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1 **Title**

2 Delta-8 desaturation activity varies among fatty acyl desaturases of teleost fish: high
3 activity in delta-6 desaturases of marine species

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23 Delta-6; Delta-8; Fatty acyl desaturase; Long-chain polyunsaturated fatty acid
24 biosynthesis; Teleost fish.

25

Summary

The benefits of dietary fish and fish oil are derived from n-3 long-chain polyunsaturated fatty acids (LC-PUFA) that have beneficial effects in a range of human diseases and pathologies such as cardiovascular and other inflammatory disorders, neural development and neurological pathologies. The precursor of n-3 LC-PUFA, 18:3n-3 does not have the same beneficial effects prompting interest in the pathways of endogenous synthesis of LC-PUFA in vertebrates. The LC-PUFA biosynthesis pathway classically involves Δ6 and Δ5 fatty acyl desaturases (Fad), but it was recently shown that Δ6 Fad in mammals also displayed Δ8 activity demonstrating a possible alternative "Δ8-pathway" for the synthesis of LC-PUFA. Our primary hypothesis was that Δ8 desaturase activity would be a common feature of vertebrate Δ6 Fads, and so the aim of the present study was to determine the ability of teleostei Fads for Δ8 desaturation activity. To this end, cDNAs for Fads from a range of freshwater, diadromous and marine teleost fish species were assayed for Δ8 activity in the heterologous yeast expression system. In summary, the present study has demonstrated that Δ8 desaturation activity was also a characteristic of fish orthologs, although the activity varied notably between freshwater/diadromous and marine fish species, with the latter possessing Fads2-like proteins with Δ8 activity far higher than mammalian FADS2. The data showed that, generally, the fish Fad are technically υ-3 desaturases, with new double bonds introduced 3C beyond a pre-existing double bond. However, the ability of zebrafish and rabbitfish Fads, previously characterised as Δ6/Δ5 bifunctional desaturases, to introduce non-methylene interrupted double bonds in 20:3n-3 and 20:2n-6 suggested that a novel combination of regioselectivity modes operates within these enzymes.

51 **Introduction**

52 Many studies have demonstrated the benefits of dietary fish and fish oil on human
53 health. These outcomes of dietary fish and fish oil are derived from their content of n-3
54 long-chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic acid (EPA; 20:5n-
55 3) and docosahexaenoic acid (DHA; 22:6n-3), that are now known to have beneficial
56 effects in a range of human diseases and pathologies such as cardiovascular and other
57 inflammatory disorders, neural development and neurological pathologies (Brouwer et
58 al., 2006; Calder, 2006; Calder and Yaqoob, 2009; Eilander et al., 2007; Ruxton et al.,
59 2007; Torrejon et al., 2007). This has prompted renewed interest in the pathways of
60 endogenous synthesis of LC-PUFA in humans, and vertebrates in general, as the
61 precursor of EPA and DHA, 18:3n-3 does not have the same beneficial effects as the n-
62 3 LC-PUFA (Brenna, 2002; Salem et al., 1999). Thus, the inefficient conversion of
63 18:3n-3 to the biologically active n-3 LC-PUFA underpins the higher efficacy of EPA
64 and DHA compared to 18:3n-3 (Brenna et al., 2009; Burdge and Calder, 2005) in
65 alleviating these conditions and highlights the importance of studying the endogenous
66 LC-PUFA biosynthesis pathways.

67 Biosynthesis of LC-PUFA in vertebrates involves desaturation and elongation of
68 precursor C_{18} PUFA (Fig. 1). Synthesis of EPA is achieved by Δ6 desaturation of
69 18:3n-3 to produce 18:4n-3 that is elongated to 20:4n-3 followed by Δ5 desaturation,
70 but DHA synthesis was believed to require two further elongation steps, a second Δ6
71 desaturation and a peroxisomal chain shortening step (Cook and McMaster, 2004). The
72 same pathway and enzymes are responsible for synthesis of n-6 LC-PUFA, arachidonic
73 acid (ARA; 20:4n-6) and 22:5n-6. The pathway for DHA synthesis from EPA via C_{24}
74 intermediates, initially described in rats (Sprecher, 2000), was also shown to operate in
75 rainbow trout, the only non-mammalian vertebrate where this pathway has been

76 confirmed (Buzzi et al., 1997), and a bifunctional Δ6/Δ5 fatty acyl desaturase (Fad) in
77 zebrafish has been shown to be capable of desaturating 24:5n-3 to 24:6n-3 (Tocher et
78 al., 2003). These studies led to the paradigm that Δ4 desaturation did not occur in
79 vertebrates and that the Sprecher pathway was ubiquitous and solely responsible for
80 DHA synthesis. However, we have recently demonstrated the presence of a Fad in a
81 teleost fish, rabbitfish (*Siganus canaliculatus*), with Δ4 activity indicating that direct
82 production of DHA by desaturation of 22:5n-3 was possible in at least some vertebrates
83 although it was unclear whether this was the only pathway for DHA production in
84 rabbitfish, or how widespread the presence of a Δ4 Fad was in vertebrates (Li et al.,
85 2010).
86 Another paradigm in the LC-PUFA pathway has been that elongation of precursor
87 18:2n-6 and 18:3n-3 resulted in the respective "dead-end" products 20:2n-6 and 20:3n-
88 3, so-called as they were not intermediates on the LC-PUFA synthesis pathway.
89 However, it was recently shown that Δ6 Fad in mammals also displayed Δ8 activity.
90 Expression of baboon Δ6 Fad (FADS2) cDNA in yeast promoted the Δ8 desaturation of
91 20:2n-6 and 20:3n-3 to 20:3n-6 and 20:4n-3, respectively (Park et al., 2009) and *Fads2*-
92 null mice lack Δ8 activity (Stroud et al., 2009). These studies demonstrate a possible
93 alternative pathway for the synthesis of EPA and ARA from 18:3n-3 and 18:2n-6,
94 respectively, which had been previously suggested with the detection of Δ8 activity in a
95 variety of mammalian cells and tissues (Albert and Coniglio, 1977; Albert et al., 1979;
96 Bardon et al., 1996; Cook et al., 1991; Nakazawa et al., 1976; Schenck et al., 1996).
97 Like Δ4 desaturation above, it is unclear how widespread among vertebrates the Δ8
98 pathway is.
99 Understanding of the biochemical and molecular mechanisms of the LC-PUFA
100 pathway in vertebrates has been advanced greatly over the last few years through

101 studies in fish species (Leaver et al., 2008). This was prompted by the unique role of
102 fish in supplying n-3 LC-PUFA in the human diet and the fact that increasing amounts
103 of market fish and seafood (50% in 2008) are now farmed (FAO, 2009). Levels of n-3
104 LC-PUFA in farmed fish have been assured by the use of marine ingredients (fish meal
105 and oil) in the feeds but these are finite, limiting and diminishing resources that must be
106 replaced for sustainable aquaculture development (Tacon and Metian, 2008). Currently,
107 the only sustainable alternatives to fish oil are vegetable oils, which can be rich in C_{18}
108 PUFA, but devoid of EPA and DHA (Naylor et al., 2009), and so fatty acid
109 compositions of fish fed diets formulated with vegetable oils are characterised by
110 increased levels of C_{18} PUFA and decreased levels of n-3 LC-PUFA, reducing their
111 nutritional value to human consumers (Tocher, 2003; Turchini et al., 2010). The LC-
112 PUFA synthesis pathway has thus been extensively studied in fish with all species
113 investigated shown to express Δ6 Fad and Elovl5 elongase activities (Tocher, 2010). In
114 contrast, many marine species appear to lack Δ5 Fad and Elovl2 activities that severely
115 limit their ability for endogenous production of LC-PUFA from C_{18} precursors (Tocher,
116 2010).

117 Our primary hypothesis was that Δ8 desaturase activity is a common feature of
118 vertebrate Δ6 Fad and that the Δ8 pathway would also offer an alternative route for the
119 endogenous production of LC-PUFA in fish. The specific aim of the present study was
120 to determine the ability of teleostei Fads for Δ8 desaturation activity. Thus, Fad
121 encoding cDNAs from a range of freshwater, diadromous and marine teleost fish
122 species were assayed for Δ8 activity in the heterologous yeast expression system.

123

124 **Materials and Methods**

125 *Phylogenetic analysis*

126 Eleven genes encoding putative Fad from teleosts including diadromous (*Salmo*
127 *salar*), freshwater (*Oncorhynchus mykiss* and *Danio rerio*), and marine (*Siganus*
128 *canaliculatus*, *Rachycentron canadum*, *Gadus morhua*, *Sparus aurata* and *Psetta*
129 *maxima*) species were investigated in the present study (Table 1). Phylogenetic analysis
130 of the amino acid (AA) sequences of the teleostei Fad was performed by constructing a
131 tree using the neighbor-joining method (Saitou and Nei, 1987), with confidence in the
132 resulting tree branch topology measured by bootstrapping through 10000 iterations. The
133 mammalian (human and baboon) FADS1 and FADS2, and the Δ8 proteins from the
134 unicellular organisms *Euglena gracilis*, *Acanthamoeba castellanii*, *Perkinsus marinus*
135 and *Pavlova salina* were also included in the phylogenetic analysis.
136
137 *Heterologous expression in yeast Saccharomyces cerevisiae*
138 Functional activities of the fish Fad proteins in the Δ6-pathway were determined
139 previously, with those activities being the basis for their nomenclature (Table 1).
140 Appropriate primers containing restriction sites allowed the amplification of the *fad*
141 open reading frames (ORF) using the high fidelity PfuTurbo DNA polymerase
142 (Stratagene, Agilent Technologies, Cheshire, UK) (Table 2). Purified ORF fragments
143 were cloned into the episomal yeast vector pYES2 (Invitrogen) and TOP10' *E. coli*
144 transformed. Positive clones containing the different Fad as inserts were extracted
145 (GenElute™ Plasmid Miniprep Kit, Sigma) and used to transform yeast (S.c.
146 EasyComp Transformation Kit, Invitrogen) in the heterologous expression assays.
147 The Δ8 activity of fish desaturases was assayed by expressing their ORF in yeast *S.*
148 *cerevisiae* grown in presence of potential substrates for Δ8 desaturation, eicosatrienoic
149 (ETA; 20:3n-3) or eicosadienoic (EDA; 20:2n-6) acids. Transformation and selection of
150 yeast with recombinant pYES2-*fad* plasmids, and yeast culture were performed as

151 described in detail previously (Agaba et al., 2004; Hastings et al., 2001). Briefly,
152 cultures of recombinant yeast were grown in *S. cerevisiae* minimal medium$^{-uracil}$
153 supplemented with either ETA or EDA. In order to directly compare the Δ8 desaturation
154 capability of the fish desaturases with the desaturation activities reported previously,
155 recombinant yeast were also grown in presence of the n-3 PUFA substrates for which
156 highest activities were obtained (Table 1). Thus, in addition to Δ8 substrates 20:3n-3
157 and 20:2n-6, yeast transformed with the Δ6Fad from *S. salar* (isoforms b and c) and *O.*
158 *mykiss*, the bifunctional Δ6Δ5Fad from *D. rerio* and *S. canaliculatus*, and the Δ6Fad
159 from the marine species *R. canadum*, *G. morhua*, *S. aurata* and *P. maxima* were also
160 grown in presence of the Δ6 substrate α-linolenic acid (18:3n-3), whereas the *S. salar*
161 Δ5Fad and *S. canaliculatus* Δ4Fad were assayed with the Δ5 substrate eicosatetraenoic
162 acid (20:4n-3) and Δ4 substrate docosapentaenoic acid (22:5n-3), respectively.
163 Eicosatrienoic and eicosadienoic acids (> 99 % pure) were obtained from Nu-Chek Prep
164 Inc. (Elysan, USA), and eicosatetraenoic and docosapentaenoic acids (> 98 – 99 %
165 pure) were purchased from Cayman Chemical Co. (Ann Arbor, USA). Alpha-linolenic
166 acid (> 99 % pure) and chemicals used to prepare the *S. cerevisiae* minimal medium^{-}
167 ^{uracil} were from Sigma Chemical Co. Ltd. (Dorset, UK). The PUFA substrates were
168 added at final concentrations of 0.5 mM (C18), 0.75 (C20) and 1.0 (C22) mM as uptake
169 efficiency decreases with increasing chain length. After two days, yeast were harvested
170 and washed for fatty acid analyses (Hastings et al., 2001). Yeast transformed with
171 pYES2 containing no insert were grown under the same conditions as a control
172 treatment.
173
174 *FAME analysis by GC-MS*

175 Total lipids were extracted from yeast samples by homogenisation in
176 chloroform/methanol (2:1, v/v) containing 0.01% BHT as antioxidant. Fatty acid methyl
177 esters were prepared, extracted, purified, and analysed by GC in order to calculate the
178 proportion of substrate fatty acid converted to elongated fatty acid product as [product
179 area/(product area +substrate area)] x 100. Identities of fatty acid peaks were based on
180 GC retention times and confirmed by GC-MS as described previously (Agaba et al.,
181 2004; Hastings et al., 2001).
182
183 **Results**
184 *Fish fatty acyl desaturase phylogenetics*
185 The phylogenetic tree comparing the deduced AA sequences of fish Fad with those of
186 mammalian FADS family members and Δ8 desaturases isolated from unicellular
187 organisms is shown in Fig. 2. All teleostei desaturases cluster together, separately from
188 mammalian FADS1 and FADS2 proteins, and more distantly from the Δ8 desaturases of
189 unicellular organisms. All fish desaturases, regardless of species and functional activity
190 previously described, are more closely related to mammalian FADS2 than FADS1.
191
192 *Determination of* Δ8 *desaturation activity of fish Fad*
193 The capability of fish Fad for Δ8 desaturation was assessed by determining the fatty
194 acid profiles of transgenic yeast grown in presence of potential Δ8 substrates ETA
195 (20:3n-3) and EDA (20:2n-6). Yeast transformed with pYES2 vector alone showed the
196 main fatty acids found in *S. cerevisiae*, namely 16:0, 16:1 isomers, 18:0 and 18:1n-9,
197 together with the exogenously added fatty acid (data not shown). This is consistent with
198 *S. cerevisiae* lacking enzymes active on PUFA substrates (Hastings et al., 2001).
199 Transgenic yeast expressing fish *fad*, however, were capable of converting the

200 exogenously added fatty acids into desaturated products, with this ability of fish Fad
201 varying among species. The Fad from freshwater fish such as rainbow trout (OmΔ6Fad)
202 or zebrafish (DrΔ6Δ5Fad), together with those from the diadromous species Atlantic
203 salmon including SsΔ6Fad_b, SsΔ6Fad_c and SsΔ5Fad, exhibited limited Δ8
204 desaturation activity (Fig. 3; Table 3). Among them, SsΔ6Fad_b showed the highest
205 conversion rates, with 4.7 % and 4.0 % of ETA and EDA, respectively, converted to
206 their corresponding Δ8 desaturated products. However, considerably higher Δ8
207 desaturation activities were observed in Fad isolated from marine species (Fig. 4; Table
208 3). The desaturases characterised previously as Δ6 enzymes from cobia (RcΔ6Fad), cod
209 (GmΔ6Fad), gilthead seabream (SaΔ6Fad) and turbot (PmΔ6Fad) were capable of
210 efficiently desaturating 20:3n-3 and 20:2n-6 at the Δ8 position, producing 20:4n-3 and
211 20:3n-6, respectively (Fig. 4). In contrast to all previous observations suggesting that
212 fish desaturases are more efficient towards n-3 PUFA substrates in comparison to n-6
213 substrates (Tocher, 2003; 2010), similar conversion rates on both Δ8 substrates 20:3n-3
214 and 20:2n-6 were observed (Table 3).

215 Irrespective of species, the Δ8 desaturation activity of all fish Fad towards ETA
216 (20:3n-3) was consistently lower than the activities towards the Δ6-pathway n-3 PUFA
217 substrates (18:3n-3, 20:4n-3 or 22:5n-3) for which the enzymes had shown highest
218 desaturase activity in previous functional assays (Δhigh) (Table 3). Excepting the
219 rabbitfish Δ4 desaturase (ScΔ4Fad), marine fish Fad had relative action Δhigh/Δ8
220 activities ranging from 1.8 (RcΔ6Fad) to 4.2 (PmΔ6Fad), whereas
221 freshwater/diadromous species values were between 12.0 (SsΔ6Fad_b) and 91.2
222 (OmΔ6Fad) (Table 3).

223 The zebrafish DrΔ6Δ5Fad and rabbitfish ScΔ6Δ5Fad require special mention in
224 relation to the desaturated products observed when they were assayed for Δ8

225 desaturation activity. These enzymes, already characterised previously as bifunctional
226 Δ6/Δ5 desaturases (Hastings et al., 2001; Li et al., 2010), also showed the capability to
227 introduce double bonds into 20:3n-3 and 20:2n-6 at the Δ8 position. Interestingly,
228 though, the corresponding Δ8 desaturation products, 20:4n-3 and 20:3n-6, were
229 subsequently desaturated to 20:5n-3 and 20:4n-6, respectively, through the activity of
230 these enzymes for Δ5 desaturation (Fig. 5). Furthermore, a third product peak was
231 detected that GC-MS of the fatty acid methyl esters identified as a second 20:4 or 20:3
232 isomer, in the case of the 20:3n-3 and 20:2n-6 Δ8 precursors, respectively (Fig. 5). This
233 indicated that the Δ8 PUFA substrates were also desaturated at position Δ6 or Δ5
234 producing non-methylene-interrupted 20:4 or 20:3 products (Fig.5). However, the small
235 size of the peaks and the lower resolution of picolinyl derivatives meant it was not
236 possible to confirm the double bond structure and so it is not known if this additional
237 product was a Δ6 or Δ5 desaturated non-methylene-interrupted isomer, specifically
238 Δ6,11,14,17-20:4 or Δ5,11,14,17-20:4 in case of the 20:3n-3 substrate. Irrespective,
239 these results reveal that DrΔ6Δ5Fad and ScΔ6Δ5Fad appear to be true multifunctional
240 desaturases capable (at least) of introducing double bonds in positions Δ5, Δ6 and Δ8 of
241 appropriate polyunsaturated fatty acyl chains.
242
243 **Discussion**
244 Preliminary evidence of the existence of Δ8 desaturation activity in vertebrates was
245 reported in tumor cell lines (Bardon et al., 1996; Cook et al., 1991; Nakazawa et al.,
246 1976), mouse liver (Schenck et al., 1996), and rat and human testes (Albert et al., 1977,
247 1979). More recently, molecular studies identified *Fads2*, the mammalian gene
248 encoding Δ6 desaturase activity, as the enzyme responsible for Δ8 desaturation (Park et
249 al., 2009; Stroud et al., 2009). This suggested that, in mammals, the biosynthesis of LC-

250 PUFA such as EPA and ARA from 18:3n-3 and 18:2n-6 can proceed through the
251 'classical' Δ6-pathway (Δ6 desaturation → elongation → Δ5 desaturation), or
252 alternatively through the Δ8-pathway (elongation → Δ8 desaturation → Δ5
253 desaturation). Previously, studies with radiolabelled 18:3n-3 and 18:2n-6 provided
254 considerable evidence for the production of the appropriate Δ8 precursors, 20:3n-3 and
255 20:2n-6, in both primary cells and cell lines from freshwater, salmonid and marine fish
256 species (Ghioni et al., 1997; Tocher and Sargent, 1990a,b; Tocher et al., 1992; Tocher,
257 1993; Tocher and Dick, 1999). Furthermore it was also shown that radioactivity was
258 recovered in 20:3n-3 and 20:2n-6 in freshwater salmonids Arctic charr (*Salvenilus*
259 *alpinus*) and rainbow trout, and marine gilthead sea bream and golden grey mullet (*Liza*
260 *aurata*) after intraperitoneal injection of radiolabelled 18:2n-6 and 18:3n-3 (Mourente
261 and Tocher, 1993a,b, 1994, 1998; Olsen et al., 1992). In all these studies there was also
262 recovery of radiolabel in 18:4n-3 and 18:3n-6 as well as 20:4n-3 and 20:3n-6 and so it
263 was not possible to determine the precise pathway for the production of 20:4n-3 or
264 20:3n-6. Therefore, there was previously no conclusive evidence for the operation of the
265 Δ8-pathway for LC-PUFA biosynthesis in fish but all the necessary fatty acid
266 components of the pathway had been observed in metabolic studies. The present study
267 provides the first evidence that the necessary enzyme activity is present in teleost fish
268 within previously identified desaturase genes.

269 The phylogenetic analysis of the desaturases investigated in the present study, as well
270 as other phylogenetic trees reported in the literature (González-Rovira et al., 2009; Jaya-
271 Ram et al., 2011; Mohd-Yusof et al., 2010; Santigosa et al., in press; Yamamoto et al.,
272 2010; Zheng et al., 2004), suggested that the Fad encoding cDNAs cloned from fish are
273 orthologs of the mammalian *FADS2*. When functionally characterised, however,
274 teleostei Fad have shown a great diversity of substrate specificities. Consistent with the

275 primary function described for mammalian FADS2, the majority of Fad cDNAs isolated
276 from fish were characterised as monofunctional Δ6 desaturases (González-Rovira et al.,
277 2009; Mohd-Yusof et al., 2010; Santigosa et al., in press; Zheng et al., 2004), although
278 genes encoding Δ5 (Hastings et al., 2005) and Δ4 desaturases (Li et al., 2010), as well as
279 bifunctional Δ6/Δ5 Fad have been also found (Hastings et al., 2001; Li et al., 2010).
280 Such 'plasticity' among fish desaturases has been hypothesised to partly obey to an
281 adaptation to natural diets with low LC-PUFA contents in freshwater environments or
282 high preformed LC-PUFA contents in marine habitats. Consequently,
283 freshwater/diadromous fish possess desaturase activities capable of both Δ6 and Δ5
284 desaturation steps required for the biosynthesis of C_{18-24} LC-PUFA (Fig. 1), whereas
285 marine fish, at least carnivorous species, apparently lack Δ5 desaturase (Hastings et al.,
286 2005; Monroig et al., 2010; Zheng et al., 2004). The present study has therefore also
287 provided evidence that the ability of FADS2-like enzymes for Δ8 desaturation,
288 previously only described in mammals, is possibly widespread in other vertebrates such
289 as teleosts. However, despite their apparent phylogenetic similarities, the effective Δ8
290 desaturase enzyme activities of fish Fads varied notably among species and so, in
291 addition, the present study has indicated that divergence between the complement of
292 desaturase enzyme activities of marine and freshwater/diadromous species is also
293 evidenced in their efficiency for Δ8 desaturation.

294 Thus, the functional analyses in recombinant yeast has demonstrated that the
295 desaturase enzymes from freshwater/diadromous species exhibit relatively low ability to
296 desaturate the appropriate LC-PUFA, 20:3n-3 and 20:2n-6, at the Δ8 position. Genes
297 encoding monofunctional Δ5 and Δ6 desaturases from rainbow trout (OmΔ6Fad) and
298 Atlantic salmon (SsΔ5Fad, SsΔ6Fad_b, SsΔ6Fad_c), but also the bifunctional zebrafish
299 Δ6/Δ5 (DrΔ6Δ5Fad), showed conversion rates below 5.0 % of total added substrate

300 fatty acid. Although relatively low, some of these rates, namely SsΔ6Fad_b (4.7-4.0 %)
301 and DrΔ6Δ5Fad (2.2-1.5 %), were nonetheless comparable to those of baboon FADS2,
302 a gene assayed in similar experimental conditions, with reported conversions of 2.8 %
303 and 0.9 % for 20:3n-3 and 20:2n-6, respectively (Park et al., 2009). These results
304 confirm that, in addition to the previously described desaturase activities, the Fad of the
305 freshwater/diadromous fish species investigated have similar capability for Δ8
306 desaturation to that reported for mammalian FADS2. In contrast, other than the Δ4
307 desaturase from rabbitfish that has no Δ6 activity, the conversion rates demonstrated by
308 marine fish Fad were one order of magnitude higher than those of
309 freshwater/diadromous species, suggesting that the alternative 'Δ8-pathway' could be
310 more active in marine fish.

311 The Fad from cobia, Atlantic cod, gilthead seabream and turbot, previously
312 characterised as Δ6 desaturases, exhibited high efficiency for Δ8 substrates, with
313 conversion rates ranging from 14.4 to 31.3 %. Comparing the Δ6 and Δ8 desaturase
314 activities of each enzyme, it is clear that the Δ6 activity is the most prominent and likely
315 primary function of these Fad from marine fish. However, the relative Δ6/Δ8 activity
316 towards n-3 PUFA substrates ranged from 1.8 (cobia RcΔ6Fad) to 4.2 (turbot
317 PmΔ6Fad), notably lower (12.0 - 91.5) than those of freshwater/diadromous fish
318 desaturases and the Δ6/Δ8 activity ratio of 23 reported for baboon FADS2 (Park et al.,
319 2009). These findings confirm that marine fish Fad have higher efficiency for Δ8
320 desaturation than freshwater/diadromous orthologs. It is not clear what has driven this
321 divergence to greater Δ8 activity and potentially more functionally important dual
322 Δ6/Δ8 activities in these marine fish desaturases. Based on our current hypothesis
323 regarding the 'plasticity' among fish desaturases and adaptation to natural diets with
324 different levels of LC-PUFA, the increased dietary intake of Δ8 PUFA substrate, such

325 as 20:3n-3, that likely occurs in marine environments, could have partly driven the
326 bifunctionalisation of marine teleost desaturases towards Δ6/Δ8 enzymes. However, it is
327 unclear what benefit this evolutionary adaptation process would have in species with
328 deficient Δ5 desaturation that limits LC-PUFA biosynthesis or, indeed, that are
329 receiving adequate dietary EPA and DHA (Bell and Tocher, 2009; Leaver et al., 2008).
330 Clearly though, our results suggest that the general concept regarding 20:3n-3 and
331 20:2n-6 as 'dead-end' metabolic products needs to be revised, at least for marine
332 teleosts (Oxley et al., 2010; Pratoomyot et al., 2008; Ruyter et al., 2003). Thus, 20:3n-3
333 and 20:2n-6 produced by the action of elongases such as Elovl5 (Guillou et al., 2010)
334 towards 18:3n-3 and 18:2n-8, are likely reincorporated into the LC-PUFA biosynthetic
335 pathway of marine species for further desaturation and elongation conversions.

336 Our findings further demonstrate that multifunctionality is an extended trait among
337 desaturases. The zebrafish Δ6/Δ5 Fad (DrΔ6Δ5Fad) was the first bifunctional desaturase
338 described (Hatings et al., 2001). Desaturases with dual function were later described in
339 filamentous fungi (Δ12 and ω3) (Damude et al., 2006), the protozoan *Acanthamoeba*
340 *castellanii* (Δ12 and Δ15) (Sayanova et al., 2006), moths (Δ11 and Δ10,12) (Serra et al.,
341 2006), basidiomycete fungus (Δ12 and Δ15) (Zhang et al., 2007) and, more recently,
342 baboon whose FADS2 desaturase was reported to possess both Δ6 and Δ8 activities
343 (Park et al., 2009). Similarly to the zebrafish desaturase, a gene isolated from the marine
344 herbivore rabbitfish was also found to encode a bifunctional Δ6/Δ5 desaturase (Li et al.,
345 2010). However, the rabbitfish ScΔ6Δ5Fad demonstrated much higher Δ8 desaturation
346 activity than DrΔ6Δ5Fad. Although Δ6 desaturation remains the preferred or primary
347 activity in both DrΔ6Δ5Fad and ScΔ6Δ5Fad, calculations of the activity ratios obtained
348 in the present study (Δ6/Δ8) and previously (Δ6/Δ5) (Hastings et al., 2001; Li et al.,
349 2010), enable us to calculate and rank the activities of the zebrafish DrΔ6Δ5Fad as Δ6 >

350 Δ5 > Δ8 and the rabbitfish ScΔ6Δ5Fad as Δ6 > Δ8 > Δ5. Furthermore, both proteins,
351 particularly the ScΔ6Δ5Fad, also desaturated 20:3n-3 (and 20:2n-6) to non methylene
352 interupted PUFA (e.g. Δ6,11,14,17-20:4 or Δ5,11,14,17-20:4). These findings confirm
353 that both desaturases have unique positional specificities hitherto not reported among
354 vertebrate fatty acyl desaturases.

355 Fatty acyl desaturases are diverse enzymes in their positional specificity (Sperling et
356 al., 2003), and are typically classified according to their apparent regioselectivity
357 (Sasata et al., 2004). The Δx desaturases introduce an unsaturation at position x counted
358 from the carboxyl end of the fatty acid substrate. The ωy desaturases introduce the
359 double bond at position y from the methyl end. A third type of desaturase, termed υ+z,
360 introduce a double bond in position z referenced from an existing double bond.
361 According to this classification, fish Fad that were reported previously as Δ6 are
362 technically υ+3 desaturases, as the new double bond is introduced 3C beyond a pre-
363 existing double bond in either potential Δ6 or Δ8 substrates. Although, υ+3
364 regioselectivity can explain the major Δ6, Δ5 and Δ8 activities of ScΔ6Δ5Fad and
365 DrΔ6Δ5Fad, their ability to introduce non-methylene interrupted double bonds in 20:3n-
366 3 and 20:2n-6 suggests that these enzymes also have a strong preference for Δx
367 desaturation. Although combined υ+z/Δx modes were described previously in other
368 membrane-bound fatty acid desaturases from plants, fungus and nematode
369 (Meesapyodsuk et al., 2007), the present findings demonstrate that a similar
370 regioselectivity mode combination is also possible in vertebrate desaturases.

371 In summary, the present study has demonstrated that the Δ8 desaturation activity first
372 reported in baboon FADS2 is also a characteristic of fish orthologs. However, the Δ8
373 desaturase activity varies notably between freshwater/diadromous and marine fish
374 species, the latter possessing Fads2-like proteins that efficiently desaturate PUFA

375 substrates in the Δ8 position. Further investigations involving site directed-mutagenesis
376 experiments are required to elucidate the specific regions and AA residues controlling
377 such differences in substrate specificity of front-end fatty acyl desaturases.
378

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384

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594 *canadum*). Aquaculture 290, 122-131.
595

596 **Figure captions**

597 Fig 1. Pathways of long-chain polyunsaturated fatty acid biosynthesis from α-linolenic
598 (18:3n-3) and linoleic (18:2n-6) acids proposed in vertebrates. Dotted arrow indicates
599 conversions only reported by a Δ4 desaturase from rabbitfish *Siganus canaliculatus* (Li
600 et al., 2010) *Elovl5 is believed to elongate 18:3n-3 and 18:2n-6 (Guillou et al., 2010).
601
602 Fig 2. Phylogenetic tree comparing putative amino acid sequences of fish fatty acyl
603 desaturases (Fad) from freshwater, diadromous and marine species, with mammalian
604 FADS and Δ8 desaturases from unicellular organisms. The tree was constructed using
605 the neighbor-joining method (Saitou and Nei, 1987) with MEGA4. The horizontal
606 branch length is proportional to amino acid substitution rate per site. The numbers
607 represent the frequencies (%) with which the tree topology presented was replicated
608 after 10000 iterations. All accession numbers are from Genbank, except for the
609 *Acanthamoeba castellanii* Δ8 desaturase obtained from Ensembl.
610
611 Fig 3. Characterisation of Δ8 desaturation activities of freshwater and diadromous fish
612 fatty acyl desaturases (Fad). The rainbow trout OmΔ6Fad (panel A) and Atlantic salmon
613 SsΔ6Fad_b (panels B) transformed into yeast *Saccharomyces cerevisiae* that were
614 grown in the presence of Δ8 substrate, eicosatrienoic acid (20:3n-3). Fatty acids were
615 extracted from yeast transformed with pYES2 vector containing the ORF of the putative
616 Fad encoding cDNA as an insert. The first four peaks are the main endogenous fatty
617 acids of *S. cerevisiae*, namely 16:0 (1), 16:1 isomers (2), 18:0 (3), and 18:1n-9 (4). The
618 substrate 20:3n-3 and its corresponding desaturated product are indicated. Vertical axis,
619 FID response; horizontal axis, retention time.
620

621 Fig 4. Characterisation of Δ8 desaturation activities of marine fish desaturases (Fad).
622 The cobia RcΔ6Fad (panel A) and Atlantic cod (GmΔ6Fad) (panels B) transformed into
623 yeast *Saccharomyces cerevisiae* were grown in the presence of Δ8 substrate,
624 eicosatrienoic acid (20:3n-3). Fatty acids were extracted from yeast transformed with
625 pYES2 vector containing the ORF of the putative Fad encoding cDNA as an insert. The
626 first four peaks are the main endogenous fatty acids of *S. cerevisiae*, namely 16:0 (1),
627 16:1 isomers (2), 18:0 (3), and 18:1n-9 (4). Substrate 20:3n-3 and its corresponding
628 desaturated product are indicated. Vertical axis, FID response; horizontal axis, retention
629 time

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631 Fig 5. Characterisation of Δ8 desaturation activities of bifunctional Δ6/Δ5 desaturases.
632 The zebrafish DrΔ6Δ5Fad (panel A) and rabbitfish ScΔ6Δ5Fad (panels B) transformed
633 into yeast *Saccharomyces cerevisiae* were grown in the presence of Δ8 substrate,
634 eicosatrienoic acid (20:3n-3). Fatty acids were extracted from yeast transformed with
635 pYES2 vector containing the ORF of the putative fatty acyl desaturase cDNA as an
636 insert. The first four peaks are the main endogenous fatty acids of *S. cerevisiae*, namely
637 16:0 (1), 16:1 isomers (2), 18:0 (3), and 18:1n-9 (4). The substrate 20:3n-3 and its
638 corresponding desaturated products are indicated. Vertical axis, FID response;
639 horizontal axis, retention time.
640 *Non-methylene interrupted 20:4 (Δ6,11,14,17-20:4 or Δ5,11,14,17-20:4).

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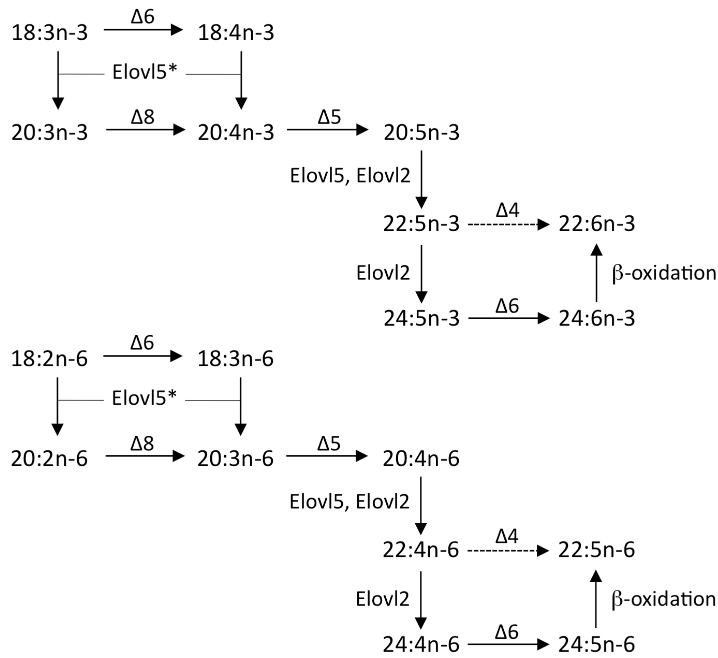
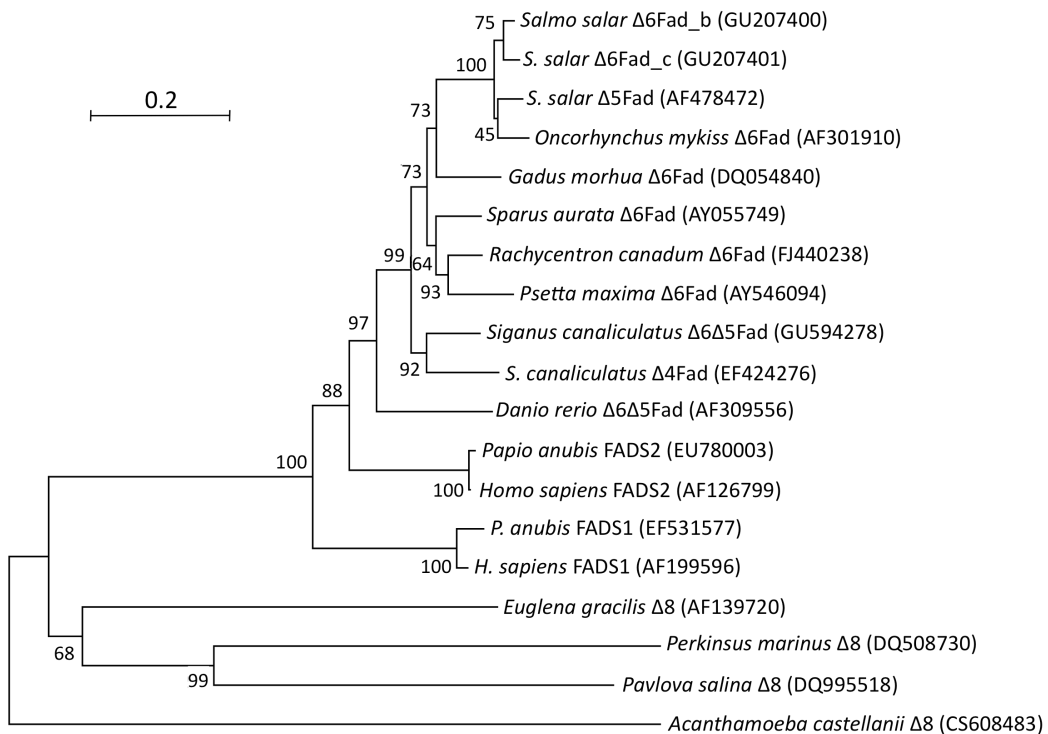
646 **Figures**

647 **Fig.1**



648

649 **Fig. 2**



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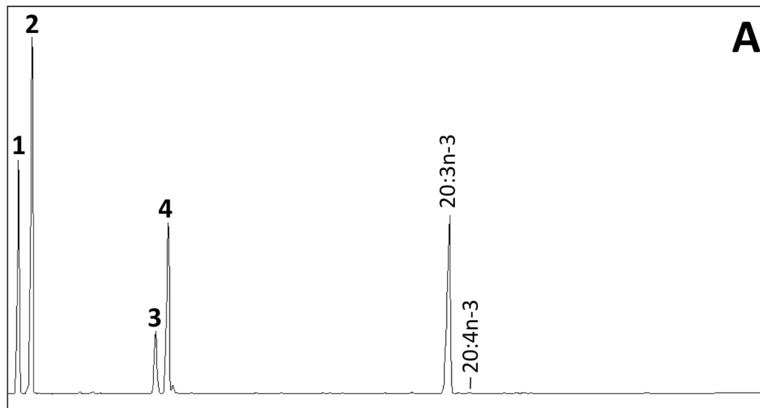
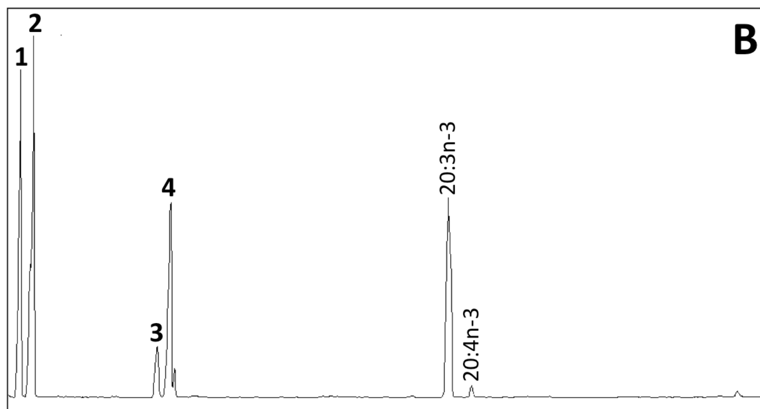
652 **Fig. 3**





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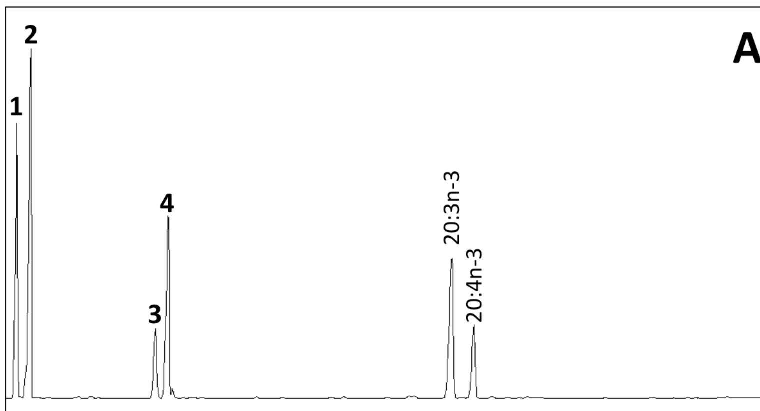
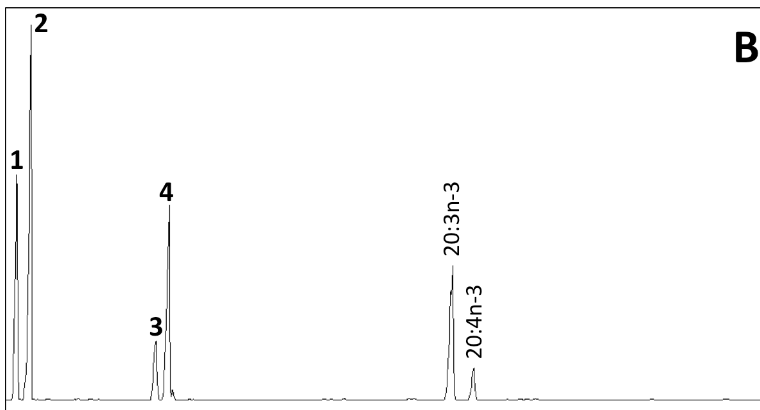
654 **Fig. 4**





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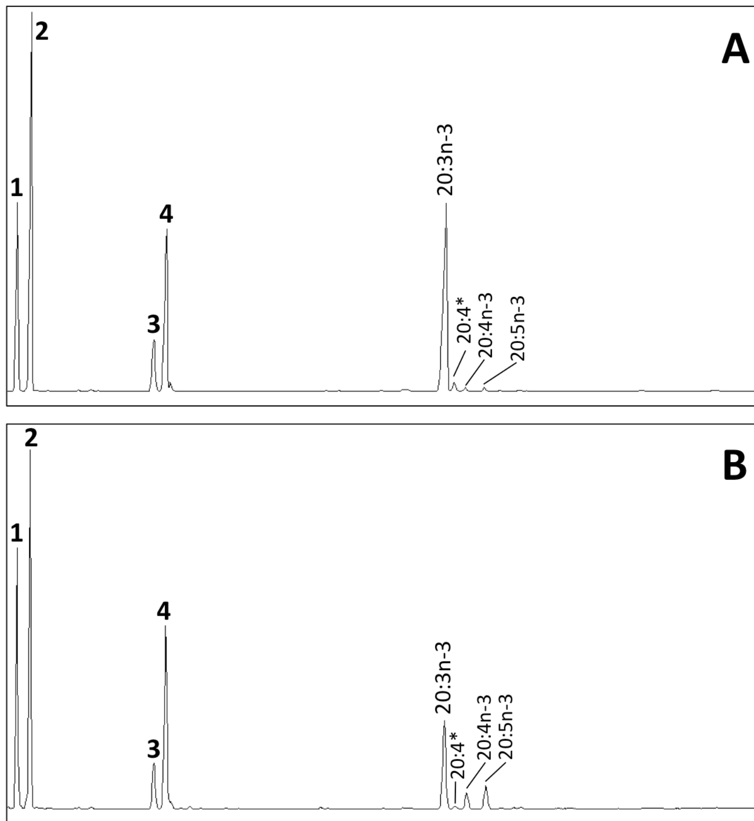
656 **Fig. 5**



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667 **Tables**

668 Table 1. Fish fatty acyl desaturases (Fad) investigated in the present study. The ability
669 for Δ8 desaturation was tested in eleven *fad* cDNAs from different species including
670 diadromous (*Salmo salar*), freshwater (*Oncorhynchus mykiss* and *Danio rerio*) and
671 marine (*Siganus canaliculatus*, *Rachycentron canadum*, *Gadus morhua*, *Sparus aurata*
672 and *Psetta maxima*) species. All Fad were functionally characterised in yeast over the
673 course of previous investigations, and their primary functions reported in the
674 corresponding publication.

Species	Common name	Desaturase name	Reported activity	Reference
Salmo salar	Atlantic salmon	SsΔ6Fad_b	Δ6	Hastings et al. (2005)
S. salar	Atlantic salmon	SsΔ6Fad_c	Δ6	Monroig et al. (2010)
S. salar	Atlantic salmon	SsΔ5Fad	Δ5	Monroig et al. (2010)
Oncorhynchus mykiss	Rainbow trout	OmΔ6Fad	Δ6	Zheng et al. (2004)
Danio rerio	Zebrafish	DrΔ6Δ5Fad	Δ6, Δ5	Hastings et al. (2001)
Siganus canaliculatus	Rabbitfish	ScΔ6Δ5Fad	Δ6, Δ5	Li et al. (2010)
S. canaliculatus	Rabbitfish	ScΔ4Fad	Δ4	Li et al. (2010)
Rachycentron canadum	Cobia	RcΔ6Fad	Δ6	Zheng et al. (2009)
Gadus morhua	Atlantic cod	GmΔ6Fad	Δ6	Tocher et al. (2006)
Sparus aurata	Gilthead seabream	SaΔ6Fad	Δ6	Zheng et al. (2004)
Psetta maxima	Turbot	PmΔ6Fad	Δ6	Zheng et al. (2004)

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Table 2. Sequence of the primer pairs used and accession number of the sequence used as reference for primer design for *fad* ORF cloning. Primers contained restriction sites (underlined) for further cloning into the yeast expression vector pYES2.

Desaturase	**Primer**	Primer sequence	Restriction site	GenBank Accession
Δ6Fad_b	SsD6bF	5'-CCCAAGCTTAGGATGGGGGGCGGAGGCC-3'	*Hind*III	GU207400
	SsD6bR	5'-CCGCTCGAGTTATTTATGGAGATATGCAT-3'	*Xho*I	
SsΔ6Fad_c	SsD6cF	5'-CCCAAGCTTAGGATGGGGGGCGGAGGCC-3'	*Hind*III	GU207401
	SsD6cR	5'-CCGCTCGAGTTATTTATGGAGATATGCAT-3'	*Xho*I	
SsΔ5Fad	SsD5F	5'-CCCAAGCTTACTATGGGGGGCGGAGGCG-3'	*Hind*III	AF478472
	SsD5R	5'- CCGCTCGAGTCATTTATGGAGATATGCAT-3'	*Xho*I	
OmΔ6Fad	OmD6F	5'-CGGAATTCAAGCTTAAGATGGGGGGCGGAGGTCA-3'	*Hind*III	AF301910
	OmD6R	5'-GCTCTAGACTCGAGTTATTTATGGAGATACGCATC-3'	*Xho*I	
DrΔ6Δ5Fad	DrD65F	5'-CCCAAGCTTACTATGGGTGGCGGAGGACAGC-3'	*Hind*III	AF309556
	DrD65R	5'-CCGCTCGAGTTATTTGTTGAGATACGC-3'	*Xho*I	
ScΔ6Δ5Fad	ScD65F	5'-CCCAAGCTTAGGATGGGAGGTGGAGGTC-3'	*Hind*III	EF424276
	ScD65R	5'-CCGTCTAGATCATTTATGGAGATATGC-3'	*Xba*I	
ScΔ4Fad	ScD4F	5'-CCCAAGCTTAGGATGGGAGGTGGAGGTC-3'	*Hind*III	GU594278
	ScD4R	5'-CCGTCTAGATCATTTATGGAGATATGC-3'	*Xba*I	
RcΔ6Fad	RcD6F	5'-CCCAAGCTTAAGATGGGAGGTGGAGGCCAGCTGAC-3'	*Hind*III	FJ440238
	RcD6R	5'-CCGCTCGAGTCATTTATGGAGATATGCATCAAGCC-3'	*Xho*I	
GmΔ6Fad	GmD6F	5'-CGGAATTCAAGCTTAAGATGGGAGGTGGAGGGCA-3'	*Hind*III	DQ054840
	GmD6R	5'-GCTCTAGACTCGAGTCACTTATGGAGATAAGCATC-3'	*Xho*I	
SaΔ6Fad	SaD6F	5'-CGGAATTCAAGCTTAAGATGGGAGGTGGAGGCCA-3'	*Hind*III	AY055749
	SaD6R	5'-GCTCTAGACTCGAGTCATTTATGGAGATAAGCATC-3'	*Xho*I	
PmΔ6Fad	PmD6F	5'-CGGAATTCAAGCTTAAGATGGGAGGTGVGAGGCCA-3'	*Hind*III	AY546094
	PmD6R	5'-GCTCTAGACTCGAGTCATTTATGGAGATATGCATC-3'	*Xho*I	

688 Table 3. Substrate conversions (percentages of total fatty acid substrate converted) of
689 transgenic yeast transformed with the fatty acyl desaturases (Fad) and grown in
690 presence of Δ8 substrates, eicosatrienoic (20:3n-3) and eicosadienoic (20:2n-6) acids.
691 Conversion rates towards the n-3 substrate for which highest activity (Δhigh) had been
692 previously reported are also shown. Thus, in addition to either 20:3n-3 or 20:2n-6, the
693 transgenic yeast were also assayed with the Δ6 substrate α-linolenic acid (18:3n-3),
694 except for *S. salar* Δ5Fad and *S. canaliculatus* Δ4Fad that were assayed with
695 eicosatetraenoic (20:4n-3) and docosapentaenoic (22:5n-3) acids, respectively.

Desaturase	n-3 Δ8 (20:3n-3 → 20:4n-3)	n-6 Δ8 (20:2n-6 → 20:3n-6)	n-3 Δhigh	n-3 Δhigh/Δ8
SsΔ6Fad_b	4.7	4.0	56.5	12.0
SsΔ6Fad_c	0.6	0.0	8.8	14.7
SsΔ5Fad	0.0	0.0	13.2	0.0
OmΔ6Fad	0.6	0.8	54.9	91.5
DrΔ6Δ5Fad*	1.5	2.2	33.6	22.4
ScΔ6Δ5Fad*	31.8	33.3	73.2	2.3
ScΔ4Fad	0.7	0.0	16.1	23.0
RcΔ6Fad	30.6	31.3	55.3	1.8
GmΔ6Fad	17.0	14.4	43.6	2.6
SaΔ6Fad	17.1	15.7	45.4	2.7
PmΔ6Fad	16.5	16.8	69.4	4.2

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697 *Conversion rates of Δ8 substrates include stepwise reactions due to multifunctional
698 desaturation abilities. For instance, the conversion rate of ScD6D5Fad on 20:3n-3
699 includes the Δ8 desaturation towards 20:4n-3, its subsequent Δ5 desaturation to 20:5n-3,
700 and also the desaturation of 20:3n-3 towards the non-methylene-interrupted products
701 Δ6,11,14,17-20:4 or Δ5,11,14,17-20:4 (see Results section).