Molecular and Functional Characterization and Expression Analysis of a Δ6 Fatty Acyl Desaturase cDNA of European Sea Bass (*Dicentrarchus labrax* L.)

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

29 **Abstract**. The extent to which fish species can produce highly unsaturated fatty acids
30 (HUFA) from C_{18} fatty acids varies with their complement of fatty acyl desaturase
31 (FAD) enzymes. Marine fish are unable to produce HUFA at a significant rate due to
32 apparent deficiencies in one or more enzymatic steps in the desaturation/elongation
33 pathway. It is not known if this is due to a lack of the genes or to tight regulation of
34 the enzymatic activity in some of the transformation steps. In the present study, we
35 report molecular cloning, cDNA, protein and functional analysis of a Δ6 FAD of
36 European sea bass (*Dicentrarchus labrax* L.), and describe its tissue expression and
37 nutritional regulation. An FAD cDNA contig sequence from brain tissue of sea bass
38 was obtained by gene walking, and full-length cDNA was obtained by amplification
39 using 5'end forward and 3'end reversed primers. The full length of the sea bass FAD
40 cDNA was 2089 bp, which included a 5'-UTR (untranslated region) of 267 bp, a 3'-
41 UTR of 484 bp and an open-reading frame (ORF) of 1338 bp, which specified a
42 protein of 445 amino acids. The mRNA size, estimated by northern blot analysis was
43 2.1 kb, consistent with the cDNA. Transient expression of Δ6-FAD-EGFP in HeLa
44 cells showed the protein compartmentalized to the endoplasmic reticulum. Functional
45 expression in yeast showed the sea bass cDNA encoded a unifunctional Δ6 FAD
46 enzyme. The sea bass FAD was more active towards 18:3n-3 with 14.5% being
47 converted to 18:4n-3 compared to 5.6% of 18:2n-6 converted to 18:3n-6. Expression
48 of the Δ6 FAD gene in sea bass tissues showed a rank order of heart, brain, ovary,
49 kidney, adipose tissue and liver as determined by RT-qPCR. Nutritional regulation of
50 gene expression was studied. Diets containing partial substitution of fish oil with
51 rapeseed or linseed oils induced up-regulation of the Δ6 FAD gene; whereas, a diet
52 containing olive oil did not influence the expression. Similarly, when fish oil was

53 partially replaced by blends of vegetable oils, one increased expression and one did

54 not.

55

56

56 **1. Introduction**

57

58 Fish are our major dietary source of n-3 highly unsaturated fatty acids (HUFA),
59 eicosapentaenoate (EPA; 20:5n-3) and docosahexaenoate (DHA; 22:6n-3)
60 (Simopoulos, 2000; Tidwell and Allan, 2002) and, with traditional fisheries
61 declining, global catches from the feed-grade fisheries that provide fish oil (FO) and
62 fish meal for aquafeed formulations have reached their sustainable limits and soon
63 there will be insufficient FO to maintain current aquaculture growth (Tacon, 2004;
64 Pike, 2005). Vegetable oils (VOs), a sustainable alternative to FO, can be rich in C_{18}
65 polyunsaturated fatty acids (PUFA) such as 18:2n-6 and 18:3n-3, but lack the n-3
66 HUFA abundant in FO (Sargent et al., 2002). The extent to which fish can convert
67 C_{18} PUFA to HUFA varies according to their capacity for fatty acyl desaturation and
68 elongation (Tocher, 2003). Marine fish are unable to produce EPA and DHA from
69 18:3n-3 at a physiologically significant rate (Sargent et al., 2002) due to apparent
70 deficient activity in one or more enzymes in the desaturation/elongation pathway
71 (Ghioni et al., 1999; Tocher and Ghioni, 1999). Thus, flesh fatty acid compositions in
72 marine fish fed VOs are characterized by increased levels of C_{18} PUFA and
73 decreased levels of n-3 HUFA, compromising their nutritional value to the human
74 consumer (Izquierdo et al., 2003; Regost et al., 2003; Mourente et al., 2005a;
75 Mourente and Bell, 2006).

76 The European sea bass (*Dicentrarchus labrax*) is among the most important
77 carnivorous marine finfish species cultured in Europe, particularly in the
78 Mediterranean region. Sea bass production in 2006 was 88.531 Tm (FAO/Globefish,
79 2007), and is still experiencing a rapid expansion. However, knowledge of its
80 nutritional requirements is still incomplete compared to other fish species, such as

81 salmonids and carps (Oliva-Teles, 2000; Kaushik, 2002; Skalli and Robin, 2004).
82 Therefore, the establishment of large-scale, sustainable sea bass culture will require
83 solutions to several nutritional issues including broodstock and larval nutrition and
84 replacement of dietary FO with alternatives in on-growing diets (Kaushik, 2004).
85 The requirement to use alternative components to FO for aquafeed formulations
86 makes necessary to determine genetic capacities and metabolic possibilities of the
87 cultivated fish for use of those new components. Especially outstanding are these
88 considerations in the case of the marine fish. In these context, our main goal is to
89 establish how the HUFA biosynthesis pathway functions in marine fish, to know
90 which are the enzymes and genes implicated in the process and to determine what
91 regulates HUFA biosynthesis and how it can be optimized to enable marine fish to
92 make effective use of dietary VOs. Recently, fatty acyl desaturases (FADs), critical
93 enzymes in the pathways for biosynthesis of long-chain HUFA from shorter chain
94 PUFA, have been cloned from several teleosts (Seiliez et al., 2001; 2003; Hasting et
95 al., 2001; 2005; Tocher et al., 2006; Zheng et al., 2005a, 2009). The cDNAs for Δ6
96 FADs have been cloned from diadromous, freshwater and marine species (Seiliez et
97 al., 2001, 2003; Zheng et al., 2005a, 2009; Tocher et al., 2006). Functional
98 characterization of the fish Δ6 FAD cDNAs showed that these enzymes were able of
99 transforming 18:3n-3 to 18:4n-3 in a heterologous yeast expression system (Zheng et
100 al., 2004a, 2005a, 2009; Tocher et al., 2006). However, relatively little is known
101 about the subcellular localization of Δ6 FAD in fish, in particular and in animals, in
102 general. In the only previous study concerning this question, Fujihara (1984)
103 demonstrated working with the Δ6 FAD of rat liver, that this enzyme was associated
104 with microsomal membranes in the cytoplasm. Production of C20 HUFA requires a
105 further Δ5 desaturation and Δ5 FAD has been cloned from Atlantic salmon (*Salmo*

106 *salar* L.), and a bifunctional Δ6/Δ5 FAD from the freshwater zebrafish (*Danio rerio*)
107 (Hasting et al., 2001; 2005). However, attempts to clone Δ5 FAD from marine fish
108 have failed (Seiliez et al., 2003; Tocher et al., 2006; Zheng et al., 2009). In
109 salmonids, clear nutritional regulation of FAD gene expression has been observed,
110 with expression of both Δ6 and Δ5 FADs up-regulated in fish fed VO compared to
111 fish fed FO (Zheng et al., 2004b, 2005 a, b). In contrast, nutritional effects are not
112 clear in marine fish (Seiliez et al., 2003; Tocher et al., 2006; Izquierdo et al., 2008).
113 Taken together, these data indicate clear differences in the HUFA synthesis pathway
114 between marine fish and fresh water or diadromous fish.
115 The specific aims of the study described here were to investigate genes of fatty acid
116 desaturation and the regulation of the HUFA biosynthetic pathway in European sea
117 bass. Thus, we describe the cDNA cloning, functional characterization, subcellular
118 localization, tissue distribution and expression of a Δ6 FAD of European sea bass that
119 would be the first, and reputedly rate-limiting, enzyme activity required for the
120 production of EPA and DHA. Moreover, this paper also describes the effects of diet
121 on the expression of the Δ6 FAD gene in liver of sea bass fed diets containing either
122 FO or diets with 60% substitution of FO with single VOs and/or blends of VOs.
123

124 **2. Materials and Methods**

125

126 2.1 cDNA Cloning

127 Total RNA was obtained from European sea bass brain tissue using Perfect RNA™,
128 Eukaryotic, Mini Kit (Eppendorf, Hamburg, Germany) following the manufacturer's
129 instructions, and contaminating genomic DNA eliminated by DNase I digestion
130 (USB, Cerdanyola, Spain). Complimentary DNA was synthesized using SuperScript

131 III RT reverse transcriptase (Invitrogen, Barcelona, Spain) primed by the
132 oligonucleotide Not I-oligo-dT, 5'-ATAAGAATGCGGCCGC(T)$_{20}$-3' which include
133 a *NotI* restriction site (underline). Reverse transcription products were RNase H
134 treated (Invitrogen, Barcelona, Spain) and cDNA was purified by filtration using a
135 Centri-sep column (Princeton Separation, Adelphia, NJ). A homolpolimeric dC tail
136 was added to the 5' end of cDNA using Terminal Deoxynucleotidyl Tranferase
137 (Invitrogen, Barcelona, Spain), and the tailed cDNA directly used for PCR
138 amplification assays.
139 Full length cDNA of putative Δ6 FAD was obtained by PCR, using a "gene walking"
140 strategy. Firstly, 3'-RACE was used to obtain a sea bass FAD cDNA fragment.
141 Nucleotide sequences of cDNAs of available fish Δ6 FADs were aligned to design a
142 degenerate forward primer, 5'-TGGTGGAAYCAYAGRCAYTTCCAGCA-3', with
143 the sequence codon usage optimized for sea bass. The reverse primer was Not I-
144 oligo-dT 5'-ATAAGAATGCGGCCGC(T)$_{20}$-3'. PCR amplification was performed
145 using EcoTaq DNA Polymerase (Ecogen, Madrid, Spain) in 50 μL, under the
146 following conditions: initial denaturation at 94 ^{o}C for 2 min, 40 cycles of
147 denaturation at 94 ^{o}C for 15 s, annealing at 57 ^{o}C for 30 s and extension at 72 ^{o}C for
148 50 s and a final extension step of 72 ^{o}C for 7 min. The PCR product was gel purified
149 and cloned into pCR 4-TOPO vector (Invitrogen, Barcelona, Spain) following the
150 manufacturer's instructions. The nucleotide sequences of five clones were determined
151 by standard dye terminator chemistry using the Big Dye Terminator v3.1 Cycle
152 Sequencing kit (Applied Biosystems, Alcobendas, Spain) in an ABI PRISM 3100
153 Genetic Analyzer (Applied Biosystems, Alcobendas, Spain). The consensus sequence
154 thus derived was used to design a specific reverse primer 5'-
155 TCTGGCTTGATGCATATCTCCA-3', which was used together with a new

156 degenerate forward primer 5'-TACACMTGGGAGGAGGTSCAG-3' to obtain an
157 overlapping PCR fragment in the 5' direction of the cDNA. For amplification, the
158 following conditions were used: initial denaturation at 94 ^{o}C for 2 min, 40 cycles of
159 denaturation at 94 ^{o}C for 15 s, annealing at 55 ^{o}C for 30 s, extension at 68 ^{o}C for 2
160 min and a final extension step of 72 ^{o}C for 7 min. After five new clones containing
161 the appropriate product were sequenced, a new specific reverse primer 5'-
162 CCAGCATAGTGGCTGATGACAC-3' was used in conjunction with a forward Not
163 I-oligo-dG primer (5'-ATAAGAAT<u>GCGGCCGC</u>TAAA$(G)_{15}$H-3') to perform 5'
164 RACE. Amplification involved an initial step at 94 ^{o}C for 2 min and 35 cycles of
165 denaturation at 94 ^{o}C for 15 s, annealing at 60 ^{o}C for 30 s, extension at 68 ^{o}C for 1
166 min and a final extension step at 72 ^{o}C for 10 min. Five new clones, containing the
167 appropriate fragment, were obtained and sequenced. All consensus sequences from
168 each of the three overlapping fragments obtained were aligned to determine a contig
169 nucleotide sequence of the sea bass putative FAD cDNA. Based on this sequence,
170 two specific primers, at the 5'-end forward 5'-
171 CCTCGAATGATCGGCTCGGAATT-3' and at the 3'-end reverse 5'-
172 CCTTTATTGTAAGACGTAGAGTTGA-3', were designed and used to PCR
173 amplify the full length cDNA for sea bass FAD, under the following conditions:
174 initial denaturation at 94 ^{o}C for 2 min, 30 cycles of denaturation at 94 ^{o}C for 30 s,
175 annealing at 50 ^{o}C for 30 s, extension at 72 ^{o}C for 2 min and a final extension step of
176 72 ^{o}C for 7 min, using KOD HOT Start DNA Polymerase (Novagen, Cerdanyola,
177 Spain). Ten clones containing the appropriate product were sequenced to
178 unequivocally confirm the complete sequence of the sea bass FAD mRNA
179 (GeneBank accession number AM746703).
180

181 2.2 Northern blot analysis

182

183 Northern blot analysis was carried out as described previously (Pendón et al., 1994).
184 Briefly, total RNA was obtained from brain using Perfect RNA^{TM}, Eukaryotic, Mini
185 Kit (Eppendorf, Hamburg, Germany) following the instruction manual. Thirty µg of
186 total RNA were denatured, electrophoresed on a 1.2 % agarose-2.2 M formaldehyde
187 gel, and transferred and fixed to a positively charged nylon membrane (Pall
188 Corporation, Florida, USA). Membrane was blocked in Church mix (Sodium
189 Phosphate Buffer 0.25M, pH 7.2, EDTA 1.0 mM, BSA 1%, SDS 7%) at 60 ^{o}C. Full-
190 length sea bass FAD cDNA was ^{32}P labeled by random priming and used as a
191 specific probe to hybridize the membrane at 60 ^{o}C over 16 h in Church mix. Two
192 washes were carried out under high-stringency conditions (0.1 x SSC, 0.5% SDS) at
193 60 ^{o}C, for 30 min. The filters were autoradiographed for 3 days with Curix RP2 film
194 (Agfa, Barcelona, Spain) at -80 °C using an intensifying screen.

195

196 2.3 DNA and protein sequence analysis

197

198 Standard DNA and protein sequence analyses were performed using BLASTn and
199 BLASTp searches (http://blast.ncbi.nlm.nih.gov). Multiple sequence alignments of
200 deduced amino acids sequence were performed using the CLUSTALw2 (1.4)
201 algorithm (http://www.ebi.ac.uk/Tools/clustalw2/). Phylogenetic and molecular
202 evolutionary analyses were conducted using *MEGA* version 4 (Tamura et al. 2007).
203 The evolutionary history was inferred using the UPGMA method (Sneath and Sokal,
204 1973). The evolutionary distances were computed using the Poisson correction
205 method (Zuckerkandl and Pauling, 1965). Conserved domains were investigated

206 using the Conserved Domain Search tool at NCBI

207 (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Secondary structure and

208 transmembrane expanded helix predictions were investigated using ANTHEPRO

209 2000 v.60 software, included in the software packages tool for secondary structure at

210 EsPAXy Proteomics Server (http://www.expasy.ch/), SVMtm Transmembrane

211 Domain Predictor bioinformatic tool (Yuan et al., 2004) in the ARC Center of

212 Excellence in Bioinformatic (http://bioinformatics.org.au/) and Predictprotein

213 software (http://www.predictprotein.org).

214

215 2.4 Functional characterization of sea bass FAD open reading frame (ORF)

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217 Functional characterization of the putative FAD cDNA ORF was performed by two

218 complementary methods: a) cellular, by determining the subcellular localization of

219 the ORF encoded protein in HeLa cells and b) biochemical, by determining the

220 enzymatic activity of the putative protein codified by heterologous expression of the

221 ORF in the yeast *Saccharomyces cerevisiae*.

222

223 2.4.1 Subcellular localization of sea bass desaturase ORF in HeLa cells

224

225 Subcellular localization of the putative FAD enzyme coded by cDNA, was analyzed

226 by transiently expression in HeLa cell of the ORF fused, at the C-terminus, to the

227 Enhanced Green Fluorescent Protein (EGFP) marker. The mean ORF of putative

228 FAD cDNA was amplified using D6LFEY-221 5'-

229 TTTTAGTGTAAGCTTCAGGTGG-3' as forward primer, which included a *Hind III*

230 restriction site (underlined), and D6LTR 5'-

231 CAACCATGGTTTTATGGAGATATGCATCAAG-3' as reversed primer, which
232 included a *Nco I* site (underlined). PCR was performed using the previously obtained
233 full length cDNA as template and a high fidelity hot start polymerase (KOD HOT
234 Start, Novagen, Cerdanyola, Spain), following the manufacturer's instructions. The
235 following conditions were used in a final volume of 50 μL: initial denaturation at 94
236 ^{o}C for 2 min, 25 cycles of denaturation at 94 ^{o}C for 30 s, annealing at 59.2 ^{o}C for 30
237 s, extension at 72 ^{o}C for 2 min and a final extension step of 72 ^{o}C for 7 min. The PCR
238 fragment was gel purified, *Hind III* and *Nco I* restricted and ligated into the similarly
239 digested and gel-purified mammalian expression vector pEGFP-N1 (Clontech,
240 Madrid, Spain). Ligation products were transformed in Top-10 *E. coli* chemically
241 competent cells (Invitrogen, Barcelona, Spain). Transformants were screened for the
242 correct construction and sequenced to confirm that variations had not taken place in
243 the nucleotide sequence. The resultant recombinant cDNA coding for the fusion
244 protein Δ6-FAD-EGFP was under the immediate early promoter region of human
245 cytomegalovirus (HCMV). The plasmid pD6DL/EGFP was purified using the
246 GenElute^{TM} HP Plasmid Midiprep Kit (Sigma-Aldrich, Tres Cantos, Spain) and used
247 to transfect HeLa cells. The pDsRed2-ER vector (Clontech, Madrid, Spain) was used
248 to localize endoplasmic reticulum in HeLa cells.
249 HeLa cell were grown on coverslips in 35-mm plates in DMEM medium
250 (Cambrex/BioWhittaker, Barcelona, Spain) supplemented with 10 % fetal bovine
251 serum until 60 % confluent, and co-transfected with pD6DL/EGFP and pDSRed2-ER
252 plasmid (3μg each plasmid per plate), using FuGENE$^{®\ HD}$ Transfection Reagent
253 (Roche, Madrid, Spain) following the instruction manual. At 24, 48 and 72 h after
254 transfection, cells were washed in phosphate-buffered saline (PBS) and fixed with 2
255 % paraformaldehyde (Sigma-Aldrich, Tres Cantos, Spain) for 10 min a room

256 temperature. Then coverslips were washed in PBS for 5 min, permeabilized with 0.1
257 % (v/v) Triton X-100 (Sigma-Aldrich, Tres Cantos, Spain) in PBS for 15 min,
258 washed in PBS for 5 min, stained with 0.2 µg/mL DAPI (Sigma-Aldrich, Tres
259 Cantos, Spain) in PBS and finally mounted in 0.02 % (v/v) glycerol in PBS. Cells
260 were observed for the presence of green fluorescent fusion protein (Δ6-FAD-EGFP)
261 and for red fluorescence of DsRed2 using a 63 x PLAN NEOFLUAR immersion oil
262 objective, mounted in a Zeiss Axiphot microscope (Carl Zeiss, Jena, Germany).
263 Images were taken using a SPOT cool digital CDD Camera (Diagnostic Instruments
264 Inc. Sterling Heights, MI, USA) and SPOT software v.4.6 (Diagnostic Instruments
265 Inc. Sterling Heights, MI, USA). Quantitative co-localization and image analyses
266 were performed using ImageJ 1.42k Software (http://rsb.info.nih.gov.ij). Pearson's
267 correlation coefficient (r_p), Overlap coefficient (r) (Manders et al., 1992) and Li's
268 Intensity Correlation Quotient (ICQ) (Li et al., 2004) were used to quantify the
269 degree of co-localization between green and red fluorescent images.
270
271 2.4.2 Heterologous expression of sea bass FAD ORF in *S. cerevisiae*.
272
273 Expression primers were designed for PCR cloning of the sea bass putative FAD
274 cDNA ORF. The forward primer, D6LFEY-221 (5'-
275 TTTTAGTGTAAGCTTCAGGTGG-3') contained a *HindIII* site (underlined) and the
276 reverse primer, D6LREY-1620 (5-AGAGAATTAGAATTCGTCATTTA-3')
277 contained an *Eco RI* site (underlined). PCR was performed using high fidelity DNA
278 polymerase (KOD HOT Start DNA Polymerase, Novagen, Cerdanyola, Spain)
279 following the manufacturer's instructions. Amplification involved an initial
280 denaturation step at 94 ^{o}C for 2 min, followed by 25 cycles of denaturation at 94 ^{o}C

281 for 30 s, annealing at 53 ^{o}C for 30 s, and extension at 72 ^{o}C for 2 min, followed by a
282 final extension at 72 ^{o}C for 7 min. The proper PCR fragment was gel purified, *Hind*
283 *III* and *Eco RI* restricted, and ligated into the similarly digested yeast expression
284 vector pYES2 (Invitrogen, Barcelona, Spain), that left the FAD ORF under the
285 inducible control of *GAL-1* promoter. Ligation product was transformed in Top10 *E.*
286 *coli* chemically competent cells (Invitrogen, Barcelona, Spain), which were screened
287 for the presence of correct construct. Selected clones were sequenced to confirm that
288 variations had not taken place in the sequence of the plasmids. Transformation of the
289 yeast *S. cerevisiae* strain InvSc1 (Invitrogen, Barcelona, Spain) with the recombinant
290 plasmids pD6D/pYES2 was carried out using the S.c. EasyComp Transformation Kit
291 (Invitrogen, Barcelona, Spain) following the manufacturer's instructions. Selection of
292 yeast containing the FAD/pYES2 constructs was on *S. cerevisiae* minimal medium
293 (SCMM) minus uracil. Culture of the recombinant yeast was carried out in SCMM^{-}
294 ^{uracil} broth as described previously (Hasting et al., 2001), using galactose induction of
295 gene expression. Each culture was supplemented with one of the following PUFA
296 substrates; 18:3n-3, 18:2n-6, 20:4n-3 and 20:3n-6, added to the yeast cultures at
297 concentrations of 0.5 mM (C_{18}) and 0.75 mM (C_{20}) as uptake efficiency decreases
298 with increasing chain length. Yeast cells were harvested, washed, dried, and lipid
299 extracted by homogenization in chloroform/methanol (2:1, by vol.) containing 0.01%
300 butylated hydroxytoluene as antioxidant as described previously (Hasting et al.,
301 2001). Fatty acid methyl esters were prepared, extracted, purified by thin layer
302 chromatography, and analyzed by gas chromatography (GC), all as described
303 previously (Hastings et al., 2001). The proportion of substrate fatty acid converted to
304 the longer chain fatty acid product was calculated from the gas chromatograms as
305 100 × [product area/(product area + substrate area)]. Unequivocal confirmation of

306 fatty acid products was obtained by GC-mass spectrometry of the picolinyl
307 derivatives as described in detail previously (Hasting et al., 2001).
308
309 2.5 RNA extraction and quantitative PCR (qPCR).
310
311 Tissue distribution and transcriptional regulation of the sea bass FAD gene was
312 studied by determining relative expression by qPCR using samples obtained from
313 two nutritional trials investigating the effect of dietary FO replacement with VOs (see
314 Mourente et al., 2005 a, b; Mourente and Bell, 2006). Samples were collected from
315 liver, brain, heart, kidney, gonad and adipose tissue and frozen immediately in liquid
316 nitrogen and stored at –80 ^{o}C prior to RNA extraction and gene expression studies.
317 Total RNA extractions were performed using Aurum^{TM}. Total RNA Mini kit (Bio-
318 Rad, Alcobendas, Spain) following the instruction manual and cDNA synthesis
319 performed using 3 μg of total RNA and iScript^{TM}cDNA Synthesis kit (Bio-Rad,
320 Alcobendas, Madrid). PCR primers were designed according to the sea bass FAD
321 sequence obtained above (accession no. AM746703), with forward and reverse
322 primers being 5'-AACTGAGGATGAAAATGTTCTCC-3' (d6-lu-1961F) and 5'-
323 CCTTTATGGTAAGACGTAGAGTTGC-3' (d6-lu-2049R) respectively, which
324 amplified a product size of 89 bp. As reference gene, primers for β-actin were
325 designed from sequence in the GeneBank database (accession no. AY148350). The
326 forward and reverse primers were 5'- CAAGATCATTGCCCCACCTGAG-3'
327 (bACTL981F) and 5'- GCAGATGTGGATCAGCAAGCAG-3' (bACTL1080R),
328 respectively, which amplified a product of 99 bp. Both FAD and β-actin amplicons
329 were verified by sequencing. All normalized data were referred to control diet (FO)
330 showing the lowest expression level of FAD gene, using the $\Delta\Delta C_T$ method (Livaka

331 and Schmittgen, 2001). Amplification of cDNA samples was carried out in a
332 MiniOpticom Real Time PCR System (Bio-Rad, Alcobendas, Madrid), using IQ™
333 SYBR® Green Supermix Kit (Bio-Rad, Alcobendas, Madrid) in 20 µL final volume
334 and the following conditions: 2 min denaturation at 94 ^{o}C, 40 cycles of 20 s at 94 ^{o}C,
335 15 s at 63 ^{o}C and 10 s at 72 ^{o}C. Samples were taken from liver: five animals per diet,
336 three replicates per animal and cDNA from 25 ng of total RNA per replicate, were
337 assayed. For tissue expression analysis, samples were taken from three animals, three
338 replicates per tissue and cDNA from 25 ng of total RNA per replicate, were assayed.
339 A melting curve was performed to confirm that a single PCR product was amplified.
340 Quantitative analyses were performed using MJ OpticonMonitor™ Analysis
341 Software v.3.1 (Bio-Rad, Alcobendas, Madrid). A standard curve using serial
342 dilutions of linearized FAD plasmid was prepared to assess reaction optimization and
343 proper quantification. To compare the expression level among tissue samples, one-
344 way analysis of variance (ANOVA) followed by Tukey's multiple comparison test
345 (Zar, 1984) ($P<0.05$) was performed.
346
347 2.6 Materials
348
349 Eicosatetraenoic (20:4n-3), (> 98-99 % pure) was purchased from Cayman Chemical
350 Co., Ann Arbor, USA. Linoleic (18:2n-6), α-linolenic (18:3n-3) and eicosatrienoic
351 (20:3n-6) acids (all >99% pure) were obtained from Sigma-Aldrich Co. Ltd. (Poole,
352 U.K.). All solvents were HPLC grade and were from Fisher Scientific
353 (Loughborough, UK).
354

355 **3. Results**

356

357 3.1 Cloning and sequence analyses of full-length cDNA for FAD of European sea
358 bass.

359

360 Three overlapping fragments for a putative Δ6 FAD of European sea bass were
361 obtained using a gene-walking strategy, a FAD contig sequence deduced, and
362 specific primers designed, at the 5'-end and 3'-ends, to amplify full length cDNA.
363 The full length of the putative sea bass FAD cDNA, was 2089 bp, including a 5'-
364 UTR of 267 bp, a 3'-UTR of 484 bp and a mean ORF of 1338 bp, which specified a
365 protein of 445 amino acids (Fig. 1A). The 5'-UTR contained eight ATG sequences
366 and 19 stop codons in the three possible reading frames upstream from the ATG
367 sequence we considered as the start codon for the Δ6 FAD protein. Analysis of the
368 predicted amino acid sequence showed that it included all the characteristic features
369 of a membrane-bound desaturase, including an N-terminal cytochrome b_5 domain
370 (amino acids 21 to 96) and a Δ6 FADS-like domain (amino acids 163 to 413) (Fig.
371 1B). The b_5 domain contains the conserved haem-binding motif H-P-G-G (Fig. 2A),
372 and the Δ6 FADS-like domain contained three conserved histidine boxes (His-box).
373 The deduced protein contains three highly hydrophobic regions, as shown by the
374 hydrophobicity plot of the protein (Fig. 2B). The prediction analysis show the protein
375 has four possible transmembrane segments (TMS) (Fig. 2A), similar to other
376 members of the desaturase family of proteins. However, an additional His-box
377 (HEKHH) (amino acids 350-354) is present in the Δ6-FADS-like domain and,
378 although the amino acid sequence of this additional His-box is not conserved among
379 species, the polarity/charge of amino acids appears to be conserved between marine

380 species (Fig. 1A). Northern blots analysis showed a unique band. The estimated size
381 for Δ6 FAD mRNA was 2.1 kb, which accurately agreed with the length of the cDNA
382 (Fig. 3). Amino acid sequence alignment of the predicted sea bass FAD with Δ6
383 FADs from other fish species indicated a high level of conservation (Fig. 1A). A pair-
384 wise comparison among fish Δ6 FAD sequences showed the amino acid sequence
385 predicted by the sea bass putative FAD shared greatest amino acid identity (94 %) to
386 Δ6 FAD of sea bream (*Sparus aurata* L.), with 83 % and 82 % identity to Δ6 FADs
387 of turbot (*Psetta maximus*) and cod (*Gadus morhua* L.), respectively (Table 1).
388 Phylogenetic analysis comparing the sea bass FAD amino acid sequence with FADs
389 from other fish species, and other organisms including mammals, clustered the sea
390 bass FAD most closely with sea bream, then turbot and cod, and more distantly from
391 freshwater species and salmonids (Fig. 4).
392
393 3.2 Heterologous expression in yeast *S. cerevisiae*
394
395 The fatty acid composition of the yeast transformed with the vector alone showed the
396 four main fatty acids normally found in *S. cerevisiae*, namely 16:0, 16:1n-7, 18:0 and
397 18:1n-9 (peaks 1, 2, 3and 4 respectively), together with the exogenously added fatty
398 acids 18:3n-3 and 18:2n-6 (peaks 5 and 7, respectively) (Fig. 5A and C). When yeast
399 transformed with the vector containing the sea bass FAD ORF were grown in the
400 presence of the Δ6 substrates, 18:3n-3 and 18:2n-6, prominent additional peaks (6
401 and 8) were observed in the fatty acid profiles (Fig. 5B and D). Based on GC
402 retention time and confirmed by GC-MS, the additional peaks associated with the
403 presence of the sea bass FAD cDNA were identified as 18:4n-3 (peak 6) (Fig. 5B)
404 and 18:3n-6 (peak 8) (Fig. 5D), corresponding to the Δ6 desaturation products of

405 18:3n-3 and 18:2n-6, respectively. All peaks were assigned as previously described
406 by Hastings et al. (2001). Approximately 14.5 % of 18:3n-3 was converted to 18:4n-3
407 and 5.6 % of 18:2n-6 was converted to 18:3n-6 in yeast transformed with the sea bass
408 FAD plasmid. No additional peaks representing desaturated fatty acid products were
409 observed in the profiles of *S. cerevisiae* transformed with sea bass Δ6 FAD and
410 incubated with 20:4n-3 or 20:3n-6 (peaks 9 and 10, respectively) (Fig.5E and F),
411 indicating the enzyme codified by the sea bass cDNA showed no Δ5 desaturase
412 activity. Similarly, the sea bass FAD cDNA did not show any Δ4 desaturase activity
413 as evidenced by the lack of additional peaks representing desaturated products of
414 22:5n-3 or 22:4n-6 (data not shown)).
415
416 3.3 Subcellular localization
417
418 HeLa cells were transfected with pD6D/EGFP plasmid to determine the subcellular
419 localization. After 48 h, the fusion protein Δ6 FAD ORF-tagged EGFP (Δ6-FAD-
420 EGFP) was highly expressed, showing a diffuse cytoplasmatic distribution
421 surrounding the nucleus that didn’t extend through the entire cytoplasm, suggesting
422 possible localization in endoplasmic reticulum (ER). Since the expression pattern
423 alone was not sufficiently clear to define the localization, an ER marker was also
424 used. Thus, HeLa cells were co-transfected with vectors expressing Δ6-FAD-EGFP
425 and DsRed2-ER, designed to specifically localize ER. All evaluated coefficients of
426 colocalization indicated that cells expressing both vectors showed a high degree of
427 co-localization of Δ6-FAD-EGFP and ER-targeted DS-Red2-ER proteins, indicating
428 that Δ6 FAD was compartmentalized to the ER (Fig. 6). Although this work was
429 focused on determining the intracellular localization of Δ6 FAD protein, we observed

430 that a high percentage of transfected cells (> 40 %) presented apoptotic nuclei (data
431 not show).
432
433 3.4 Expression of Δ6 FAD in sea bass
434
435 Expression of the sea bass Δ6 FAD gene was examined in various tissues from fish
436 fed a diet containing FO. The expression level in six different tissues was expressed
437 as mean of absolute copy number (± SD) of Δ6 FAD transcripts present in 25 ng of
438 total RNA (Whelan et al., 2003). The highest level of Δ6 FAD gene expression was
439 observed in heart, brain and ovary, while kidney, adipose tissue and liver showed
440 considerably lower expression levels (Fig. 7). The effects of dietary fatty acid
441 composition on the expression of the Δ6 FAD were determined in liver from sea bass
442 fed FO or diets with partial replacement of FO with VOs (Fig. 8). The expression of
443 Δ6 FAD was up-regulated in fish fed diets with 60 % of the FO replaced with
444 rapeseed or linseed oils, but not olive oil (Fig. 8A). Moreover, in sea bass fed FO
445 compared to 60 % substitution of FO with two different blends of the same three
446 VOs, the expression of Δ6 FAD was only significantly up-regulated in fish fed one of
447 the blends (Fig. 8B)
448
449 **4. Discussion**
450
451 The study reported here revealed that European sea bass express a fatty acid
452 desaturase as a unique mRNA of 2.1 kB, was expressed in heart > brain > ovary >
453 kidney > adipose tissue > liver, showed intracellular localization in ER when
454 expressed in HeLa cells and was confirmed as a Δ6 FAD when its activity/specificity

455 was determined in a yeast expression system. Comparing the protein sequence with
456 that of a range of other FADs of fish showed the sea bass Δ6 FAD sequence to be
457 more similar to the Δ6 FADs previously cloned from other marine fish, turbot, cod
458 and, especially, gilthead sea bream (Seiliez et al., 2003; Zheng et al., 2004; Tocher et
459 al., 2006). Phylogenetic analysis of the fish FAD sequences reflected classical
460 phylogeny (Fig 4), showing the Acanthopterygia (cichlids, perciformes and
461 pleuronectiformes) line, that includes sea bass and sea bream (both perciformes), with
462 cod (Paracanthopterygii; Gadiformes) as a branch and further separated from both the
463 carp and zebrafish (Ostariophysi; cyprinids), and salmonids (Salmoniformes;
464 salmonidae) (Nelson, 1994).
465 Northern blot analysis of total RNA from brain of sea bass, showed a unique signal
466 for Δ6 FAD transcript with an approximate size of 2.1 kb. Same results were obtained
467 when different tissues were assayed (data not show). Unlike sea bass, two Δ6 FAD
468 transcripts, with an approximate size of 3.8 and 1.8 kb, were detected in sea bream
469 tissues (Seiliez et al., 2003). This difference can be due to the low astringent
470 hybridization and washing conditions used in the assay (Seiliez et al., 2003).
471 However, when Northern blots analysis of total RNA from sea bream was carried out
472 in our lab under higher astringent conditions (60 ^{o}C, 0.1 x SSC, 0.5% SDS), only one
473 transcript of about 3.7 kb for Δ6 FAD was detected (data not show).
474 In order to functionally characterize Δ6 FAD protein we have determined its
475 enzymatic activity and specificity and its subcellular localization. Up to now the
476 functional characterization of fish desaturases has consisted on the
477 determination/characterization of its enzymatic activity, assuming that its subcellular
478 localization was the appropriate one. Subcellular localization is a key functional
479 characteristic of proteins. For proper functioning, the protein has to be translocated to

480 the correct intra- or extracellular compartments in a soluble form or attached to a
481 membrane. To demonstrate the subcelular localization of a protein is time consuming,
482 requiring the use of specific antibodies or how in our case, by transfection and
483 transitory expression of the cDNA fused to a reporter gene, like the GFP. The protein
484 code by the cDNA we have cloned showed an unequivocally fatty acid delta-6
485 desaturase activity when it was assayed in a heterologous system. However, these
486 assays do not show its subcellular localization. To affirm that the cDNA code by a Δ6
487 fatty acyl desaturase enzyme, the protein must be localized at their appropriate
488 subcellular compartment, the endoplasmic reticulum (ER), to perform their desired
489 function. In this work we have demonstrated that the fusion protein Δ6-FAD-EGFP
490 was localized in the ER when transiently expressed into HeLa cells. It is the first time
491 that this determination was made. At the same time, we observed a high percentage of
492 apoptotic nuclei in cells presenting high levels of Δ6-FAD-EGFP expression.
493 Although the explanation for this fact is not clear, it may be due to over-expression
494 effect of the fusion protein or, like it has been described previously for Atlantic
495 salmon, desaturated product of this enzyme could induce apoptosis, decreasing levels
496 of linoleic acids (LA; 18:2 n-6) contained in phospholipids (PLs) associated with
497 mitochondrial membranes and increasing, therefore, susceptibility of PLs to
498 peroxidation (Todorcevic et al., 2009).
499 Along with the cloning of Δ6 FADs cDNAs of sea bream, turbot and cod, the work
500 described has confirmed that marine fish have, and express the gene required for the
501 first activity in the HUFA biosynthesis pathway, Δ6 desaturation, and the protein is
502 localized in the proper intracellular compartment, the ER. Then, deficiencies in the
503 pathway in marine fish would be at a subsequent step, such as chain elongation and/or
504 Δ5 desaturation. This is consistent with biochemical data suggesting deficiencies in

505 these steps in turbot and sea bream cell lines (Ghioni et al., 1999; Tocher and Ghioni,
506 1999). However, despite expressing an apparently active Δ6 FAD, the activity of the
507 HUFA biosynthesis pathway in both hepatocytes and enterocytes in sea bass was very
508 low (Mourente and Dick 2002; Mourente et al., 2005b), and considerably lower than
509 the activities measured in salmon hepatocytes and enterocytes (Tocher et al., 2002;
510 Zheng et al., 2005). Indeed, the activities in sea bass were too low to accurately
511 quantify individual products in hepatocytes, although in enterocytes it was confirmed
512 that only Δ6 desaturated products were observed. Thus, the major product of 18:3n-3
513 desaturation was 18:4n-3, and little HUFA (defined as $\geq C_{20}$ and ≥ 3 double bonds)
514 and negligible EPA and DHA were produced in sea bass (Mourente et al., 2005b). In
515 contrast, functional expression indicated that the sea bass Δ6 FAD displayed Δ6
516 enzymatic activity towards 18:3n-3 (almost 15 % conversion) in the yeast system.
517 This fact clearly shows that, although 18:3n-3 and 18:2n-2 are substrates for the
518 enzyme *in vitro* or in a yeast functional assay, they are not good substrates for the Δ6
519 FAD enzyme in marine fishes. It is also clear that it is essential that gene expression
520 at the protein level should also be studied to confirm the extent of translation of the
521 *Δ6 FAD* gene in sea bass tissues and other marine fishes. However, in the same yeast
522 system, the salmon Δ6 FAD showed over 60% conversion of 18:3n-3 (Zheng et al.,
523 2005a) and the cod Δ6 gave 33 % conversion (Tocher et al., 2006). Furthermore,
524 conversion of 18:2n-6 by the sea bass Δ6 FAD was only 5.6 % in the yeast expression
525 system, compared to 14 % for the salmon Δ6 FAD (Zheng et al., 2005a). Therefore,
526 consistent with the salmon expressing higher HUFA synthesis activities than marine
527 fish (Tocher et al., 2006), the salmon Δ6 FAD was more active than sea bass Δ6 FAD
528 in a comparative yeast system. However this comparison is limited by the
529 consideration that heterologous expression systems will be using the endogenous

530 yeast translation machinery with rather different usage codon between yeast and
531 fishes. Thus, it also would be necessary to estimate the quantity of protein that this
532 heterologous system is able to synthesize for each species studied to accurately
533 compare the obtained conversion rates.
534 The expression of the sea bass Δ6 FAD was higher in heart, brain and ovary and
535 smaller in kidney, adipose tissue and liver. Why heart show high levels of expression
536 of Δ6 FAD gen? And what is the physiological significance of this fact? The answer
537 to both questions could be that heart is the most important neuromotor organ and the
538 maintenance of the n-3 LC PUFA level on the motoneuron inervating cells is critical
539 for the correct functioning of this organ. In cod, Δ6 FAD expression was highest in
540 brain and to a lesser extent in liver, kidney, intestine, red muscle and gill, and at much
541 lower levels in white muscle, spleen and heart (Tocher et al., 2006). In salmon, the
542 expression of both Δ6 and Δ5 FADs were highest in intestine, liver and brain (Zheng
543 et al., 2005). Mammalian Δ6 and Δ5 FADs also show relatively high expression in
544 liver, brain, heart and kidney (Cho et al., 1999a; 1999b). A third desaturase gene in
545 humans (FADS3), with as yet unknown function, shows highest expression in brain,
546 heart and liver (Marquardt et al., 2000). In contrast, intestine does not appear to be a
547 site of high desaturase expression in mammals (Leonard et al., 2000).
548 Mammalian FAD genes have been demonstrated to be subject to nutritional
549 regulation. The expression of Δ6 FAD in liver was increased in mice fed triolein
550 (18:1n-9), an EFA-deficient diet, compared to mice fed corn oil, a diet rich in the
551 PUFA18:2n-6 (Cho et al., 1999a). Similarly, the expression of both Δ6 and Δ5 FADs
552 was 4-fold higher in rats fed a fat-free diet or a diet containing triolein compared to
553 that in rats fed high levels of PUFA either as safflower oil (18:2n-6) or menhaden oil
554 (EPA and DHA) (Cho et al., 1999b). Similar results have been obtained in salmonids,

555 with dietary linseed oil (rich in 18:3n-3) increasing the expression of Δ6 FAD in liver
556 of rainbow trout, and Δ5 FAD in liver of Atlantic salmon compared to levels in fish
557 fed diets containing FO (Seiliez et al., 2001; Zheng et al., 2004b). Furthermore,
558 expression levels of both Δ6 and Δ5 FADs were increased in liver of salmon fed a
559 VO blend (rich in C_{18} PUFA) compared to levels in fish fed FO (Zheng et al.,
560 2005a,b). In the present study, the expression of Δ6 FAD in sea bass liver was higher
561 in fish fed linseed and rapeseed oils compared to that in fish fed FO; whereas, dietary
562 olive oil did not significantly increase expression. Similarly contrasting results were
563 obtained with sea bass fed two different blends of the same three VOs, with the
564 expression of Δ6 FAD only significantly up-regulated in fish fed one of the blends.
565 The reason for these conflicting data are not clear, but may be related to the fact that
566 the Δ6 FAD is only expressed at low levels in sea bass liver, and has not been
567 determined in other tissues regarding ingestion of dietary VOs. Certainly, conflicting
568 data have been reported previously in marine fish. In cod, expression of Δ6 FAD was
569 slightly, but not significantly, increased in liver and intestine of fish fed a VO blend
570 compared to fish fed FO (Tocher et al., 2006). However, expression of Δ6 FAD in sea
571 bream liver was higher in fish fed a HUFA-free diet compared to that in fish fed a
572 HUFA-rich diet (Seiliez et al., 2003). Similarly, the expression of the Δ6 FAD gene
573 was increased in sea bream larvae fed linseed oil and, especially, soybean and
574 rapeseed oils, compared to larvae fed a FO diet (Izquierdo et al. 2008). There is also
575 conflicting data in marine fish when comparing FAD expression and activity of the
576 HUFA biosynthesis pathway. Thus, although increased expression of Δ6 FAD was
577 observed in liver of sea bass fed some VOs or VO blends, the activity of the HUFA
578 biosynthetic pathway has been previously investigated in sea bass and nutritional up-
579 regulation due to dietary inclusion of VOs or VO blends has not been observed in

580 hepatocytes (Mourente and Dick, 2002; Mourente et al., 2005b), although increased
581 activity was reported in pyloric caecal enterocytes of fish fed VO blends (Mourente et
582 al., 2005a). In cod, where expression of Δ6 FAD was not significantly increased in
583 liver and intestine of fish fed a VO blend compared to fish fed FO, there was
584 similarly no significant effect on HUFA biosynthetic activity in either hepatocytes or
585 enterocytes (Tocher et al., 2006). In contrast, FAD expression was correlated with
586 enzyme activities in mice fed EFA-deficient versus corn oil diets (Cho et al., 1999a)
587 and in salmon fed FO versus VO diets (Zheng et al., 2004; 2005b). It is likely that the
588 conflicting data in both FAD expression and HUFA biosynthetic activity in marine
589 fish are due to the low levels of expression and enzyme activity recorded in marine
590 fish tissues compared to salmonids (Tocher et al., 2006).
591
592 **5. Conclusions**
593
594 The study described here has demonstrated that European sea bass have and express a
595 fatty acid desaturase gene, the product of which is localized in the ER and shows
596 clear Δ6 FAD activity in a heterologous yeast expression system. These data support
597 the hypothesis that the poor ability of marine fish, such as sea bass, to synthesize
598 HUFA is not due to lack of a Δ6 FAD. The sea bass Δ6 FAD was highly expressed in
599 heart, brain and ovary. Kidney, adipose tissue and liver showed considerable lower
600 expression levels of Δ6 FAD. The expression of the Δ6 FAD may be under some
601 form of nutritional regulation, being increased in hepatocytes of fish fed diets
602 containing VOs compared to fish fed FO, although the data are not consistent. Further
603 studies are required to conclusively determine why C_{18} PUFA are not substrates for
604 the enzyme in sea bass: a low expression of the gene, an high and strict enzymatic

605 activity control or some other factor (genetics, hormonal, environmental) can be the
606 reason why the Δ6 FAD appears to be barely functional in European sea bass, as in
607 other marine fish species. Sea bass has the genetic capacity to carry out the first step
608 of the transformation of the C18 PUFA in HUFAs. However metabolic possibility to
609 carry out this conversion is shrunk or handicapped.

610

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612

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619

620 **7. References**

621

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814
815
816 **LEGENDS TO FIGURES**

817 **FIG. 1.** Comparison of the deduced amino acid sequence of the Δ6 FAD from
818 European sea bass (*Dicentrarchus labrax*)(Dl) with that of Δ6 FADs from gilthead
819 sea bream (*Sparus aurata*) (Sa), Atlantic cod (*Gadus morhua*) (Ac), Atlantic salmon
820 (*Salmon salar*) (As) and common carp (*Ciprinus carpio*) (Cc). (A) Deduced amino
821 acid sequences of FADs were aligned using ClustalX. Identical residues are shaded
822 black and residues conserved in four proteins are shaded grey. A conserved domain
823 search of the deduced amino acid sequence of the sea bass Δ6 FAD was carried out
824 against the Conserved Domain Database at NCBI. (B) Two domains were found: a
825 cytochrome b_5 domain (Cyt-b5) at the N-terminus (amino acids 21-96) and a Δ6-
826 FADS-like domain at the C-terminus (amino acid 163-413).

827 **FIG. 2.** Transmembrane segments prediction profiles of the amino acid sequence for
828 sea bass Δ6 FAD. The amino acid sequence exhibited all the characteristics features
829 of FADs: (A) a conserved haem-binding motif H-P-G-G (designed with a black
830 triangle) in the cytochrome b_5 domain and three sequences-conserved histidine
831 cluster boxes (indicated with a black triangle) in the Δ6-FADS-like domain. (B) The
832 prediction analysis for transmembrane segments show the protein has three highly
833 hydrophobic regions, as shown the hydrophobicity plot of the protein, containing four
834 possible transmembrane expanded segments (indicated with a grey box labeled with
835 TMS).
836
837 **FIG.3.** Northern blot analysis of Δ6 FAD mRNA from brain of European sea bass.
838 Total RNA (30 µg) were used to determine the mRNA size of the Δ6 FAD. As probe,
839 Δ6 FAD full-length ^{32}P radiolabelled cDNA was used. Hybridization and washing of
840 the blot were carried out at high stringency (see material and methods). After 3 days
841 of exposure, a single band of approximately 2.1 kb was observed. Sizes of RNA
842 standards are indicated.
843
844 **FIG.4.** Phylogenetic tree of European sea bass Δ6 FAD and FADs from other fish
845 species (Atlantic salmon, zebrafish, cherry salmon, rainbow trout, sea bream,
846 common carp, turbot and tilapia), mammals (mouse and human), fungus (*Mortierella*
847 *alpina*, *Pythium irregular*, *Mucor circineloides*), algae (*Phaeodactylum tricornotum*,
848 *Thraustochytrium sp.*), amoeba (*Dictyostelium discoideum*) and nematode
849 (*Caenorhabditis elegan*s). The tree topology presented was replicated after 1000
850 bootstrap iterations. The optimal tree is shown (next to the branches). The
851 evolutionary distances are in the units of the number of amino acid substitutions per
852 site. Sequences marked with an asterisk are not functionally characterized. The Δ9
853 FAD sequence from *Mortirella alpine* was used, as an out-group sequence, to
854 construct a rooted tree. Protein accession numbers: Δ5 Atlantic salmon AF478472,
855 Δ5 Human AF199596, Δ5 Mouse AB072976, Δ5 *Mortierella alpina* AF067654, Δ5
856 *Caenorhabditis elegans* AF078796, Δ5 *Dictyostelium discoideum* AA37090, Δ5
857 *Phaeodactylum tricornutum* AY082392, Δ5 *Phytium irregulare* AAL13311, Δ5
858 Thaustochytrium sp AAM09687, Des1* Cherry salmon AB070444, Des2* Cherry
859 salmon AB074149, Δ6 *Phytium irregulare* AAL133100, Δ6 *Phaeodactylum*
860 *tricornutum* AY082393, Δ6 *Mucor circinelloides* BAB69055, Δ6 *Caenorhabditis*

861 *elegans* AF031477, Δ6 *Mortierella alpina* AF110510, Δ6 Mouse AF126798, Δ6
862 Human AF126799, Δ6 *Dicentrarchus labrax* AM746703, Δ5/Δ6 Zebrafish
863 AF309556, Δ6 Atlantic cod DQ054840, Δ6 Common carp AF309557, Δ6 Turbot
864 AY546094, Des* Nile tilapia AB069727, Δ6 *Sparus aurata* AY055749, Δ6 Rainbow
865 trout AF301910 and Δ6 Atlantic salmon AY458652.
866
867 **FIG.5.** Functional characterization of the European sea bass FAD by heterologous
868 expression in yeast (*S. cerevisiae*). Panels A and C show the fatty acids extracted
869 from yeast transformed with pYES vector without insert and grown in the presence of
870 18:3n-3 and 18:2n-6, respectively. Panels B, D, E and F show the fatty acids
871 composition of yeast transformed with pYES vector containing the putative
872 desaturase ORF and grown in the presence of 18:3n-3, 18:2n-6, 20:4n-3 and 20:3n-6,
873 respectively. The first four peaks in all panels are the main endogenous fatty acids of
874 *S. cerevisiae,* namely 16:0 (1), 16:1n-7 (2), 18:0 (3) and 18:1n-9 (with 18:1n-7 as
875 shoulder) (4). Peak 5 in panels A and B, and peak 7 in panels C and D are the
876 exogenously added substrate fatty acids, 18:3n-3 and 18:2n-6, respectively. Peaks 6
877 and 8 in panels B and D were identified as the resultant desaturated products, namely
878 18:4n-3 and 18:3n-6, respectively. Peaks 9 and 10 in panels E and F are the
879 exogenously added substrate fatty acids, 20:4n-3 and 20:3n-6, respectively. Vertical
880 axis: FID response; horizontal axis: retention time.
881
882 **FIG.6.** Subcellular localization of Δ6 FAD from European sea bass in HeLa cells.
883 The plasmid coding for the fusion protein D-6-D-EGFP was transiently co-
884 transfected into HeLa cells with a plasmid construct designed to localize the
885 endoplasmic reticulum (pDsRed2-ER, Clontech, Madrid, Spain). Forty-eight h after
886 transfection green channel (panel A) and red channel (panel B) fluorescent images
887 were taken for the same field. DAPI was used to localize the nucleus (blue channel,
888 panel C). Overlay composed of three channels (panel D) shows in yellow pixels
889 where the green (D6D-EGFP protein) and red (DsRed2 protein) channels match. To
890 quantify the degree of co-localization, intensity correlation coefficient-based (ICCB)
891 analyses were performed, using JACoP tool included in Image J software (v. 1.42k)
892 (http://rsb.info.nih.gov/ij). In E), Pearson's and Overlap coefficients and Li's
893 Intensity Correlation Quotient (IQC) for ten analyzed images, are shown as mean ±

894 SD. Maximum (completed co-localization) and minimum (exclusion) values for each
895 coefficient are indicated.
896
897 **FIG. 7.** Tissue transcription of Δ6 FAD in European sea bass. Transcript (mRNA)
898 copy numbers were determined by quantitative real-time PCR (qPCR) as described in
899 the Methods section. Results expressed as means of absolute copy number (± SD)
900 (n=3) of FAD transcripts in 25 ng of total RNA. Letters show significant differences
901 ($P < 0.05$) among tissues as determined by one-way ANOVA followed by Tukey's
902 multiple comparison test (Zar, 1984).
903
904 **FIG. 8.** Effect of dietary VO on the expression of Δ6 FAD in liver from European sea
905 bass. Relative expression was determined by quantitative PCR (qPCR) and
906 normalized as described in the Materials and Methods Section. Results are referred to
907 kidney expression (1.0) and expressed as means ± SD (n = 5). A) Effect of 60 %
908 substitution of dietary FO with rapeseed oil (RO), linseed oil (LO) and olive oil
909 (OO). B) Effect of 60 % substitution of dietary FO with blends of VOs (see material
910 and methods). Superscript letter denotes a significant effect of diet as determined by
911 one way ANOVA analysis and subsequent Tukey's multiple comparison test ($P <$
912 0.05) (Zar, 1984).
913
914

914

915

917

TABLE 1

lentity matrix showing the percentage of identical amino acids residues in the protein :quences of fish and human Δ6 FAD

	Atlantic salmon Δ6	*Rainbow trout Δ6*	*Sea bream Δ6*	*Nile Tilapia Δ6*	*Turbot Δ6*	*Common carp Δ6*	*Atlantic cod Δ6*	*Zebrafish Δ6*	*Human Δ6*
Sea Bass Δ6	78	77	**94**	76	**83**	65	**82**	67	64
Atlantic salmon Δ6		94	78	71	73	65	76	66	66
Rainbow trout Δ6			77	72	73	65	77	66	65
Sea Bream Δ6				76	84	65	82	67	64
***Nile Tilapia* Δ6**					73	61	72	63	61
Turbot Δ6						66	77	67	62
Common carp Δ6							67	89	62
Atlantic cod Δ6								70	63
Zebrafish Δ6									64

934

936

FIG. 1

A

```
Dl      MGGGGQLTEPGESGSR---------RAGGVYTWEEVQSHCNRNDQWLVIDRKVYDITHWAKGHPGGHRVISHYAGE
Sa      MGGGGQLTEPGEPGSR---------RAGGVYTWEEVQSHSSRNDQWLVIDRKVYNVTKWAKRHPGGFRVINHYAGE
Ac      MGGGGQLTEPVETSACGG-------RAASVYTWDEVQKHCHRNDQWLVINRKVYNVTQWAKRHPGGLRVISHYAGE
As      MGGGGQQNDSGEPAKGDRGGPGGGLGGSAVYTWEEVQRHSHRGDQWLVIDRKVYNITQWAKRHPGGIRVISHFAGE
Cc      MGGGGQQTDRITGTNG----------RFGTYTWEEVQKHTKFGDQWIEVERKVYNVSQWVKRHPGGVRILGHYAGE

Dl      DATEAFTAFHPNLKFVQKFLKPLLIGELAATEPSQDRNKNAAIIQDFYTLRAQAESEGLFKAQPLFFCLHLGHILL
Sa      DATEAFTAFHPDLKFVQKFLKPLLIGELAATEPSQDRNKNAAVIQDFHTLRAQAESDGLFRAQPLFFCLHLGHILL
Ac      DATEAFLAFHPNPKLVQKFLKPLLIGELAVTEPSQDRNKNAAVVEDFQALRTRAEGLGLFQAQPLFFCLHLGHILL
As      DATDAFVAFHPNPNFVRKFLKPLLIGELAPTEPSQDHGKNAVLVQDFQALRNRVEREGLLRARPLFFSLYLGHILL
Cc      DATEAFTAFHPDLPLVRKYMKPLLIGELEASEPSQDRQKNAALVEDFRALRERLEAEGCFKTQPLFLILHLSHILL

Dl      LEALAWLIIWLWGTSWTLTFLCSIMLATAQSQAGWLQHDFGHLSVFKKSSWNHMLHKFVIGHLKGASANWWNHRHF
Sa      LEALAWLIIWLWGTSWTLTFLISIILATAQTQAGWLQHDFGHLSVFKKSSWNHILHKFVIGHLKGASANWWNHRHF
Ac      LELLAWMSVWLWGTGWRTTLLCSFILAVAQAQAGWLQHDFGHLSVFKLSRWNHIFHKFIIGHLKGASGNWWNHRHF
As      LEALALGLLWVWGTSWSLTLLCSLMLATSQSQAGWLQHDYGHLSVCKKSSWNHVLHKFVIGHLKGASANWWNHRHF
Cc      LEAIALMMVWYLGTGWINTAIVAVLLATAQSQAEWLQHDFGHLSVFKTSRWNHLVHKFVVGHIKGASAGRWNHRHF

Dl      QHHAKPNIFSKDPDVNMLHIFVVGATQPVEYGIKKIKYMPYHHQHQYFLLVGPPLLIPVYFHIQIIRTMISRRDWV
Sa      QHHAKPNIFSKDPDVNMLHIFVLGDTQPVEYGIKKIKYLPYHHQHQYFLLVGPPLLIPVYFHIQIIRTMISRHDWV
Ac      QHHAKPNVFSKDPDVNMLHVFVVGDIQPVEYGIKKIKYMPYHHQHQYFFLVGPPLLIPVYFHIQILRAMFSRRDWV
As      QHHAKPNVLSKDPDVNMLHVFVLGDKQPVEYGIKKLKYMPYHHQHQYFFLIGPPLLIPVFFTIQIFQTMFSQRNWV
Cc      QHHAKPNVFKKDPDVNMLNAFVAGKVQPVEYGVKKIKHLPYNHQHKYFFFIGPPLLIPVYFQFQIFHNMIAHGLWV

Dl      DLAWSMSYYLRYLCCYVPLYGLFGSLALISFVRFLESHWFVWVTQMNHLPMDIDHEKHHDWLTMQLQATCNIEKSS
Sa      DLAWSMSYYLRYLCCYVPLYGLFGSVALISFVRFLESHWFVWVTQMNHLPMDIDHEKHHDWLTMQLQATCNIEKSV
Ac      DLAWSMSYYLRYFCCYAPFYGLLGSVALISFVRFLESHWFVWVTQMNHLPMNIDHEKQQDWLSMQLSATCNIEQSC
As      DLAWSMTFYLRFFCSYYPFFGFFGSVALITFVRFLESHWFVWVTQMNHLPMEIDHERHQDWLTMQLSGTCNIEQST
Cc      DLAWCISYYVRYFLCYTQYYGVFWAVILFNFVRFLKSHWFVWVTQMSHIPMQIDYEKHQDRLSMQLVATCNIEQSS

Dl      FNDWFSGHLNFQIEHHLFPTMPRHNYHLVAPLVHALCEKHGIPYQVKTMWQGLVDVIRSLKNSGDLWLDAYLHK
Sa      FNDWFSGHLNFQIEHHLFPTMPRHNYHLVAPLVHALCEKHGIPYQVKTMWQGIVDVIRSLKNSGDLWLDAYLHK
Ac      FNDWFSGHLNFQIEHHLFPTMPRHNYQVLAPLVRALCEKHSIPYQEKTLWRGVADVVRSLKNSGDLWMDAYLHK
As      FNDWFSGHLNFQIEHHLFPTMPRHNYHLVAPLVRTLCEKHGIPYQVKTLQKAIIDVVRSLKKSGDLWLDAYLHK
Cc      FNDWFSGHLNFQIEHHLFPTMPRHNYWRAAPHVRELCAKYGIKYQEKTLQGAFADVVRSLEKSGEIWLDAYLNE
```

B

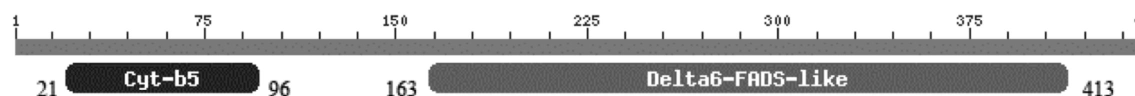

FIG 2

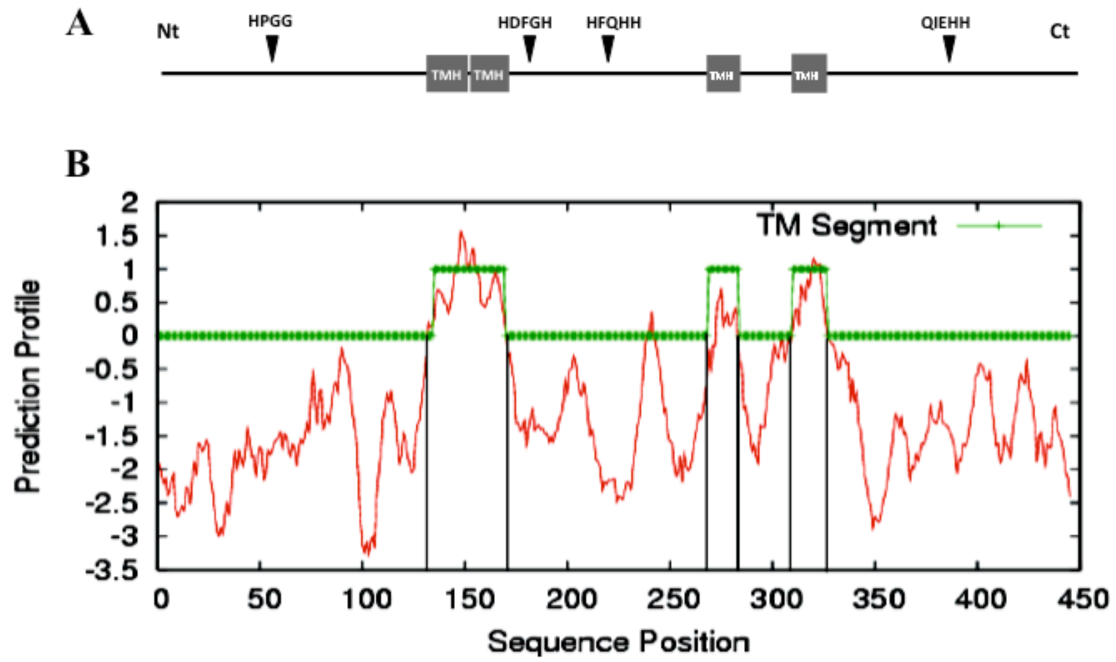

FIG 3

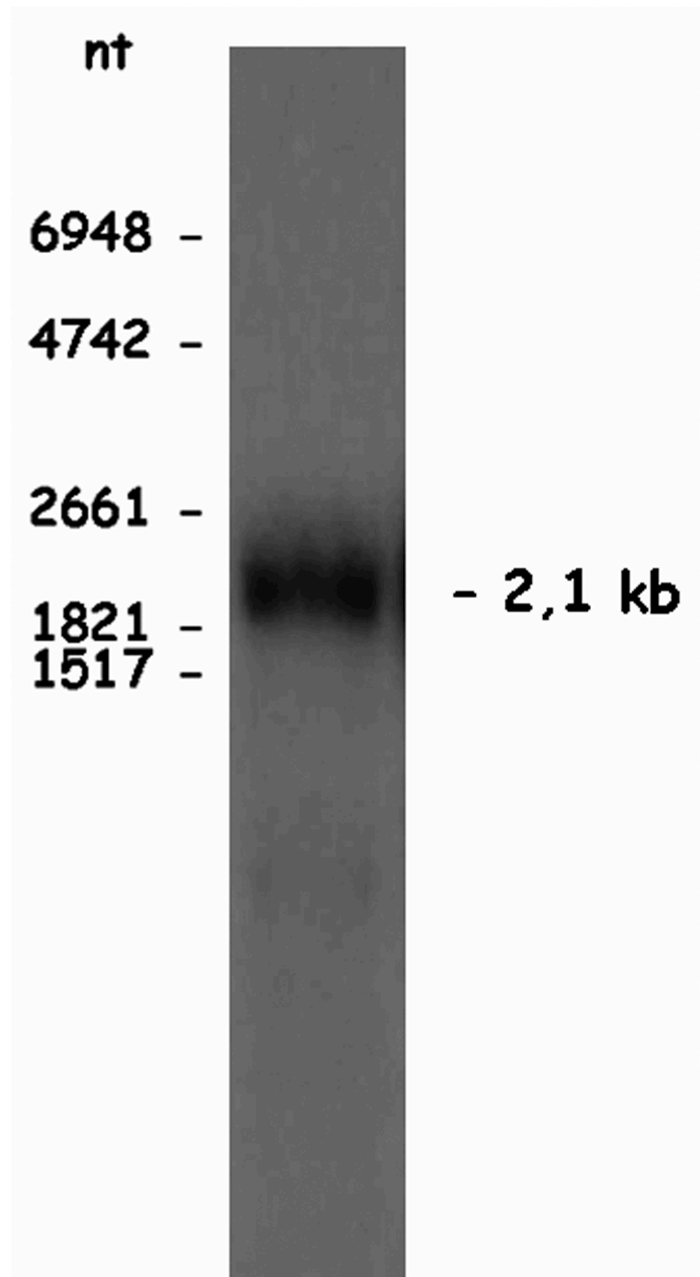

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Fig 4

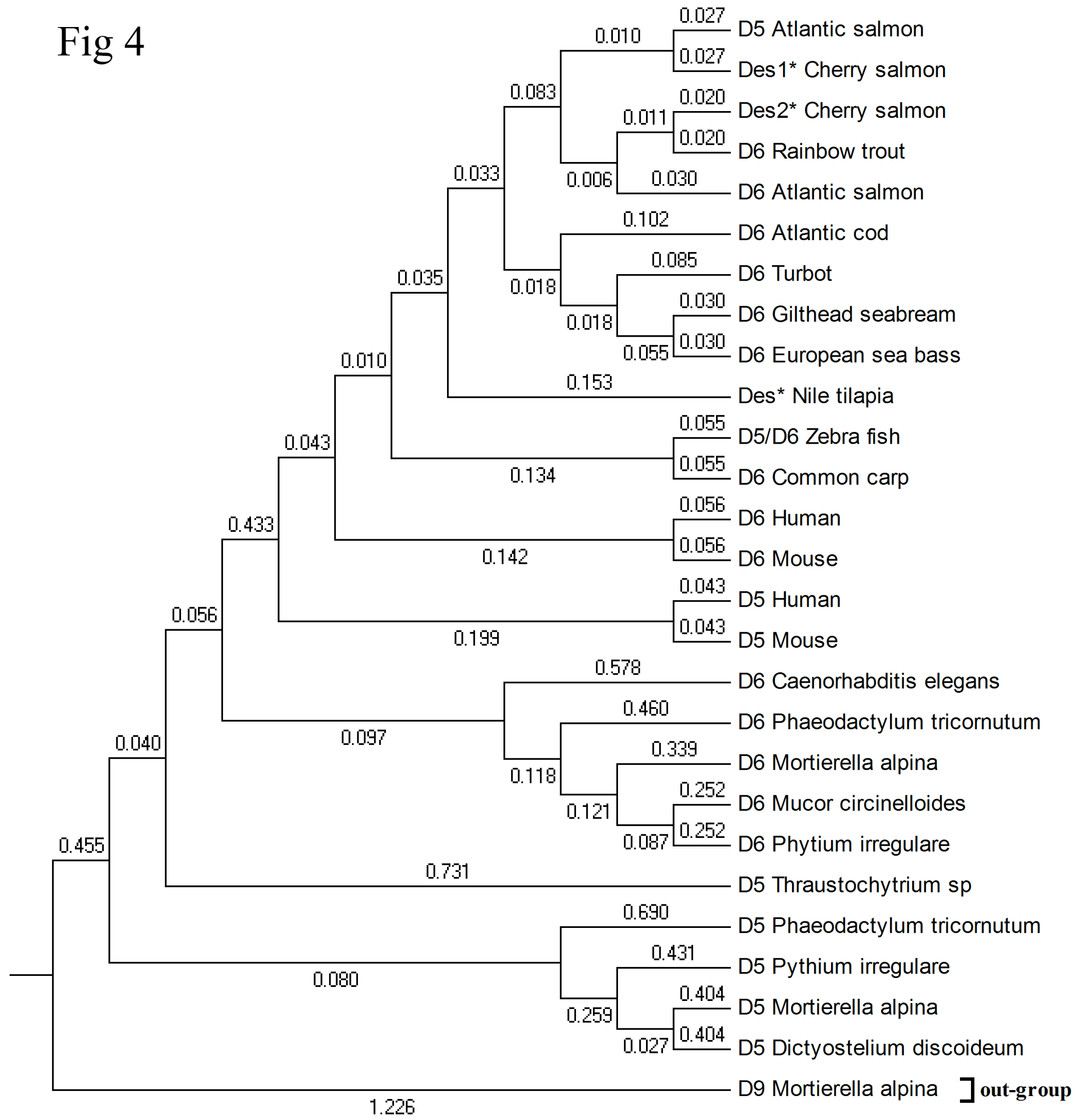

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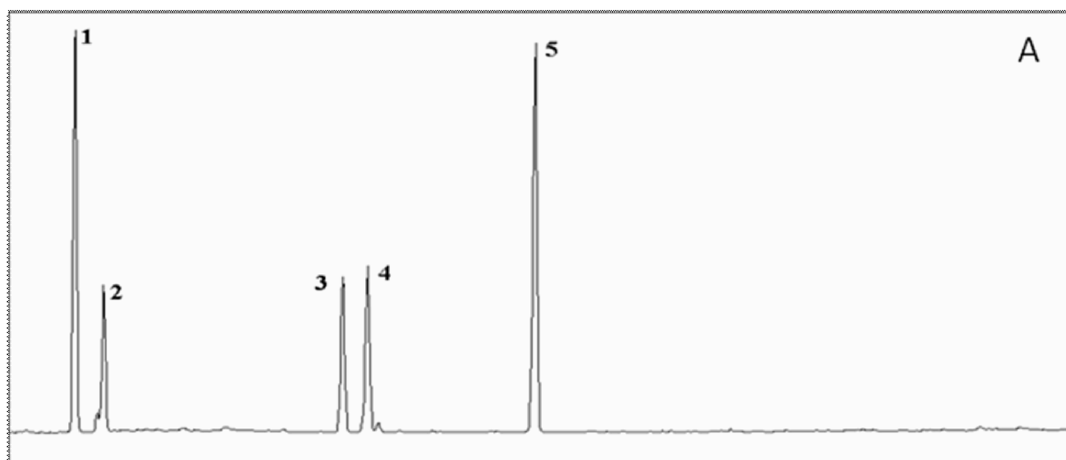
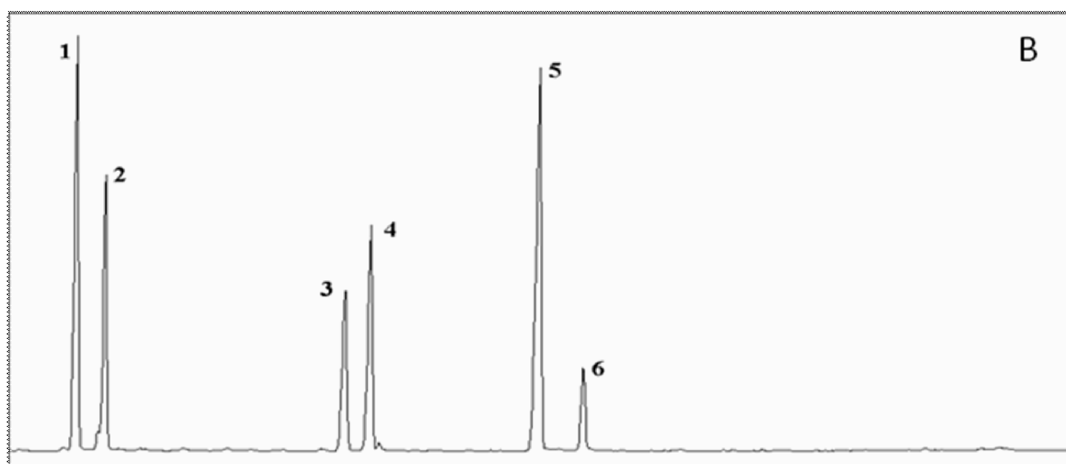
FIG. 5





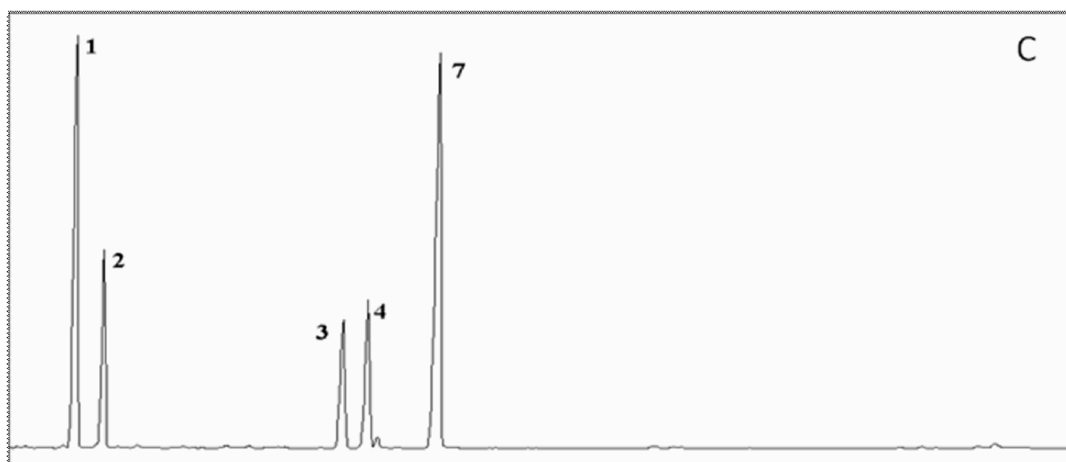


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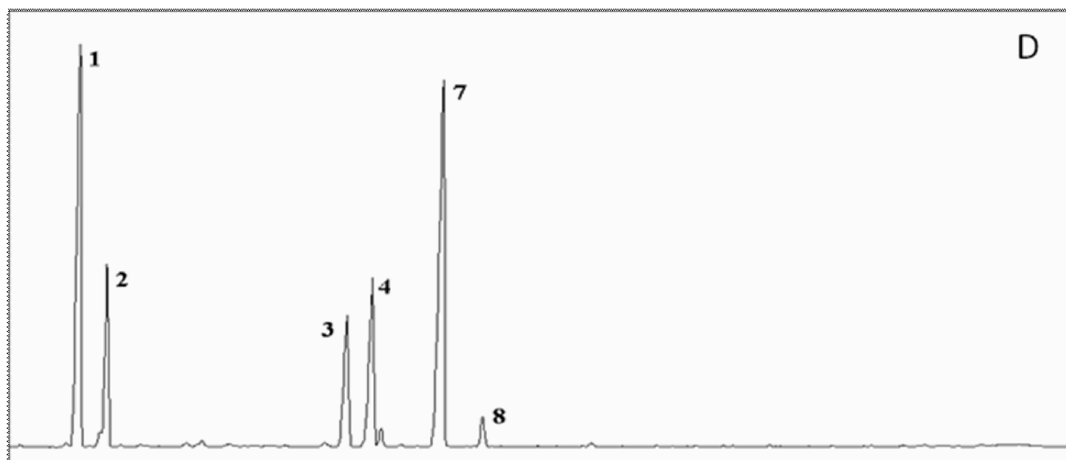
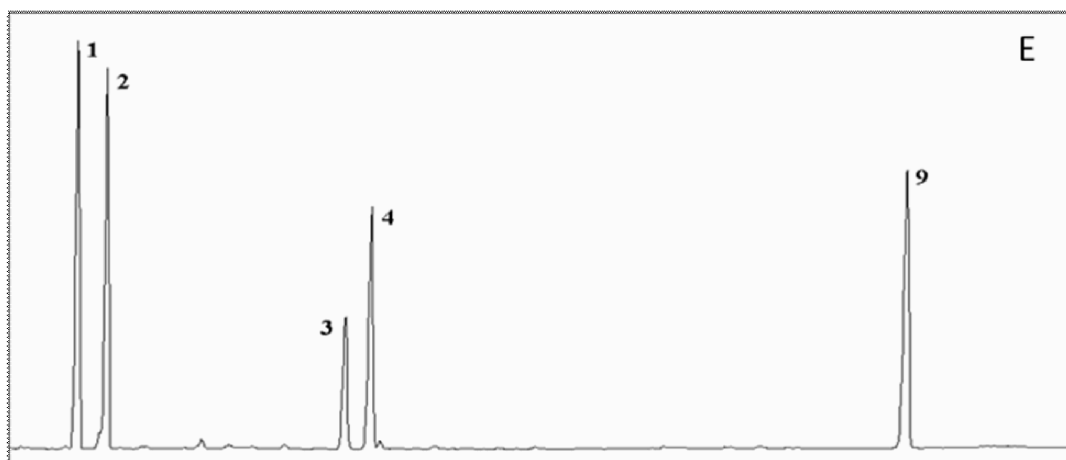
FIG. 5





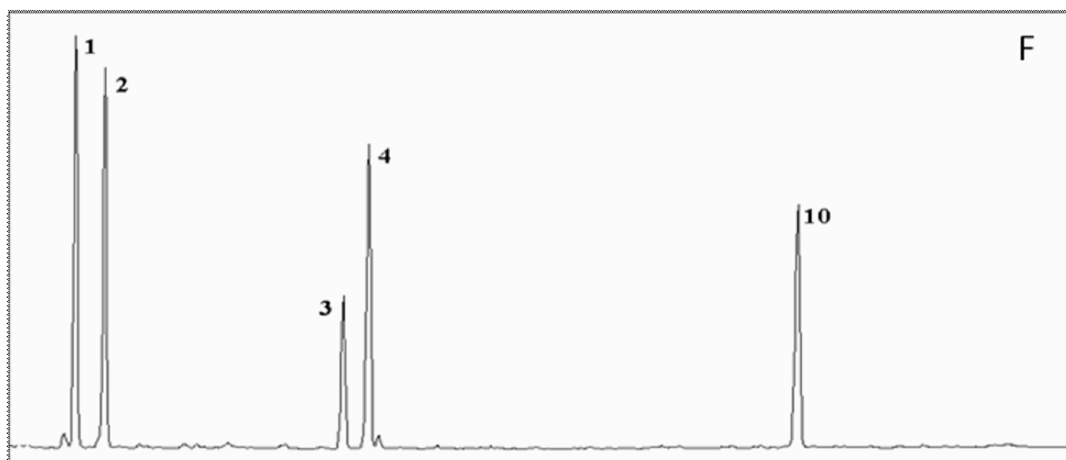


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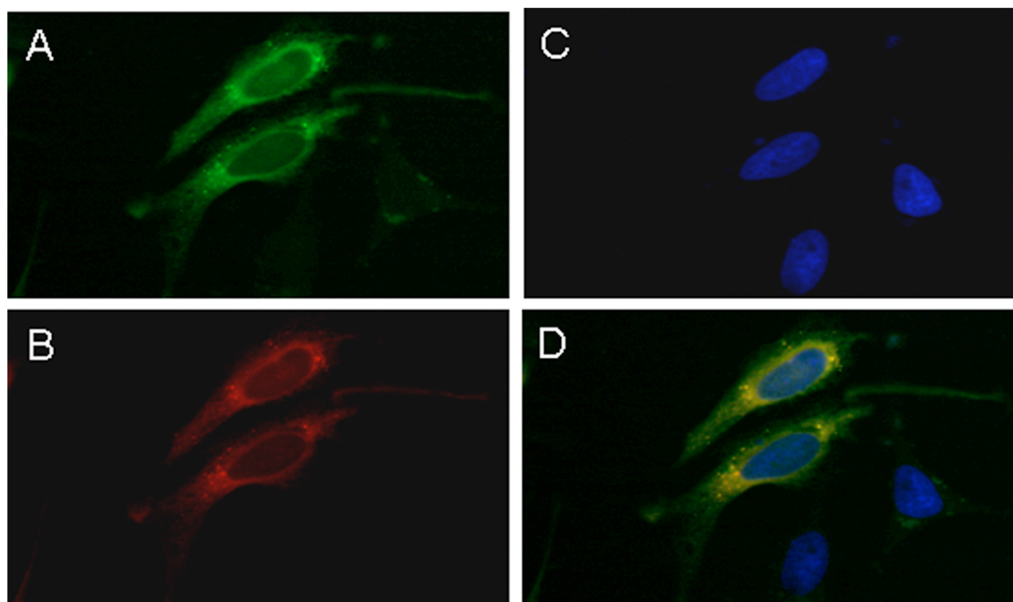
FIG. 6

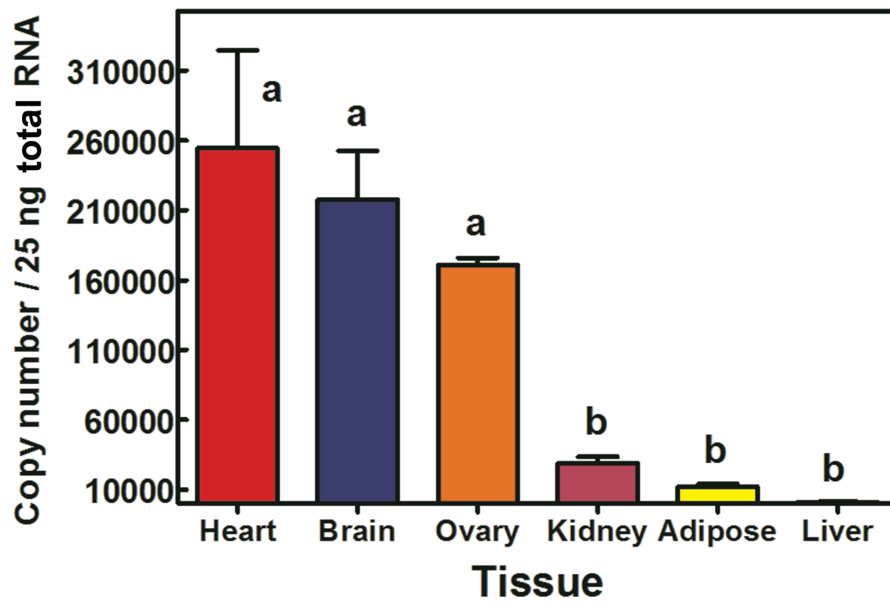


E

Quantitative Colocalization

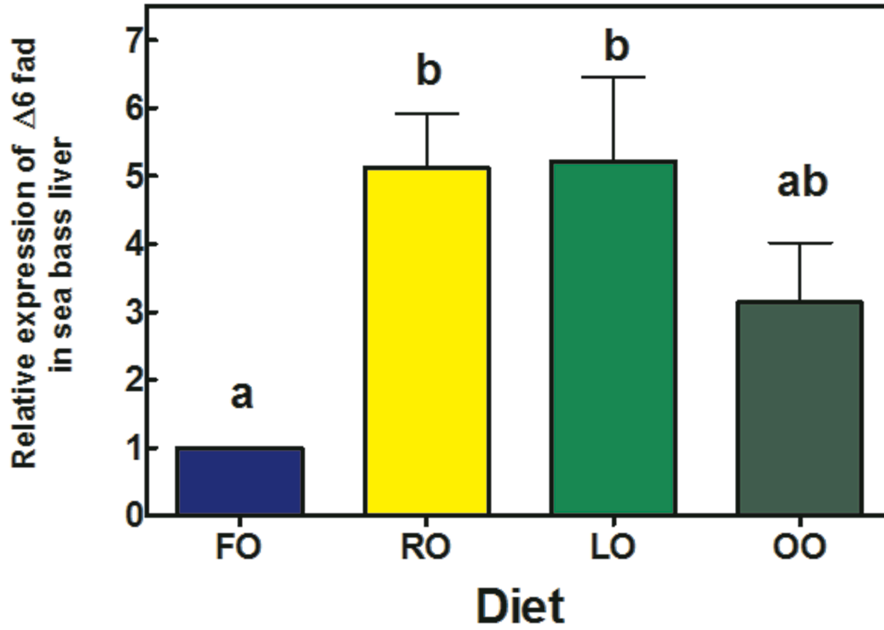
Pearson's (-1.0, 1.0)	**Overlap** (0.0, 1.0)	**Li's ICQ** (-0.5, 0.5)
0.94 ± 0.03	0.91 ± 0.06	0.34 ± 0.09

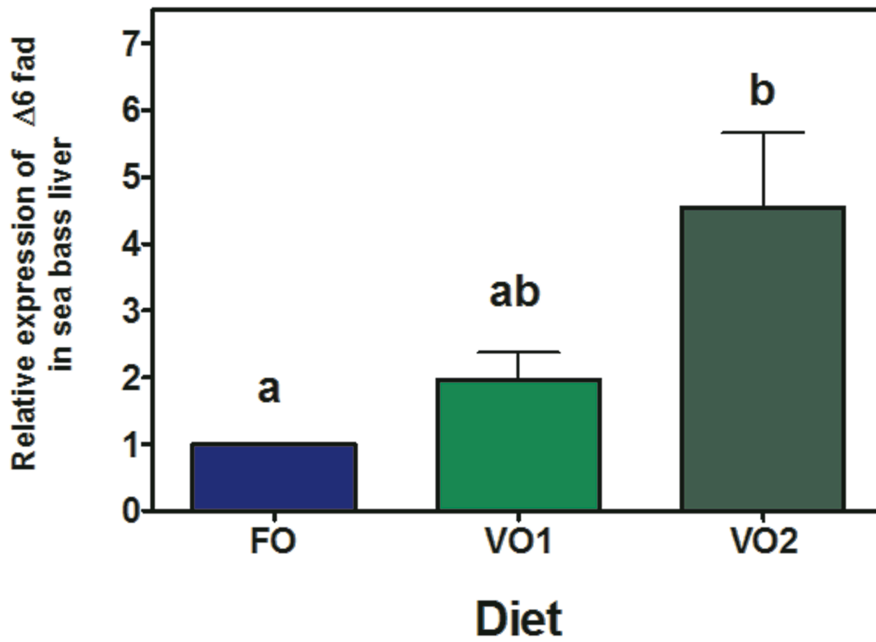
Fig. 7



990

Fig. 8





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