

Expression of fatty acyl desaturase and elongase genes, and evolution of
DHA:EPA ratio during development of unfed larvae of Atlantic bluefin
tuna (*Thunnus thynnus* L.)

Sofia Morais^{a*}, Gabriel Mourente^b, Aurelio Ortega^c, Jamie A. Tocher^a and Douglas
R. Tocher^a

^a *Institute of Aquaculture, University of Stirling, FK9 4LA Stirling, Scotland, UK*

^b *Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz,
11510 Puerto Real, Cádiz, Spain*

^c *Planta Experimental de Cultivos Marinos, Instituto Español de Oceanografía (IEO), 30860
Puerto de Mazarrón (Murcia), Spain*

* Corresponding author: Tel.: 00441786467993; fax: 00441786472133

E-mail address: sofia.morais@stir.ac.uk

19 ABSTRACT

20 The concentration of n-3 long-chain polyunsaturated fatty acids (LC-PUFA) in neural
21 tissues is known to be crucial for effective prey capture from the time of first feeding in marine fish
22 larvae. Furthermore, tissues of tunids, including Atlantic bluefin tuna, have relatively high levels of
23 DHA (docosahexaenic acid, 22:6n-3) and a high ratio of DHA:EPA (eicosapentaenoic acid; 20:5n-
24 3) compared to most other species. Although the lipid biochemistry underpinning the high
25 DHA:EPA ratio in tuna is unclear, it has been generally assumed that they must selectively
26 accumulate and retain DHA in their tissues. In the present study, we investigated lipid and fatty acid
27 metabolism during early development of Atlantic bluefin tuna and determined the changes in lipid
28 content, lipid class composition and total, phospholipid and neutral lipid fatty acid profiles in unfed
29 larvae during yolk sac utilisation. In addition, we studied the LC-PUFA biosynthesis pathway by
30 quantifying expression of fatty acyl desaturase and elongase genes. To this end, we cloned and
31 functionally characterized two cDNAs by heterologous expression in yeast, showing them to code
32 for a $\Delta 6$ desaturase and Elovl5 elongase, respectively, that could both be involved in the conversion
33 of EPA to DHA. The level of DHA was maintained, but the proportion of EPA declined, and so the
34 DHA:EPA ratio increased in bluefin tuna larvae during yolk sac utilization. Although this could be
35 the result of relative retention of DHA during a period of generally high fatty acid oxidation and
36 utilization, there was also a great increase in desaturase and elongase expression with larval
37 development. This suggests that increased activity of these enzymes is important for the normal
38 development of tuna larvae related to the provision of adequate DHA for the formation of
39 biomembranes, particularly in neural (brain and eye) tissues.

40

41 *Keywords:* Yolk sac larvae, lipid content, lipid classes, fatty acid composition, cDNA, gene
42 expression.

43

44 **1. Introduction**

45 Scombrid fish comprise species of high commercial value such as bonitos and tunas. One of
46 the most iconic species is the Atlantic bluefin tuna (ABT), *Thunnus thynnus* (Linnaeus 1758),
47 which has relevant ecological, recreational and commercial importance in Atlantic and
48 Mediterranean ecosystems (Rodríguez-Roda, 1964; Rey, 1999; Fromentin and Powers, 2005). The
49 ABT fishery has become a highly profitable activity with the development of the sushi-sashimi
50 market in Japan increasing the demand for high quality fish, stimulating very high prices and, in
51 consequence, increasing regulated and unregulated fishing effort (Fromentin and Powers, 2005). In
52 recent years considerable research effort has been undertaken in order to develop aquaculture of
53 these large pelagic migratory fish species (Wexler et al., 2003; Margulies et al., 2007; Masuma et
54 al., 2008). Determining nutritional requirements, particularly at larval stages, will be a key task
55 (Mourente and Tocher, 2003, 2009).

56 The importance of lipids for growth and development of fish is based on their key roles as
57 sources of metabolic energy and as essential components of tissue and cell membranes. In most fish
58 species, lipids are favoured as an energy source compared to proteins and carbohydrates, and are
59 characterized by high contents of long-chain polyunsaturated fatty acids (LC-PUFA) essential for
60 cell membrane structure and function. The reserves of lipid in fish eggs are used by the developing
61 embryo and subsequent larvae for energy metabolism, as structural components of biomembranes
62 and precursors of lipid-derived hormones (Sargent, 1995; Wiegand, 1996; Sargent et al., 1989,
63 2002). Lipid reserves in teleost fish eggs are stored as lipoproteins in yolk reserves and, in some
64 species, as discrete or fragmented oil globules. Yolk lipoproteins contain primarily phospholipids,
65 mainly phosphatidylcholine and phosphatidylethanolamine, showing high levels of polyunsaturated
66 fatty acids (PUFA), particularly the n-3 LC-PUFA, eicosapentaenoic acid (20:5n-3; EPA) and
67 docosahexaenoic acid (22:6n-3; DHA). Tuna eggs contain a single discrete oil globule, comprised
68 of neutral lipids, primarily triacylglycerol (TAG), steryl ester and/or wax ester, rich in both
69 monounsaturated fatty acids (MUFA) and n-3 LC-PUFA (Wiegand, 1996; Ortega and Mourente,

70 2010). The LC-PUFA have well-established roles in membrane structure and function as well as
71 energy sources during embryonic and early larval development of marine fish (Sargent et al., 2002).
72 The composition and metabolism of lipid and fatty acids during embryogenesis and yolk-sac larvae
73 development can give useful information of the nutritional requirements during early larval stages
74 (Sargent et al., 2002). This approach can provide insights that can be applied in the development of
75 live feed enrichments and/or artificial feeds (Tocher, 2003).

76 Tuna tissues have a relatively high level of DHA and a high DHA:EPA ratio (Tocher, 2003;
77 Mourente and Tocher, 2009). However, all marine fish studied to date have only very limited ability
78 to biosynthesize LC-PUFA and so have an absolute requirement for dietary EPA and DHA (Tocher,
79 2003). The fatty acid profile of lipids from eggs of wild-caught tunas shows high levels of DHA,
80 possibly suggesting that tunas may have a high requirement for this fatty acid (Nichols et al., 1998;
81 Mourente and Tocher, 2009; Ortega and Mourente, 2010). Although the lipid biochemistry
82 underpinning the high level of DHA and the high DHA:EPA ratio in tuna is unclear, it has been
83 generally assumed that tuna must selectively accumulate and retain DHA in their tissues (Ishihara
84 and Saito, 1996; Saito et al., 1996; Mourente and Tocher, 2009). Tuna are top predators of the
85 benthic-pelagic trophic web and the concentration of n-3 LC-PUFA in neural tissues is known to be
86 crucial for effective prey capture from the time of first feeding (Bell et al., 1995; Mourente and
87 Tocher, 2009).

88 Long-chain PUFA can be biosynthesized from short-chain (C_{18}) PUFA in reactions
89 catalysed by fatty acyl desaturase (Fad) and elongase (Elovl) enzymes. Understanding of the
90 biochemical and molecular mechanisms of LC-PUFA biosynthesis in fish has advanced in recent
91 years and several Fads have been characterized in various fish species. With the exception of a
92 zebrafish bifunctional $\Delta 6/\Delta 5$ Fad (Hastings et al., 2001) and an Atlantic salmon (*Salmo salar*) $\Delta 5$
93 Fad (Hastings et al., 2005), all have been $\Delta 6$ Fads (Zheng et al., 2004, 2005, 2009; Tocher et al.,
94 2006; Gonzales-Rovira et al., 2009). Biochemical studies in fish cell lines (Ghioni et al., 1999;
95 Tocher and Ghioni, 1999) led to the hypothesis that some fish may be unable to biosynthesize LC-

96 PUFA because they lack specific genes in the pathway (Leaver et al., 2008). However, although $\Delta 6$
97 Fads cDNAs have been cloned from all fish species studied so far, and all showed significant
98 activity in heterologous yeast expression systems, $\Delta 6$ Fad expression and activity are very low in
99 Atlantic cod (*Gadus morhua*) tissues compared to expression and activity of $\Delta 6$ Fad in salmon
100 tissues (Tocher et al., 2006). Elongase cDNAs have been cloned from freshwater species, zebrafish
101 (*Danio rerio*), common carp (*Cyprinus carpio*) and tilapia (*Oreochromis nilotica*); salmonids,
102 Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*); and marine species, Atlantic cod (*Gadus*
103 *morhua*), turbot (*Psetta maxima*), gilthead sea bream (*Sparus aurata*), cobia (*Rathycentron*
104 *canadum*) and Southern bluefin tuna (*Thunnus maccoyii*) (Agaba et al., 2004, 2005; Hastings et al.,
105 2005; Zheng et al., 2009; Gregory et al., 2010).

106 In the present study, we investigated lipid and fatty acid metabolism during early larval
107 development of ABT. Specifically, we describe the changes in total lipid content, lipid class
108 composition and total, phospholipid and neutral lipid fatty acid profiles in unfed ABT larvae during
109 yolk sac utilisation. Moreover, we studied the LC-PUFA biosynthesis pathway by quantifying
110 expression of Fad and Elovl genes. To this end, we cloned and functionally characterized the
111 cDNAs for these genes by heterologous expression in yeast, showing them to be a $\Delta 6$ Fad and
112 Elovl5, respectively. The results were discussed in relation to the maintenance of DHA levels and
113 the evolution of DHA:EPA ratios in tuna tissues, and the physiological role for these genes in ABT
114 development.

115

116 **2. Materials and Methods**

117

118 *2.1 Atlantic bluefin tuna larvae*

119 Atlantic bluefin tuna eggs came from a broodstock composed of 35 fish with an estimated
120 mean body weight of 100 kg. The broodstock were kept in captivity for 3 years in a floating cage
121 located at El Gorguel, off Cartagena coast, SE Spain and were fed on frozen fish, mainly mackerel.

122 On the 26th and 27th June 2009, 15 captive-reared ABT broodstock fish were induced to mature
123 and reproduce by means of GnRHa implants, and an egg collector, 500 µm mesh screen size, was
124 placed around the cage. Spawning started on June 29th and ceased on July 15th. Over 126 million
125 eggs were collected, with a hatching rate of 84.4% ±13.5%.

126 Collected eggs were transported in a 500 l plastic tank supplied with pure oxygen to the IEO
127 – Mazarrón aquaculture facilities and placed in 100 L tanks with gentle oxygenation and flow
128 through sterilized sea water in order to clean them. After 1 h, aeration and water flow were stopped
129 to separate buoyant (viable) from non-buoyant (non-viable) eggs, and fertilized eggs collected and
130 incubated at an initial egg density of 1100 eggs per L in 1000 L cylindro-conical tanks. Incubation
131 was carried out at 25-26 °C, 37 ppt and continuous photoperiod, with a light intensity of 100 lux.
132 An upwelling flow-through, with gentle aeration in order to keep oxygen levels next to saturation,
133 was employed.

134

135 *2.2 Sample collection and dry weight determination*

136 As described above, the larvae were obtained from the first ever spawning of captive-reared
137 ABT broodstock as part of the EU FP7 project “SELFDOTT”. Due to the unique and extremely
138 limited nature of this resource, most larvae were retained for the first research into larval rearing
139 (juvenile production) of this species and so only unfed larvae were available for the present study.
140 Eggs hatched 9th July (0 days post-hatch, 0 DPH) and the larvae were maintained in the conditions
141 described above. Hatching rate in this batch was close to 94 %, and survival of hatched larvae after
142 1, 2, 3 and 4 days was 69 %, 46 %, 12 % and 4 %, respectively. Thus, the final sample was obtained
143 at 4 days post-hatch (4 DPH) as survival was too low at 5 DPH to obtain viable samples. Each day,
144 three samples of 5-10,000 larvae were collected from the incubator. Water from the incubator was
145 filtered with a 250 µm mesh screen net in order to concentrate the larvae. Larvae were washed with
146 distilled water and excess water blotted through the screen with filter paper. Sampled larvae were
147 collected in cryotubes, frozen in liquid nitrogen and stored at -80°C until analysis. Replicates of

148 preweighed samples (approximately 50 mg wet weight) were maintained at 110°C for 24 h. The dry
149 weights were determined after cooling *in vacuo* for at least 1 h.

150

151 2.3 Lipid content, lipid class composition and fatty acid analysis

152 Total lipid of ABT larvae was extracted from triplicate pooled samples according to the
153 method of Folch et al. (1957). Approximately 1 g of ABT larvae was placed in 20 ml of ice-cold
154 chloroform/methanol (2:1, by vol) and homogenized with an Ultra-Turrax tissue disrupter (Fisher
155 Scientific, Loughborough, U.K.). The non-lipid and lipid layers were separated by addition of 5 ml
156 of 0.88 % (w/v) KCl and allowed to separate on ice for 1 h. The upper non-lipid layer was aspirated
157 and the lower lipid layer dried under oxygen-free nitrogen. The lipid content was determined
158 gravimetrically after drying overnight in a vacuum desiccator.

159 Lipid class composition was determined by high-performance thin-layer chromatography
160 (HPTLC) using 10 x 10 cm plates (VWR, Lutterworth, England). Approximately 2 mg of total lipid
161 was applied as 2 mm streaks and the plates developed in methyl acetate/isopropanol/
162 chloroform/methanol/0.25 % aqueous KCl (25:25:25:10:9, by vol.) to two-thirds up the plate. After
163 desiccation for 20 min, the plate was fully developed with isohexane/diethyl ether/acetic acid
164 (85:15:1, by vol.). The lipid classes were visualized by charring at 160 °C for 15 min after spraying
165 with 3 % (w/v) aqueous cupric acetate containing 8 % (v/v) phosphoric acid and quantified by
166 densitometry using a CAMAG-3 TLC scanner (version Firmware 1.14.16) (Henderson and Tocher,
167 1992). Scanned images were recorded automatically and analyzed by computer using winCATS
168 Planar Chromatography Manager (version 1.2.0).

169 Fatty acid methyl esters (FAME) were prepared from total lipid, total phospholipids and
170 total neutral lipids by acid-catalyzed transesterification at 50 °C for 16 h according to the method of
171 Christie (1993). Phospholipids and neutral lipids were separated by TLC, and extraction and
172 purification of FAME was carried out as described by Tocher and Harvie (1988). The FAME were
173 separated and quantified by gas-liquid chromatography (Carlo Erba Vega 8160, Milan, Italy) using

174 a 30m x 0.32 mm i.d. capillary column (CP Wax 52CB, Chrompak, London, U.K.) and on-column
175 injection at 50°C. Hydrogen was used as carrier gas and temperature programming was from 50 °C
176 to 150 °C at 40 °C min⁻¹ and then to 230 °C at 2.0 °C min⁻¹. Individual methyl esters were identified
177 by comparison with known standards and by reference to published data (Ackman, 1980; Tocher
178 and Harvie, 1988). Data were collected and processed using Chromcard for Windows (version 1.19)

179

180 2.4 Cloning of putative fatty acid desaturase and elongase from ABT larvae

181 Sequences corresponding to the open reading frame (ORF) of *fads* and *elovls* from several fish
182 species were aligned and primers designed on conserved regions (Table 1). GenBank accession
183 numbers of the sequences used in these alignments were AY055749 (*Sparus aurata*), DQ054840
184 (*Gadus morhua*), AY546094 (*Scophthalmus maximus*), NM_001123575 (*Salmo salar*) and
185 AF301910 (*Oncorhynchus mykiss*) for $\Delta 6fad$; and GQ204105 (*Thunnus maccoyii*), AY660879
186 (*Sparus aurata*), AY660881 (*Gadus morhua*), AF465520 (*Scophthalmus maximus*),
187 NM_001123567 and NM_001136552 (*Salmo salar*), DQ067616 (*Oncorhynchus masou*) for *elovl5*-
188 like transcripts. Fragments were obtained by reverse transcription polymerase chain reaction (RT-
189 PCR) (GoTaq® Colorless Master Mix; Promega, Southampton, U.K.) on cDNA synthesised (as
190 described below) from 1 µg of total RNA pooled from 0 to 3 DPH larvae using the conserved
191 region (CR) primers, in the case of elongase, using also primers designed on the sequence of the
192 closely related species *T. maccoyii* (Tm). After sequencing (CEQ-8800 Beckman Coulter Inc.,
193 Fullerton, U.S.A.), the ORF fragments were further extended by 3' rapid amplification of cDNA
194 ends (RACE) PCR (FirstChoice® RLM-RACE kit, Ambion, Applied Biosystems, Warrington,
195 U.K.), to obtain the sequence of the 3' UTR. The specific primers used for 3' RACE are shown in
196 Table 1. The *Thunnus thynnus* sequences corresponding to the putative $\Delta 6fad$ and *elovl5* were
197 assembled using BioEdit (Tom Hall, Ibis Therapeutics, CA) and deposited in the GenBank database
198 under accession numbers HQ214238 and HQ214237, respectively.

199

200 2.5 Sequence and phylogenetic analysis

201 The deduced amino acid sequences of the newly cloned ABT *Δ6fad* and *elovl5* cDNAs were
202 aligned with different fatty acyl desaturases and elongases of *Caenorhabditis elegans*, *Mortierella*
203 *alpina*, several fish species, human and mouse (accession numbers shown in Figs 1 and 2) using
204 ClustalW (BioEdit). Phylogenetic analysis was then conducted using the neighbor-joining method
205 (Saitou and Nei, 1987) in MEGA version 4 (Tamura et al., 2007), with confidence in the resulting
206 tree branch topology measured by bootstrapping through 10,000 iterations. Comparison of amino
207 acid and cDNA sequences of ABT and Southern bluefin tuna (SBT, *Thunnus maccoyii*) was
208 performed using BLAST (bl2seq; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

209

210 2.6 Functional characterisation of cDNAs by heterologous expression in *Saccharomyces cerevisiae*

211 PCR fragments corresponding to the ORFs of the putative *Δ6fad* and *elovl5* were amplified from
212 3DPH larvae cDNA using sense and antisense primers containing either a *SacI* or *XhoI* digestion
213 site, respectively (Table 1). In the case of *Δ6fad*, a two stage (semi-nested) PCR had to be
214 performed, first using primers CR-D6DES-F4 and Tt-D6DES-R4 (Table 1), located at the start of
215 the ORF and in the 3' UTR, respectively. For this transcript, PCR was performed using the
216 HotStarTaq DNA Polymerase with Q-solution (Qiagen, West Sussex, U.K.), while the putative
217 *elovl5* ORF was amplified in a single step using the high fidelity PfuTurbo® DNA Polymerase
218 (Stratagene, Agilent Technologies, Cheshire, U.K.), using 35 cycles of amplification with the
219 primers and annealing temperatures shown in Table 1 and following manufacturer's instructions.
220 The DNA fragments were then purified (Illustra GFX™ PCR DNA and gel band purification kit,
221 GE Healthcare Life Sciences, Buckinghamshire, U.K.) and digested with the corresponding
222 restriction endonucleases (New England BioLabs, Herts, U.K.), purified again and ligated into a
223 similarly restricted pYES2 yeast expression vector (Invitrogen, Paisley, U.K.). Ligation products
224 were used to transform Top10F' *Escherichia coli* competent cells (Invitrogen) which were screened
225 for the presence of recombinants. The purified plasmids (GenElute™ Plasmid Miniprep Kit, Sigma)

226 containing the ORFs (confirmed by sequencing) were then used to transform *Saccharomyces*
227 *cerevisiae* competent cells (S.c. EasyComp Transformation Kit, Invitrogen). Transformation and
228 selection of yeast with recombinant *putative* $\Delta 6fad$ -pYES2 and *elovl5*-pYES2 plasmids, yeast
229 culture and fatty acid analysis were performed as described in detail previously (Hastings et al.,
230 2001; Agaba et al., 2004; Zheng et al., 2005). Briefly, cultures of recombinant yeast were grown in
231 *S. cerevisiae* minimal medium^{-uracil} supplemented with one of the following fatty acid substrates:
232 18:3n-3, 18:2n-6, 20:4n-3, 20:3n-6, 22:5n-3 or 22:4n-6 for Fad; and 18:4n-3, 18:3n-6, 20:5n-3,
233 20:4n-6, 22:5n-3 or 22:4n-6 for Elovl. Fatty acids were added to the yeast cultures at final
234 concentrations of 0.5 (C18), 0.75 (C20) and 2.0 (C22) mM. After 2-days, yeast were harvested,
235 washed, and lipid extracted. The FAME were analyzed by GC and the proportion of substrate fatty
236 acid converted to desaturated or elongated fatty acid product was calculated as [product
237 area/(product area + substrate area)] x 100.

238

239 2.7 RNA extraction and quantitative real time PCR (qPCR)

240 Samples originating from a single pool of larvae collected each day from 0 to 4 DPH were
241 rapidly disrupted in TRI-Reagent (Ambion, Applied Biosystems) using an Ultra-Turrax
242 homogenizer (Fisher Scientific, Loughborough, U.K.). Total RNA was extracted by organic
243 solvent, according to manufacturer's instructions (Ambion, Applied Biosystems), and RNA quality
244 and quantity assessed by electrophoresis (Bioanalyser 2100, Agilent Technologies, Santa Clara,
245 U.S.A.) and spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, U.S.A.).
246 One μ g of total RNA per sample was reverse transcribed into cDNA using a VersoTM cDNA kit
247 (ABgene, Surrey, U.K.), following manufacturer's instructions, and using a mixture of random
248 hexamers and anchored oligo-dT (3:1, v/v). Negative controls (containing no enzyme) were
249 performed to later check for genomic DNA contamination.

250 For quantitative real time PCR (qPCR), primers were designed for both transcripts using the
251 Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). In addition, two

reference genes, the elongation factor-1 α (*elf-1 α* ; GenBank accession number: FM995222) and β -*actin* (GU046791) were quantified (Table 1). Relative quantification was performed in triplicate using a Quantica machine (Techne, Cambridge, U.K.) and the amplification efficiency of each primer pair assessed by serial dilutions of cDNA pooled from the samples being quantified. The amplifications were carried out in a final volume of 20 μ l containing 2 μ l (for the reference genes) or 5 μ l (for larvae samples) diluted (1/20) cDNA, 0.5 μ M of each primer and 10 μ l AbsoluteTM QPCR SYBR[®] Green mix (ABgene). In addition, amplifications were carried out with a systematic negative control (NTC-non template control, containing no cDNA). The qPCR profiles contained an initial activation step at 95 °C for 15 min, followed by 30 to 40 cycles: 15 s at 95 °C, 15 s at the annealing T_m and 30 s at 72 °C. After the amplification phase, a melt curve of 0.5 °C increments from 75 °C to 90 °C was performed, enabling confirmation of the amplification of a single product in each reaction. The qPCR product sizes and the presence of primer-dimers in the NTC were checked by agarose gel electrophoresis. In addition, qPCR product identities were confirmed by sequencing.

Results are given as expression values (obtained from the standard curve performed with cDNA serial dilutions) normalized by the average value of the two reference genes and are expressed as a relative ratio between each sampling day and the expression at 0 DPH.

2.8 Materials

The fatty acids, 22:5n-3, 22:4n-6 and 20:4n-3 (all 98–99% pure), were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA), and 18:2n-6, 18:3n-3, 18:4n-3, 18:3n-6, 20:3n-6, 20:5n-3 and 20:4n-6 acids (all 99% pure), BHT and chemicals used to prepare the *S. cerevisiae* minimal medium^{-uracil} were from Sigma-Aldrich Co. Ltd. (Poole, UK). Solvents were HPLC grade and from Fisher Scientific (Loughborough, UK).

2.9 Statistical analysis

Results are presented as means \pm SD ($n = 3$) except for gene expression data, where a single pool of larvae was analysed. The data were checked for homogeneity of variances using the Bartlett test and, where necessary, arc-sin transformed before further statistical analysis. Differences between mean values were analyzed by one-way analysis of variance (ANOVA), followed when pertinent by a multiple comparison test (Tukey). Differences were reported as statistically significant when $P < 0.05$ (Zar, 1999).

3. Results

3.1 Sequence analyses of ABT larvae desaturase and elongase cDNAs

The overlapping fragments of a putative *fad* and *elovl* that were obtained by classic RT-PCR and 3'-RACE-PCR amplification from ABT cDNA were assembled into a sequence of 1817 bp and 1170 bp, respectively (gb|HQ214238 and gb|HQ214237, respectively). These sequences included ORFs of 1338 bp for $\Delta 6fad$ and 885 bp for *elovl5*, encoding predicted proteins of 446 and 294 amino acids, respectively. Comparison of these sequences with those of *fad* and *elovl* cDNAs (GenBank accession nos. HM032095 and GQ204105, respectively) of the closely related SBT (*Thunnus maccoyii*) revealed practically complete sequence conservation. In fact, both $\Delta 6fad$ and *elovl5* ORF sequences are 99% identical, being translated into a 100% identical Fad protein or 99% identical Elovl5 (Thr in ABT instead of Met in SBT in position 85). The phylogenetic trees of both $\Delta 6$ Fad (Fig. 1) and Elovl5 (Fig. 2) also reveal this, with both ABT and SBT protein sequences clustering together. In addition, the ABT putative $\Delta 6$ Fad clustered closely with other marine fish $\Delta 6$ Fads, including those of gilthead seabream, European seabass, turbot, cobia and Asian seabass, and more distantly from other fish Fads (including $\Delta 5$ Fads and $\Delta 5/\Delta 6$ Fads) and mammalian $\Delta 6$ Fads (Fig. 1). The phylogenetic tree constructed for the Elovl proteins (Fig. 2) has clearly separated the vertebrate Elovl5, Elovl2 and Elovl4 proteins, with the ABT and SBT Elovl5s clustering more

302 closely with gilthead seabream, turbot and tilapia Elovl5's, followed by salmonid Elovl5 proteins
303 and more distantly with the mammalian Elovl5 proteins.

304

305 3.2 Functional characterisation of ABT fatty acyl desaturase and elongase

306 The fatty acid composition of untransformed yeast *S.cerevisiae* shows four main fatty acids,
307 namely 16:0, 16:1n-7, 18:0 and 18:1n-9 (Hastings et al., 2001), numbered 1-4 in Figs. 3 and 4.
308 When yeast, transformed with the ABT Fad cDNA insert, was grown in the presence of $\Delta 6$
309 desaturase substrate 18:3n-3, two additional peaks were observed in the GC traces, corresponding to
310 the exogenously added fatty acid, 18:3n-3 and its desaturated product, 18:4n-3 (Fig. 3A). The GC
311 traces of Fad-transformed yeast grown in the presence of $\Delta 5$ and $\Delta 4$ Fad substrates showed peaks
312 for the exogenously added fatty acids, 20:4n-3 and 22:5n-3, but no desaturated products, 20:5n-3
313 and 22:6n-3, respectively (Figs. 3B and 3C). Identical results were obtained in parallel experiments
314 incubating Fad-transformed yeast with n-6 PUFA, with 18:3n-6 being produced from 18:2n-6, but
315 no products obtained with either 20:3n-6 or 22:4n-6 substrates. In this heterologous assay system,
316 the ABT desaturase showed a preference for the n-3 fatty acid substrates, with approximately 31%
317 of 18:3n-3 converted to 18:4n-3, and 20% of 18:2n-6 converted to 18:3n-6 (Table 2). When
318 yeast, transformed with the ABT Elovl cDNA insert, was grown in the presence of fatty acid
319 substrate for C₁₈ elongation, four additional peaks were observed in the GC trace, corresponding to
320 the exogenously added fatty acid, 18:4n-3, its immediate elongated product, 20:4n-3, and the
321 further elongated products, 22:4n-3 and 24:4n-3 (Fig. 4A). The GC trace also showed a large peak
322 of 18:1n-7 (peak 5) indicating elongation of endogenous 16:1n-7, and peaks were also observed
323 that corresponded to 20:1n-9 and 20:1n-7 (peaks 6), indicating elongation of 18:1n-9 and 18:1n-7
324 (Figs. 4A-C). Similarly, the GC traces of Elovl-transformed yeast grown in the presence of
325 substrate for C₂₀ elongation showed additional peaks corresponding to the exogenously added fatty
326 acid, 20:5n-3, its immediate elongation product, 22:5n-3, and the further elongated product,
327 24:5n-3 (Fig. 4B). Transformed yeast grown in the presence of substrate for C₂₂ elongation showed

328 additional peaks corresponding the exogenously added fatty acid, 22:5n-3, and its elongated
329 product, 24:5n-3 (Fig. 4C). Again, similar results were obtained with the corresponding n-6 PUFA
330 substrates. In this heterologous expression system, the ABT elongase showed similar activity
331 towards C₁₈ and C₂₀ fatty acid substrates, with much lower activity towards C₂₂ substrates (Table 2).
332 There was no preference between n-3 and n-6 PUFA substrates for C₁₈ and C₂₀ elongation, but
333 there may be a slight preference for n-3 with C₂₂ elongation (Table 2).

334

335 *3.3 Dry mass, lipid content and lipid class composition of unfed ABT larvae*

336 Yolk sac larvae of ABT showed a significant decrease of around 29 % in dry mass from
337 hatching (DPH0) to DPH4 (Table 3). During the same period, total lipid content decreased by
338 approximately 27 %, on a dry mass basis. The lipid class composition of ABT yolk sac larvae at
339 hatching was predominantly neutral lipids (~77 %), primarily steryl ester/wax ester, TAG and
340 cholesterol, with 23 % total polar lipids, primarily phosphatidylcholine, phosphatidylethanolamine,
341 phosphatidylserine and phosphatidylinositol. The proportion of neutral lipids declined to 59 % by 4
342 DPH due to decreased percentages of both TAG and steryl ester/wax ester. Over the same period,
343 the proportions of membrane lipid increased with total polar lipid and cholesterol rising to 41 % and
344 22 %, respectively (Table 3). Consequently, the nutritional index (TAG/cholesterol) decreased
345 significantly from 1.9 to 0.3.

346

347 *3.4 Fatty acid composition*

348 Total lipids of ABT yolk sac larvae at hatching were characterized by 26 % saturated fatty
349 acids (primarily 16:0, followed by 18:0 and 14:0), 33 % MUFA (primarily 18:1n-9) and 34 %
350 PUFA, primarily the n-3 LC-PUFA, DHA (19 %) and EPA (6 %) with a DHA:EPA ratio of 3.0
351 (Table 4). From hatching to DPH4, the proportion of saturated fatty acids increased to 32 %
352 whereas total MUFA decreased to 27 %, mainly due to increased percentages of 18:0, and
353 decreased percentages of almost all MUFA. There were no significant changes in the proportions of

354 n-3, n-6 or total PUFA during this period of development but the percentages of arachidonic acid
355 (ARA; 20:4n-6) and DHA significantly increased, whereas the proportion of EPA decreased (Table
356 4), such that the DHA:EPA ratio in total lipid increased to 5.0 by DPH4 (Fig.5).

357 Total polar lipids of ABT yolk sac larvae at hatching were characterized by 36 % saturated
358 fatty acids (primarily 16:0 and 18:0), 19 % MUFA (primarily 18:1n-9) and 39 % PUFA, primarily
359 DHA (27 %) and EPA (6 %) with a DHA:EPA ratio of 4.7 (Table 5). There were few major effects
360 of development on polar lipid fatty acid composition from hatching to DPH4, other than a slight but
361 significant increased proportion of total n-6 PUFA, and decreased percentage of EPA (down to 3.7
362 %) with a trend for increased DHA that resulted in the DHA:EPA ratio significantly increasing to
363 7.9 (Table 5, Fig.5). In contrast, there were no overall changes in the proportions of total saturated
364 fatty acids, MUFA or PUFA, including EPA and DHA, in total neutral lipids between DPH0 and
365 DPH4 (Table 6). Similarly, the DHA:EPA ratio was constant at around 2.4-2.5 in neutral lipids
366 (Fig.5).

367

368 3.5 Expression of *Δ6 Fad* and *Elovl5* elongase in ABT larvae

369 Quantitative PCR data for each gene were normalized using elongation factor 1 α (*elf1 α*) and
370 β -actin as reference genes. The expression of *Δ6fad* and *elovl5* elongase were very similar during
371 development (inanimation) of ABT yolk sac larvae. There was quantitative increased expression of
372 over 8-fold for both genes in only 24 h, from DPH0 to DPH1. In the following 24 h the level of the
373 expression remained the same but from DPH2 to DPH4, the expression of *Δ6fad* and *elovl5*
374 increased greatly to attain levels of expression around 23- and 27-fold greater than at DPH0,
375 respectively (Fig.5).

376

377 4. Discussion

378

379 The present study is the first report of lipid and fatty acid metabolism in ABT and has focussed
380 specifically on a critical time in marine fish development, such as larvae during the period of yolk
381 sac utilization. Although nutrient utilization and metabolism during early larval development has
382 been a relatively well-studied area in fish there are few comparative data on scombrid species
383 (Weigand, 1996). Fish of the order Perciformes, to which *T. thynnus* (Perciformes, Scombridae)
384 belongs, generally produce eggs with oil globules, showing variability in the egg or oocyte lipid
385 class compositions but generally with neutral lipids predominating over polar lipids (Wiegand,
386 1996). Previously, we have reported lipid and fatty acid compositions of wild ABT eggs (Ortega
387 and Mourente, 2010). The newly-hatched yolk sac larvae obtained from captive ABT showed
388 higher dry mass and total lipid content, and a higher proportion of total neutral lipids, and lower
389 polar lipids, than wild ABT eggs. Importantly, however, the nutritional index (TAG/cholesterol)
390 was almost 50 % lower in yolk sac larvae from the captive ABT than in wild ABT eggs (Ortega and
391 Mourente, 2010). The ABT yolk sac larvae at hatching also showed higher proportions of
392 monoenes and lower proportions of PUFA and a lower DHA:EPA ratio than the total lipid fatty
393 profile of eggs of wild caught ABT (Ortega and Mourente, 2010). These differences in apparent
394 quality parameters (TAG/cholesterol, PUFA level and DHA:EPA ratio) of the larvae from captive
395 ABT compared to eggs from wild ABT suggest that this is an area worthy of future research effort.

396 The decreasing total lipid content, and the decreased proportions of neutral lipids, particularly
397 TAG but also steryl ester/wax ester, showed that lipid was utilized during this early stage of yolk
398 sac larval development in ABT. Phospholipid, TAG or wax ester, exclusively, sequentially or in
399 combination, are all used as energy sources by fish embryos with the pattern varying with species
400 (Sargent et al., 1989, 2002; Wiegand, 1996). Thus, ABT showed a pattern of lipid metabolism
401 during early development similar to that of marine larval fish from temperate waters whose eggs
402 contain high levels of total lipids, including an oil globule, and which preferentially utilize neutral
403 lipids as the primary energy source (Weigand et al., 1996; Sargent et al., 2002). Studies of fatty acid
404 depletion in developing embryos and early larvae of a range of Perciform species have found

405 preferential catabolism of MUFAs along with preferential retention of DHA, ARA and specific
406 saturated fatty acids, usually 16:0 or 18:0 (Mourete and Vazquez, 1996; Wiegand et al., 1996;
407 Mourente et al., 1999; Sargent et al., 2002; Ortega and Mourente, 2010). This reflects the essential
408 structural role of DHA in membranes, the importance of ARA in eicosanoid production and specific
409 roles of saturated fatty acids in the sn-1 position of structural phospholipids.

410 In the present study the ABT larvae were not fed and so were, in effect, starving and the
411 changes in lipids have to be interpreted in that context. Therefore, one explanation for the changes
412 observed in the larval fatty acid composition, showing an increasing DHA:EPA ratio, can be
413 advanced simply in terms of differential oxidation and retention of DHA. The results show total
414 lipid and neutral lipid decreased during the development period and so fatty acids were undoubtedly
415 being utilized for energy. Although it is known that both EPA and DHA can be oxidized in fish, at
416 least salmon, when in dietary excess (Stubhaug et al., 2007), DHA is known to be the fatty acid that
417 is most preferentially retained (Sargent et al., 2002). Whether this is a true active retention or
418 whether it is due to DHA being more slowly and inefficiently oxidized is not clear (Tocher, 2003).
419 The results with the ABT larvae showed that the proportion of polar lipid increased with time and,
420 as polar lipid had a higher DHA content than neutral lipid, the proportion of DHA in total lipid
421 would consequently increase, as observed. In addition however, whereas DHA content in neutral
422 lipid remained constant there was a trend for increased proportions of DHA in polar lipids
423 suggesting that DHA released by hydrolysis of TAG could be then reesterified in polar lipid as
424 observed in previous studies on larval development in marine fish (Tocher et al., 1985; Fraser et al.,
425 1988). So the fact that the content of DHA relative to total fatty acids tends to increase in both total
426 lipid and polar lipid would be consistent with the known phenomenon of retention of DHA.

427 The present study also represents the first investigation of LC-PUFA biosynthesis in ABT and
428 reports the cloning of cDNAs for two important genes in the pathway. Heterologous expression in
429 the yeast *S. cerevisiae* demonstrated that the ABT cDNAs coded for a $\Delta 6$ Fad and an Elovl5
430 elongase. Alignment and phylogenetic analyses showed that the ABT cDNAs sequences were very

431 similar to sequences present in the database for Fad and Elovl cDNAs from SBT (Gregory et al.,
432 2010; Schuller et al., 2010). The remarkable level of sequence conservation between the *Thunnus*
433 species was far greater than observed previously between any other teleost species including closely
434 related salmonids (Zheng et al., 2004; Monroig et al., 2010). Furthermore, the functional data
435 obtained with the ABT elongase in the present study were qualitatively identical and quantitatively
436 very similar to the results previously reported for SBT elongase, which was also shown by
437 expression in yeast to be an Elovl5 (Gregory et al., 2010). This is not surprising considering the
438 ORF and translated protein sequences of the *Thunnus* elongases were 99 % identical with only one
439 amino acid difference. The ABT and SBT Fad ORF sequences were also 99 % identical but 100 %
440 identical at the protein level and so, although not functionally characterized, it is certain that the
441 SBT desaturase is also a $\Delta 6$ Fad as shown for the ABT desaturase in the present study.

442 The presence of the *$\Delta 6$ fad* and *elovl5* genes and the role of $\Delta 6$ Fad and Elovl5 activities in LC-
443 PUFA biosynthesis in marine fish species has been the subject of some discussion and speculation
444 (Zheng et al., 2009; Tocher, 2010). Thus, *$\Delta 6$ fad* and *elovl5* cDNAs have both been isolated from
445 several marine species including Atlantic cod, turbot, gilthead sea bream, Asian sea bass (*Lates*
446 *calcarifer*), and cobia (Seiliez et al., 2003; Zheng et al., 2004; Tocher et al., 2006; Mohd-Yusof et
447 al., 2010), all carnivorous, largely piscivorous, species with trophic levels of greater than 3
448 (Kaushik and Troell, 2010). As such, all these species will normally obtain high levels of EPA and
449 DHA, and rather low levels of 18:3n-3 and 18:2n-6, in their natural diet and so endogenous LC-
450 PUFA biosynthesis from C₁₈ precursors is redundant. Consistent with this, LC-PUFA synthesis in
451 hepatocytes or enterocytes from marine species including cod, and European and Asian sea bass, is
452 very low (Mourente et al., 2005; Tocher et al., 2006; Mohd-Yusof et al., 2010). However, the role
453 of $\Delta 6$ Fad in the pathway from EPA to DHA (desaturation of C₂₄ PUFA intermediates), along with
454 the high expression of *$\Delta 6$ fad* and *elovl5* in brain in cod, cobia and Asian sea bass, has suggested an
455 alternative role for these enzymes in marine fish, based on a requirement to maintain membrane
456 DHA levels, particularly in neural tissues at times of high demand such as embryonic and larval

development (Zheng et al., 2005, 2009; Tocher et al., 2006; Mohd-Yusof et al., 2010). The data from the present study in ABT showing increased expression of *Δ6fad* and *elovl5* during yolk sac larval development is consistent with this hypothesis. Interestingly, the Elov12 elongase, which has high activity towards C₂₂ PUFA would be the more important Elov1 in this pathway for production of C₂₄ PUFA intermediates, but Elov12 has only been isolated from Atlantic salmon and not from any marine fish (Morais et al., 2009). However, Elov15 enzymes in fish have activity towards C₂₂ PUFA (Zheng et al., 2004, 2009; Mohd-Yusof et al., 2010), in contrast to mammalian Elov15 that has very low or no activity towards C₂₂ PUFA (Leonard et al., 2000; Inagaki et al., 2002).

The above data also offer a further possible explanation for the increasing DHA:EPA ratio observed in total lipid in the ABT larvae. As this increasing ratio was due solely to changes in the DHA:EPA ratio in polar lipid and not neutral lipid, this may suggest that there is an active conversion of EPA to DHA. The *fad* and *elovl* gene expression data are consistent with this, but it is not possible to confirm this from the data available. It is probable that the situation is one where both mechanisms could contribute to the effect. As the larvae were starving in the present study, it is likely that the observed changes in DHA:EPA ratio were more the consequence of retention of DHA through relatively lower oxidation and channeling towards reesterification in polar lipid. In natural development with exogenous feeding, the increased expression of *fad* and *elovl* genes and consequent increased conversion of EPA to DHA would be an important pathway to help satisfy the high requirement for DHA necessary for the rapid development of neural tissue (brain and eye) at this time (Mourete, 2003). It was previously speculated that the high DHA:EPA ratio in tuna tissues in general was consistent with selective metabolism of EPA, through both conversion to DHA via elongation and desaturation and also by catabolism via β -oxidation (Tocher, 2003).

In conclusion, the data in the present study with ABT larvae show that the increasing DHA:EPA ratio is most likely the result of retention of DHA in polar lipids and selective catabolism of EPA although the increasing expression of the genes of LC-PUFA biosynthetic pathway, *Δ6fad* and *elovl5*, offers an alternative mechanism. Irrespective of the primary mechanism for increased

483 DHA:EPA ratio in the present study with unfed larvae, the increasing expression of *Δ6fad* and
484 *elovl5* suggests that endogenous biosynthesis of DHA may be important during normal early
485 development in ABT larvae.

486

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493

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646

646 Figure legends

647 **Fig. 1.** Phylogenetic tree comparing ABT D6Fad with other Fad protein orthologues. The tree was
648 constructed using the Neighbour Joining method with MEGA4. The horizontal branch length is
649 proportional to amino acid substitution rate per site. The numbers represent the frequencies (%)
650 with which the tree topology presented was replicated after 10,000 iterations.

651
652 **Fig. 2.** Phylogenetic tree comparing ABT Elovl5 with other Elovl-like protein orthologues. The tree
653 was constructed using the Neighbour Joining method with MEGA4. The horizontal branch
654 length is proportional to amino acid substitution rate per site. The numbers represent the
655 frequencies (%) with which the tree topology presented was replicated after 10,000 iterations.

656
657 **Fig.3.** Functional characterization of the Atlantic bluefin tuna fatty acyl desaturase in transgenic
658 yeast (*Saccharomyces cerevisiae*) grown in the presence of $\Delta 6$ substrate 18:3n-3 (A), $\Delta 5$
659 substrate 20:4n-3 (B) and $\Delta 4$ substrate 22:5n-3 (C). Fatty acids were extracted from yeast
660 transformed with pYES2 vector containing the ORF of the fatty acyl desaturase cDNA as an
661 insert. The first four peaks in all panels are the main endogenous fatty acids of *S. cerevisiae*,
662 namely 16:0 (1), 16:1n-7 (2), 18:0 (3), and 18:1n-9 (4). Vertical axis, FID response; horizontal
663 axis, retention time.

664
665 **Fig.4.** Functional characterisation of Atlantic bluefin tuna fatty acyl elongase in transgenic
666 *Saccharomyces cerevisiae* grown in the presence of fatty acid substrates 18:4n-3 (panel A),
667 20:5n-3 (B), and 22:5n-3 (C). Fatty acids were extracted from yeast transformed with pYES2
668 vector containing the ORF of the fatty acyl elongase cDNA as an insert. Peaks 1-4 are as
669 described in legend to Figure 3. Peak 5 corresponds to 18:1n-7 arising from the elongation of
670 endogenous 16:1n-7 and peaks 6 correspond to 20:1n-9 and 20:1n-7, resulting from the
671 elongation of endogenous 18:1n-9 and 18:1n-7. In each panel the supplemented substrate fatty

acid is marked with an asterisk and the remaining named peaks are elongated products. Vertical axis, FID response; horizontal axis, retention time.

Fig.5. The effects of time after hatching (DPH) on the expression of $\Delta 6$ fatty acyl desaturase (*D6fad*) and fatty acyl elongase 5 (*elovl5*) mRNAs (upper panel) and the DHA:EPA ratio in total, polar and neutral lipids (lower panel) in unfed yolk sac larvae of Atlantic bluefin tuna. Columns within each lipid class with a different letter are significantly different (ANOVA, Tukey, $p < 0.05$). DHA, docosahexaenoic acid (22:6n-3); EPA, eicosapentaenoic acid (20:5n-3).

684 Table 1. Sequence, annealing temperature (T_m) and size of the fragment produced by the primer
685 pairs used for cloning, functional characterization (restriction sites for *SacI* and *XhoI* underlined),
686 and quantitative PCR (qPCR).

Aim	Transcript	Primer	Primer sequence	Fragment	T _m
ORF cloning	<i>Δ6fad</i>	CR-D6DES-F4	ATGGGAGGTGGAGGCCA	1134 bp	58°C
		CR-D6DES-R3	TCCGCTGAACCAAGTCGTTGA		
	<i>elovl5</i>	Tm-Elo5-F1	GGTTACACAGCCGCGTTCT	520 bp	58°C
		CR-Elo5-R2	AGTTCATAACGAACCACCAGAT		
		CR-Elo5-F1	GCTCTACAATCTGGGCCTC	612 bp	58°C
		Tm-Elo5-R1	GGAAAGCCATTCTGATGCTC		
3'RACE	<i>Δ6fad</i>	Tt-D6DES-F2 ¹	ACCGCCAACACCAGTACTTC	1032 bp	60°C
		Tt-D6DES-F3 ²	CCGTGCACTGTGTGAGAAAC	602 bp	60°C
	<i>elovl5</i>	Tt-Elo5-F2 ¹	GGGATGGCTGTACTTCCAAA	479 bp	60°C
		Tm-Elo5-F5 ²	CCAAATAGGTTACATGGTCACA CTC	464 bp	60°C
Functional characterization	<i>Δ6fad</i>	CR-D6DES-F4	ATGGGAGGTGGAGGCCA	1465 bp	58°C
		Tt-D6DES-R4	GAAGCAAAGATCATTCCCACA		
		TtD6DESVF	CCC <u>GAGCTCA</u> ATATGGGTGGTG GAGGCCAGC	1341 bp	50°C
		TtD6DESVR	CCG <u>CTCGAGT</u> CATTTATGAAGA TATGCATC		
	<i>elovl5</i>	TtElo5VF	CCC <u>GAGCTCA</u> AAAATGGAGACTT TCAATTATAAACTGAACA	885 bp	60°C
		TtElo5VR	CCG <u>CTCGAGT</u> CAATCCACCCGC AGTTTCT		
qPCR	<i>Δ6fad</i>	Tt-qD6DES-F	CCGTGCACTGTGTGAGAAAC	152 bp	60°C
		Tt-qD6DES-R	CAGTGTAAGCGATAAAATCAG CTG		
	<i>elovl5</i>	Tt-qElo5-F	CCACGCTAGCATGCTGAATA	236 bp	60°C
		Tt-qElo5-R	ATGGCCATATGACTGCACAC		
	<i>elf-1α</i>	Tt-qEF1-F	CCCCTGGACACAGAGACTTC	119 bp	60°C
		Tt-qEF1-R	GCCGTTCTTGGAGATAACCAG		
	<i>β-actin</i>	Tt-qBACT-F	ACCCACACAGTGCCCATCTA	155 bp	61°C
		Tt-qBACT-R	TCACGCACGATTTCCCTCT		

687 ¹Outer and ²Inner Primers used for 3' RACE with the FirstChoice RLM-RACE kit.

688

689

690 Table 2. Functional characterization of Atlantic bluefin tuna (*Thunnus thynnus* L.) fatty acyl
 691 desaturase (Fad) and fatty acyl elongase (Elovl) in yeast *Saccharomyces cerevisiae*.

FA substrate	Product	Conversion	Activity
<u>Fad</u>			
18:3n-3	18:4n-3	31.2	Δ6
18:2n-6	18:3n-6	19.7	Δ6
<u>Elovl</u>			
18:4n-3	20:4n-3	56.9	C18→20
	22:4n-3	19.3	C20→22
	24:4n-3	1.4	C22→24
	Total	77.6	
18:3n-6	20:3n-6	54.8	C18→20
	22:3n-6	22.4	C20→22
	24:3n-6	2.1	C22→24
	Total	79.3	
20:5n-3	22:5n-3	61.4	C20→22
	24:5n-3	12.5	C22→24
	Total	73.9	
20:4n-6	22:4n-6	60.0	C20→22
	24:4n-6	7.0	C22→24
	Total	67.0	
22:5n-3	24:5n-3	8.6	C22→24
22:4n-6	24:4n-6	3.3	C22→24

692 Conversion is expressed as a percentage of total fatty acid (FA) substrate converted to
 693 desaturated/elongated products.

694

694 Table 3. Dry mass (percentage), total lipid content (percentage live mass and dry mass) and lipid
695 class composition (percentage of total lipid) of Atlantic bluefin tuna (*Thunnus thynnus* L.) unfed
696 yolk sac larvae from 0 to 4 days post hatch (DPH).

	DPH0	DPH1	DPH2	DPH3	DPH4
Dry mass (%)	8.4 ± 1.8 ^{ab}	8.9 ± 0.3 ^a	9.8 ± 0.1 ^b	8.4 ± 0.3 ^a	6.3 ± 0.9 ^c
Total Lipid (% live mass)	2.4 ± 0.4 ^a	2.3 ± 0.2 ^a	2.4 ± 0.1 ^a	1.5 ± 0.1 ^b	1.3 ± 0.1 ^b
Total Lipid (% dry mass)	29.9 ± 2.3 ^a	25.9 ± 1.9 ^{ab}	22.8 ± 1.8 ^b	19.2 ± 0.3 ^b	19.3 ± 0.8 ^b
<u>Lipid Class</u>					
Phosphatidylcholine	10.4 ± 0.3 ^a	13.4 ± 0.8 ^{bc}	12.1 ± 1.4 ^{ab}	13.9 ± 1.1 ^{bc}	15.9 ± 0.8 ^c
Phosphatidylethanolamine	4.6 ± 0.2 ^a	7.4 ± 0.3 ^b	7.0 ± 0.5 ^b	10.0 ± 0.3 ^c	11.1 ± 0.3 ^d
Phosphatidylserine	2.1 ± 0.4 ^a	4.1 ± 0.4 ^b	3.7 ± 0.2 ^b	5.8 ± 0.2 ^c	7.7 ± 0.2 ^d
Phosphatidylinositol	2.6 ± 0.1	2.0 ± 1.0	2.6 ± 0.2	2.6 ± 0.1	2.6 ± 0.2
Phosphatidic acid/cardioplin	1.0 ± 0.3	1.8 ± 0.8	1.0 ± 0.4	1.5 ± 0.2	1.7 ± 0.1
Sphingomyelin	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.7 ± 0.2
Lyso-Phosphatidylcholine	0.7 ± 0.1 ^a	0.9 ± 0.2 ^a	0.5 ± 0.1 ^b	1.1 ± 0.2 ^{ab}	0.9 ± 0.3 ^{ab}
Total polar	22.6 ± 1.3 ^a	30.7 ± 1.5 ^b	27.8 ± 1.6 ^b	35.9 ± 1.5 ^c	40.8 ± 2.0 ^d
Total neutral	77.4 ± 1.2 ^a	69.2 ± 1.5 ^b	72.2 ± 1.6 ^b	64.1 ± 1.5 ^c	59.1 ± 2.0 ^d
Cholesterol	11.8 ± 0.8 ^a	15.2 ± 0.2 ^b	16.1 ± 1.6 ^b	21.2 ± 0.9 ^c	22.2 ± 0.8 ^c
Triacylglycerol (TAG)	23.4 ± 1.5 ^a	18.9 ± 0.7 ^b	19.3 ± 2.2 ^b	9.0 ± 1.2 ^c	7.3 ± 0.3 ^c
Steryl/wax ester	37.2 ± 0.5 ^a	29.7 ± 1.2 ^b	31.6 ± 1.1 ^b	29.1 ± 1.0 ^b	23.5 ± 0.6 ^c
Free fatty acid	4.7 ± 0.5	5.3 ± 0.9	4.8 ± 1.3	4.7 ± 0.9	6.0 ± 0.5
TAG:Cholesterol	1.9 ± 0.2 ^a	1.2 ± 0.0 ^b	1.2 ± 0.2 ^b	0.4 ± 0.1 ^c	0.3 ± 0.0 ^c

697 Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Values bearing different
698 superscript letter are significantly different (P<0.05).

699

699 Table 4. Fatty acid composition (percentage of total fatty acids) of total lipid from unfed Atlantic
700 bluefin tuna (*Thunnus thynnus* L.) yolk-sac larvae from 0 to 4 days post hatch (DPH).

Fatty acid	DPH0				DPH1				DPH2				DPH3				DPH4			
14:0	2.5	±	0.1	^a	2.2	±	0.2	^{ab}	2.0	±	0.4	^{abc}	1.6	±	0.1	^{bc}	1.5	±	0.2	^c
16:0	16.8	±	0.8		18.8	±	1.0		19.1	±	0.8		18.7	±	0.8		19.3	±	1.4	
18:0	5.6	±	0.5	^c	6.9	±	0.5	^{bc}	7.4	±	0.3	^{bc}	9.0	±	0.6	^{ab}	10.2	±	1.5	^a
Total saturated ¹	25.6	±	1.4	^b	28.6	±	1.7	^{ab}	29.2	±	1.2	^{ab}	30.1	±	1.3	^{ab}	31.9	±	2.8	^a
16:1n-7	4.9	±	0.5	^a	4.3	±	0.2	^a	4.1	±	0.2	^a	3.2	±	0.2	^b	2.9	±	0.4	^b
18:1n-9	18.1	±	1.4	^a	17.8	±	0.6	^b	17.9	±	0.4	^b	16.2	±	0.4	^b	16.1	±	1.3	^b
18:1n-7	3.9	±	0.8	^a	3.1	±	0.1	^{ab}	3.1	±	0.1	^{ab}	2.9	±	0.2	^{ab}	2.6	±	0.2	^b
20:1n-9	3.3	±	0.4		3.4	±	0.1		2.9	±	1.3		3.1	±	0.1		3.0	±	0.1	
22:1	2.1	±	0.3		2.1	±	0.1		2.0	±	0.2		1.7	±	0.1		1.6	±	0.2	
24:1n-9	0.5	±	0.2		0.4	±	0.1		0.4	±	0.2		0.7	±	0.5		0.6	±	0.4	
Total monoenes ²	33.0	±	0.7	^a	31.2	±	0.8	^a	30.6	±	1.8	^{ab}	28.0	±	1.1	^b	27.0	±	2.3	^b
18:2n-6	2.0	±	0.2	^a	1.6	±	0.1	^b	1.5	±	0.1	^b	1.5	±	0.1	^b	1.3	±	0.0	^b
20:4n-6	1.2	±	0.1	^c	1.5	±	0.0	^{bc}	1.6	±	0.1	^b	2.0	±	0.2	^a	2.2	±	0.2	^a
Total n-6PUFA ³	4.2	±	0.1		4.1	±	0.2		4.2	±	0.2		4.6	±	0.3		4.6	±	0.2	
18:3n-3	0.8	±	0.1	^a	0.7	±	0.1	^{ab}	0.6	±	0.1	^{ab}	0.5	±	0.1	^b	0.5	±	0.1	^b
18:4n-3	1.1	±	0.1	^a	1.0	±	0.0	^{ab}	0.9	±	0.1	^{ab}	0.7	±	0.1	^{bc}	0.5	±	0.2	^c
20:4n-3	0.7	±	0.1	^a	0.6	±	0.0	^a	0.6	±	0.0	^a	0.5	±	0.0	^{ab}	0.4	±	0.1	^b
20:5n-3	6.4	±	0.3	^a	6.2	±	0.2	^a	5.8	±	0.2	^{ab}	5.1	±	0.1	^{bc}	4.7	±	0.4	^c
22:5n-3	1.7	±	0.1		1.7	±	0.1		1.6	±	0.1		1.6	±	0.1		1.5	±	0.2	
22:6n-3	19.3	±	0.8	^c	20.6	±	0.5	^{bc}	20.6	±	0.9	^{bc}	22.7	±	0.8	^{ab}	23.0	±	0.9	^a
Total n-3PUFA ⁴	30.2	±	1.3		30.8	±	0.8		30.3	±	0.8		31.2	±	0.8		30.8	±	1.4	
DHA:EPA	3.0	±	0.1	^c	3.3	±	0.0	^{bc}	3.6	±	0.3	^b	4.4	±	0.1	^a	5.0	±	0.5	^a
Total PUFA	34.4	±	1.3		34.9	±	1.0		34.5	±	0.9		35.8	±	1.0		35.4	±	1.4	

701 Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Values bearing different
702 superscript letters are significantly different (P<0.05). ¹, Totals include 15:0, 20:0 and 22:0; ²,
703 Totals include 16:1n-9 and 20:1n-7; ³, Totals include 18:3n-6, 20:2n-6, 20:3n-6 and 22:5n-6; ⁴,
704 Totals include 20:3n-3; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA,
705 polyunsaturated fatty acid.

706

707

Table 5. Fatty acid composition (percentage of total fatty acids) of total polar lipids from unfed Atlantic bluefin tuna (*Thunnus thynnus* L.) yolk-sac larvae from 0 to 4 days post hatch (DPH).

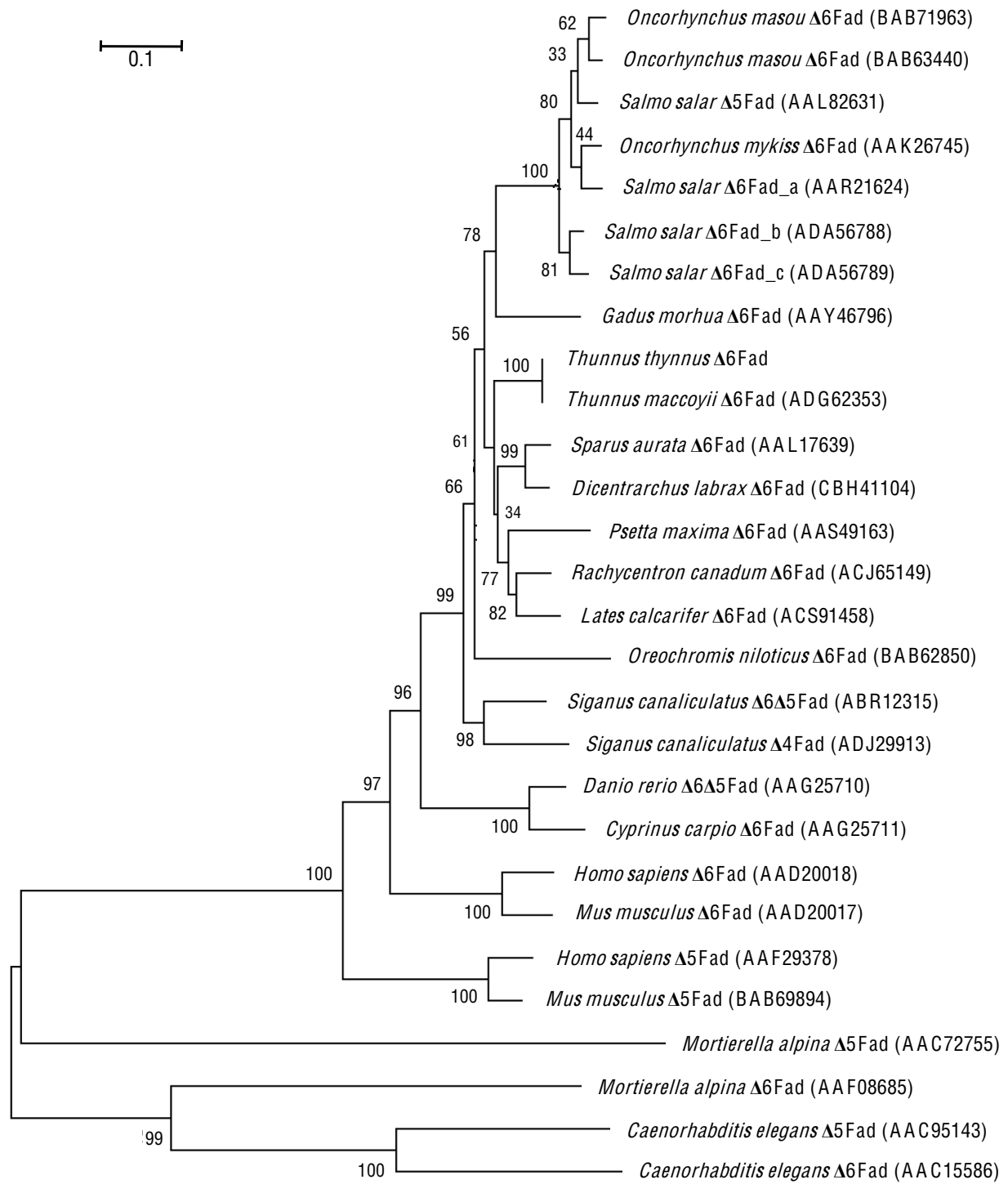
Fatty acid	DPH0	DPH1	DPH2	DPH3	DPH4
14:0	1.6 ± 0.2	1.3 ± 0.2	1.3 ± 0.3	1.2 ± 0.5	1.3 ± 0.1
16:0	21.1 ± 0.6	22.5 ± 0.2	21.9 ± 2.5	19.5 ± 1.6	19.7 ± 0.0
18:0	11.7 ± 1.6 ^b	12.6 ± 0.6 ^{ab}	14.1 ± 0.8 ^a	11.9 ± 0.5 ^{ab}	14.0 ± 0.1 ^a
Total saturated ¹	35.8 ± 1.8 ^{ab}	37.3 ± 0.9 ^{ab}	38.8 ± 2.9 ^a	33.5 ± 0.2 ^b	36.3 ± 0.1 ^{ab}
16:1n-7	3.6 ± 1.3	2.6 ± 0.4	2.7 ± 0.8	2.2 ± 0.4	2.6 ± 0.0
18:1n-9	10.0 ± 1.4	9.3 ± 0.0	9.1 ± 0.3	9.1 ± 0.4	9.5 ± 0.1
18:1n-7	2.0 ± 0.1	2.3 ± 0.2	2.2 ± 0.2	2.1 ± 0.1	2.1 ± 0.4
20:1n-9	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.0	1.4 ± 0.1
22:1	1.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.0	0.7 ± 0.1	0.4 ± 0.0
24:1n-9	1.1 ± 0.4	1.5 ± 1.1	1.1 ± 0.5	1.9 ± 1.6	1.3 ± 0.4
Total monoenes ²	19.4 ± 3.0	17.7 ± 1.2	17.2 ± 1.2	17.3 ± 1.5	17.3 ± 0.6
18:2n-6	1.2 ± 0.4	1.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.0	1.1 ± 0.0
20:4n-6	2.3 ± 0.4	2.1 ± 0.3	2.1 ± 0.1	2.4 ± 0.2	2.6 ± 0.0
22:5n-6	0.5 ± 0.1 ^b	0.6 ± 0.1 ^{ab}	0.6 ± 0.0 ^{ab}	0.7 ± 0.0 ^a	0.7 ± 0.0 ^a
Total n-6PUFA ³	4.4 ± 0.1 ^{bc}	4.3 ± 0.1 ^{cd}	4.1 ± 0.0 ^d	4.6 ± 0.0 ^{ab}	4.8 ± 0.1 ^a
18:3n-3	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
18:4n-3	0.4 ± 0.1 ^a	0.3 ± 0.0 ^{ab}	0.2 ± 0.0 ^b	0.2 ± 0.0 ^b	0.2 ± 0.0 ^b
20:4n-3	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.0
20:5n-3	5.8 ± 0.8 ^a	5.0 ± 0.7 ^{ab}	4.0 ± 0.1 ^b	4.3 ± 0.5 ^b	3.7 ± 0.1 ^b
22:5n-3	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.0	1.3 ± 0.0
22:6n-3	26.8 ± 4.1	27.8 ± 4.0	27.2 ± 1.1	30.0 ± 2.5	29.1 ± 1.0
Total n-3PUFA ⁴	34.7 ± 5.1	35.1 ± 4.9	33.4 ± 1.2	36.4 ± 2.9	34.8 ± 1.1
DHA:EPA	4.7 ± 0.1^e	5.5 ± 0.0^d	6.8 ± 0.0^c	7.1 ± 0.2^b	7.9 ± 0.0^a
Total PUFA	39.1 ± 4.9	39.3 ± 4.9	37.5 ± 1.2	41.0 ± 2.9	38.5 ± 1.1

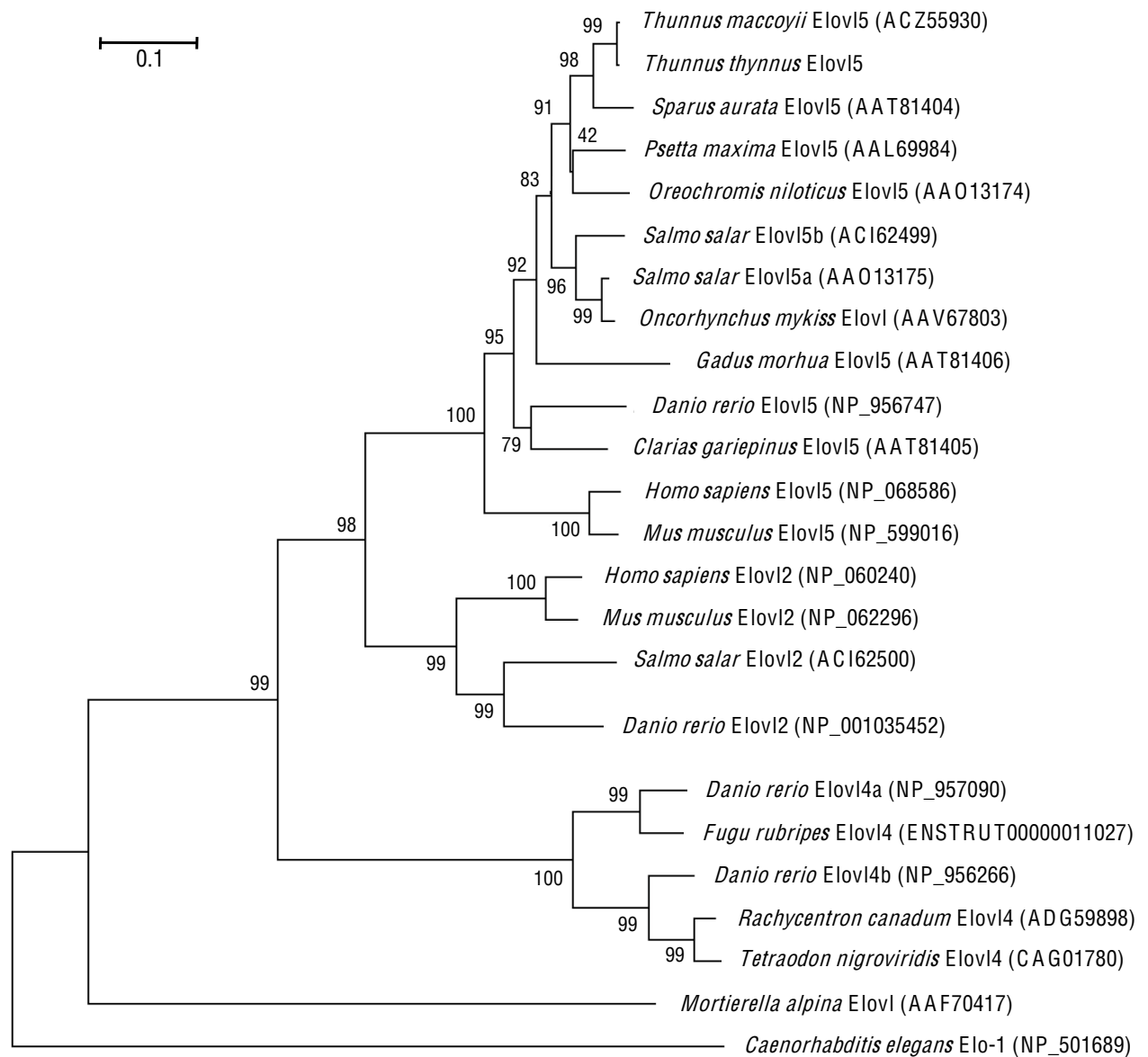
Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Values bearing different superscript letters are significantly different (P<0.05). ¹, Totals include 15:0, 20:0 and 22:0; ², Totals include 16:1n-9 and 20:1n-7; ³, Totals include 18:3n-6, 20:2n-6, 20:3n-6 and 22:5n-6; ⁴, Totals include 20:3n-3; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid.

Table 6. Fatty acid composition (percentage of total fatty acids) of total neutral lipids from unfed Atlantic bluefin tuna (*Thunnus thynnus* L.) yolk-sac larvae from 0 to 4 days post hatch (DPH).

Fatty acid	DPH0			DPH1			DPH2			DPH3			DPH4		
14:0	3.3	±	0.1	3.3	±	0.1	3.2	±	0.3	3.3	±	0.2	3.5	±	0.2
16:0	14.5	±	0.8	15.5	±	0.6	15.5	±	0.1	15.0	±	1.2	14.3	±	0.4
18:0	4.2	±	0.4 ^c	5.6	±	0.1 ^a	5.7	±	0.3 ^a	5.8	±	0.1 ^a	4.9	±	0.0 ^b
Total saturated ¹	22.8	±	1.6	25.1	±	0.6	25.3	±	0.3	24.8	±	1.4	23.6	±	0.8
16:1n-7	6.1	±	0.1 ^{ab}	6.1	±	0.1 ^{ab}	6.2	±	0.1 ^{ab}	5.8	±	0.0 ^b	6.5	±	0.4 ^a
18:1n-9	22.7	±	1.3 ^{ab}	23.6	±	0.5 ^a	24.6	±	0.5 ^a	23.3	±	0.5 ^{ab}	21.5	±	0.4 ^b
18:1n-7	3.1	±	0.0	3.5	±	0.5	3.2	±	0.3	3.2	±	0.1	3.6	±	0.1
20:1n-9	3.9	±	0.1 ^b	4.5	±	0.1 ^a	4.7	±	0.1 ^a	4.7	±	0.1 ^a	4.1	±	0.1 ^b
22:1	2.7	±	0.1 ^b	3.1	±	0.0 ^a	3.0	±	0.0 ^a	3.1	±	0.0 ^a	2.8	±	0.1 ^b
24:1n-9	1.0	±	0.8	0.8	±	0.4	0.8	±	0.2	0.7	±	0.1	0.6	±	0.1
Total monoenes ²	39.7	±	0.6 ^b	41.7	±	0.9 ^a	42.6	±	0.5 ^a	41.0	±	0.6 ^{ab}	39.4	±	0.3 ^b
18:2n-6	2.0	±	0.1	1.9	±	0.0	2.1	±	0.1	2.0	±	0.1	2.3	±	0.3
20:4n-6	0.6	±	0.0 ^c	0.8	±	0.0 ^b	0.8	±	0.1 ^b	1.0	±	0.0 ^a	1.0	±	0.1 ^a
Total n-6PUFA ³	3.4	±	0.0 ^b	3.6	±	0.1 ^b	3.8	±	0.4 ^b	4.0	±	0.1 ^b	5.0	±	0.6 ^a
18:3n-3	0.9	±	0.0	0.9	±	0.0	1.0	±	0.1	0.9	±	0.1	0.9	±	0.0
18:4n-3	1.5	±	0.1 ^a	1.3	±	0.0 ^b	1.2	±	0.0 ^b	1.3	±	0.1 ^b	1.3	±	0.0 ^b
20:4n-3	1.0	±	0.1	0.8	±	0.0	0.8	±	0.1	0.8	±	0.0	0.8	±	0.1
20:5n-3	6.0	±	0.7 ^a	5.4	±	0.1 ^{ab}	5.0	±	0.0 ^b	5.3	±	0.2 ^{ab}	5.9	±	0.3 ^{ab}
22:5n-3	2.0	±	0.2 ^a	1.8	±	0.1 ^{ab}	1.6	±	0.0 ^b	1.7	±	0.1 ^{ab}	1.8	±	0.1 ^{ab}
22:6n-3	15.2	±	1.2 ^a	12.5	±	0.4 ^b	11.8	±	0.0 ^b	13.6	±	1.3 ^{ab}	14.0	±	0.5 ^{ab}
Total n-3PUFA ⁴	26.5	±	2.3 ^a	22.8	±	0.7 ^{ab}	21.4	±	0.1 ^b	23.6	±	1.8 ^{ab}	24.7	±	0.9 ^{ab}
DHA:EPA	2.5	±	0.1	2.3	±	0.1	2.4	±	0.0	2.6	±	0.1	2.4	±	0.0
Total PUFA	29.9	±	2.3 ^a	26.4	±	0.6 ^{ab}	25.2	±	0.5 ^b	27.5	±	1.8 ^{ab}	29.7	±	0.4 ^a

Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. Values bearing different superscript letters are significantly different (P<0.05). ¹, Totals include 15:0, 20:0 and 22:0; ², Totals include 16:1n-9 and 20:1n-7; ³, Totals include 18:3n-6, 20:2n-6, 20:3n-6 and 22:5n-6; ⁴, Totals include 20:3n-3; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid.

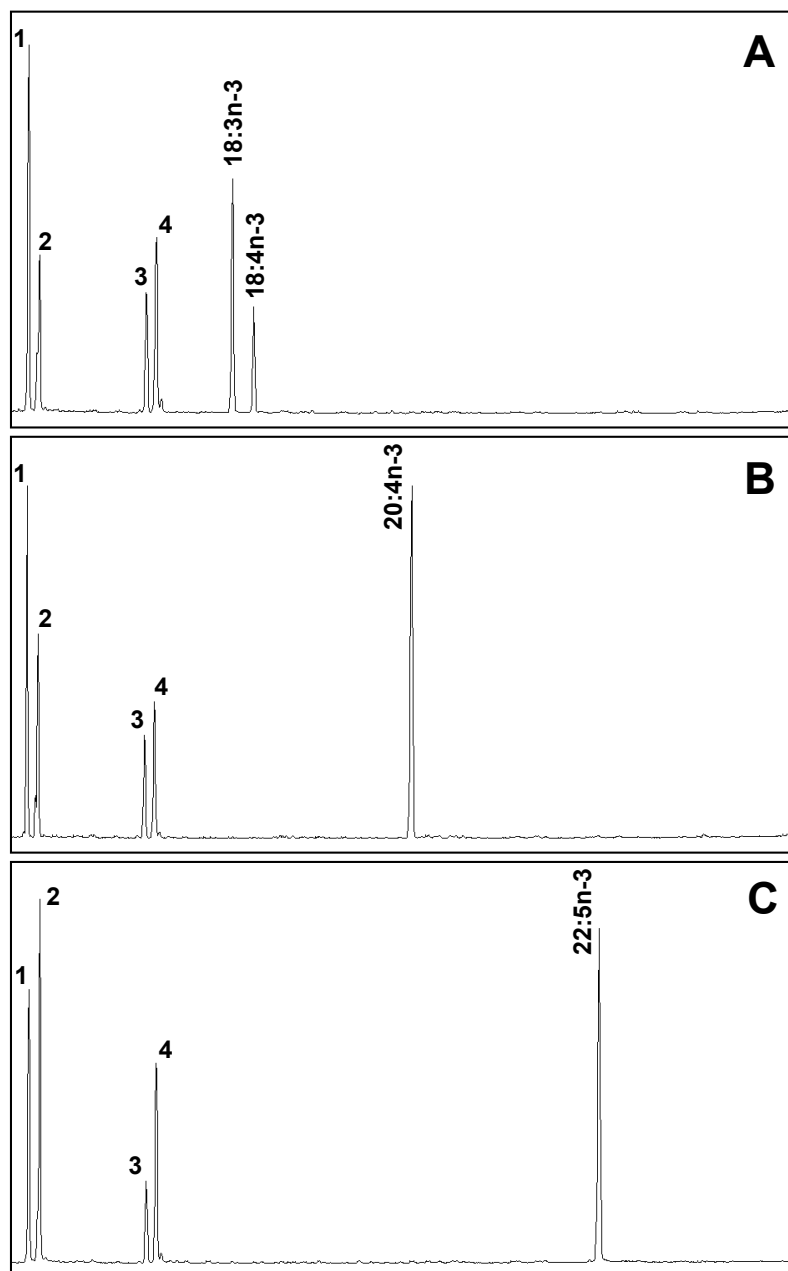




728 Fig.3.

730

732



734 Fig.4.

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