

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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22 **Keywords**

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

25

## 26 **Summary**

27 The benefits of dietary fish and fish oil are derived from n-3 long-chain polyunsaturated  
28 fatty acids (LC-PUFA) that have beneficial effects in a range of human diseases and  
29 pathologies such as cardiovascular and other inflammatory disorders, neural  
30 development and neurological pathologies. The precursor of n-3 LC-PUFA, 18:3n-3  
31 does not have the same beneficial effects prompting interest in the pathways of  
32 endogenous synthesis of LC-PUFA in vertebrates. The LC-PUFA biosynthesis pathway  
33 classically involves  $\Delta 6$  and  $\Delta 5$  fatty acyl desaturases (Fad), but it was recently shown  
34 that  $\Delta 6$  Fad in mammals also displayed  $\Delta 8$  activity demonstrating a possible alternative  
35 “ $\Delta 8$ -pathway” for the synthesis of LC-PUFA. Our primary hypothesis was that  $\Delta 8$   
36 desaturase activity would be a common feature of vertebrate  $\Delta 6$  Fads, and so the aim of  
37 the present study was to determine the ability of teleostei Fads for  $\Delta 8$  desaturation  
38 activity. To this end, cDNAs for Fads from a range of freshwater, diadromous and  
39 marine teleost fish species were assayed for  $\Delta 8$  activity in the heterologous yeast  
40 expression system. In summary, the present study has demonstrated that  $\Delta 8$  desaturation  
41 activity was also a characteristic of fish orthologs, although the activity varied notably  
42 between freshwater/diadromous and marine fish species, with the latter possessing  
43 Fads2-like proteins with  $\Delta 8$  activity far higher than mammalian FADS2. The data  
44 showed that, generally, the fish Fad are technically  $\nu$ -3 desaturases, with new double  
45 bonds introduced 3C beyond a pre-existing double bond. However, the ability of  
46 zebrafish and rabbitfish Fads, previously characterised as  $\Delta 6/\Delta 5$  bifunctional  
47 desaturases, to introduce non-methylene interrupted double bonds in 20:3n-3 and 20:2n-  
48 6 suggested that a novel combination of regioselectivity modes operates within these  
49 enzymes.

50

## 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<sub>18</sub> PUFA (Fig. 1). Synthesis of EPA is achieved by  $\Delta$ 6 desaturation of  
69 18:3n-3 to produce 18:4n-3 that is elongated to 20:4n-3 followed by  $\Delta$ 5 desaturation,  
70 but DHA synthesis was believed to require two further elongation steps, a second  $\Delta$ 6  
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<sub>24</sub>  
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  $\Delta 6/\Delta 5$  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  $\Delta 4$  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  $\Delta 4$  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  $\Delta 4$  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  $\Delta 6$  Fad in mammals also displayed  $\Delta 8$  activity.  
90 Expression of baboon  $\Delta 6$  Fad (FADS2) cDNA in yeast promoted the  $\Delta 8$  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  $\Delta 8$  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  $\Delta 8$  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  $\Delta 4$  desaturation above, it is unclear how widespread among vertebrates the  $\Delta 8$   
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<sub>18</sub>  
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<sub>18</sub> 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  $\Delta 6$  Fad and Elovl5 elongase activities (Tocher, 2010). In  
114 contrast, many marine species appear to lack  $\Delta 5$  Fad and Elovl2 activities that severely  
115 limit their ability for endogenous production of LC-PUFA from C<sub>18</sub> precursors (Tocher,  
116 2010).

117 Our primary hypothesis was that  $\Delta 8$  desaturase activity is a common feature of  
118 vertebrate  $\Delta 6$  Fad and that the  $\Delta 8$  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  $\Delta 8$  desaturation activity. Thus, Fad  
121 encoding cDNAs from a range of freshwater, diadromous and marine teleost fish  
122 species were assayed for  $\Delta 8$  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  $\Delta 8$  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  $\Delta 6$ -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  $\Delta 8$  activity of fish desaturases was assayed by expressing their ORF in yeast *S.*  
148 *cerevisiae* grown in presence of potential substrates for  $\Delta 8$  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<sup>-uracil</sup>  
153 supplemented with either ETA or EDA. In order to directly compare the  $\Delta 8$  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  $\Delta 8$  substrates 20:3n-3  
157 and 20:2n-6, yeast transformed with the  $\Delta 6$ Fad from *S. salar* (isoforms b and c) and *O.*  
158 *mykiss*, the bifunctional  $\Delta 6\Delta 5$ Fad from *D. rerio* and *S. canaliculatus*, and the  $\Delta 6$ Fad  
159 from the marine species *R. canadum*, *G. morhua*, *S. aurata* and *P. maxima* were also  
160 grown in presence of the  $\Delta 6$  substrate  $\alpha$ -linolenic acid (18:3n-3), whereas the *S. salar*  
161  $\Delta 5$ Fad and *S. canaliculatus*  $\Delta 4$ Fad were assayed with the  $\Delta 5$  substrate eicosatetraenoic  
162 acid (20:4n-3) and  $\Delta 4$  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<sup>-</sup>  
167 <sup>uracil</sup> 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  $\Delta 8$  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  $\Delta 8$  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 $\Delta 8$ desaturation activity of fish Fad*

193 The capability of fish Fad for  $\Delta 8$  desaturation was assessed by determining the fatty  
194 acid profiles of transgenic yeast grown in presence of potential  $\Delta 8$  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 (Om $\Delta$ 6Fad)  
202 or zebrafish (Dr $\Delta$ 6 $\Delta$ 5Fad), together with those from the diadromous species Atlantic  
203 salmon including Ss $\Delta$ 6Fad\_b, Ss $\Delta$ 6Fad\_c and Ss $\Delta$ 5Fad, exhibited limited  $\Delta$ 8  
204 desaturation activity (Fig. 3; Table 3). Among them, Ss $\Delta$ 6Fad\_b showed the highest  
205 conversion rates, with 4.7 % and 4.0 % of ETA and EDA, respectively, converted to  
206 their corresponding  $\Delta$ 8 desaturated products. However, considerably higher  $\Delta$ 8  
207 desaturation activities were observed in Fad isolated from marine species (Fig. 4; Table  
208 3). The desaturases characterised previously as  $\Delta$ 6 enzymes from cobia (Rc $\Delta$ 6Fad), cod  
209 (Gm $\Delta$ 6Fad), gilthead seabream (Sa $\Delta$ 6Fad) and turbot (Pm $\Delta$ 6Fad) were capable of  
210 efficiently desaturating 20:3n-3 and 20:2n-6 at the  $\Delta$ 8 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  $\Delta$ 8 substrates 20:3n-3  
214 and 20:2n-6 were observed (Table 3).

215 Irrespective of species, the  $\Delta$ 8 desaturation activity of all fish Fad towards ETA  
216 (20:3n-3) was consistently lower than the activities towards the  $\Delta$ 6-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 ( $\Delta$ high) (Table 3). Excepting the  
219 rabbitfish  $\Delta$ 4 desaturase (Sc $\Delta$ 4Fad), marine fish Fad had relative action  $\Delta$ high/ $\Delta$ 8  
220 activities ranging from 1.8 (Rc $\Delta$ 6Fad) to 4.2 (Pm $\Delta$ 6Fad), whereas  
221 freshwater/diadromous species values were between 12.0 (Ss $\Delta$ 6Fad\_b) and 91.2  
222 (Om $\Delta$ 6Fad) (Table 3).

223 The zebrafish Dr $\Delta$ 6 $\Delta$ 5Fad and rabbitfish Sc $\Delta$ 6 $\Delta$ 5Fad require special mention in  
224 relation to the desaturated products observed when they were assayed for  $\Delta$ 8

225 desaturation activity. These enzymes, already characterised previously as bifunctional  
226  $\Delta 6/\Delta 5$  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  $\Delta 8$  position. Interestingly,  
228 though, the corresponding  $\Delta 8$  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  $\Delta 5$  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  $\Delta 8$  precursors, respectively (Fig. 5). This  
233 indicated that the  $\Delta 8$  PUFA substrates were also desaturated at position  $\Delta 6$  or  $\Delta 5$   
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  $\Delta 6$  or  $\Delta 5$  desaturated non-methylene-interrupted isomer, specifically  
238  $\Delta 6,11,14,17-20:4$  or  $\Delta 5,11,14,17-20:4$  in case of the 20:3n-3 substrate. Irrespective,  
239 these results reveal that Dr $\Delta 6\Delta 5$ Fad and Sc $\Delta 6\Delta 5$ Fad appear to be true multifunctional  
240 desaturases capable (at least) of introducing double bonds in positions  $\Delta 5$ ,  $\Delta 6$  and  $\Delta 8$  of  
241 appropriate polyunsaturated fatty acyl chains.

242

## 243 **Discussion**

244 Preliminary evidence of the existence of  $\Delta 8$  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  $\Delta 6$  desaturase activity, as the enzyme responsible for  $\Delta 8$  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’  $\Delta 6$ -pathway ( $\Delta 6$  desaturation  $\rightarrow$  elongation  $\rightarrow$   $\Delta 5$  desaturation), or  
252 alternatively through the  $\Delta 8$ -pathway (elongation  $\rightarrow$   $\Delta 8$  desaturation  $\rightarrow$   $\Delta 5$   
253 desaturation). Previously, studies with radiolabelled 18:3n-3 and 18:2n-6 provided  
254 considerable evidence for the production of the appropriate  $\Delta 8$  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  $\Delta 8$ -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  $\Delta 6$  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  $\Delta 5$  (Hastings et al., 2005) and  $\Delta 4$  desaturases (Li et al., 2010), as well as  
279 bifunctional  $\Delta 6/\Delta 5$  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  $\Delta 6$  and  $\Delta 5$   
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  $\Delta 5$  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  $\Delta 8$  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  $\Delta 8$   
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  $\Delta 8$  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  $\Delta 8$  position. Genes  
297 encoding monofunctional  $\Delta 5$  and  $\Delta 6$  desaturases from rainbow trout (Om $\Delta 6$ Fad) and  
298 Atlantic salmon (Ss $\Delta 5$ Fad, Ss $\Delta 6$ Fad\_b, Ss $\Delta 6$ Fad\_c), but also the bifunctional zebrafish  
299  $\Delta 6/\Delta 5$  (Dr $\Delta 6\Delta 5$ Fad), showed conversion rates below 5.0 % of total added substrate

300 fatty acid. Although relatively low, some of these rates, namely Ss $\Delta$ 6Fad\_b (4.7-4.0 %)   
301 and Dr $\Delta$ 6 $\Delta$ 5Fad (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  $\Delta$ 8   
306 desaturation to that reported for mammalian FADS2. In contrast, other than the  $\Delta$ 4   
307 desaturase from rabbitfish that has no  $\Delta$ 6 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 ‘ $\Delta$ 8-pathway’ could be   
310 more active in marine fish.

311 The Fad from cobia, Atlantic cod, gilthead seabream and turbot, previously   
312 characterised as  $\Delta$ 6 desaturases, exhibited high efficiency for  $\Delta$ 8 substrates, with   
313 conversion rates ranging from 14.4 to 31.3 %. Comparing the  $\Delta$ 6 and  $\Delta$ 8 desaturase   
314 activities of each enzyme, it is clear that the  $\Delta$ 6 activity is the most prominent and likely   
315 primary function of these Fad from marine fish. However, the relative  $\Delta$ 6/ $\Delta$ 8 activity   
316 towards n-3 PUFA substrates ranged from 1.8 (cobia Rc $\Delta$ 6Fad) to 4.2 (turbot   
317 Pm $\Delta$ 6Fad), notably lower (12.0 - 91.5) than those of freshwater/diadromous fish   
318 desaturases and the  $\Delta$ 6/ $\Delta$ 8 activity ratio of 23 reported for baboon FADS2 (Park et al.,   
319 2009). These findings confirm that marine fish Fad have higher efficiency for  $\Delta$ 8   
320 desaturation than freshwater/diadromous orthologs. It is not clear what has driven this   
321 divergence to greater  $\Delta$ 8 activity and potentially more functionally important dual   
322  $\Delta$ 6/ $\Delta$ 8 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  $\Delta$ 8 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  $\Delta 6/\Delta 8$  enzymes. However, it is  
327 unclear what benefit this evolutionary adaptation process would have in species with  
328 deficient  $\Delta 5$  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 Elov15 (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  $\Delta 6/\Delta 5$  Fad (Dr $\Delta 6\Delta 5$ Fad) was the first bifunctional desaturase  
338 described (Hastings et al., 2001). Desaturases with dual function were later described in  
339 filamentous fungi ( $\Delta 12$  and  $\omega 3$ ) (Damude et al., 2006), the protozoan *Acanthamoeba*  
340 *castellanii* ( $\Delta 12$  and  $\Delta 15$ ) (Sayanova et al., 2006), moths ( $\Delta 11$  and  $\Delta 10,12$ ) (Serra et al.,  
341 2006), basidiomycete fungus ( $\Delta 12$  and  $\Delta 15$ ) (Zhang et al., 2007) and, more recently,  
342 baboon whose FADS2 desaturase was reported to possess both  $\Delta 6$  and  $\Delta 8$  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  $\Delta 6/\Delta 5$  desaturase (Li et al.,  
345 2010). However, the rabbitfish Sc $\Delta 6\Delta 5$ Fad demonstrated much higher  $\Delta 8$  desaturation  
346 activity than Dr $\Delta 6\Delta 5$ Fad. Although  $\Delta 6$  desaturation remains the preferred or primary  
347 activity in both Dr $\Delta 6\Delta 5$ Fad and Sc $\Delta 6\Delta 5$ Fad, calculations of the activity ratios obtained  
348 in the present study ( $\Delta 6/\Delta 8$ ) and previously ( $\Delta 6/\Delta 5$ ) (Hastings et al., 2001; Li et al.,  
349 2010), enable us to calculate and rank the activities of the zebrafish Dr $\Delta 6\Delta 5$ Fad as  $\Delta 6 >$

350  $\Delta 5 > \Delta 8$  and the rabbitfish Sc $\Delta 6\Delta 5$ Fad as  $\Delta 6 > \Delta 8 > \Delta 5$ . Furthermore, both proteins,  
351 particularly the Sc $\Delta 6\Delta 5$ Fad, also desaturated 20:3n-3 (and 20:2n-6) to non methylene  
352 interrupted PUFA (e.g.  $\Delta 6,11,14,17-20:4$  or  $\Delta 5,11,14,17-20:4$ ). 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  $\Delta x$  desaturases introduce an unsaturation at position x counted  
358 from the carboxyl end of the fatty acid substrate. The  $\omega y$  desaturases introduce the  
359 double bond at position y from the methyl end. A third type of desaturase, termed  $\nu+z$ ,  
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  $\Delta 6$  are  
362 technically  $\nu+3$  desaturases, as the new double bond is introduced 3C beyond a pre-  
363 existing double bond in either potential  $\Delta 6$  or  $\Delta 8$  substrates. Although,  $\nu+3$   
364 regioselectivity can explain the major  $\Delta 6$ ,  $\Delta 5$  and  $\Delta 8$  activities of Sc $\Delta 6\Delta 5$ Fad and  
365 Dr $\Delta 6\Delta 5$ Fad, 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  $\Delta x$   
367 desaturation. Although combined  $\nu+z/\Delta x$  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  $\Delta 8$  desaturation activity first  
372 reported in baboon FADS2 is also a characteristic of fish orthologs. However, the  $\Delta 8$   
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  $\Delta 8$  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

### 379 **Acknowledgements**

380 This research and OM were supported by a Marie Curie Intra European Fellowship  
381 within the 7th European Community Framework Programme (PIEF-GA-2008-220929,  
382 COBIAGENE), with additional support from The Royal Society-National Natural  
383 Science Foundation of China joint grant (JP090748 and 31011130156).

384

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595

596 **Figure captions**



597 Fig 1. Pathways of long-chain polyunsaturated fatty acid biosynthesis from  $\alpha$ -linolenic  
598 (18:3n-3) and linoleic (18:2n-6) acids proposed in vertebrates. Dotted arrow indicates  
599 conversions only reported by a  $\Delta 4$  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  $\Delta 8$  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*  $\Delta 8$  desaturase obtained from Ensembl.  
610

611 Fig 3. Characterisation of  $\Delta 8$  desaturation activities of freshwater and diadromous fish  
612 fatty acyl desaturases (Fad). The rainbow trout Om $\Delta 6$ Fad (panel A) and Atlantic salmon  
613 Ss $\Delta 6$ Fad\_b (panels B) transformed into yeast *Saccharomyces cerevisiae* that were  
614 grown in the presence of  $\Delta 8$  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  $\Delta 8$  desaturation activities of marine fish desaturases (Fad).  
622 The cobia Rc $\Delta 6$ Fad (panel A) and Atlantic cod (Gm $\Delta 6$ Fad) (panels B) transformed into  
623 yeast *Saccharomyces cerevisiae* were grown in the presence of  $\Delta 8$  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

630

631 Fig 5. Characterisation of  $\Delta 8$  desaturation activities of bifunctional  $\Delta 6/\Delta 5$  desaturases.  
632 The zebrafish Dr $\Delta 6\Delta 5$ Fad (panel A) and rabbitfish Sc $\Delta 6\Delta 5$ Fad (panels B) transformed  
633 into yeast *Saccharomyces cerevisiae* were grown in the presence of  $\Delta 8$  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 ( $\Delta 6,11,14,17$ -20:4 or  $\Delta 5,11,14,17$ -20:4).

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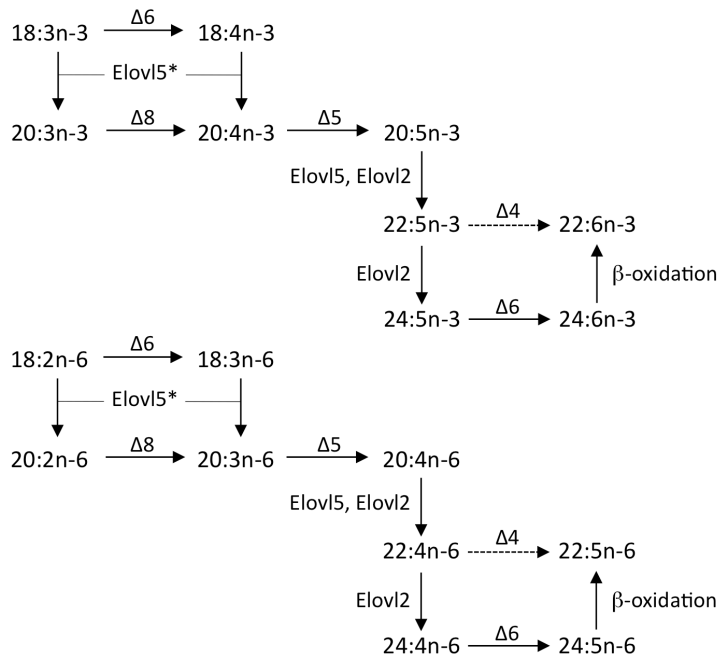
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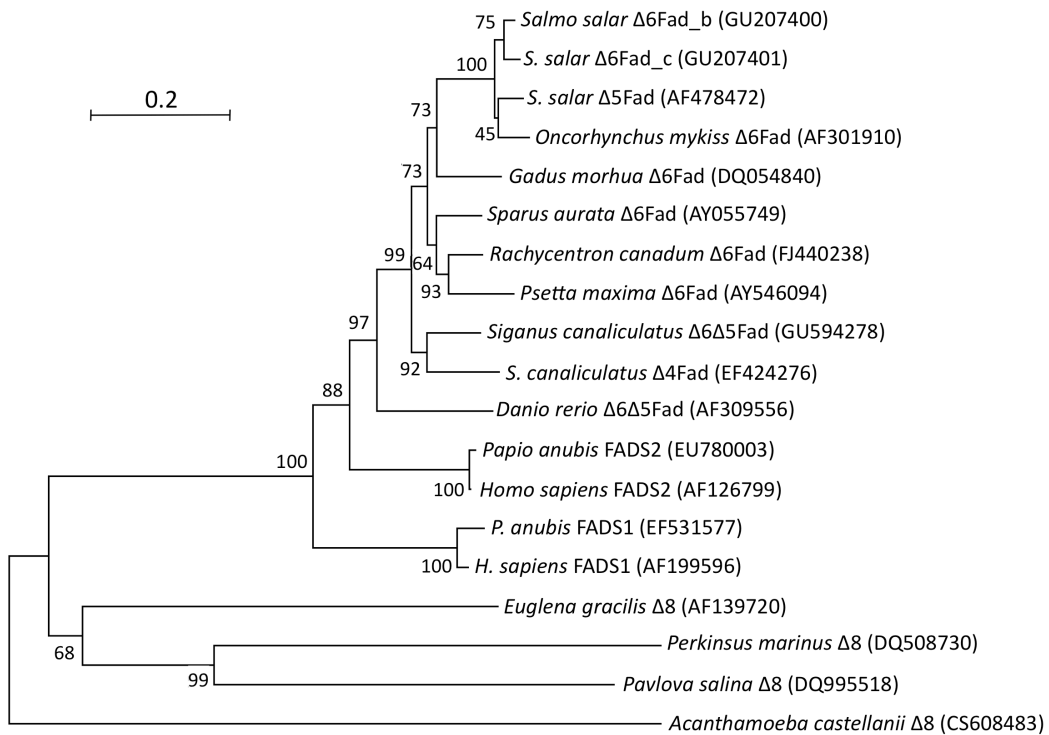
646 **Figures**

647 **Fig.1**



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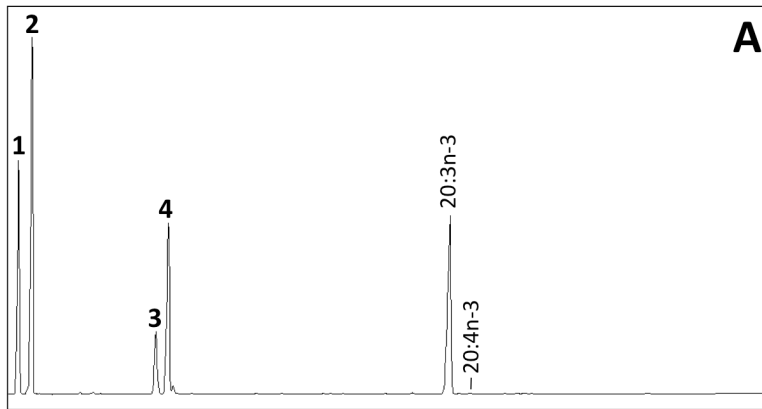
649 **Fig. 2**



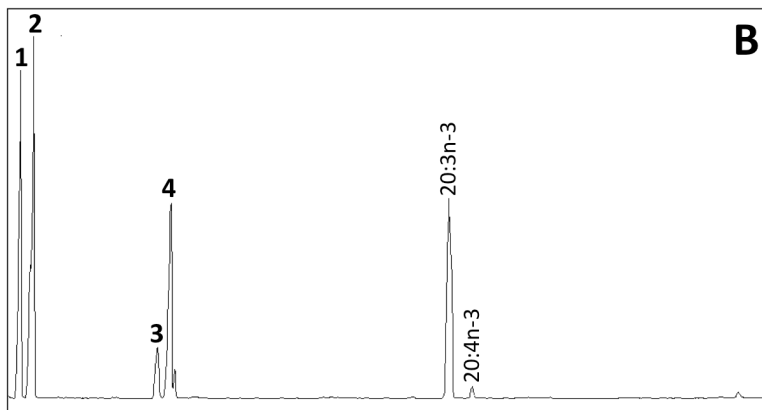
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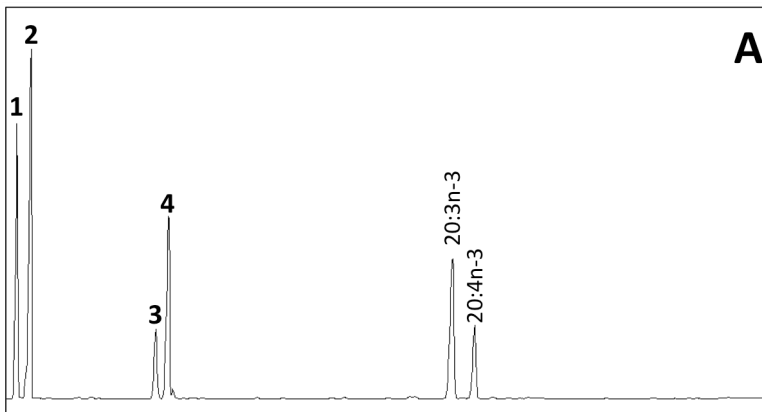
652 **Fig. 3**



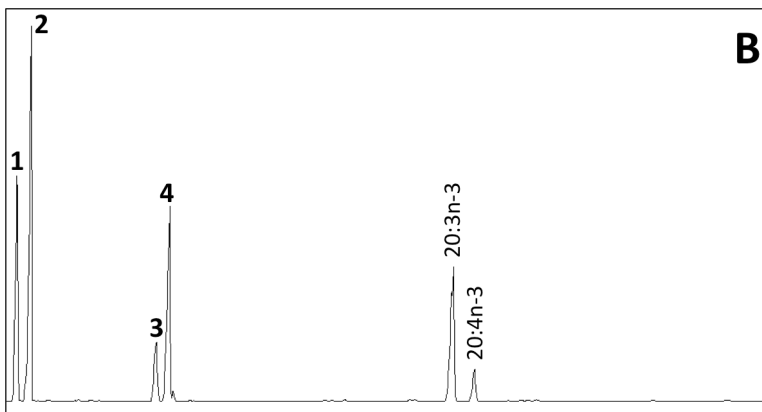
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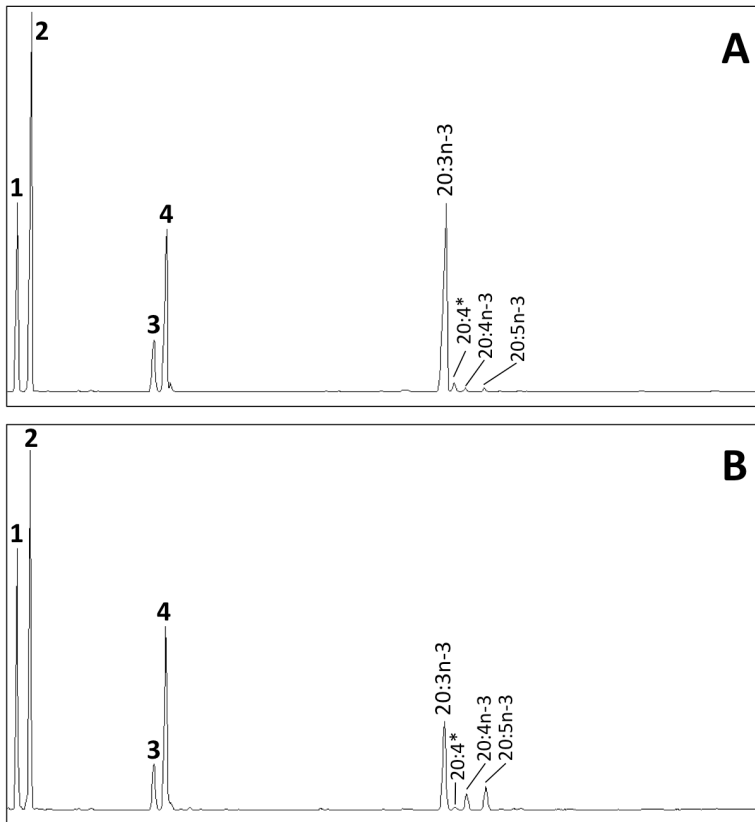
654 **Fig. 4**



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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  $\Delta 8$  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
<i>Salmo salar</i>	Atlantic salmon	Ss $\Delta 6$ Fad_b	$\Delta 6$	Hastings et al. (2005)
<i>S. salar</i>	Atlantic salmon	Ss $\Delta 6$ Fad_c	$\Delta 6$	Monroig et al. (2010)
<i>S. salar</i>	Atlantic salmon	Ss $\Delta 5$ Fad	$\Delta 5$	Monroig et al. (2010)
<i>Oncorhynchus mykiss</i>	Rainbow trout	Om $\Delta 6$ Fad	$\Delta 6$	Zheng et al. (2004)
<i>Danio rerio</i>	Zebrafish	Dr $\Delta 6\Delta 5$ Fad	$\Delta 6$ , $\Delta 5$	Hastings et al. (2001)
<i>Siganus canaliculatus</i>	Rabbitfish	Sc $\Delta 6\Delta 5$ Fad	$\Delta 6$ , $\Delta 5$	Li et al. (2010)
<i>S. canaliculatus</i>	Rabbitfish	Sc $\Delta 4$ Fad	$\Delta 4$	Li et al. (2010)
<i>Rachycentron canadum</i>	Cobia	Rc $\Delta 6$ Fad	$\Delta 6$	Zheng et al. (2009)
<i>Gadus morhua</i>	Atlantic cod	Gm $\Delta 6$ Fad	$\Delta 6$	Tocher et al. (2006)
<i>Sparus aurata</i>	Gilthead seabream	Sa $\Delta 6$ Fad	$\Delta 6$	Zheng et al. (2004)
<i>Psetta maxima</i>	Turbot	Pm $\Delta 6$ Fad	$\Delta 6$	Zheng et al. (2004)

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

Desaturase	Primer	Primer sequence	Restriction site	GenBank Accession
iΔ6Fad_b	SsD6bF	5'-CCCAAGCTTAGGATGGGGGCGGAGGCC-3'	<i>Hind</i> III	GU207400
	SsD6bR	5'-CCGCTCGAGTTATTTATGGAGATATGCAT-3'	<i>Xho</i> I	
SsΔ6Fad_c	SsD6cF	5'-CCCAAGCTTAGGATGGGGGCGGAGGCC-3'	<i>Hind</i> III	GU207401
	SsD6cR	5'-CCGCTCGAGTTATTTATGGAGATATGCAT-3'	<i>Xho</i> I	
SsΔ5Fad	SsD5F	5'-CCCAAGCTTACTATGGGGGCGGAGGCG-3'	<i>Hind</i> III	AF478472
	SsD5R	5'-CCGCTCGAGTCATTTATGGAGATATGCAT-3'	<i>Xho</i> I	
OmΔ6Fad	OmD6F	5'-CGGAATTC AAGCTT AAGATGGGGGCGGAGGTCA-3'	<i>Hind</i> III	AF301910
	OmD6R	5'-GCTCTAGACTCGAGTTATTTATGGAGATACGCATC-3'	<i>Xho</i> I	
DrΔ6Δ5Fad	DrD65F	5'-CCCAAGCTTACTATGGGTGGCGGAGGACAGC-3'	<i>Hind</i> III	AF309556
	DrD65R	5'-CCGCTCGAGTTATTTGTTGAGATACGC-3'	<i>Xho</i> I	
ScΔ6Δ5Fad	ScD65F	5'-CCCAAGCTTAGGATGGGAGGTGGAGGTC-3'	<i>Hind</i> III	EF424276
	ScD65R	5'-CCGCTCTAGATCATTATGGAGATATGC-3'	<i>Xba</i> I	
ScΔ4Fad	ScD4F	5'-CCCAAGCTTAGGATGGGAGGTGGAGGTC-3'	<i>Hind</i> III	GU594278
	ScD4R	5'-CCGCTCTAGATCATTATGGAGATATGC-3'	<i>Xba</i> I	
RcΔ6Fad	RcD6F	5'-CCCAAGCTT AAGATGGGAGGTGGAGGCCAGCTGAC-3'	<i>Hind</i> III	FJ440238
	RcD6R	5'-CCGCTCGAGTCATTTATGGAGATATGCATCAAGCC-3'	<i>Xho</i> I	
GmΔ6Fad	GmD6F	5'-CGGAATTC AAGCTT AAGATGGGAGGTGGAGGGCA-3'	<i>Hind</i> III	DQ054840
	GmD6R	5'-GCTCTAGACTCGAGTCACTTATGGAGATAAGCATC-3'	<i>Xho</i> I	
SaΔ6Fad	SaD6F	5'-CGGAATTC AAGCTT AAGATGGGAGGTGGAGGCCA-3'	<i>Hind</i> III	AY055749
	SaD6R	5'-GCTCTAGACTCGAGTCATTTATGGAGATAAGCATC-3'	<i>Xho</i> I	
PmΔ6Fad	PmD6F	5'-CGGAATTC AAGCTT AAGATGGGAGGTGAGAGGCCA-3'	<i>Hind</i> III	AY546094
	PmD6R	5'-GCTCTAGACTCGAGTCATTTATGGAGATATGCATC-3'	<i>Xho</i> I	

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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  $\Delta 8$  substrates, eicosatrienoic (20:3n-3) and eicosadienoic (20:2n-6) acids.  
 691 Conversion rates towards the n-3 substrate for which highest activity ( $\Delta$ high) 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  $\Delta 6$  substrate  $\alpha$ -linolenic acid (18:3n-3),  
 694 except for *S. salar*  $\Delta 5$ Fad and *S. canaliculatus*  $\Delta 4$ Fad that were assayed with  
 695 eicosatetraenoic (20:4n-3) and docosapentaenoic (22:5n-3) acids, respectively.

Desaturase	n-3 $\Delta 8$ (20:3n-3 $\rightarrow$ 20:4n-3)	n-6 $\Delta 8$ (20:2n-6 $\rightarrow$ 20:3n-6)	n-3 $\Delta$ high	n-3 $\Delta$ high/ $\Delta 8$
Ss $\Delta 6$ Fad_b	4.7	4.0	56.5	12.0
Ss $\Delta 6$ Fad_c	0.6	0.0	8.8	14.7
Ss $\Delta 5$ Fad	0.0	0.0	13.2	0.0
Om $\Delta 6$ Fad	0.6	0.8	54.9	91.5
Dr $\Delta 6\Delta 5$ Fad*	1.5	2.2	33.6	22.4
Sc $\Delta 6\Delta 5$ Fad*	31.8	33.3	73.2	2.3
Sc $\Delta 4$ Fad	0.7	0.0	16.1	23.0
Rc $\Delta 6$ Fad	30.6	31.3	55.3	1.8
Gm $\Delta 6$ Fad	17.0	14.4	43.6	2.6
Sa $\Delta 6$ Fad	17.1	15.7	45.4	2.7
Pm $\Delta 6$ Fad	16.5	16.8	69.4	4.2

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697 \*Conversion rates of  $\Delta 8$  substrates include stepwise reactions due to multifunctional  
 698 desaturation abilities. For instance, the conversion rate of Sc $\Delta 6\Delta 5$ Fad on 20:3n-3  
 699 includes the  $\Delta 8$  desaturation towards 20:4n-3, its subsequent  $\Delta 5$  desaturation to 20:5n-3,  
 700 and also the desaturation of 20:3n-3 towards the non-methylene-interrupted products  
 701  $\Delta 6,11,14,17$ -20:4 or  $\Delta 5,11,14,17$ -20:4 (see Results section).