

Highly Unsaturated Fatty Acid Synthesis in Vertebrates: New Insights with the Cloning and Characterisation of a $\Delta 6$ Desaturase of Atlantic Salmon

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Abbreviations: FO, fish oil; HUFA, highly unsaturated fatty acids (carbon chain length $\geq C_{20}$ with ≥ 3 double bonds); ORF, open reading frame; Q-PCR, quantitative (real-time) polymerase chain reaction; RACE, rapid amplification of cDNA ends; UTR, untranslated region; VO, vegetable oil.

1 **ABSTRACT:** Fish are an important source of the n-3 highly unsaturated fatty acids (HUFA),
2 eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids that are crucial to the health of higher
3 vertebrates. The synthesis of HUFA involves enzyme-mediated desaturation, and a $\Delta 5$ fatty acyl
4 desaturase cDNA has been cloned from Atlantic salmon (*Salmo salar*) and functionally
5 characterized previously. Here we report cloning and functional characterisation of a $\Delta 6$ fatty acyl
6 desaturase of Atlantic salmon, and describe its genomic structure, tissue expression and nutritional
7 regulation. A salmon genomic library was screened with a salmon $\Delta 5$ desaturase cDNA and
8 positive recombinant phage isolated and subcloned. The full-length cDNA for the putative fatty
9 acyl desaturase was shown to comprise 2106bp containing an ORF of 1365 bp specifying a protein
10 of 454 amino acids (GenBank accession no. AY458652). The protein sequence included three
11 histidine boxes, two transmembrane regions, and an N-terminal cytochrome b₅ domain containing
12 the haem-binding motif HPGG, all of which are characteristic of microsomal fatty acid desaturases.
13 Functional expression showed that this gene possessed predominantly $\Delta 6$ desaturase activity.
14 Screening and sequence analysis of the genomic DNA of a single fish revealed that the $\Delta 6$
15 desaturase gene comprised 13 exons in 7965 bp of genomic DNA. Quantitative real time PCR assay
16 of gene expression in Atlantic salmon showed that both $\Delta 6$ and $\Delta 5$ fatty acyl desaturase genes, and
17 a fatty acyl elongase gene, were highly expressed in intestine, liver and brain, and less so in kidney,
18 heart, gill, adipose tissue, muscle and spleen. Furthermore, expression of both $\Delta 6$ and $\Delta 5$ fatty acyl
19 desaturase genes in intestine, liver, red muscle and adipose tissue was higher in salmon fed a diet
20 containing vegetable oil than in fish fed a diet containing fish oil.

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23 Highly unsaturated fatty acids (HUFA), arachidonate (AA; 20:4n-6), eicosapentaenoate (EPA;
24 20:5n-3) and docosahexaenoate (DHA; 22:6n-3), are crucial to the health and normal development
25 of higher vertebrates (1-3). Fish are the most important source of n-3 HUFA for humans, but, with
26 fisheries in decline, an increasing proportion of fish is being provided by rapidly expanding
27 aquaculture (4). Paradoxically, aquaculture is itself dependent upon fisheries for the provision of
28 fishmeals and oils traditionally used in the feed formulations (5). Their use ensured the high
29 nutritional quality of farmed fish through the high levels of n-3 HUFA that fish oil and meal
30 provided. However, feed-grade fisheries have reached sustainable limits. Along with concern over
31 organic contaminants in fish oil, this has dictated that alternatives to fish oil must be found if
32 aquaculture is to continue to expand and supply more of the global demand for fish (6).

33 The only practical, sustainable alternative to fish oils is vegetable oils, which are rich in C₁₈
34 PUFA but devoid of the n-3 HUFA abundant in fish oils (7). Consequently, tissue fatty acid
35 compositions in fish fed vegetable oils are characterised by increased levels of C₁₈ PUFA and
36 decreased levels of n-3 HUFA, which may reduce their nutritional value to the human consumer
37 (8). The extent to which fish can convert C₁₈ PUFA to HUFA varies, associated with their
38 complement of fatty acid desaturase enzymes. Although Atlantic salmon (*Salmo salar* L.) are
39 capable of producing DHA from 18:3n-3, and so express the necessary desaturase activities, the
40 production is insufficient to maintain n-3 HUFA in fish fed vegetable oils at levels found in fish fed
41 fish oils (9-11). Our primary hypothesis is that understanding the molecular basis of HUFA
42 biosynthesis and its regulation in fish will enable us to optimise the activity of the pathway to
43 ensure efficient and effective use of vegetable oils in aquaculture whilst maintaining the nutritional
44 quality of farmed fish for the consumer.

45 $\Delta 5$ and $\Delta 6$ fatty acyl desaturases and elongases are critical enzymes in the pathways for the
46 biosynthesis of HUFA. In recent years, significant progress has been made in characterizing fatty
47 acid desaturases involved in HUFA synthesis (12). Full-length cDNAs for $\Delta 6$ desaturases have been
48 isolated from the filamentous fungus *Mortierella alpina* (13), the nematode *Caenorhabditis elegans*
49 (14), rat (15), mouse and human (16). Fatty acid $\Delta 5$ desaturase genes have been isolated from *M.*
50 *alpina* (17) *C. elegans* (18,19) and human (20,21). Moreover, we have reported isolation of a cDNA
51 of zebrafish (*Danio rerio*, GenBank accession no. AF309556), with high similarity to mammalian
52 $\Delta 6$ desaturase genes. Functional analysis by heterologous expression in the yeast *Saccharomyces*
53 *cerevisiae* indicated that the zebrafish gene was unique in that the cDNA encoded an enzyme
54 having both $\Delta 6$ and $\Delta 5$ desaturase activities (22). Putative fatty acid desaturase cDNAs have now
55 also been isolated and cloned from rainbow trout (*Oncorhynchus mykiss*, GenBank accession no.
56 AF301910) (23) and gilthead seabream (*Sparus aurata*, GenBank accession no. AY055749) (24).
57 Functional analysis showed that these two desaturase genes, along with cDNAs recently cloned

58 from common carp (*Cyprinus carpio*, GenBank accession no. AF309557) and turbot (*Psetta*
59 *maximus*, GenBank accession no. AF301910) encoded basically unfunctional $\Delta 6$ fatty acid
60 desaturase enzymes responsible for the first and possibly rate-limiting step in the biosynthesis of
61 HUFA from 18:3n-3 and 18:2n-6 (25). Recently, a full-length cDNA for a desaturase containing
62 1365bp encoding 454 amino acid residues has been cloned from Atlantic salmon (GenBank
63 accession no. AF478472). Functional analysis showed that this gene was primarily a $\Delta 5$ desaturase
64 with virtually no $\Delta 6$ activity (26). Therefore, it was presumed that other fatty acid desaturase genes
65 should be present in Atlantic salmon.

66 The objectives of the study described here were first to clone and functionally characterize a $\Delta 6$
67 desaturase gene of Atlantic salmon, second to describe its genomic structure and third to place it in
68 evolutionary and physiological contexts. Therefore we detail the exon/intron organization of a
69 salmon $\Delta 6$ desaturase gene, describe the expression profile of both $\Delta 6$ and $\Delta 5$ fatty acyl desaturase
70 and fatty acyl elongase genes in various tissues, and demonstrate nutritional regulation of the fatty
71 acyl desaturase genes.

72 **MATERIALS AND METHODS**

73 *Putative desaturase cloning and its genomic organization.* An Atlantic salmon genomic DNA
74 library constructed previously with the lambda FIX II/Xho I partial fill-in vector kit (Stratagene, La
75 Jolla, CA, USA) was probed with a full-length salmon $\Delta 5$ fatty acyl desaturase cDNA (GenBank
76 accession no. AF478472). Inserts of positive recombinant phages were isolated and subcloned into
77 the pBluescript KS II vector for sequencing (Stratagene, La Jolla, CA, USA). The full putative
78 desaturase genomic nucleotide sequence was assembled using BioEdit version 5.0.6 (Tom Hall,
79 Department of Microbiology, North Carolina State University, USA).

80 Total RNA was extracted from liver tissue of Atlantic salmon fed a standard extruded diet
81 based on fish meal and fish oil using TRIzol® reagent (GibcoBRL, NY, U.S.A.). 3' RACE cDNA
82 was synthesized using MMLV reverse transcriptase (Promega, Madison, WI, U.S.A) primed by the
83 oligonucleotide, T7PolyT, 5'-TACGACTCACTATAGGGCGTGCAGTTTT TTTTTTTT-3'. The
84 specific sense primer, D6P31, 5'-CAGGGGTGGGCCCGGTGGAGGGCTA-3' was designed for
85 3'RACE PCR based on the genomic sequence described above. This was used in conjunction with
86 T7PolyT primer for the RACE PCR isolation of the salmon desaturase cDNA fragment predicted to
87 contain the 3' UTR. PCR amplification was performed using the Hotstar Taq master kit (Qiagen,
88 Crowley, West Sussex, UK) and involved an initial denaturation step at 95 °C for 15 min, followed

89 by 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C
90 for 3 min. Final extension at 72 °C was for 10 min. 5'-RACE-cDNA was synthesized using the
91 SMART™ RACE cDNA amplification kit (Clontech, NJ, U.S.A). The primer, SD6PPR3, 5'-
92 GTCGCATTCCATCCCAATCC-3' was designed according to the 3'RACE PCR fragment
93 sequence. This was used in conjunction with universal primer mix (UPM): long 5'-
94 CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' and short 5'-
95 CTAATACGACTCACTATAGGGC-3' to perform 5' RACE PCR using high fidelity DNA
96 polymerase (Roche Diagnostics Ltd., Lewes, East Sussex, UK). Amplification involved an initial
97 step at 95 °C for 1 min and 70°C for 3 min, and 4 cycles of denaturation at 95 °C for 15 s, annealing
98 at 62 °C for 1 min and extension at 72 °C for 1min and 30 s, followed by 27 cycles of denaturation
99 at 95 °C for 15s, annealing at 56 °C for 30s and extension at 72 °C for 1min and 30 s. The final
100 extension at 72 °C was for 10 min.

101 All RACE PCR products were cloned into the pBluescript KS II⁺ vector for sequencing. The
102 3' and 5' RACE PCR fragment sequences were aligned to assemble the full nucleotide sequence of
103 the putative desaturase cDNA using BioEdit version 5.0.6. The assembled putative fatty acyl
104 desaturase cDNA sequence and its genomic DNA sequence were aligned to assign consensus donor
105 and acceptor splice recognition sequences.

106

107 *Heterologous expression of desaturase ORFs in Saccharomyces cerevisiae.* PCR amplification
108 was carried out to clone the salmon putative desaturase cDNA ORF. Sense primer, D6RF2, 5'-
109 ATGGGGGGCGGAGGCCAGCAGAATGATTCAG -3', and antisense primer, D6RR1, 5'-
110 ATGCGATGGATTAAATCCCG -3' (located in the 3'UTR) were designed for first round PCR
111 after comparing nucleotide sequences of this putative cDNA and the $\Delta 5$ desaturase cDNA.
112 Expression primers were designed for a second round of PCR. The sense primer, SalpYESFOR, 5'-
113 CCCAAGCTTACTATGGGGGGCGGAGGCC-3' contains a *HindIII* site (underlined) and
114 antisense primer, SalPYESREV2, 5'- CCGCTCGAGTCATTTATGGAGATATGCAT-3' contains
115 an *XhoI* site (underlined). PCR was performed using high fidelity DNA polymerase (Roche
116 Diagnostics Ltd., Lewes, East Sussex, UK) following the manufacturer's instructions.
117 Amplification involved an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of
118 denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min and 30 s
119 followed by a final extension at 72 °C for 10 min.

120 Following PCR, the DNA fragments were restricted with the appropriate enzymes, *HindIII* and
121 *XhoI*, and ligated into the similarly digested yeast expression vector pYES2 (Invitrogen Ltd,
122 Paisley, UK). Ligation products were then used to transform Top10F' *E. coli* competent cells
123 (Invitrogen Ltd, Paisley, UK) which were screened for the presence of recombinants.
124 Transformation of the yeast *S. cerevisiae* (strain InvSc1) with the recombinant plasmids was carried
125 out using the S.c.EasyComp Transformation Kit (Invitrogen Ltd, Paisley, UK). Selection of yeast
126 containing the desaturase/pYES2 constructs was on *S. cerevisiae* minimal medium (SCMM) minus
127 uracil. Culture of the recombinant yeast was carried out in SCMM^{-uracil} broth as described
128 previously (22), using galactose induction of gene expression. Each culture was supplemented with
129 one of the following PUFA substrates; α -linolenic acid (18:3n-3), linoleic acid (18:2n-6),
130 eicosatetraenoic acid (20:4n-3), dihomo- γ -linoleic acid (20:3n-6), docosapentaenoic acid (22:5n-3)
131 and docosatetraenoic acid (22:4n-6). PUFA were to added to the yeasy cultures at concentrations of
132 0.5 mM (C₁₈), 0.75 mM (C₂₀) and 1 mM (C₂₂) as uptake efficiency decreases with increasing chain
133 length. Yeast cells were harvested, washed, dried, and lipid extracted by homogenisation in
134 chloroform/methanol (2:1, by vol.) containing 0.01% butylated hydroxytoluene (BHT) as
135 antioxidant as described previously (22). Fatty acid methyl esters (FAME) were prepared,
136 extracted, purified by thin layer chromatography (TLC), and analysed by gas chromatography (GC),
137 all as described previously (22). The proportion of substrate fatty acid converted to the longer chain
138 fatty acid product was calculated from the gas chromatograms as $100 \times [\text{product area}/(\text{product area}$
139 $+ \text{substrate area})]$. Unequivocal confirmation of fatty acid products was obtained by GC-mass
140 spectrometry of the picoliny derivatives as described in detail previously (22).

141
142 *Salmon tissue RNA extraction and quantitative real time PCR (Q-PCR)*. Tissue expression profiles
143 and effects of diet were investigated in Atlantic salmon that had been fed one of two diets from first
144 feeding. The diets consisted of a control in which fish oil (FO) was the only added oil and an
145 experimental diet in which 75% of the FO was replaced by a vegetable oil blend (VO) containing
146 rapeseed, palm and linseed oils in a 3.7 : 2 : 1 ratio. Both diets were fishmeal based and contained
147 48% protein, 26% lipid, 7% moisture and 8% ash as determined by proximate analyses. The fatty
148 acid compositions of the diets (6 mm pellet) are given in Table 1. The diets were prepared by the
149 Nutreco Aquaculture Research Centre, Stavanger, Norway and formulated to satisfy the nutritional
150 requirements of salmonid fish (27).

151 Fish were sampled in November 2003, six months after seawater transfer, following 18 months
152 on the diets, at which point the weights of the fish fed the FO and VO diets were $1250.0 \pm 84.9\text{g}$
153 and $1280.0 \pm 79.4\text{g}$, respectively. Eight fish per dietary treatment were sampled and liver, brain,
154 heart, kidney, gill, intestine (pyloric caeca), spleen, white and red muscle and adipose tissue were

155 collected, frozen immediately in liquid nitrogen and subsequently stored at -80°C before extraction.
156 Total RNA extraction was performed as described above. Five μg of total RNA was reverse
157 transcribed into cDNA using M-MLV reverse transcriptase first strand cDNA synthesis kit
158 (Promega UK, Southampton, UK). Gene expression of the fatty acyl $\Delta 6$ and $\Delta 5$ desaturase, and
159 fatty acyl elongase genes in tissue from individual salmon fed the different diets was studied by
160 quantitative RT-PCR (Q-PCR). β -Actin was used for normalization of mRNA levels. The PCR
161 primers were designed according to $\Delta 6$ desaturase (accession no. AY458652), and the published $\Delta 5$
162 desaturase (accession no. AF478472), elongase (accession no. AY170327) and β -actin (accession
163 no. AF012125) cDNA sequences. For the $\Delta 6$ desaturase, the forward primer was 5'-
164 CCCAGACGTTTGTGTCAG-3', and the reverse primer was 5'-
165 CCTGGATTGTTGCTTTGGAT-3'. For the $\Delta 5$ desaturase, the forward primer was 5'-
166 GTGAATGGGGATCCATAGCA-3', and the reverse primer was 5'-
167 AAACGAACGGACAACCAGA-3'. For the elongase, the forward and reverse primers were 5'-
168 TGATTTGTGTTCCAAATGGC-3' and 5'-CTCATGACGGGAACCT CAAT-3', respectively. For
169 β -actin, 5'-ACATCAAGGAGAAGCTGTGC-3' and 5'-GACAACGGAACCTCTCGTTA-3' were
170 the forward and reverse primers, respectively. PCR products sizes were 181,192, 219 and 141bp,
171 respectively. The linearised plasmid DNA containing the target sequence for each gene was
172 quantified to generate a standard curve of known copy number. Amplification of cDNA samples
173 and DNA standards was carried out using SYBR Green PCR Kit (Qiagen, Crawley, West Sussex,
174 UK) and the following conditions: 15 min denaturation at 95°C , 45 cycles of 15 s at 94°C , 15 s at
175 55°C and 30 s at 72°C . This was followed by product melt to confirm single PCR products.
176 Thermal cycling and fluorescence detection were conducted in a Rotor-Gene 3000 system (Corbett
177 Research, Cambridge, UK). The copy numbers of the specific genes in the sample, normalised to
178 total RNA, was used to compare expression levels between different tissues, and the ratios of copy
179 numbers between the target genes and β -actin were calculated and used to compare the gene
180 expression levels in fish fed the two diets.

181 *Sequence analysis.* Nucleotide sequences were determined by standard dye terminator chemistry
182 using a Perkin Elmer ABI-377 DNA sequencer following the manufacturer's protocols (Perkin
183 Elmer, Applied Biosystems). Deduced amino acid sequences of desaturases from various species
184 were aligned using ClustalX and sequence phylogenies were predicted using the Neighbour Joining
185 method (28). Confidence in the resulting phylogenetic tree branch topology was measured by
186 bootstrapping through 1000 iterations.

187 *Materials.* Eicosatetraenoic (20:4n-3), docosapentaenoic (22:5n-3) and docosatetraenoic (22:4n-6)
188 acids (all > 98-99% pure) were purchased from Cayman Chemical Co., Ann Arbor, U.S.A.
189 Linoleic (18:2n-6), α -linolenic (18:3n-3), eicosatrienoic (20:3n-6) acids (all >99% pure), BHT,
190 1,1'-carbonyldiimidazole, 2,2-dimethoxypropane, fatty acid-free BSA, galactose, 3-
191 (hydroxymethyl) pyridine, HBSS, nitrogen base, raffinose, tergitol NP-40 and uracil dropout
192 medium were obtained from Sigma Chemical Co. Ltd., Dorset, UK. TLC (20 x 20 cm x 0.25 mm)
193 plates pre-coated with silica gel 60 (without fluorescent indicator) were purchased from Merck,
194 Darmstadt, Germany. All solvents were HPLC grade and were from Fisher Scientific,
195 Loughborough, U.K.

196

197 **RESULTS**

198

199 *Sequence analyses.* The full length of the putative salmon desaturase cDNA (mRNA), as
200 determined by 5' and 3' RACE PCR, was shown to be 2106bp which included a 5'-UTR of 284bp
201 and a 3'-UTR of 457bp. Sequencing revealed that the cDNA included an ORF of 1365 bp, which
202 specified a protein of 454 amino acids (GenBank accession no. AY458652). The protein sequence
203 included all the characteristic features of microsomal fatty acid desaturases, including three
204 histidine boxes and an N-terminal cytochrome b₅ domain containing the haem-binding motif, H-P-
205 G-G (Fig.1). The protein sequence also contained two transmembrane regions. These features are
206 similar to those of other fatty acid desaturase genes including salmon Δ 5 desaturase, the zebrafish
207 Δ 6/ Δ 5 desaturase, and the human Δ 5 (GenBank accession no. AF126799) and Δ 6 (GenBank
208 accession no. AF199596) desaturases. However, the new salmon desaturase, like the salmon Δ 5
209 desaturase and the rainbow trout Δ 6 desaturase sequences, had an insertion of 10 amino acid
210 residues at the N-terminal end.

211 A pair-wise comparison was made between fish and human desaturase sequences. The amino
212 acid sequence predicted by the salmon putative (Δ 6) desaturase ORF shows 91% identity to the
213 salmon Δ 5 desaturase, and 94% identity to the trout Δ 6 desaturase. The salmon cDNA shows 65%
214 identity to that of the zebrafish Δ 6/ Δ 5 desaturase, and 65 and 58% identity to the human Δ 6 and Δ 5
215 cDNAs, respectively.

216 A phylogenetic tree was constructed on the basis of the amino acid sequence alignments
217 between the salmon fatty acyl desaturases, and 15 other desaturases of fish and mammals (Fig 2).
218 The phylogenetic analysis clustered the new Atlantic salmon putative desaturase sequence with the
219 Atlantic salmon Δ 5 desaturase, rainbow trout Δ 6 desaturase and other, as yet uncharacterised,
220 masou (cherry) salmon (*Oncorhynchus masou*) desaturase genes, but closest to the trout Δ 6
221 desaturase. The salmonid desaturases clustered more closely with turbot, sea bream and tilapia

222 (*Oreochromis nilotica*) desaturases, than with carp $\Delta 6$ desaturase and zebrafish $\Delta 5/\Delta 6$ desaturase.
223 All of the fish desaturase genes clustered together, and closer to the mammalian (mouse and human)
224 $\Delta 6$ desaturases than to the mammalian $\Delta 5$ desaturases.

225

226 *Functional characterisation.* The salmon desaturase cDNA was functionally characterized by
227 determining the fatty acid profiles of transformed *S. cerevisiae* containing either the pYES vector
228 alone or the vector with the salmon desaturase cDNA insert, grown in the presence of a variety of
229 potential fatty acid substrates, including $\Delta 6$ substrates (18:2n-6 and 18:3n-3), $\Delta 5$ substrates (20:3n-6
230 and 20:4n-3) and $\Delta 4$ substrates (22:4n-6 and 22:5n-3). The fatty acid composition of the yeast
231 transformed with the vector alone showed the four main fatty acids normally found in *S. cerevisiae*,
232 namely 16:0, 16:1n-7, 18:0 and 18:1n-9, together with the exogenously derived fatty acids. This is
233 consistent with *S. cerevisiae* not possessing $\Delta 5$ or $\Delta 6$ fatty acid desaturase activities (Figs. 3 and 4).
234 The most prominent additional peaks were observed in the profiles of transformed yeast grown in
235 the presence of the $\Delta 6$ desaturase substrates, 18:3n-3 and 18:2n-6 (Fig.3). Based on GC retention
236 time and confirmed by GC-MS, the additional peaks associated with the presence of the salmon
237 desaturase cDNA were identified as 18:4n-3 (Fig.3B) and 18:3n-6 (Fig.3D), corresponding to the
238 $\Delta 6$ desaturation products of 18:3n-3 and 18:2n-6, respectively. Approximately, 60.1% of 18:3n-3
239 was converted to 18:4n-3 and 14.4% of 18:2n-6 was converted to 18:3n-6 in yeast transformed with
240 the salmon desaturase (Table 2). However, a very small additional peak representing desaturated
241 fatty acid product, as confirmed by GC-MS, was observed in the lipids of *S. cerevisiae* transformed
242 with the desaturase cDNA when the transformed yeast was incubated with 20:4n-3 (Figs.4A and B).
243 About 2.3% of 20:4n-3 (n-3 $\Delta 5$ activity) was desaturated by the salmon clone, but no product of
244 desaturation of the 20:3n-6 substrate was detected, indicating no significant n-6 $\Delta 5$ desaturase
245 activity. The desaturase cDNA did not express any $\Delta 4$ desaturase activity as evidenced by the lack
246 of any observable additional peaks representing desaturated products of 22:5n-3 or 22:4n-6 (data
247 not shown). Overall, therefore, the results showed that the salmon desaturase cDNA encoded
248 enzyme was essentially a $\Delta 6$ fatty acyl desaturase, with only a very low level of $\Delta 5$ desaturase
249 activity, and no $\Delta 4$ desaturase activity.

250

251 *Genomic structure.* The alignment of the $\Delta 6$ fatty acyl desaturase cDNA and the genomic
252 sequences revealed 13 exons spanning 7965 bp of genomic DNA as illustrated in Table 3.

253

254 *Fatty acid desaturase and elongase gene expression in salmon tissues.* To identify which tissues
255 were likely to contribute to HUFA synthesis in the Atlantic salmon, reverse transcription Q-PCR
256 was used to examine the tissue distribution of $\Delta 6$ and $\Delta 5$ fatty acyl desaturase and fatty acyl

257 elongase mRNAs. The results showed that the three genes were expressed in all tissues examined,
258 with highest expression in terms of the absolute copy numbers (mean \pm SD, n =8) in intestine,
259 followed by liver and brain (Fig.5). In comparison to the Δ 5 desaturase, the transcript copy
260 abundance for the Δ 6 desaturase was higher in these tissues with higher expression, but lower in
261 tissues with lower expression, other than kidney. The transcript copy abundance for fatty acyl
262 elongase was much lower than that for the Δ 6 and Δ 5 desaturases in all tissues.

263 The ratios of copy numbers between the target genes and β -actin were determined (means \pm
264 SD, n = 4), and the fold difference between the mean value of target gene expression in the tissue of
265 fish fed VO calculated relative to the expression in tissues of fish fed FO (Fig 6). The results
266 revealed that Δ 6 and Δ 5 fatty acyl desaturase gene expression in liver and red muscle of fish fed VO
267 was significantly increased compared to fish fed the FO diet, whereas the expression of both
268 desaturases in heart and spleen, and Δ 5 in gill and kidney was decreased in fish fed VO (Fig.6).
269 Expression of both desaturases in intestine and adipose tissue was also higher in fish fed VO,
270 although with the high variation these effects were below the level of statistical significance.
271 However, feeding VO decreased the expression of the fatty acyl elongase gene in most tissues,
272 significantly so in heart, gill, brain, adipose, spleen and kidney (Fig.6).

273

274 **DISCUSSION**

275

276 Several fish desaturases have been cloned and functionally characterised in recent years. These are
277 the bifunctional zebrafish enzyme showing both Δ 6 and Δ 5 desaturase activity (22), an Atlantic
278 salmon desaturase that was shown to be predominantly an n-3 Δ 5 desaturase (26), and common
279 carp, rainbow trout, gilthead seabream and turbot desaturases that were all shown to be
280 predominantly Δ 6 desaturases (25). The bifunctional nature of the Δ 6/ Δ 5 desaturase of zebrafish
281 suggested that it may be a prototypic or ancestral progenitor desaturase (22,29). But the subsequent
282 characterisation of several essentially unifunctional Δ 6 fish desaturases and the salmon Δ 5
283 desaturase indicates that the zebrafish enzyme might be atypical.

284 The study described here has further increased our knowledge of PUFA desaturases in fish.
285 The cloning and functional characterisation of a predominantly Δ 6 desaturase gene makes the
286 Atlantic salmon the first fish species to be shown to have separate and distinct genes for Δ 6 and Δ 5
287 desaturases, as reported previously for *C. elegans* (14,18,19) and human (16,20,21). The salmon Δ 6
288 desaturase clone also showed measurable, but very low, levels of Δ 5 activity, and thus was similar
289 to other fish Δ 6 desaturases of carp, trout, seabream and turbot (25). But, unlike the zebrafish
290 desaturase, which showed very significant Δ 5 desaturase activity at around 70% of the Δ 6 activity

291 (22), the n-3 $\Delta 5$ activity in the salmon cDNA product was only 3.8% of the $\Delta 6$ activity. It is likely
292 that the level of $\Delta 5$ desaturase activity measured is of limited physiological significance.

293 The study described here also clearly showed that the salmon $\Delta 6$ desaturase has a marked
294 preference for the n-3 substrate 18:3n-3 over the n-6 substrate 18:2n-6. A similar preference for n-3
295 fatty acid substrates rather than n-6 substrates upon heterologous expression in yeast was observed
296 previously with the zebrafish $\Delta 6/\Delta 5$ desaturase, salmon $\Delta 5$ desaturase (22,26), and trout, seabream,
297 carp and turbot $\Delta 6$ desaturases (25). These data are consistent with earlier enzymological studies
298 investigating the desaturation of ^{14}C -labelled fatty acid substrates in primary hepatocytes (9),
299 primary brain astrocytes (30) and established cell lines (31). Therefore, it appears that greater
300 activity towards n-3 PUFA may be a characteristic of fish fatty acyl desaturases. In contrast,
301 functional characterisation of $\Delta 6$ desaturases of other organisms including nematode, mammals,
302 fungi, mosses and higher plants failed to show a preference for either 18:3n-3 or 18:2n-6 substrates,
303 although recently $\Delta 6$ desaturases have been identified in *Primula* sp. which have a preference for n-
304 3 substrates (32). However, data of these kinds obtained from heterologous expression can only be
305 regarded as semi-quantitative as there are likely to be differences between fatty acids in, for
306 example, their uptake into organisms such as yeasts (33).

307 The present study shows unequivocally that distinct $\Delta 6$ and $\Delta 5$ desaturase genes exist in Atlantic
308 salmon, as is the case in humans, and possibly in mammals in general. However, the two salmon
309 cDNAs are very similar in that the predicted amino acid sequence encoded by the $\Delta 6$ cDNA is 91%
310 identical with that encoded by the $\Delta 5$ desaturase cDNA. In contrast, in human and *C. elegans*, the
311 two functional $\Delta 6$ and $\Delta 5$ desaturases share an amino acid identity of only 62% (20) and 45% (19),
312 respectively. Whether or not distinct $\Delta 6$ and $\Delta 5$ desaturase genes evolved from a common ancestral
313 desaturase progenitor, these data suggest that the process occurred or began more recently in the
314 evolution of Atlantic salmon than in the evolutions of human and *C. elegans*. In this regard it is
315 pertinent to note that the Atlantic salmon is partially tetraploid, with the tetraploidisation event
316 thought to have occurred 25-100 million years ago (34). However, evolution of desaturases in
317 Atlantic salmon and in fish in general remains a subject for speculation. Study of further fatty acid
318 desaturase genes of fish are indicated, and certainly other desaturases are likely to be identified in
319 fish species such as carp and trout, which have the ability to produce DHA from 18:3n-3 (35). But,
320 in marine species such as sea bream and turbot, the search for $\Delta 5$ desaturases will be particularly
321 intriguing as these species lack the ability to produce EPA and DHA from 18:3n-3. This is
322 attributed to deficiencies in $\Delta 5$ desaturation in sea bream, but to C_{18-20} elongation in turbot (36,37).

323 The salmon $\Delta 6$ desaturase showed no $\Delta 4$ desaturase activity, perhaps as expected based upon
324 the functional characterisation of all fish and mammalian $\Delta 6$ and $\Delta 5$ desaturases reported to date
325 (22,25,26,38). This is consistent with the hypothesis that the synthesis of DHA from EPA in both

326 mammals and fish proceeds via elongation to 24:5n-3 followed by a $\Delta 6$ desaturation rather than via
327 $\Delta 4$ desaturation of 22:5n-3 (35,39). Heterologous expression studies of human and rat $\Delta 6$
328 desaturases showed that the same enzymes are active on C₁₈ and C₂₄ fatty acids (33,40), and the
329 bifunctional zebrafish desaturase was also capable of desaturating C₂₄ fatty acids (41). It will be
330 interesting to determine the activities of all animal $\Delta 6$ desaturases towards C₂₄ fatty acid substrates.
331 In contrast to higher animals, production of DHA via a pathway including $\Delta 4$ desaturation appears
332 to operate in some lower organisms such as *Thraustochytrium* sp. (42), and the algae *Euglena*
333 *gracilis* (43) and *Pavlova lutheri* (44).

334 Genomic characterization showed that the salmon $\Delta 6$ desaturase comprised 13 exons, which is
335 one more than that reported for the human $\Delta 6$ desaturase (45). The additional exon in the salmon
336 gene is a small 25 bp exon at the extreme 5' end. The remaining exons are homologous to the 12
337 exons in the human $\Delta 6$ desaturase, except that exon 2 of the salmon gene is 30 bp longer than exon
338 1 in the human gene, corresponding to the additional 10 amino acids found in most salmonid
339 desaturases. However, the remaining exons are exactly the same size as their equivalents in the
340 human gene, and splice and acceptor sites are interrupted at similar nucleotide positions, even
341 though the lengths of the introns are quite different. In human, there is evidence that the desaturase
342 gene cluster has arisen by gene duplication. This is on the basis that the exon organization is nearly
343 identical in the three family members, with each gene consisting of 12 exons and splice and
344 acceptor sites interrupted at identical nucleotide positions within highly conserved codons (45).
345 Further work on the genomic organisation of fish desaturases may help to clarify the significance of
346 the additional exon in salmon and the possible evolutionary history of desaturases, as sequence
347 alignments alone are not conclusive (46).

348 The phylogenetic sequence analyses grouped the fish desaturases largely as expected based on
349 classical phylogeny with the carp and zebrafish (Ostariophysi; cyprinids), trout and salmon
350 (Salmoniformes; salmonidae), and tilapia, sea bream and turbot (Acanthopterygia; cichlids,
351 perciformes and pleuronectiformes) appearing in three distinct clusters (47). However, the cloning
352 of Atlantic salmon $\Delta 6$ desaturase has revealed that both $\Delta 6$ and $\Delta 5$ desaturases in salmonids contain
353 additional amino acids by comparison with those of other species, having chain lengths of 454
354 amino acids (or 452 as in cherry salmon Des2) compared to 444 for the cyprinid (carp and
355 zebrafish) and human desaturases (16,20,22,23,26). Furthermore, it has been reported that the
356 desaturase cDNAs encode proteins of 445 amino acids in seabream (24) and turbot (25), one more
357 residue than in cyprinid and human desaturases. These data support our previous observation that
358 differences in polypeptide length are not in these cases related to function (25).

359 Q-PCR revealed that the expression of fatty acyl desaturase genes was highest in intestine, liver
360 and brain, and lower in heart, gill, white and red muscle, kidney, spleen and adipose tissue.

361 Previously, using RT-PCR, it was shown that $\Delta 6$ desaturase of rainbow trout and sea bream was
362 expressed in intestinal tissue (23,24). In the present study, salmon intestinal tissue had levels of $\Delta 6$
363 and $\Delta 5$ expression 3- and 1.5-fold greater than liver. Similarly, expression of $\Delta 6$ and $\Delta 5$ in intestine
364 was 7.2- and 1.9-fold greater than in brain. Therefore these results suggest that intestine, the first
365 organ to encounter dietary fatty acids, has the capacity to play an important role in the primary
366 processing of dietary fatty acids via desaturation. Cho et al. (20) reported that human liver
367 expressed 4-5 times more $\Delta 5$ desaturase, and 12 times more $\Delta 6$ desaturase than brain. Our results
368 show that salmon liver contained 2.4 times more $\Delta 6$ desaturase mRNA than brain, and the $\Delta 5$
369 desaturase mRNA levels in liver and brain were similar. Regardless of which gene has the higher
370 level of mRNA, the observation that all tissues investigated express detectable levels of $\Delta 6$ and $\Delta 5$
371 desaturase and elongase mRNAs is consistent with the important roles that desaturase and elongase
372 enzymes play in maintaining cellular membrane HUFA. That intestine expressed such high levels of
373 both $\Delta 6$ and $\Delta 5$ desaturase is consistent with data from *in vitro* enzyme assays in isolated
374 enterocytes (48,49), and *in vivo* stable isotope studies (50,51), which have shown enterocytes and
375 intestine to be sites of significant HUFA synthesis in salmonids. The level of $\Delta 6$ desaturase mRNA
376 in highly expressing tissues was substantially greater than the amount of $\Delta 5$ desaturase mRNA, but
377 the level of $\Delta 6$ desaturase mRNA in lower expressing tissues was lower than the amount of $\Delta 5$
378 desaturase mRNA. In comparison, a study of the relative abundance of $\Delta 6$ and $\Delta 5$ desaturase
379 mRNA in various human tissues revealed that the level of $\Delta 6$ desaturase mRNA in 8 different
380 tissues was significantly greater than the amount of $\Delta 5$ desaturase mRNA (20). This observation is
381 particularly interesting because $\Delta 6$ is often considered the enzyme which catalyses the rate-limiting
382 step in the synthesis of HUFA (52).

383 The results of this study show that the expression of $\Delta 6$ and $\Delta 5$ fatty acid desaturases is under
384 nutritional regulation in Atlantic salmon. Thus, the expression of these genes was higher in liver
385 and red muscle (and possibly intestine and adipose tissue) of salmon fed diets containing C₁₈
386 PUFA-rich vegetable oil compared to fish fed diets containing HUFA-rich fish oil. Although $\Delta 6$
387 desaturase is regarded as the main rate-limiting step in the HUFA biosynthesis pathway,
388 $\Delta 6$ desaturase is reported to also be under nutritional regulation in mammals (53). In a previous
389 study, the expression and activity of fatty acyl elongase appeared to be nutritionally regulated in
390 Atlantic salmon (54). That study showed that dietary linseed oil increased the expression of both $\Delta 5$
391 fatty acid desaturase and elongase genes in salmon liver (54). Similar effects of dietary linseed oil
392 had been reported previously, with the liver transcript level of $\Delta 6$ desaturase being higher in trout
393 fed linseed oil compared to in trout fed fish oil (23). However, the present study showed the
394 expression and activity of the elongase decreased in most tissues of salmon fed diets containing the
395 vegetable oil blend compared to fish fed diets containing fish oil. The precise reason for the

396 different responses in elongase gene expression is unclear, but may be related to differences in the
397 fatty acid profiles of the linseed oil and VO blend diets. In the present trial, the total n-3HUFA
398 level in the diet in which the VO blend replaced 75% of the FO was over 8%, which compares well
399 with 9% HUFA in the diet in the previous trial in which 25% of the FO was replaced by linseed oil,
400 a level of replacement which did not increase elongase activity (54). Elongase activity was only
401 increased by diets in which 50-100% of FO was replaced with linseed oil, resulting in much lower
402 levels of n-3HUFA (54).

403 In conclusion, the study reported here has identified and characterised a $\Delta 6$ desaturase gene in
404 Atlantic salmon. It had measurable, but very low, levels of $\Delta 5$ desaturase activity. The salmon $\Delta 6$
405 desaturase gene comprises 13 exons, one more than the human $\Delta 6$ and $\Delta 5$ desaturases. $\Delta 6$ and $\Delta 5$
406 desaturases and elongase genes were expressed in various tissues of salmon, and highly expressed
407 in liver, intestine and brain. Both $\Delta 6$ and $\Delta 5$ desaturase gene expression in intestine, liver, red
408 muscle and adipose tissue were significantly increased in salmon fed vegetable oil compared to in
409 fish fed fish oil.

410

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412

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573

574 **Fig.1.** Comparison of the deduced amino acid sequence of $\Delta 6$ and $\Delta 5$ polyunsaturated fatty acyl
 575 desaturases from Atlantic salmon with that of desaturases from trout, zebrafish and human.
 576 Identical residues are shaded black and similar residues are shaded grey. Identity/similarity
 577 shading was based on the BLOSUM62 matrix and the cut off for shading was 75%. The
 578 cytochrome b_5 -like domain is dot-underlined, the two transmembrane regions are dash underlined,
 579 the three histidine-rich domains are solid underlined and asterisks on the top mark the haem-
 580 binding motif, H-P-G-G.

581 **Fig.2.** Phylogenetic tree of $\Delta 6$ and $\Delta 5$ desaturases from salmon, and desaturases from other fish
 582 species (zebrafish, cherry salmon, rainbow trout, seabream, common carp, turbot and tilapia),
 583 mammals (mouse and human), fungus (*Mortierella alpina*) and nematode (*Caenorhabditis*
 584 *elegans*). The tree was constructed using the N-J method using *CLUSTALX* and *NJPLLOT*. The
 585 horizontal branch length is proportional to amino acid substitution rate per site. The numbers
 586 represent the frequencies with which the tree topology presented here was replicated after 1000
 587 bootstrap iterations. Sequences marked with an asterisk are not functionally characterized.

588
 589 **Fig.3.** Functional expression of the Atlantic salmon putative fatty acyl desaturase in transgenic
 590 yeast (*Saccharomyces cerevisiae*) grown in the presence of $\Delta 6$ substrates, 18:3n-3 and 18:2n-6.
 591 Fatty acids were extracted from yeast transformed with pYES vector without insert (A and C) or
 592 containing the putative fatty acid desaturase cDNA insert (B and D). The first four peaks in panels
 593 A-D are the main endogenous fatty acids of *S. cerevisiae*, namely 16:0 (1), 16:1n-7 (2), 18:0 (3) and
 594 18:1n-9 (with 18:1n-7 as shoulder) (4). Peak 5 in panels A and B, and peak 7 in panels C and D are
 595 the exogenously added substrate fatty acids, 18:3n-3 and 18:2n-6, respectively. Peaks 6 and 8 in
 596 panels B and D were identified as the resultant desaturated products, namely 18:4n-3 and 18:3n-6,
 597 respectively. Vertical axis, FID response; horizontal axis, retention time.

598
 599 **Fig.4.** Functional expression of the Atlantic salmon putative fatty acyl desaturase in transgenic
 600 yeast (*Saccharomyces cerevisiae*) grown in the presence of $\Delta 5$ substrates, 20:4n-3 and 20:3n-6.
 601 Fatty acids were extracted from yeast transformed with pYES vector without insert (A and C) or
 602 containing the putative fatty acid desaturase cDNA insert (B and D). The first four peaks in panels
 603 A-D are as described in legend to Fig.3. Peak 9 in panels A and B, and peak 11 in panels C and D
 604 are the exogenously added substrate fatty acids, 20:4n-3 and 20:3n-6, respectively. Peak 10 in
 605 panel B was identified as the resultant desaturated product of 20:4n-3, namely 20:5n-3. Vertical
 606 axis, FID response; horizontal axis, retention time.

607

608

609 **Fig. 5.** Tissue distribution of fatty acid $\Delta 6$ and $\Delta 5$ desaturase and elongase genes in Atlantic salmon.
610 Transcript (mRNA) copy number was determined by real-time quantitative PCR (Q-PCR) as
611 described in the Materials and Methods Section. Results are expressed as the copy numbers in
612 250ng of total RNA and are means \pm SEM (n = 4). L, liver; H, heart; G, gill; WM, white muscle;
613 RM, red muscle; I, intestine; B, brain; A, adipose; S, spleen; K, kidney.

614

615 **Fig.6.** Effect of dietary vegetable oil on the expression of fatty acid $\Delta 6$ and $\Delta 5$ desaturase and
616 elongase genes in tissues from Atlantic salmon. Transcript (mRNA) copy number was determined
617 by real-time quantitative RT-PCR (Q-PCR) as described in the Materials and Methods Section. The
618 ratios of copy numbers between the target genes and β -actin were calculated as means \pm SEM (n =
619 4). Results are expressed as the fold differences by comparison of mean values in fish fed the
620 vegetable oil diet compared to those in fish fed the fish oil diet (FO = 1). L, liver; H, heart; G, gill;
621 WM, white muscle; RM, red muscle; I, intestine; B, brain; A, adipose; S, spleen ; K, kidney.

Table 1
Fatty Acid Composition (Percentage of Total Fatty Acids) of Diets

	FO	VO
14:0	6.1	2.4
16:0	14.7	16.0
18:0	2.8	3.3
Total saturated ¹	24.3	21.9
16:1n-7 ²	5.0	2.0
18:1n-9	13.5	35.2
18:1n-7	2.5	2.3
20:1n-9 ³	10.4	3.6
22:1n-11 ⁴	14.9	4.8
24:1n-9	0.7	0.3
Total monoenes	47.0	48.2
18:2n-6	4.0	11.8
20:4n-6	0.5	0.2
Total n-6 PUFA ⁵	5.1	12.2
18:3n-3	1.1	8.5
18:4n-3	2.4	0.8
20:4n-3	0.7	0.2
20:5n-3	6.7	2.8
22:5n-3	1.1	0.4
22:6n-3	10.4	4.5
Total n-3 PUFA ⁶	22.4	17.3
Total PUFA ⁷	28.7	29.9

Data are the means of two samples. FO, fish oil; PUFA, polyunsaturated fatty acids; VO, vegetable oil blend.¹totals contain 15:0 present at up to 0.5%; ²contains 16:1n-9; ³contains 20:1n-11 and 20:1n-7; ⁴contains 22:1n-9; ⁵totals contain 18:3n-6, 20:2n-6, 20:3n-6 and 22:5n-6 present at up to 0.2%; ⁶totals contain 20:3n-3 present at up to 0.1%; ⁷totals contain C₁₆ PUFA.

Table 2
Functional Characterisation of Salmon Fatty Acid Desaturase cDNA
Clone in the Yeast *Saccharomyces cerevisiae*

PUFA substrates	Products	Desaturase Activity	Conversion rate (%)
α -Linolenic acid (18:3n-3)	18: 4n-3	$\Delta 6$	60.1
Linoleic acid (18:2n-6)	18: 3n-6	$\Delta 6$	14.4
Eicosatetraenoic acid (20:4n-3)	20: 5n-3	$\Delta 5$	2.3
Dihomo- γ -linoleic acid (20:3n-6)	20: 4n-6	$\Delta 5$	0
Docosapentaenoic acid (22:5n-3)	22: 6n-3	$\Delta 4$	0
Docosatetraenoic acid (22:4n-6)	22: 5n-6	$\Delta 4$	0

Conversion rates represent the proportion of substrate fatty acid converted to the longer chain fatty acid product, calculated from the gas chromatograms as $100 * [\text{product area} / (\text{product area} + \text{substrate area})]$.

PUFA, polyunsaturated fatty acid.

Table 3**Exon and Intron Boundaries of Atlantic Salmon $\Delta 6$ Fatty Acyl Desaturase**

Exon	Size (bp)	3' splice acceptor	5' splice donor	Intron size (bp)
1	25 ^a		..AATATTGgtgagtg..	698
2	496 ^b	..tttcagCTGGCCC..	..TGCCACGgtcagta..	1127
3	111	..ttttagGACGCAT..	..GAAAAATgtgagga..	744
4	198	..catacagGCAGTAC..	..GTCTCAGgtaccat..	228
5	102	..ctctcagTCCCAGG..	..CCTAAAGgtaggct..	345
6	126	..ttccagGGTGCCT..	..TGTAGAGgtagtta..	515
7	61	..attcagTATGGTA..	..TTCCTCAgtaagtc..	128
8	77	..cttcagTTGGACC..	..CTGGGTGgtgagat..	303
9	98	..tgtgaagGATCTGG..	..TCGTCAGgtaaagt..	161
10	97	..tatatagGTTTTTG..	..CATGCAGgtaacat..	1011
11	80	..gtcttagTTGAGTG..	..AACACCAgtaagtg..	383
12	126	..ctcccagTCTGTTT..	..TTGTCAGgtaagtg..	216
13	509 ^c	..tctccagGTCACTG..		

^aExon is a 5'-UTR of 25 bp

^bExon includes a 5'-UTR of 259 bp.

^cExon includes a 3'-UTR of 457 bp.

Fig. 1

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                                                                 ****
Atlantic salmon D6  MCGCGQONDSCPEPAKGDRCGPGGGLGCSAVYTWEVQQRHSHRCDQWLVIDRKYNIITQQAARHPGCGIRVI 70
Atlantic salmon D5  MCGCGQQTESSEPAKGDGLEPDGGQGGCSAVYTWEVQQRHSHRSDQWLVIDRKYNIITQQAARHPGCGIRVI 70
Rainbow trout D6   MCGCGQQTESSEPAKGDGVGPDGGRCGCSAVYTWEVQQRHCHRSQWLVIDRKYNIITQQAARHPGCGIRVI 70
Zebrafish D5/D6   MCGCGQQTDRITDTNG-----RFSSTTWEVQQRHTKHGDQWVWVERKYNIWVQVVERHPGCLDIL 60
Human D6          MCRGCMQCEGAAAREVVS-----VPTFSWEELQREHLRTRDLVIDRKYNIITKOSIQHPGCGQIRVI 60
Human D5          MAPDPLAAETAQAQGLTP-----RYFTQDEVAQRSGCEEKWLVIDRKYNIITSEFTLRHPGCGSRVI 59
.....

Atlantic salmon D6  SHFACEDATDAFWAFHPNPNFWRKFLKPLLLIGELAPTEPSQDHCKNAVLVQDFQALRNRVERECLLRARP 140
Atlantic salmon D5  SHFACEDATEAFSAFHLDANFWRKFLKPLLLIGELAPTEPSQDHCKNAALVQDFQALRDRHVERECLLRARL 140
Rainbow trout D6   SHFACEDATDAFWAFHPDPNFWRKFLKPLLLIGELATEPSQDHCKNAVLVQDFQALRDRVERECLLRARP 140
Zebrafish D5/D6   CHFACEDATEAFTAFAHPNLQLWRKYLKPLLLICELEASPSQDRQKNAALVEDFQALRERLEARGCCFKTQP 130
Human D6          CHFACEDATDAFBAFHPDLEFWCKFLKPLLLIGELAPEPSQDHCKNSKITEDFQALRKTAEQMMNLFKTNH 130
Human D5          SHFACQDATDPEWAFHINKLWRKYMNLSLLIGELSPQPSFEPTKMKELTDEFRELRATVERMGLMKANH 129
.....

Atlantic salmon D6  LFFSLYLCHILLLEALALGLLQVWCTSWSLTLLCSLMLATSQSQACWLQHDYCHLSVCKKSSUMHVLHKKF 210
Atlantic salmon D5  LFFSLYLCHILLLEALALGLLQVWCTSWSLTLLCSLMLATSQSQACWLQHDYCHLSVCKKSSUMHKLHKKF 210
Rainbow trout D6   LFFSLYLCHILLLEALALGLLQVWCTSWSLTLLCSLMLATSQSQACWLQHDYCHLSVCKTSSUMHVLHKKF 210
Zebrafish D5/D6   LFFALHLCHELLLEAIAFMVWYFCTGQINTLIVAVILATAQSQACWLQHDYCHLSVFKTSUMHVLHKKF 200
Human D6          VFFLLLLAHLILALESIAWFTVFFFCNGQIPLTLITAFVLATSQAAQACWLQHDYCHLSVYRKPUMHVLHKKF 200
Human D5          VFFLLYLLHILLLDGAAWLTLQVWCTSLPFLLCVALLSAVQAQACWLQHDYCHLSVFFSTSRUMHLLHKKF 199
.....

Atlantic salmon D6  VICHLKCASAMWMMHRRHFQHHAKPNVLSKDPDVMMLH-VFVLCGRQPVEYCGIKRKLKYPMPYHQHQYFFFLI 279
Atlantic salmon D5  VICHLKCASAMWMMHRRHFQHHAKPNVFRKDPDINSLP-VFVLCDTQPVEYCGIKRKLKYPMPYHQHQYFFFLI 279
Rainbow trout D6   VICHLKCASAMWMMHRRHFQHHAKPNVFSKDPDVMNSLH-VFVLCGRQPVEYCGIKRKLKYPMPYHQHQYFFFLI 279
Zebrafish D5/D6   VICHLKCASACWMMHRRHFQHHAKPNIFRKPDPDVMMLN-AFVWCVQVQVVEYCGIKRKLKYPMPYHQHQYFFFLI 269
Human D6          VICHLKCASAMWMMHRRHFQHHAKPNIFRKPDPDVMMLH-VFVLCGRQPVEYCGIKRKLKYPMPYHQHQYFFFLI 269
Human D5          VICHLKCAPASWMMHMHFQHHAKPMCFRKPDPDIMMHPFFALCKILSWELCKQKRYMPYMHQHQYFFFLI 269
.....

Atlantic salmon D6  GPPLLIPVFFFTIQIFQTMFSQRNVDLAWMSMTFYLRFFCSYYPFFCFFGSVALITFVRFLESHWVFWVVTQ 349
Atlantic salmon D5  GPPLLIPVFFFTIQIFRTMFSQRDQVDLAWMSMTFYLRFFCCYYPFFCFFGSVALISEVRFLESHWVFWVVTQ 349
Rainbow trout D6   GPPLWIPVFFFTIQIFQTMFSQRNVDLAWAMTFYLRFFCCYYPFFCFFGSVALISEVRFLESHWVFWVVTQ 349
Zebrafish D5/D6   GPPLLIPVVFQFQIEHNMISHGMQVDLLQCSYYPVRFELCYTQFYCVFWAIIIFNFVRFMESHWVFWVVTQ 339
Human D6          GPPLLIPMYFQYQIIMTMIVHRNVDLAWAVSYIRFFITYIPFYCILCALLFLNFRFLESHWVFWVVTQ 339
Human D5          GPPALLPLYEQWYLEYFVVIQRKQVDLAWMITFYVRFELTYVPELLCLKAFGLFFIVRFLESHWVFWVVTQ 339
.....

Atlantic salmon D6  MNHLPMEIDHERHQDWLTMQLSCTCNIHQSTFNDWFSCHLNFIQIEHHLFPTMPRHNYHLVAPLVRTLCEK 419
Atlantic salmon D5  MNHLPMEIDHERHQDWLTMQLSATCNIHQSTFNDWFSCHLNFIQIEHHLFPTMPRHNYHLVAPLVRTLCEK 419
Rainbow trout D6   MNHLPMEIDHERHQDWLTMQLSATCNIHQSTFNDWFSCHLNFIQIEHHLFPTMPRHNYHLVAPLVRALCEK 419
Zebrafish D5/D6   MSHLPMNIDYERNDQWLSMQLWATCNIHQSAFNDWFSCHLNFIQIEHHLFPTVPRHNYWRAAPVRALCEK 409
Human D6          MNHLPMEIDQRAYRDWFSQLTATCNWEQSEFNDWFSCHLNFIQIEHHLFPTMPRHNLHKIAPLWKSLECAK 409
Human D5          MNHLPMHIDHD RNMDWVSTQLQATCNWHRSAFNDWFSCHLNFIQIEHHLFPTMPRHNYHKVAPLWQSLCAK 409
.....

Atlantic salmon D6  HCLPYQVKTQKRAIIDVVRSLKKSGLWLDAYLHK 454
Atlantic salmon D5  HCVPYQVKTQKGMTDVVRSLKKSGLWLDAYLHK 454
Rainbow trout D6   HCLPYQVKTQKRAIIDVVGSLKKSGLWLDAYLHK 454
Zebrafish D5/D6   YGVRYQEKTLYCAFADIIIRSLKKSGLWLDAYLHK 444
Human D6          HCLPYQEKPLLRALLDIIIRSLKKSGLWLDAYLHK 444
Human D5          HCLPYQSRPLLSAFADIIIRSLKKSGLWLDAYLHQ 444

```

Fig. 2.

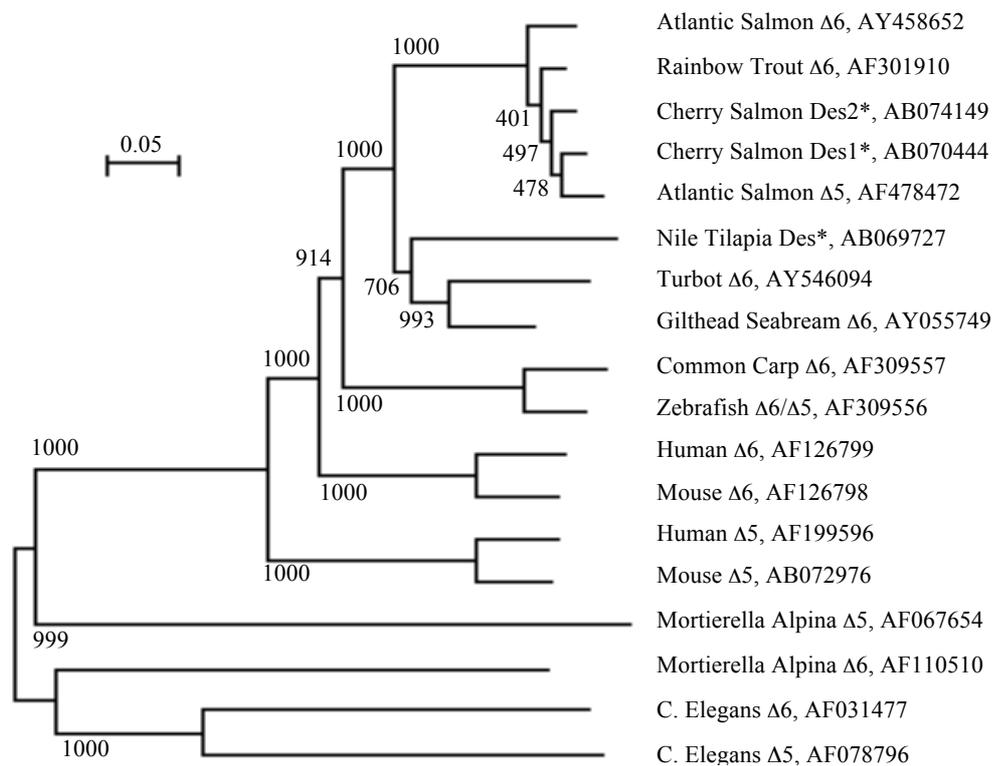


Fig.3.

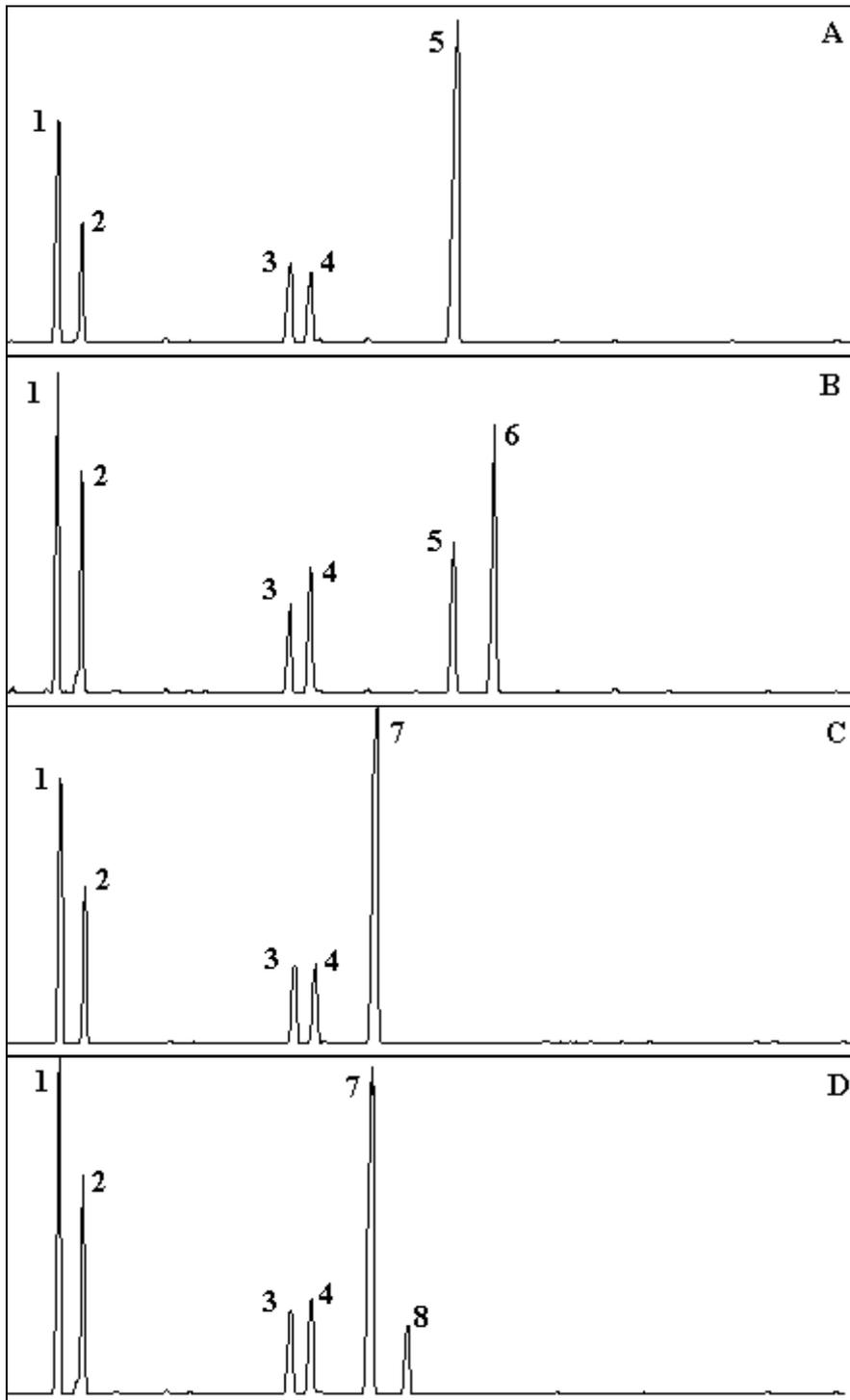


Fig.4.

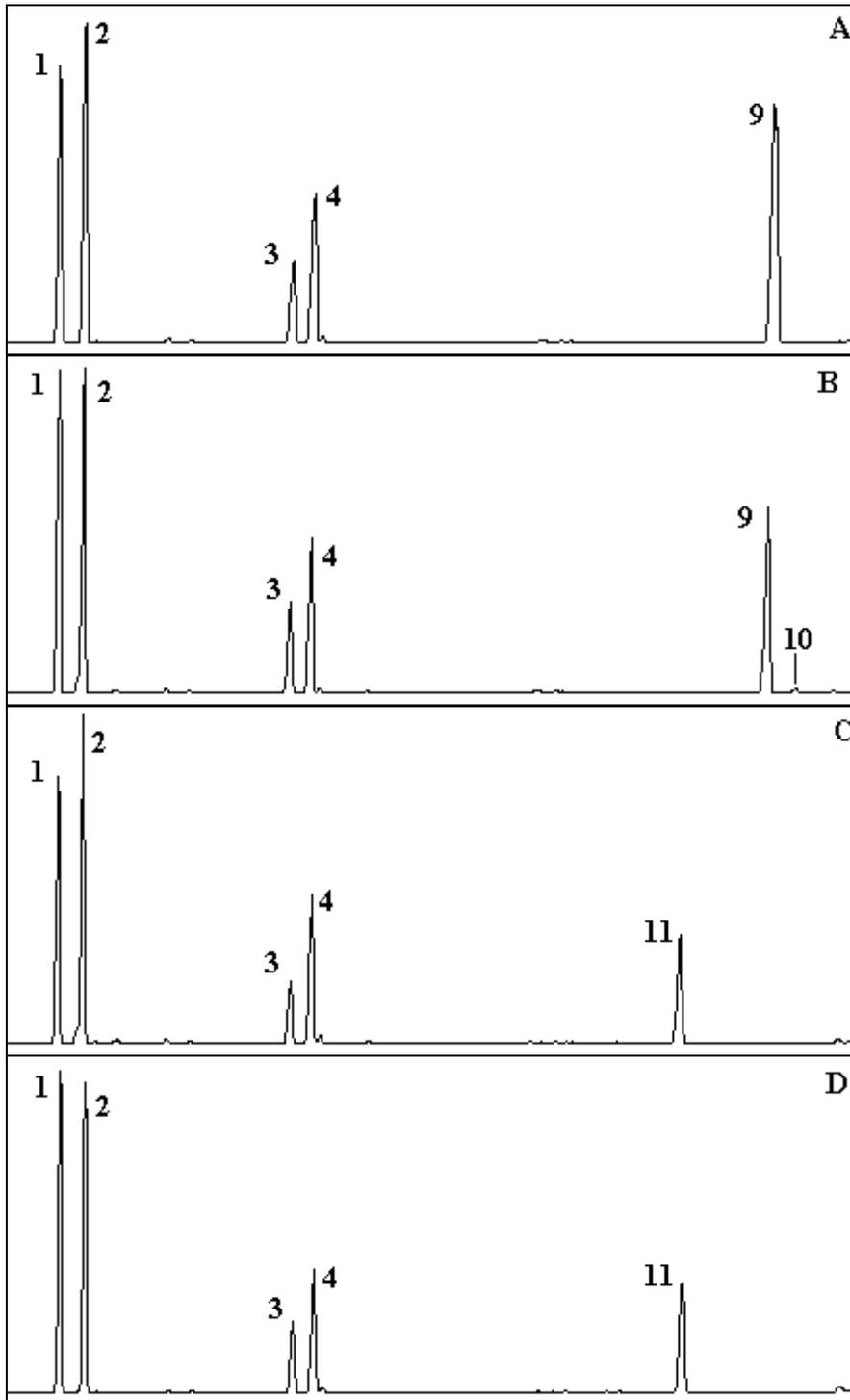


Fig. 5

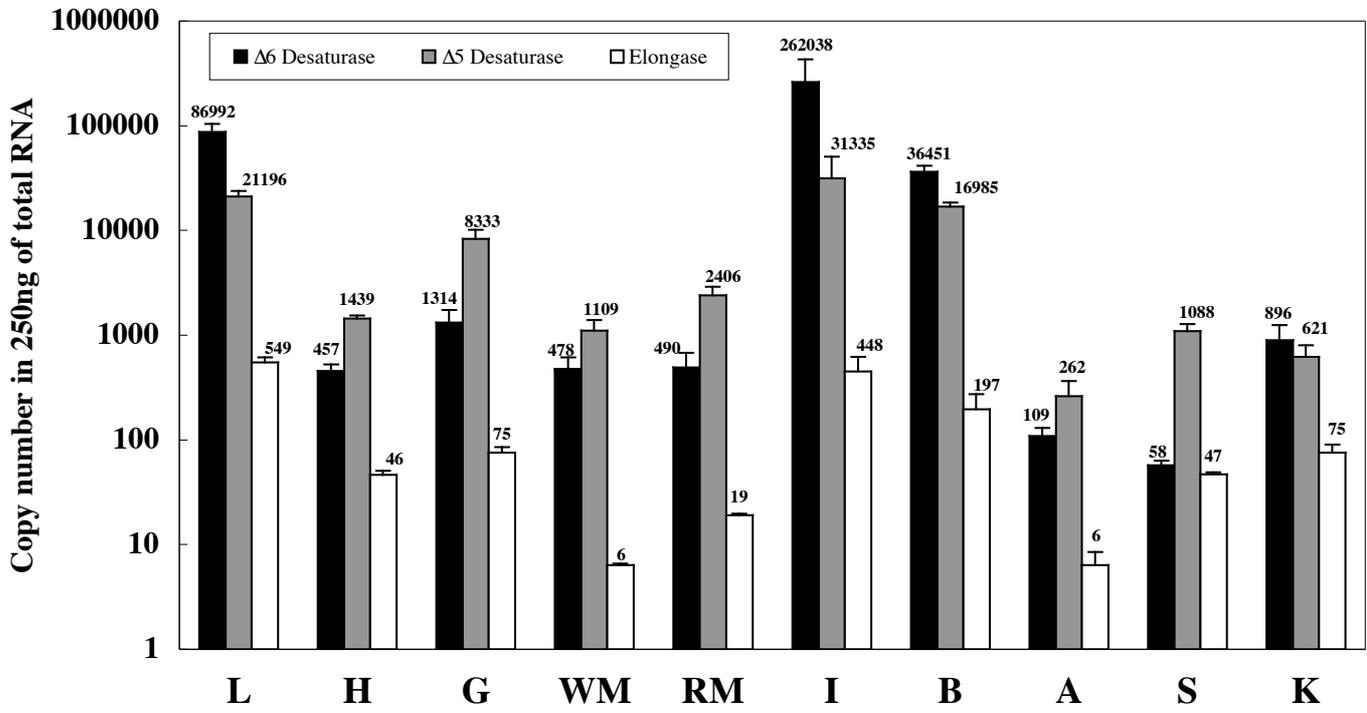


Fig.6.

