the biosynthesis of saturated very long-chain fatty acids (VLC-FA). Yeast (*S. cerevisiae*) transformed with empty pYES2 vector (A) or pYES2 containing the ORF of *elovl4* (B) as insert were grown in the presence of lignoceric acid (24:0), and the fatty acid composition was determined. Substrate 24:0 ("*") and its corresponding elongated products are indicated accordingly. Vertical axis, MS response; horizontal axis, retention time.

Fig. 5. Role of cobia Elovl4 in the biosynthesis of very long-chain fatty acids (VLC-PUFA). Yeast (*S. cerevisiae*) transformed with pYES2 vector containing the ORF of

691 *elovl4* as insert were grown in the presence of PUFA substrates 22:5n-3 (A) and 22:4n-6
692 (B), and the fatty acid composition was determined. Substrates ("*") and their
693 corresponding elongated products are indicated accordingly. Vertical axis, MS
694 response; horizontal axis, retention time.
695
696 Fig. 6. RT-PCR analyses of the temporal expression patterns of the previously cloned
697 Δ6*fad* and *elvol5* (Zheng et al., 2009), and the newly isolated *elovl4* during cobia early
698 development (0 to 84 hpf). hpf, hours post-fertilization; NTC, no template control.
699
700 Fig. 7. RT-PCR analyses showing the spatial expression of *elovl4* in cobia adults.
701 Expression of the housekeeping gene *β-actin* is also shown.
702

Table 1. Sequence of the primer pairs used, size of the fragment produced and accession number of the sequence used as reference for primer design, for *elovl4* ORFs cloning and reverse transcriptase PCR (RT-PCR) performed in cobia embryos and adult tissues.

Aim	Transcript	Primer	Primer sequence	Fragment	Accession No[1].
ORF cloning	*elovl4*	COBE4U5F	5'-TGAGAGGAGCAGGGCATCAA-3'	1082 bp	HM026361
		COBE4U3R	5'-TCCTTCCCTACCCTCCATCCT-3'		
		COBE4VF	5'-CCCAAGCTTAGGATGGAGGTTGTAACACAT-3'	946 bp	
		COBE4VR	5'-CCGCTCGAGTCTTCCTTCTTTACTCCCT-3'		
RT-PCR	*Δ6fad*	COBDESF	5'-ATCTGTTTCCTACGATGCCA-3'	531 bp	FJ440238
		COBDESR	5'-AGCTGGGATTGTCAGGGTAA-3'		
	elovl5	COBELO5F	5'-GGTGGTACTACTTCTCCAAGC-3'	594 bp	FJ440239
		COBELO5R	5'-CCTAGCAGCATTTGCTAACAC-3'		
	elovl4	COBELO4F	5'-TGCCTGTACCTGCTCTTCCT-3'	446 bp	HM026361
		COBELO4R	5'-GCCAGGCCATAGTAACCGTA-3'		
	ß-actin	COBACTF	5'-GATCCTGACAGAGCGTGG-3'	132 bp	EU266539
		COBACTR	5'-GAAGAGGAGGAGGCAGC-3'		

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

702

Table 2. Functional characterisation of cobia Elovl4 elongase: Role in biosynthesis of very long-chain saturated fatty acids (FA). Results are expressed as an area percentage of total saturated FA C ≥ 24 found in yeast transformed with either cobia *elovl4* ORF or empty pYES2 vector (Control).

FA	Control	Elovl4
24:0*	10.2	5.8
26:0	79.7	41.8
28:0	7.9	33.9
30:0	1.5	14.5
32:0	0.7	2.9
34:0	0.0	0.8
36:0	0.0	0.2

* Lignoceric acid used as exogenously added substrate.

Table 3. Functional characterisation of cobia Elovl4 elongase: conversions on polyunsaturated fatty acid (FA) substrates. Results are expressed as a percentage of total FA substrate converted to elongated product. Percentage of stepwise conversion into intermediary products of the elongation pathway is also shown.

FA substrate	Product	Elovl4	Activity
20:5n-3	22:5n-3	9.7	C20→22
	24:5n-3	3.7	C22→24
	26:5n-3	0.4	C24→26
	28:5n-3	0.2	C26→28
	30:5n-3	1.5	C28→30
	32:5n-3	12.0	C30→32
	34:5n-3	5.5	C32→34
	36:5n-3	0.2	C34→36
	Total	33.1	
20:4n-6	22:4n-6	11.1	C20→22
	24:4n-6	6.5	C22→24
	26:4n-6	1.0	C24→26
	28:4n-6	0.5	C26→28
	30:4n-6	6.4	C28→30
	32:4n-6	23.4	C30→32
	34:4n-6	5.3	C32→34
	36:4n-6	0.5	C34→36
	Total	54.6	
22:5n-3	24:5n-3	3.9	C22→24
	26:5n-3	0.5	C24→26
	28:5n-3	0.1	C26→28
	30:5n-3	1.9	C28→30
	32:5n-3	18.3	C30→32
	34:5n-3	9.0	C32→34
	36:5n-3	0.3	C34→36
	Total	34.1	
22:4n-6	24:4n-6	2.9	C22→24
	26:4n-6	0.6	C24→26
	28:4n-6	0.2	C26→28
	30:4n-6	4.9	C28→30
	32:4n-6	25.2	C30→32
	34:4n-6	6.4	C32→34
	36:4n-6	0.7	C34→36
	Total	40.9	
22:6n-3	24:6n-3	1.3	C22→24
	26:6n-3	0.0	C24→26
	28:6n-3	0.0	C26→28
	30:6n-3	0.2	C28→30
	32:6n-3	2.3	C30→32
	34:6n-3	0.6	C32→34
	36:6n-3	0.0	C34→36
	Total	4.3	

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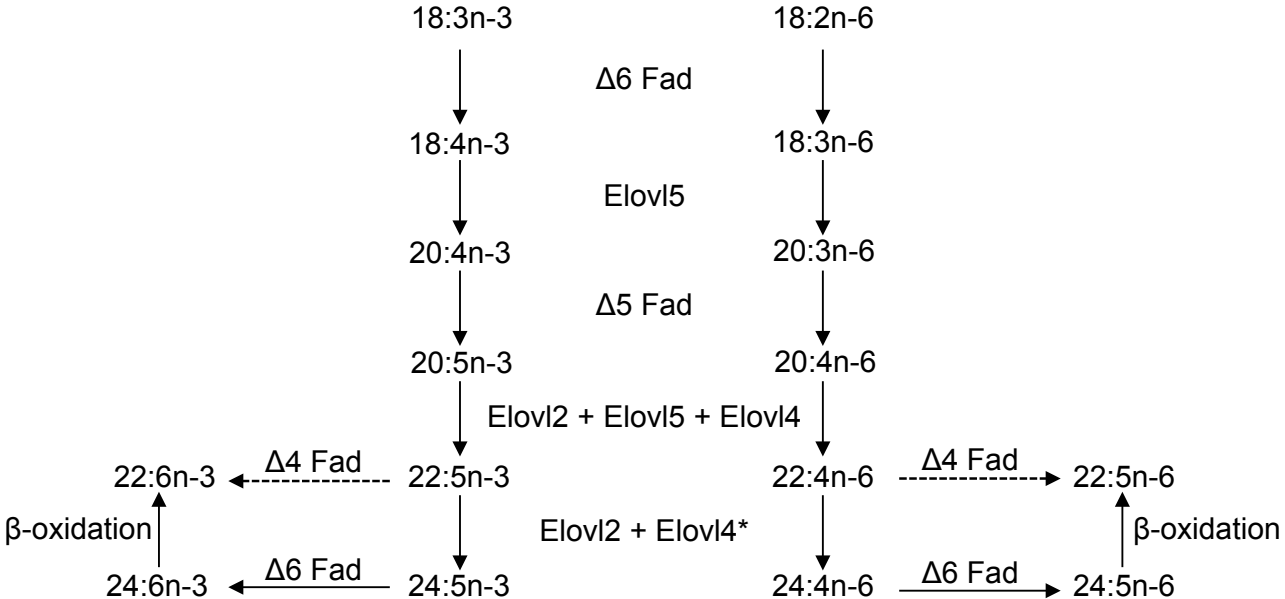
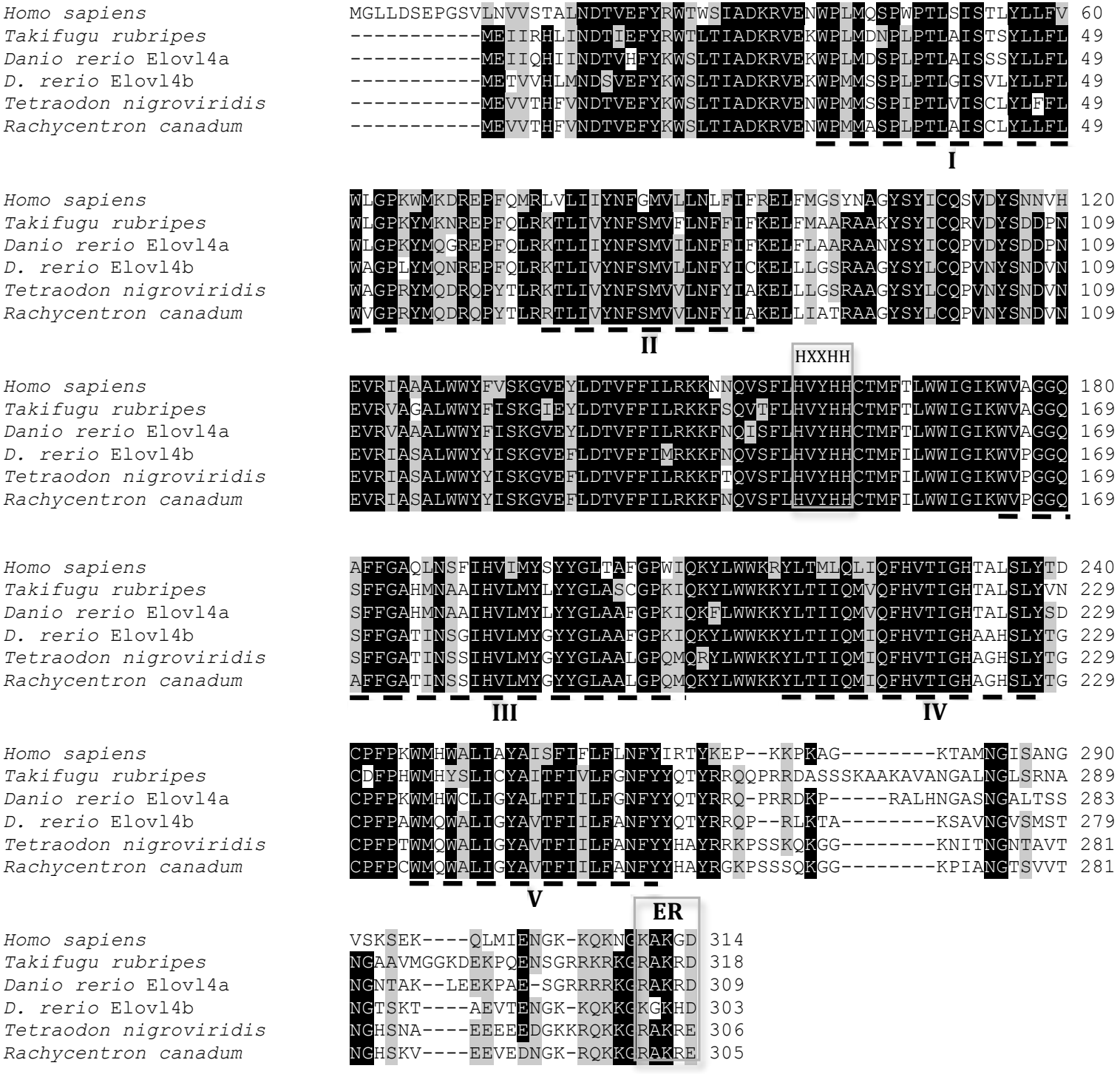
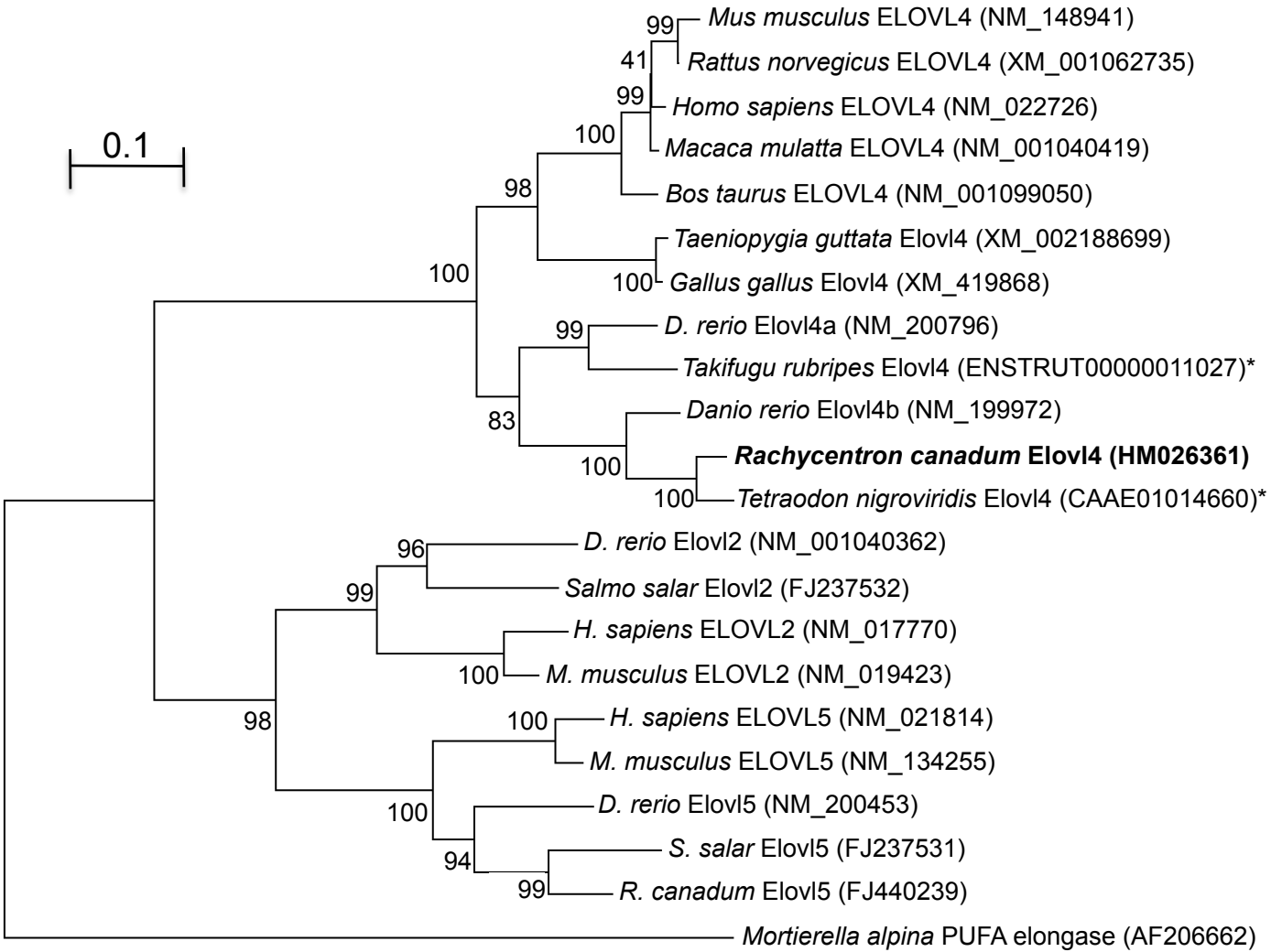
Table 4. Fatty acid composition of cobia embryos at different stages of development. Results are expressed in percentage of total fatty acids.

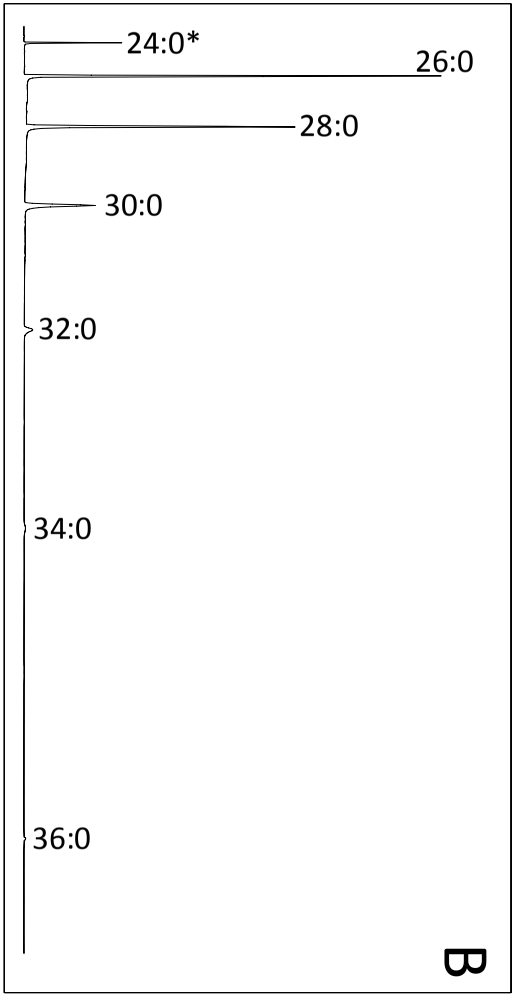
Fatty acid	*0 hpf*	*24 hpf*	*48 hpf*	*72 hpf*
14:0	1.5	1.4	1.2	1.3
15:0	0.3	0.3	0.3	0.3
16:0	17.6	18.4	17.1	19.5
18:0	4.3	5.0	4.9	6.4
20:0	0.1	0.2	0.2	0.3
Total saturated	23.7	25.4	23.9	27.8
16:1n-9	0.3	0.4	0.3	0.3
16:1n-7	5.7	5.7	5.2	5.1
18:1n-9	12.9	13.3	12.7	13.5
18:1n-7	3.5	3.6	3.5	3.8
20:1[1]	0.9	1.1	1.3	1.0
22:1[2]	0.3	0.6	0.4	0.3
24:1n-9	0.3	0.5	0.4	0.4
Total monounsaturated	23.9	25.1	23.8	24.4
C_{16} PUFA	0.6	0.6	1.0	0.6
18:2n-6	2.6	2.6	2.6	2.6
18:3n-6	0.2	0.2	0.2	0.2
20:2n-6	0.1	0.2	0.2	0.2
20:3n-6	0.4	0.2	0.3	0.4
20:4n-6	2.6	2.5	2.9	3.1
22:4n-6	0.2	0.2	0.2	0.2
22:5n-6	0.6	0.5	0.6	0.6
Total n-6 PUFA	6.8	6.4	7.0	7.3
18:3n-3	0.5	0.5	0.5	0.5
18:4n-3	0.6	0.5	0.5	0.4
20:3n-3	0.1	0.1	0.1	0.1
20:4n-3	0.5	0.5	0.5	0.4
20:5n-3	10.5	9.5	9.7	8.6
22:5n-3	1.2	2.3	2.4	2.2
22:6n-3	26.0	22.2	25.0	23.7
Total n-3 PUFA	39.5	35.6	38.7	35.9

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


18:3n-3
18:2n-6
Δ6 Fad
18:4n-3
18:3n-6
Elovl5
20:4n-3
20:3n-6
Δ5 Fad
20:5n-3
20:4n-6
Elovl2 + Elovl5 + Elovl4
22:6n-3
Δ4 Fad
22:5n-3
22:4n-6
Δ4 Fad
22:5n-6
β-oxidation
Elovl2 + Elovl4*
β-oxidation
24:6n-3
Δ6 Fad
24:5n-3
24:4n-6
Δ6 Fad
24:5n-6


Homo sapiens
Takifugu rubripes
Danio rerio Elovl4a
D. rerio Elovl4b
Tetraodon nigroviridis
Rachycentron canadum
I
II
HXXHH
III
IV
V
ER


0.1
99
Mus musculus ELOVL4 (NM_148941)
41
Rattus norvegicus ELOVL4 (XM_001062735)
99
Homo sapiens ELOVL4 (NM_022726)
100
Macaca mulatta ELOVL4 (NM_001040419)
98
Bos taurus ELOVL4 (NM_001099050)
Taeniopygia guttata Elovl4 (XM_002188699)
100
100
Gallus gallus Elovl4 (XM_419868)
99
D. rerio Elovl4a (NM_200796)
Takifugu rubripes Elovl4 (ENSTRUT00000011027)*
83
Danio rerio Elovl4b (NM_199972)
100
Rachycentron canadum Elovl4 (HM026361)
100
Tetraodon nigroviridis Elovl4 (CAAE01014660)*
96
D. rerio Elovl2 (NM_001040362)
99
Salmo salar Elovl2 (FJ237532)
H. sapiens ELOVL2 (NM_017770)
100
M. musculus ELOVL2 (NM_019423)
98
100
H. sapiens ELOVL5 (NM_021814)
M. musculus ELOVL5 (NM_134255)
100
D. rerio Elovl5 (NM_200453)
94
S. salar Elovl5 (FJ237531)
99
R. canadum Elovl5 (FJ440239)
Mortierella alpina PUFA elongase (AF206662)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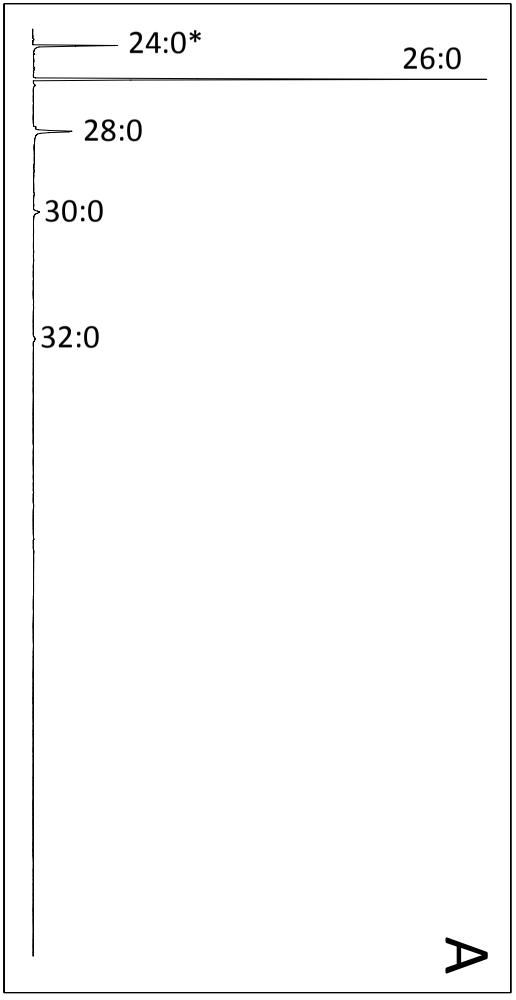
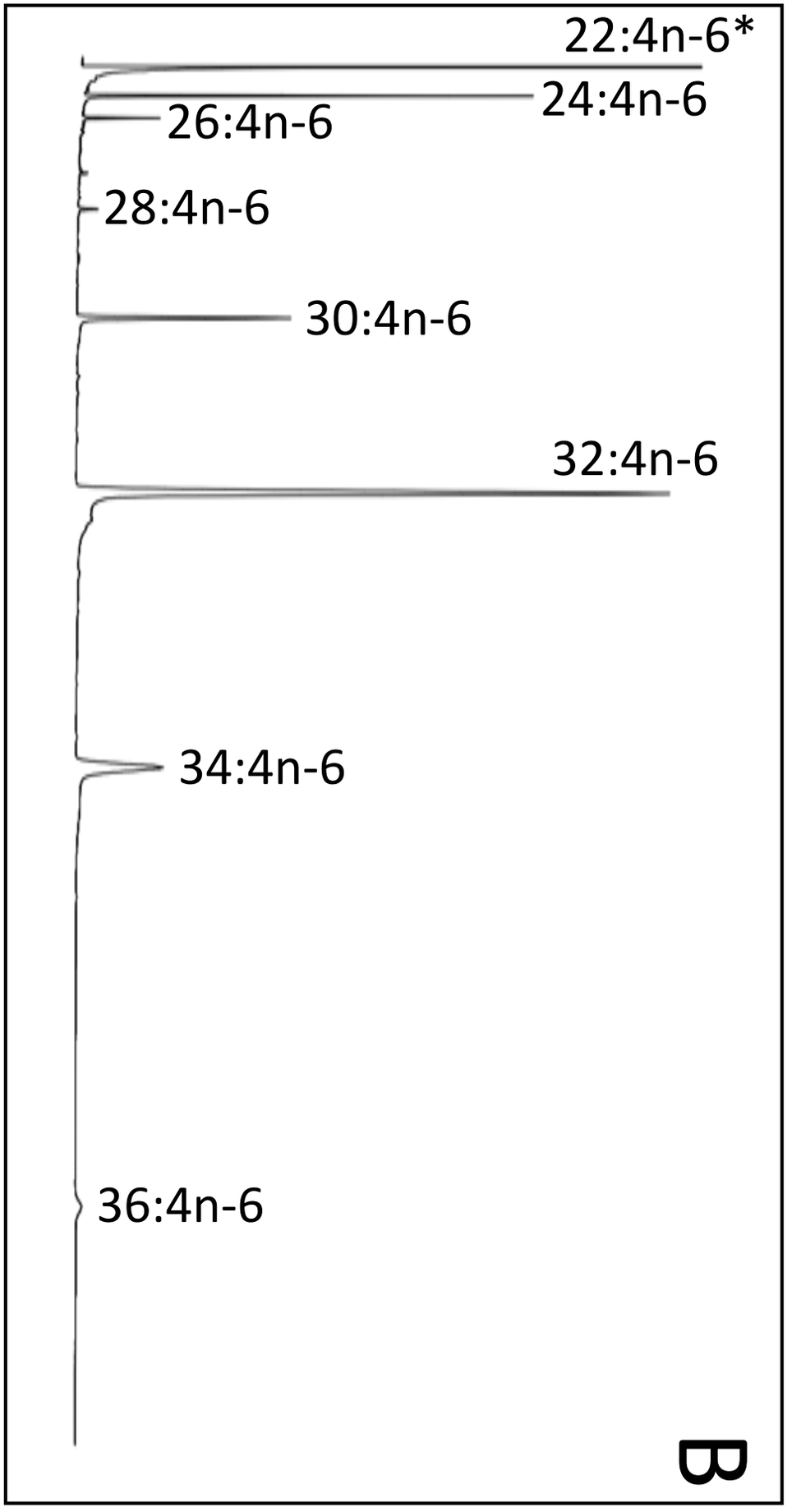
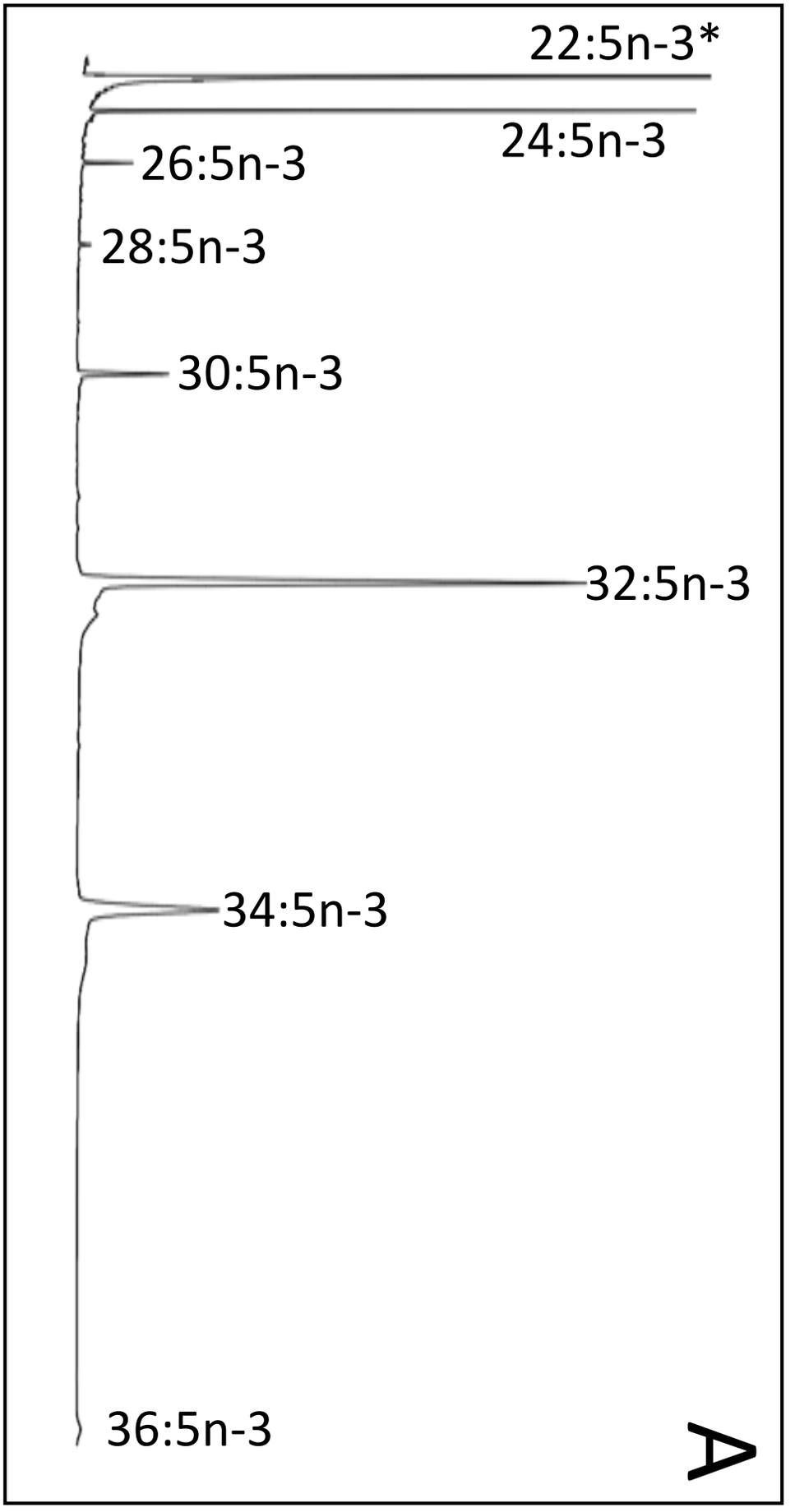
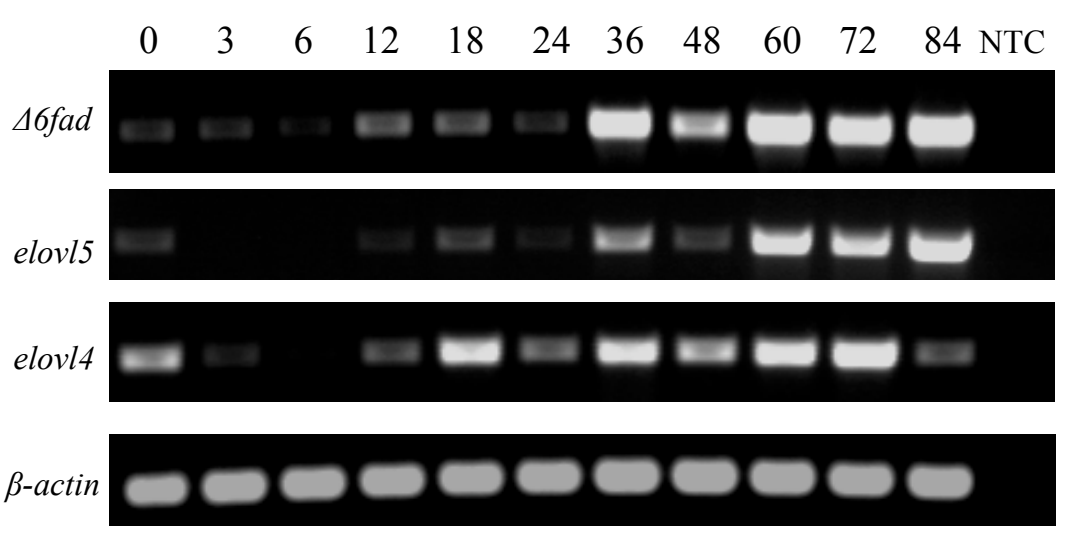
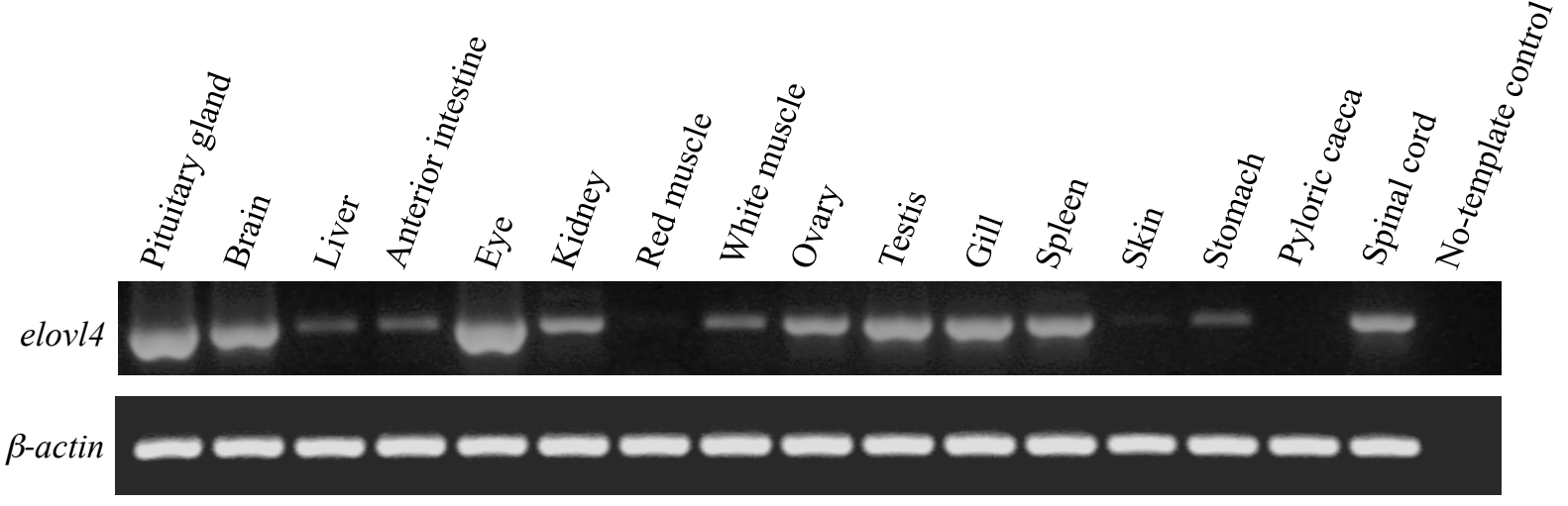
26:0
28:0
30:0
32:0
34:0
36:0
B


24:0*
26:0
28:0
30:0
32:0
A


22:4n-6*
24:4n-6
26:4n-6
28:4n-6
30:4n-6
32:4n-6
34:4n-6
36:4n-6
B


22:5n-3*
24:5n-3
26:5n-3
28:5n-3
30:5n-3
32:5n-3
34:5n-3
36:5n-3
A


0
3
6
12
18
24
36
48
60
72
84
NTC
Δ6fad
elovl5
elovl4
β-actin


Pituitary gland
Brain
Liver
Anterior intestine
Eye
Kidney
Red muscle
White muscle
Ovary
Testis
Gill
Spleen
Skin
Stomach
Pyloric caeca
Spinal cord
No-template control
elovl4
β-actin