

**Molecular and Functional Characterization and
Expression Analysis of a $\Delta 6$ 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 $\geq C_{20}$ 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 C₁₈ 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 $\Delta 6$ 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 $\Delta 6$ -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 $\Delta 6$ 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 $\Delta 6$ 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 $\Delta 6$ 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.

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1. Introduction

Fish are our major dietary source of n-3 highly unsaturated fatty acids (HUFA), eicosapentaenoate (EPA; 20:5n-3) and docosahexaenoate (DHA; 22:6n-3) (Simopoulos, 2000; Tidwell and Allan, 2002) and, with traditional fisheries declining, global catches from the feed-grade fisheries that provide fish oil (FO) and fish meal for aquafeed formulations have reached their sustainable limits and soon there will be insufficient FO to maintain current aquaculture growth (Tacon, 2004; Pike, 2005). Vegetable oils (VOs), a sustainable alternative to FO, can be rich in C₁₈ polyunsaturated fatty acids (PUFA) such as 18:2n-6 and 18:3n-3, but lack the n-3 HUFA abundant in FO (Sargent et al., 2002). The extent to which fish can convert C₁₈ PUFA to HUFA varies according to their capacity for fatty acyl desaturation and elongation (Tocher, 2003). Marine fish are unable to produce EPA and DHA from 18:3n-3 at a physiologically significant rate (Sargent et al., 2002) due to apparent deficient activity in one or more enzymes in the desaturation/elongation pathway (Ghioni et al., 1999; Tocher and Ghioni, 1999). Thus, flesh fatty acid compositions in marine fish fed VOs are characterized by increased levels of C₁₈ PUFA and decreased levels of n-3 HUFA, compromising their nutritional value to the human consumer (Izquierdo et al., 2003; Regost et al., 2003; Mourente et al., 2005a; Mourente and Bell, 2006).

The European sea bass (*Dicentrarchus labrax*) is among the most important carnivorous marine finfish species cultured in Europe, particularly in the Mediterranean region. Sea bass production in 2006 was 88.531 Tm (FAO/Globefish, 2007), and is still experiencing a rapid expansion. However, knowledge of its 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 $\Delta 6$
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 $\Delta 6$ 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 $\Delta 6$ 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 $\Delta 6$ FAD of rat liver, that this enzyme was associated
104 with microsomal membranes in the cytoplasm. Production of C20 HUFA requires a
105 further $\Delta 5$ desaturation and $\Delta 5$ FAD has been cloned from Atlantic salmon (*Salmo*

salar L.), and a bifunctional $\Delta 6/\Delta 5$ FAD from the freshwater zebrafish (*Danio rerio*) (Hasting et al., 2001; 2005). However, attempts to clone $\Delta 5$ FAD from marine fish have failed (Seiliez et al., 2003; Tocher et al., 2006; Zheng et al., 2009). In salmonids, clear nutritional regulation of FAD gene expression has been observed, with expression of both $\Delta 6$ and $\Delta 5$ FADs up-regulated in fish fed VO compared to fish fed FO (Zheng et al., 2004b, 2005 a, b). In contrast, nutritional effects are not clear in marine fish (Seiliez et al., 2003; Tocher et al., 2006; Izquierdo et al., 2008). Taken together, these data indicate clear differences in the HUFA synthesis pathway between marine fish and fresh water or diadromous fish.

The specific aims of the study described here were to investigate genes of fatty acid desaturation and the regulation of the HUFA biosynthetic pathway in European sea bass. Thus, we describe the cDNA cloning, functional characterization, subcellular localization, tissue distribution and expression of a $\Delta 6$ FAD of European sea bass that would be the first, and reputedly rate-limiting, enzyme activity required for the production of EPA and DHA. Moreover, this paper also describes the effects of diet on the expression of the $\Delta 6$ FAD gene in liver of sea bass fed diets containing either FO or diets with 60% substitution of FO with single VOs and/or blends of VOs.

2. Materials and Methods

2.1 cDNA Cloning

Total RNA was obtained from European sea bass brain tissue using Perfect RNATM, Eukaryotic, Mini Kit (Eppendorf, Hamburg, Germany) following the manufacturer's instructions, and contaminating genomic DNA eliminated by DNase I digestion (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 homopolimeric dC tail
 136 was added to the 5' end of cDNA using Terminal Deoxynucleotidyl Transferase
 137 (Invitrogen, Barcelona, Spain), and the tailed cDNA directly used for PCR
 138 amplification assays.

139 Full length cDNA of putative $\Delta 6$ 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 $\Delta 6$ FADs were aligned to design a
 142 degenerate forward primer, 5'-TGGTGGAAAYCAYAGRCAYTTCCAGCA-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 μ L, under the
 146 following conditions: initial denaturation at 94 °C for 2 min, 40 cycles of
 147 denaturation at 94 °C for 15 s, annealing at 57 °C for 30 s and extension at 72 °C for
 148 50 s and a final extension step of 72 °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

degenerate forward primer 5'-TACACMTGGGAGGAGGTSCAG-3' to obtain an overlapping PCR fragment in the 5' direction of the cDNA. For amplification, the following conditions were used: initial denaturation at 94 °C for 2 min, 40 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, extension at 68 °C for 2 min and a final extension step of 72 °C for 7 min. After five new clones containing the appropriate product were sequenced, a new specific reverse primer 5'-CCAGCATAGTGGCTGATGACAC-3' was used in conjunction with a forward Not I-oligo-dG primer (5'-ATAAGAATGCGGCCGCTAAA(G)₁₅H-3') to perform 5' RACE. Amplification involved an initial step at 94 °C for 2 min and 35 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, extension at 68 °C for 1 min and a final extension step at 72 °C for 10 min. Five new clones, containing the appropriate fragment, were obtained and sequenced. All consensus sequences from each of the three overlapping fragments obtained were aligned to determine a contiguous nucleotide sequence of the sea bass putative FAD cDNA. Based on this sequence, two specific primers, at the 5'-end forward 5'-CCTCGAATGATCGGCTCGGAATT-3' and at the 3'-end reverse 5'-CCTTTATTGTAAGACGTAGAGTTGA-3', were designed and used to PCR amplify the full length cDNA for sea bass FAD, under the following conditions: initial denaturation at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 2 min and a final extension step of 72 °C for 7 min, using KOD HOT Start DNA Polymerase (Novagen, Cerdanyola, Spain). Ten clones containing the appropriate product were sequenced to unequivocally confirm the complete sequence of the sea bass FAD mRNA (GeneBank accession number AM746703).

2.2 Northern blot analysis

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

2.3 DNA and protein sequence analysis

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

using the Conserved Domain Search tool at NCBI (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Secondary structure and transmembrane expanded helix predictions were investigated using ANTHEPRO 2000 v.60 software, included in the software packages tool for secondary structure at EsPAXy Proteomics Server (<http://www.expasy.ch/>), SVMtm Transmembrane Domain Predictor bioinformatic tool (Yuan et al., 2004) in the ARC Center of Excellence in Bioinformatic (<http://bioinformatics.org.au/>) and Predictprotein software (<http://www.predictprotein.org>).

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

Functional characterization of the putative FAD cDNA ORF was performed by two complementary methods: a) cellular, by determining the subcellular localization of the ORF encoded protein in HeLa cells and b) biochemical, by determining the enzymatic activity of the putative protein codified by heterologous expression of the ORF in the yeast *Saccharomyces cerevisiae*.

2.4.1 Subcellular localization of sea bass desaturase ORF in HeLa cells

Subcellular localization of the putative FAD enzyme coded by cDNA, was analyzed by transiently expression in HeLa cell of the ORF fused, at the C-terminus, to the Enhanced Green Fluorescent Protein (EGFP) marker. The ORF of putative FAD cDNA was amplified using D6LFEY-221 5'-TTTTAGTGTAAGCTTCAGGTGG-3' as forward primer, which included a *Hind III* 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 μ L: initial denaturation at 94
236 $^{\circ}$ C for 2 min, 25 cycles of denaturation at 94 $^{\circ}$ C for 30 s, annealing at 59.2 $^{\circ}$ C for 30
237 s, extension at 72 $^{\circ}$ C for 2 min and a final extension step of 72 $^{\circ}$ 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 Δ 6-FAD-EGFP was under the immediate early promoter region of human
245 cytomegalovirus (HCMV). The plasmid pD6DL/EGFP was purified using the
246 GenEluteTM 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 (3 μ g 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

temperature. Then coverslips were washed in PBS for 5 min, permeabilized with 0.1 % (v/v) Triton X-100 (Sigma-Aldrich, Tres Cantos, Spain) in PBS for 15 min, washed in PBS for 5 min, stained with 0.2 µg/mL DAPI (Sigma-Aldrich, Tres Cantos, Spain) in PBS and finally mounted in 0.02 % (v/v) glycerol in PBS. Cells were observed for the presence of green fluorescent fusion protein (Δ6-FAD-EGFP) and for red fluorescence of DsRed2 using a 63 x PLAN NEOFLUAR immersion oil objective, mounted in a Zeiss Axiphot microscope (Carl Zeiss, Jena, Germany). Images were taken using a SPOT cool digital CDD Camera (Diagnostic Instruments Inc. Sterling Heights, MI, USA) and SPOT software v.4.6 (Diagnostic Instruments Inc. Sterling Heights, MI, USA). Quantitative co-localization and image analyses were performed using ImageJ 1.42k Software (<http://rsb.info.nih.gov/ij>). Pearson's correlation coefficient (r_p), Overlap coefficient (r) (Manders et al., 1992) and Li's Intensity Correlation Quotient (ICQ) (Li et al., 2004) were used to quantify the degree of co-localization between green and red fluorescent images.

2.4.2 Heterologous expression of sea bass FAD ORF in *S. cerevisiae*.

Expression primers were designed for PCR cloning of the sea bass putative FAD cDNA ORF. The forward primer, D6LFEY-221 (5'-TTTTAGTGTAAGCTTCAGGTGG-3') contained a *HindIII* site (underlined) and the reverse primer, D6LREY-1620 (5'-AGAGAATTAGAAATTCGTCATTTA-3') contained an *EcoRI* site (underlined). PCR was performed using high fidelity DNA polymerase (KOD HOT Start DNA Polymerase, Novagen, Cerdanyola, Spain) following the manufacturer's instructions. Amplification involved an initial denaturation step at 94 °C for 2 min, followed by 25 cycles of denaturation at 94 °C

for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 7 min. The proper PCR fragment was gel purified, *Hind* III and *Eco* RI restricted, and ligated into the similarly digested yeast expression vector pYES2 (Invitrogen, Barcelona, Spain), that left the FAD ORF under the inducible control of *GAL-1* promoter. Ligation product was transformed in Top10 *E. coli* chemically competent cells (Invitrogen, Barcelona, Spain), which were screened for the presence of correct construct. Selected clones were sequenced to confirm that variations had not taken place in the sequence of the plasmids. Transformation of the yeast *S. cerevisiae* strain InvSc1 (Invitrogen, Barcelona, Spain) with the recombinant plasmids pD6D/pYES2 was carried out using the S.c. EasyComp Transformation Kit (Invitrogen, Barcelona, Spain) following the manufacturer's instructions. Selection of yeast containing the FAD/pYES2 constructs was on *S. cerevisiae* minimal medium (SCMM) minus uracil. Culture of the recombinant yeast was carried out in SCMM^{uracil} broth as described previously (Hasting et al., 2001), using galactose induction of gene expression. Each culture was supplemented with one of the following PUFA substrates; 18:3n-3, 18:2n-6, 20:4n-3 and 20:3n-6, added to the yeast cultures at concentrations of 0.5 mM (C₁₈) and 0.75 mM (C₂₀) as uptake efficiency decreases with increasing chain length. Yeast cells were harvested, washed, dried, and lipid extracted by homogenization in chloroform/methanol (2:1, by vol.) containing 0.01% butylated hydroxytoluene as antioxidant as described previously (Hasting et al., 2001). Fatty acid methyl esters were prepared, extracted, purified by thin layer chromatography, and analyzed by gas chromatography (GC), all as described previously (Hastings et al., 2001). The proportion of substrate fatty acid converted to the longer chain fatty acid product was calculated from the gas chromatograms as $100 \times [\text{product area}/(\text{product area} + \text{substrate area})]$. Unequivocal confirmation of

306 fatty acid products was obtained by GC-mass spectrometry of the picolinyll
307 derivatives as described in detail previously (Hasting et al., 2001).

308

309 2.5 RNA extraction and quantitative PCR (qPCR).

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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°C prior to RNA extraction and gene expression studies.
317 Total RNA extractions were performed using AurumTM. 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 iScriptTMcDNA 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 $\Delta\Delta\text{C}_T$ method (Livaka

and Schmittgen, 2001). Amplification of cDNA samples was carried out in a MiniOpticom Real Time PCR System (Bio-Rad, Alcobendas, Madrid), using IQTM SYBR[®] Green Supermix Kit (Bio-Rad, Alcobendas, Madrid) in 20 µL final volume and the following conditions: 2 min denaturation at 94 °C, 40 cycles of 20 s at 94 °C, 15 s at 63 °C and 10 s at 72 °C. Samples were taken from liver: five animals per diet, three replicates per animal and cDNA from 25 ng of total RNA per replicate, were assayed. For tissue expression analysis, samples were taken from three animals, three replicates per tissue and cDNA from 25 ng of total RNA per replicate, were assayed. A melting curve was performed to confirm that a single PCR product was amplified. Quantitative analyses were performed using MJ OpticonMonitorTM Analysis Software v.3.1 (Bio-Rad, Alcobendas, Madrid). A standard curve using serial dilutions of linearized FAD plasmid was prepared to assess reaction optimization and proper quantification. To compare the expression level among tissue samples, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (Zar, 1984) (P<0.05) was performed.

2.6 Materials

Eicosatetraenoic (20:4n-3), (> 98-99 % pure) was purchased from Cayman Chemical Co., Ann Arbor, USA. Linoleic (18:2n-6), α-linolenic (18:3n-3) and eicosatrienoic (20:3n-6) acids (all >99% pure) were obtained from Sigma-Aldrich Co. Ltd. (Poole, U.K.). All solvents were HPLC grade and were from Fisher Scientific (Loughborough, UK).

3. Results

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

Three overlapping fragments for a putative $\Delta 6$ FAD of European sea bass were obtained using a gene-walking strategy, a FAD contig sequence deduced, and specific primers designed, at the 5'-end and 3'-ends, to amplify full length cDNA. The full length of the putative sea bass FAD cDNA, was 2089 bp, including a 5'-UTR of 267 bp, a 3'-UTR of 484 bp and a mean ORF of 1338 bp, which specified a protein of 445 amino acids (Fig. 1A). The 5'-UTR contained eight ATG sequences and 19 stop codons in the three possible reading frames upstream from the ATG sequence we considered as the start codon for the $\Delta 6$ FAD protein. Analysis of the predicted amino acid sequence showed that it included all the characteristic features of a membrane-bound desaturase, including an N-terminal cytochrome b_5 domain (amino acids 21 to 96) and a $\Delta 6$ FADS-like domain (amino acids 163 to 413) (Fig. 1B). The b_5 domain contains the conserved haem-binding motif H-P-G-G (Fig. 2A), and the $\Delta 6$ FADS-like domain contained three conserved histidine boxes (His-box). The deduced protein contains three highly hydrophobic regions, as shown by the hydrophobicity plot of the protein (Fig. 2B). The prediction analysis show the protein has four possible transmembrane segments (TMS) (Fig. 2A), similar to other members of the desaturase family of proteins. However, an additional His-box (HEKHH) (amino acids 350-354) is present in the $\Delta 6$ -FADS-like domain and, although the amino acid sequence of this additional His-box is not conserved among species, the polarity/charge of amino acids appears to be conserved between marine

species (Fig. 1A). Northern blots analysis showed a unique band. The estimated size for $\Delta 6$ FAD mRNA was 2.1 kb, which accurately agreed with the length of the cDNA (Fig. 3). Amino acid sequence alignment of the predicted sea bass FAD with $\Delta 6$ FADs from other fish species indicated a high level of conservation (Fig. 1A). A pairwise comparison among fish $\Delta 6$ FAD sequences showed the amino acid sequence predicted by the sea bass putative FAD shared greatest amino acid identity (94 %) to $\Delta 6$ FAD of sea bream (*Sparus aurata* L.), with 83 % and 82 % identity to $\Delta 6$ FADs of turbot (*Psetta maximus*) and cod (*Gadus morhua* L.), respectively (Table 1). Phylogenetic analysis comparing the sea bass FAD amino acid sequence with FADs from other fish species, and other organisms including mammals, clustered the sea bass FAD most closely with sea bream, then turbot and cod, and more distantly from freshwater species and salmonids (Fig. 4).

3.2 Heterologous expression in yeast *S. cerevisiae*

The fatty acid composition of the yeast transformed with the vector alone showed the four main fatty acids normally found in *S. cerevisiae*, namely 16:0, 16:1n-7, 18:0 and 18:1n-9 (peaks 1, 2, 3 and 4 respectively), together with the exogenously added fatty acids 18:3n-3 and 18:2n-6 (peaks 5 and 7, respectively) (Fig. 5A and C). When yeast transformed with the vector containing the sea bass FAD ORF were grown in the presence of the $\Delta 6$ substrates, 18:3n-3 and 18:2n-6, prominent additional peaks (6 and 8) were observed in the fatty acid profiles (Fig. 5B and D). Based on GC retention time and confirmed by GC-MS, the additional peaks associated with the presence of the sea bass FAD cDNA were identified as 18:4n-3 (peak 6) (Fig. 5B) and 18:3n-6 (peak 8) (Fig. 5D), corresponding to the $\Delta 6$ desaturation products of

18:3n-3 and 18:2n-6, respectively. All peaks were assigned as previously described by Hastings et al. (2001). Approximately 14.5 % of 18:3n-3 was converted to 18:4n-3 and 5.6 % of 18:2n-6 was converted to 18:3n-6 in yeast transformed with the sea bass FAD plasmid. No additional peaks representing desaturated fatty acid products were observed in the profiles of *S. cerevisiae* transformed with sea bass $\Delta 6$ FAD and incubated with 20:4n-3 or 20:3n-6 (peaks 9 and 10, respectively) (Fig.5E and F), indicating the enzyme codified by the sea bass cDNA showed no $\Delta 5$ desaturase activity. Similarly, the sea bass FAD cDNA did not show any $\Delta 4$ desaturase activity as evidenced by the lack of additional peaks representing desaturated products of 22:5n-3 or 22:4n-6 (data not shown)).

3.3 Subcellular localization

HeLa cells were transfected with pD6D/EGFP plasmid to determine the subcellular localization. After 48 h, the fusion protein $\Delta 6$ FAD ORF-tagged EGFP ($\Delta 6$ -FAD-EGFP) was highly expressed, showing a diffuse cytoplasmatic distribution surrounding the nucleus that didn't extend through the entire cytoplasm, suggesting possible localization in endoplasmic reticulum (ER). Since the expression pattern alone was not sufficiently clear to define the localization, an ER marker was also used. Thus, HeLa cells were co-transfected with vectors expressing $\Delta 6$ -FAD-EGFP and DsRed2-ER, designed to specifically localize ER. All evaluated coefficients of colocalization indicated that cells expressing both vectors showed a high degree of co-localization of $\Delta 6$ -FAD-EGFP and ER-targeted DS-Red2-ER proteins, indicating that $\Delta 6$ FAD was compartmentalized to the ER (Fig. 6). Although this work was focused on determining the intracellular localization of $\Delta 6$ FAD protein, we observed

that a high percentage of transfected cells (> 40 %) presented apoptotic nuclei (data not show).

3.4 Expression of $\Delta 6$ FAD in sea bass

Expression of the sea bass $\Delta 6$ FAD gene was examined in various tissues from fish fed a diet containing FO. The expression level in six different tissues was expressed as mean of absolute copy number (\pm SD) of $\Delta 6$ FAD transcripts present in 25 ng of total RNA (Whelan et al., 2003). The highest level of $\Delta 6$ FAD gene expression was observed in heart, brain and ovary, while kidney, adipose tissue and liver showed considerably lower expression levels (Fig. 7). The effects of dietary fatty acid composition on the expression of the $\Delta 6$ FAD were determined in liver from sea bass fed FO or diets with partial replacement of FO with VO's (Fig. 8). The expression of $\Delta 6$ FAD was up-regulated in fish fed diets with 60 % of the FO replaced with rapeseed or linseed oils, but not olive oil (Fig. 8A). Moreover, in sea bass fed FO compared to 60 % substitution of FO with two different blends of the same three VO's, the expression of $\Delta 6$ FAD was only significantly up-regulated in fish fed one of the blends (Fig. 8B)

4. Discussion

The study reported here revealed that European sea bass express a fatty acid desaturase as a unique mRNA of 2.1 kB, was expressed in heart > brain > ovary > kidney > adipose tissue > liver, showed intracellular localization in ER when expressed in HeLa cells and was confirmed as a $\Delta 6$ FAD when its activity/specificity

was determined in a yeast expression system. Comparing the protein sequence with that of a range of other FADs of fish showed the sea bass $\Delta 6$ FAD sequence to be more similar to the $\Delta 6$ FADs previously cloned from other marine fish, turbot, cod and, especially, gilthead sea bream (Seiliez et al., 2003; Zheng et al., 2004; Tocher et al., 2006). Phylogenetic analysis of the fish FAD sequences reflected classical phylogeny (Fig 4), showing the Acanthopterygia (cichlids, perciformes and pleuronectiformes) line, that includes sea bass and sea bream (both perciformes), with cod (Paracanthopterygii; Gadiformes) as a branch and further separated from both the carp and zebrafish (Ostariophysi; cyprinids), and salmonids (Salmoniformes; salmonidae) (Nelson, 1994).

Northern blot analysis of total RNA from brain of sea bass, showed a unique signal for $\Delta 6$ FAD transcript with an approximate size of 2.1 kb. Same results were obtained when different tissues were assayed (data not show). Unlike sea bass, two $\Delta 6$ FAD transcripts, with an approximate size of 3.8 and 1.8 kb, were detected in sea bream tissues (Seiliez et al., 2003). This difference can be due to the low stringent hybridization and washing conditions used in the assay (Seiliez et al., 2003). However, when Northern blots analysis of total RNA from sea bream was carried out in our lab under higher stringent conditions (60 °C, 0.1 x SSC, 0.5% SDS), only one transcript of about 3.7 kb for $\Delta 6$ FAD was detected (data not show).

In order to functionally characterize $\Delta 6$ FAD protein we have determined its enzymatic activity and specificity and its subcellular localization. Up to now the functional characterization of fish desaturases has consisted on the determination/characterization of its enzymatic activity, assuming that its subcellular localization was the appropriate one. Subcellular localization is a key functional characteristic of proteins. For proper functioning, the protein has to be translocated to

the correct intra- or extracellular compartments in a soluble form or attached to a membrane. To demonstrate the subcellular localization of a protein is time consuming, requiring the use of specific antibodies or how in our case, by transfection and transitory expression of the cDNA fused to a reporter gene, like the GFP. The protein code by the cDNA we have cloned showed an unequivocally fatty acid Δ -6 desaturase activity when it was assayed in a heterologous system. However, these assays do not show its subcellular localization. To affirm that the cDNA code by a Δ -6 fatty acyl desaturase enzyme, the protein must be localized at their appropriate subcellular compartment, the endoplasmic reticulum (ER), to perform their desired function. In this work we have demonstrated that the fusion protein Δ -6-FAD-EGFP was localized in the ER when transiently expressed into HeLa cells. It is the first time that this determination was made. At the same time, we observed a high percentage of apoptotic nuclei in cells presenting high levels of Δ -6-FAD-EGFP expression. Although the explanation for this fact is not clear, it may be due to over-expression effect of the fusion protein or, like it has been described previously for Atlantic salmon, desaturated product of this enzyme could induce apoptosis, decreasing levels of linoleic acids (LA; 18:2 n-6) contained in phospholipids (PLs) associated with mitochondrial membranes and increasing, therefore, susceptibility of PLs to peroxidation (Todorovic et al., 2009).

Along with the cloning of Δ -6 FADs cDNAs of sea bream, turbot and cod, the work described has confirmed that marine fish have, and express the gene required for the first activity in the HUFA biosynthesis pathway, Δ -6 desaturation, and the protein is localized in the proper intracellular compartment, the ER. Then, deficiencies in the pathway in marine fish would be at a subsequent step, such as chain elongation and/or Δ -5 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 $\Delta 6$ 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 $\Delta 6$ 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 $\Delta 6$ FAD displayed $\Delta 6$
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 $\Delta 6$
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 $\Delta 6$ FAD gene in sea bass tissues and other marine fishes. However, in the same yeast
522 system, the salmon $\Delta 6$ FAD showed over 60% conversion of 18:3n-3 (Zheng et al.,
523 2005a) and the cod $\Delta 6$ gave 33 % conversion (Tocher et al., 2006). Furthermore,
524 conversion of 18:2n-6 by the sea bass $\Delta 6$ FAD was only 5.6 % in the yeast expression
525 system, compared to 14 % for the salmon $\Delta 6$ 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 $\Delta 6$ FAD was more active than sea bass $\Delta 6$ 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 $\Delta 6$ 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 $\Delta 6$ 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 innervating cells is critical
539 for the correct functioning of this organ. In cod, $\Delta 6$ 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 $\Delta 6$ and $\Delta 5$ FADs were highest in intestine, liver and brain (Zheng
543 et al., 2005). Mammalian $\Delta 6$ and $\Delta 5$ 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 $\Delta 6$ 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 $\Delta 6$ and $\Delta 5$ 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 $\Delta 6$ FAD in liver
556 of rainbow trout, and $\Delta 5$ 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 $\Delta 6$ and $\Delta 5$ FADs were increased in liver of salmon fed a
559 VO blend (rich in C₁₈ PUFA) compared to levels in fish fed FO (Zheng et al.,
560 2005a,b). In the present study, the expression of $\Delta 6$ 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 $\Delta 6$ 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 $\Delta 6$ 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 $\Delta 6$ 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 $\Delta 6$ 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 $\Delta 6$ 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 $\Delta 6$ 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

hepatocytes (Mourente and Dick, 2002; Mourente et al., 2005b), although increased activity was reported in pyloric caecal enterocytes of fish fed VO blends (Mourente et al., 2005a). In cod, where expression of $\Delta 6$ FAD was not significantly increased in liver and intestine of fish fed a VO blend compared to fish fed FO, there was similarly no significant effect on HUFA biosynthetic activity in either hepatocytes or enterocytes (Tocher et al., 2006). In contrast, FAD expression was correlated with enzyme activities in mice fed EFA-deficient versus corn oil diets (Cho et al., 1999a) and in salmon fed FO versus VO diets (Zheng et al., 2004; 2005b). It is likely that the conflicting data in both FAD expression and HUFA biosynthetic activity in marine fish are due to the low levels of expression and enzyme activity recorded in marine fish tissues compared to salmonids (Tocher et al., 2006).

5. Conclusions

The study described here has demonstrated that European sea bass have and express a fatty acid desaturase gene, the product of which is localized in the ER and shows clear $\Delta 6$ FAD activity in a heterologous yeast expression system. These data support the hypothesis that the poor ability of marine fish, such as sea bass, to synthesize HUFA is not due to lack of a $\Delta 6$ FAD. The sea bass $\Delta 6$ FAD was highly expressed in heart, brain and ovary. Kidney, adipose tissue and liver showed considerable lower expression levels of $\Delta 6$ FAD. The expression of the $\Delta 6$ FAD may be under some form of nutritional regulation, being increased in hepatocytes of fish fed diets containing VOs compared to fish fed FO, although the data are not consistent. Further studies are required to conclusively determine why C_{18} PUFA are not substrates for the enzyme in sea bass: a low expression of the gene, an high and strict enzymatic

activity control or some other factor (genetics, hormonal, environmental) can be the reason why the $\Delta 6$ FAD appears to be barely functional in European sea bass, as in other marine fish species. Sea bass has the genetic capacity to carry out the first step of the transformation of the C18 PUFA in HUFAs. However metabolic possibility to carry out this conversion is shrunk or handicapped.

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LEGENDS TO FIGURES

FIG. 1. Comparison of the deduced amino acid sequence of the $\Delta 6$ FAD from European sea bass (*Dicentrarchus labrax*)(DI) with that of $\Delta 6$ FADs from gilthead sea bream (*Sparus aurata*) (Sa), Atlantic cod (*Gadus morhua*) (Ac), Atlantic salmon (*Salmon salar*) (As) and common carp (*Ciprinus carpio*) (Cc). (A) Deduced amino acid sequences of FADs were aligned using ClustalX. Identical residues are shaded black and residues conserved in four proteins are shaded grey. A conserved domain search of the deduced amino acid sequence of the sea bass $\Delta 6$ FAD was carried out against the Conserved Domain Database at NCBI. (B) Two domains were found: a cytochrome *b*₅ domain (Cyt-b₅) at the N-terminus (amino acids 21-96) and a $\Delta 6$ -FADS-like domain at the C-terminus (amino acid 163-413).

FIG. 2. Transmembrane segments prediction profiles of the amino acid sequence for sea bass $\Delta 6$ FAD. The amino acid sequence exhibited all the characteristics features of FADs: (A) a conserved haem-binding motif H-P-G-G (designed with a black triangle) in the cytochrome b_5 domain and three sequences-conserved histidine cluster boxes (indicated with a black triangle) in the $\Delta 6$ -FADS-like domain. (B) The prediction analysis for transmembrane segments show the protein has three highly hydrophobic regions, as shown the hydrophobicity plot of the protein, containing four possible transmembrane expanded segments (indicated with a grey box labeled with TMS).

FIG.3. Northern blot analysis of $\Delta 6$ FAD mRNA from brain of European sea bass. Total RNA (30 μ g) were used to determine the mRNA size of the $\Delta 6$ FAD. As probe, $\Delta 6$ FAD full-length 32 P radiolabelled cDNA was used. Hybridization and washing of the blot were carried out at high stringency (see material and methods). After 3 days of exposure, a single band of approximately 2.1 kb was observed. Sizes of RNA standards are indicated.

FIG.4. Phylogenetic tree of European sea bass $\Delta 6$ FAD and FADs from other fish species (Atlantic salmon, zebrafish, cherry salmon, rainbow trout, sea bream, common carp, turbot and tilapia), mammals (mouse and human), fungus (*Mortierella alpina*, *Pythium irregular*, *Mucor circinelloides*), algae (*Phaeodactylum tricornutum*, *Thraustochytrium* sp.), amoeba (*Dictyostelium discoideum*) and nematode (*Caenorhabditis elegans*). The tree topology presented was replicated after 1000 bootstrap iterations. The optimal tree is shown (next to the branches). The evolutionary distances are in the units of the number of amino acid substitutions per site. Sequences marked with an asterisk are not functionally characterized. The $\Delta 9$ FAD sequence from *Mortierella alpina* was used, as an out-group sequence, to construct a rooted tree. Protein accession numbers: $\Delta 5$ Atlantic salmon AF478472, $\Delta 5$ Human AF199596, $\Delta 5$ Mouse AB072976, $\Delta 5$ *Mortierella alpina* AF067654, $\Delta 5$ *Caenorhabditis elegans* AF078796, $\Delta 5$ *Dictyostelium discoideum* AA37090, $\Delta 5$ *Phaeodactylum tricornutum* AY082392, $\Delta 5$ *Phytium irregulare* AAL13311, $\Delta 5$ *Thaustochytrium* sp AAM09687, Des1* Cherry salmon AB070444, Des2* Cherry salmon AB074149, $\Delta 6$ *Phytium irregulare* AAL133100, $\Delta 6$ *Phaeodactylum tricornutum* AY082393, $\Delta 6$ *Mucor circinelloides* BAB69055, $\Delta 6$ *Caenorhabditis*

elegans AF031477, $\Delta 6$ *Mortierella alpina* AF110510, $\Delta 6$ Mouse AF126798, $\Delta 6$ Human AF126799, $\Delta 6$ *Dicentrarchus labrax* AM746703, $\Delta 5/\Delta 6$ Zebrafish AF309556, $\Delta 6$ Atlantic cod DQ054840, $\Delta 6$ Common carp AF309557, $\Delta 6$ Turbot AY546094, Des* Nile tilapia AB069727, $\Delta 6$ *Sparus aurata* AY055749, $\Delta 6$ Rainbow trout AF301910 and $\Delta 6$ Atlantic salmon AY458652.

FIG.5. Functional characterization of the European sea bass FAD by heterologous expression in yeast (*S. cerevisiae*). Panels A and C show the fatty acids extracted from yeast transformed with pYES vector without insert and grown in the presence of 18:3n-3 and 18:2n-6, respectively. Panels B, D, E and F show the fatty acids composition of yeast transformed with pYES vector containing the putative desaturase ORF and grown in the presence of 18:3n-3, 18:2n-6, 20:4n-3 and 20:3n-6, respectively. The first four peaks in all panels are the main endogenous fatty acids of *S. cerevisiae*, namely 16:0 (1), 16:1n-7 (2), 18:0 (3) and 18:1n-9 (with 18:1n-7 as shoulder) (4). Peak 5 in panels A and B, and peak 7 in panels C and D are the exogenously added substrate fatty acids, 18:3n-3 and 18:2n-6, respectively. Peaks 6 and 8 in panels B and D were identified as the resultant desaturated products, namely 18:4n-3 and 18:3n-6, respectively. Peaks 9 and 10 in panels E and F are the exogenously added substrate fatty acids, 20:4n-3 and 20:3n-6, respectively. Vertical axis: FID response; horizontal axis: retention time.

FIG.6. Subcellular localization of $\Delta 6$ FAD from European sea bass in HeLa cells. The plasmid coding for the fusion protein D-6-D-EGFP was transiently co-transfected into HeLa cells with a plasmid construct designed to localize the endoplasmic reticulum (pDsRed2-ER, Clontech, Madrid, Spain). Forty-eight h after transfection green channel (panel A) and red channel (panel B) fluorescent images were taken for the same field. DAPI was used to localize the nucleus (blue channel, panel C). Overlay composed of three channels (panel D) shows in yellow pixels where the green (D6D-EGFP protein) and red (DsRed2 protein) channels match. To quantify the degree of co-localization, intensity correlation coefficient-based (ICCB) analyses were performed, using JACoP tool included in Image J software (v. 1.42k) (<http://rsb.info.nih.gov/ij/>). In E), Pearson's and Overlap coefficients and Li's Intensity Correlation Quotient (IQC) for ten analyzed images, are shown as mean \pm

SD. Maximum (completed co-localization) and minimum (exclusion) values for each coefficient are indicated.

FIG. 7. Tissue transcription of $\Delta 6$ FAD in European sea bass. Transcript (mRNA) copy numbers were determined by quantitative real-time PCR (qPCR) as described in the Methods section. Results expressed as means of absolute copy number (\pm SD) (n=3) of FAD transcripts in 25 ng of total RNA. Letters show significant differences ($P < 0.05$) among tissues as determined by one-way ANOVA followed by Tukey's multiple comparison test (Zar, 1984).

FIG. 8. Effect of dietary VO on the expression of $\Delta 6$ FAD in liver from European sea bass. Relative expression was determined by quantitative PCR (qPCR) and normalized as described in the Materials and Methods Section. Results are referred to kidney expression (1.0) and expressed as means \pm SD (n = 5). A) Effect of 60 % substitution of dietary FO with rapeseed oil (RO), linseed oil (LO) and olive oil (OO). B) Effect of 60 % substitution of dietary FO with blends of VOs (see material and methods). Superscript letter denotes a significant effect of diet as determined by one way ANOVA analysis and subsequent Tukey's multiple comparison test ($P < 0.05$) (Zar, 1984).

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TABLE 1

Identity matrix showing the percentage of identical amino acids residues in the protein sequences of fish and human $\Delta 6$ FAD

| | <i>Atlantic salmon $\Delta 6$</i> | <i>Rainbow trout $\Delta 6$</i> | <i>Sea bream $\Delta 6$</i> | <i>Nile Tilapia $\Delta 6$</i> | <i>Turbot $\Delta 6$</i> | <i>Common carp $\Delta 6$</i> | <i>Atlantic cod $\Delta 6$</i> | <i>Zebrafish $\Delta 6$</i> | <i>Human $\Delta 6$</i> |
|--|--|--|--|---|---|--|---|--|--|
| Sea Bass $\Delta 6$ | 78 | 77 | 94 | 76 | 83 | 65 | 82 | 67 | 64 |
| Atlantic salmon $\Delta 6$ | | 94 | 78 | 71 | 73 | 65 | 76 | 66 | 66 |
| Rainbow trout $\Delta 6$ | | | 77 | 72 | 73 | 65 | 77 | 66 | 65 |
| Sea Bream $\Delta 6$ | | | | 76 | 84 | 65 | 82 | 67 | 64 |
| Nile Tilapia $\Delta 6$ | | | | | 73 | 61 | 72 | 63 | 61 |
| Turbot $\Delta 6$ | | | | | | 66 | 77 | 67 | 62 |
| Common carp $\Delta 6$ | | | | | | | 67 | 89 | 62 |
| Atlantic cod $\Delta 6$ | | | | | | | | 70 | 63 |
| Zebrafish $\Delta 6$ | | | | | | | | | 64 |

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FIG. 1

A

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Dl  MGGGGQLTEPGESGSR-----RAGGVYTWEEVQSHCNRNQDQWLVIDRKVYDITHWAKGHPGGRVISHYAGE
Sa  MGGGGQLTEPGEPGSR-----RAGGVYTWEEVQSHSSRNQDQWLVIDRKVYNVTKWAKRHPGGERVINHYAGE
Ac  MGGGGQLTEPVETSACGG-----RAASVYTWDEVQKHCHRNQDQWLVINRKVYNVTQWAKRHPGGLRVISHYAGE
As  MGGGGQONDSCEPAKGDRCGGPGGLGGSNAVYTWEEVQRHSHRGDQWLVIDRKVYNITQWAKRHPGGRVISHFACE
Cc  MGGGGQQTDRITGTNG-----RFGTYTWEEVQKHTKFGDQWIEVERKVYNVSQWVKRHPGGVRILGHYAGE

Dl  DATEAFTAFHPNLKFVQKFLKPLLIGELAAEPSQDRNKNAAIQDFYTLRAQAESEGLFKAQPLEFCLHLGHILL
Sa  DATEAFTAFHPDLKFVQKFLKPLLIGELAAEPSQDRNKNAAVIQDFHTLRAQAESDGLFRAQPLEFCLHLGHILL
Ac  DATEAFLAFHPNPKLVQKFLKPLLIGELAVTEPSQDRNKNAAVVEDFQALRTRAEGGLGFQAQPLEFCLHLGHILL
As  DATDAFVAFHPNPNFVRKFLKPLLIGELAPTEPSQDHGKNAVLVQDFQALRNVRVEREGLLRARPLEFSLYLGHILL
Cc  DATEAFTAFHPDLPLVRKYMKPLLIGELEASEPSQDRQKNAALVEDFRALRERLEAEGCFKTOPLLELILHSHILL

Dl  LEALAWLIITLWGTSWTLLTFLCSIMLATAQSQACWLQHDFGHLSVEKKSSWNHMLHKEFVIGHLKGASANWNNHRHF
Sa  LEALAWLIITLWGTSWTLLTFLISIIILATAQTOACWLQHDFGHLSVEKKSSWNHILHKEFVIGHLKGASANWNNHRHF
Ac  LELLAWMSVTLWGTSWRTLLCSFILAVACAQACWLQHDFGHLSVEKLSRWNNHIEHKEFIIIGHLKGASGNWNNHRHF
As  LEALALGLITLWGTSWSLTLCSIMLATSQSQACWLQHDYGHLSVCKKSSWNHVLHKEFVIGHLKGASANWNNHRHF
Cc  LEAIALMMVTLGTGWINTAIVAVLLATAQSQAEWLQHDFGHLSVEKTSRWNNHLVHKEFVVGHIKGSAGRWNHRHF

Dl  QHHAKPNI FSKDPDVNMLHIFVVGATQPV EYCIKKIKYMPYHHQH OYFLLVGPPLLIPVYFHIQIIRTMISR RDWV
Sa  QHHAKPNI FSKDPDVNMLHIFVLGDTQPV EYCIKKIKYLPYHHQH OYFLLVGPPLLIPVYFHIQIIRTMISR HDWV
Ac  QHHAKPNV FSKDPDVNMLHIFVVGDIQPV EYCIKKIKYMPYHHQH OYFLLVGPPLLIPVYFHIQILRAMFSR RDWV
As  QHHAKPNV LSKDPDVNMLHIFVLCDKQPV EYCIKKIKYMPYHHQH OYFLLIGPPLLIPVFFTIQIEQTMF SQRNVV
Cc  QHHAKPNV FKKDPDVNMLNAFVACKVQPV EYCVKKIKHLPLYHHQH KYFFFI GPPLLIPVYFQFQIEFHNMI AHGLWV

Dl  DLAWSMSYLLRYLCCYVPLYCLFGSLALISFVRELESHWFVWVTQMNHLPMDIDHEKHHDWLTMQLOATCNIEKSS
Sa  DLAWSMSYLLRYLCCYVPLYCLFGSVALISFVRELESHWFVWVTQMNHLPMDIDHEKHHDWLTMQLOATCNIEKSV
Ac  DLAWSMSYLLRYFCCYAPFYCLLGSVALISFVRELESHWFVWVTQMNHLPMDIDHEKQODWLSMQLSATCNIEQSC
As  DLAWSMTEYLRFFCSYYPFFCLFGSVALITFVRELESHWFVWVTQMNHLPMEIDHERHQDWLTMQLSGTCNIEQST
Cc  DLAWCISYVRYFLCYTQYYEVFWAVILFNFVRELKSHWFVWVTQMSHIPMQIDYEKHQDRLSMQLVATCNIEQSS

Dl  FNDWFSGHLNFQIEHHLFPTMPRHNYHLVAPLVHALCEKHGIPYQVKTMWQGLVDVIRSLKNSGDLWLDAYLHK
Sa  FNDWFSGHLNFQIEHHLFPTMPRHNYHLVAPLVHALCEKHGIPYQVKTMWQGIVDVIRSLKNSGDLWLDAYLHK
Ac  FNDWFSGHLNFQIEHHLFPTMPRHNYQVLAPLVRALCEKHSIPYQEKTLWRGVADVVRSLKNSGDIWMDAYLHK
As  FNDWFSGHLNFQIEHHLFPTMPRHNYHLVAPLVRTLCEKHGIPYQVKTLQKAIIDVVRSLKKSGLWLDAYLHK
Cc  FNDWFSGHLNFQIEHHLFPTMPRHNYWRAAPHVRELCAKYCIKYQEKTLQGAFAADVVRSLKSGEITWLDAYLNE

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B

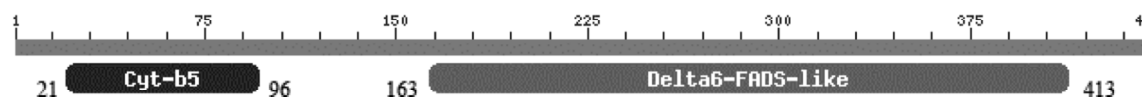


FIG 2

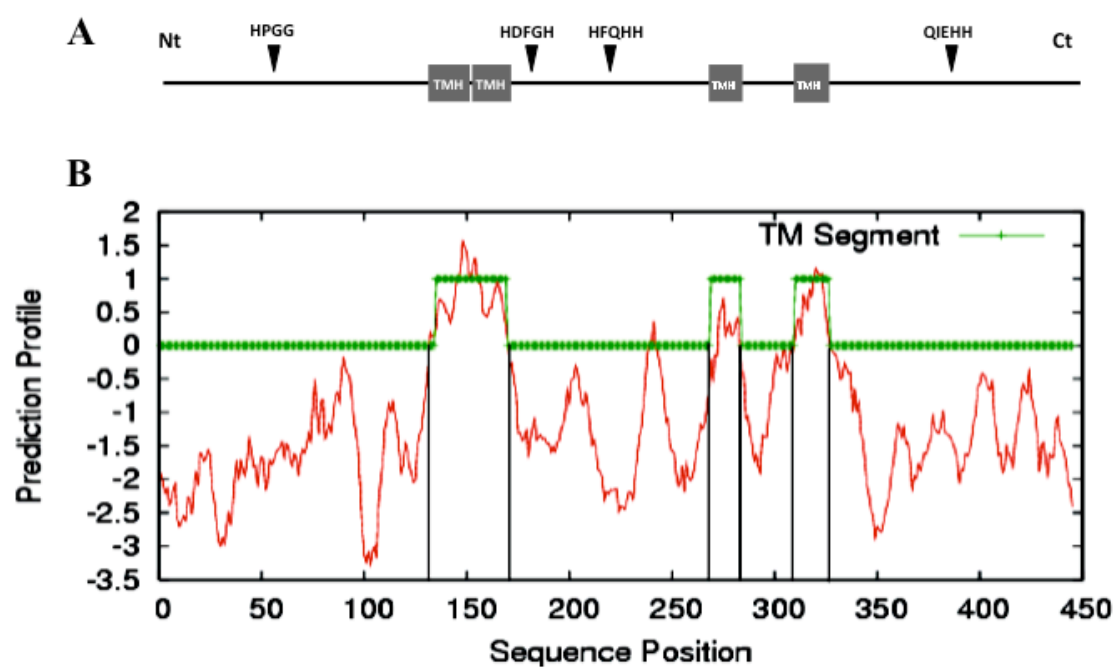
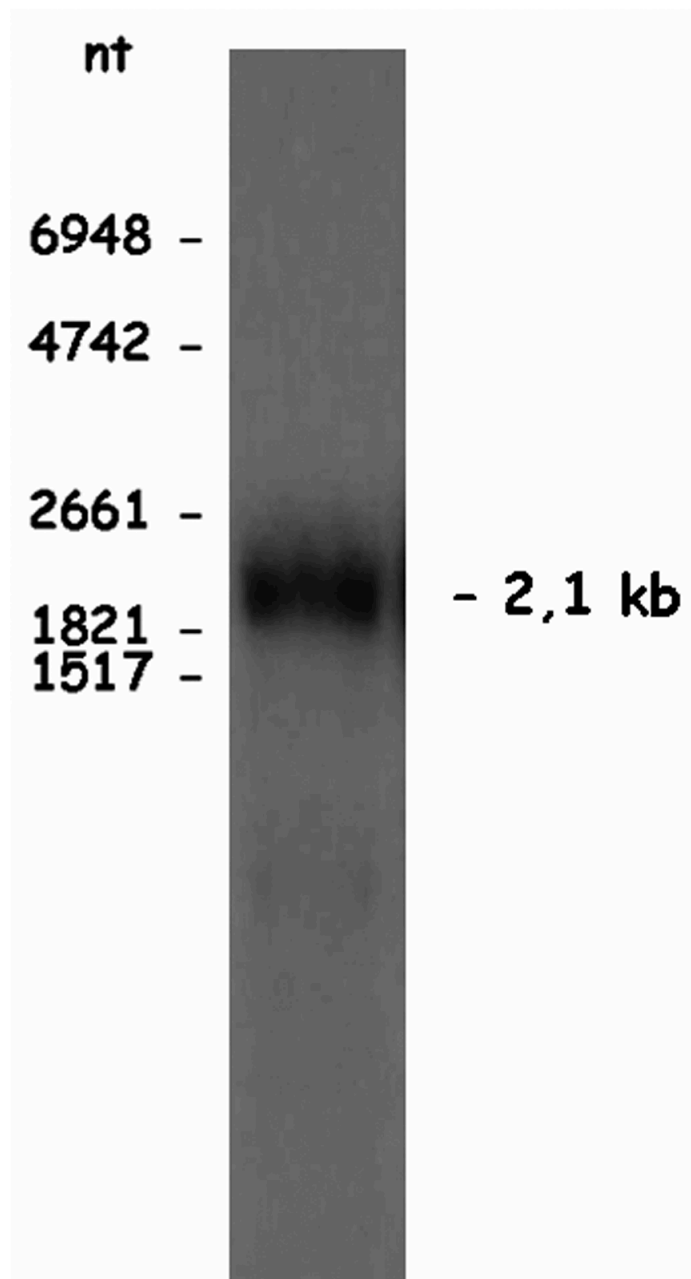
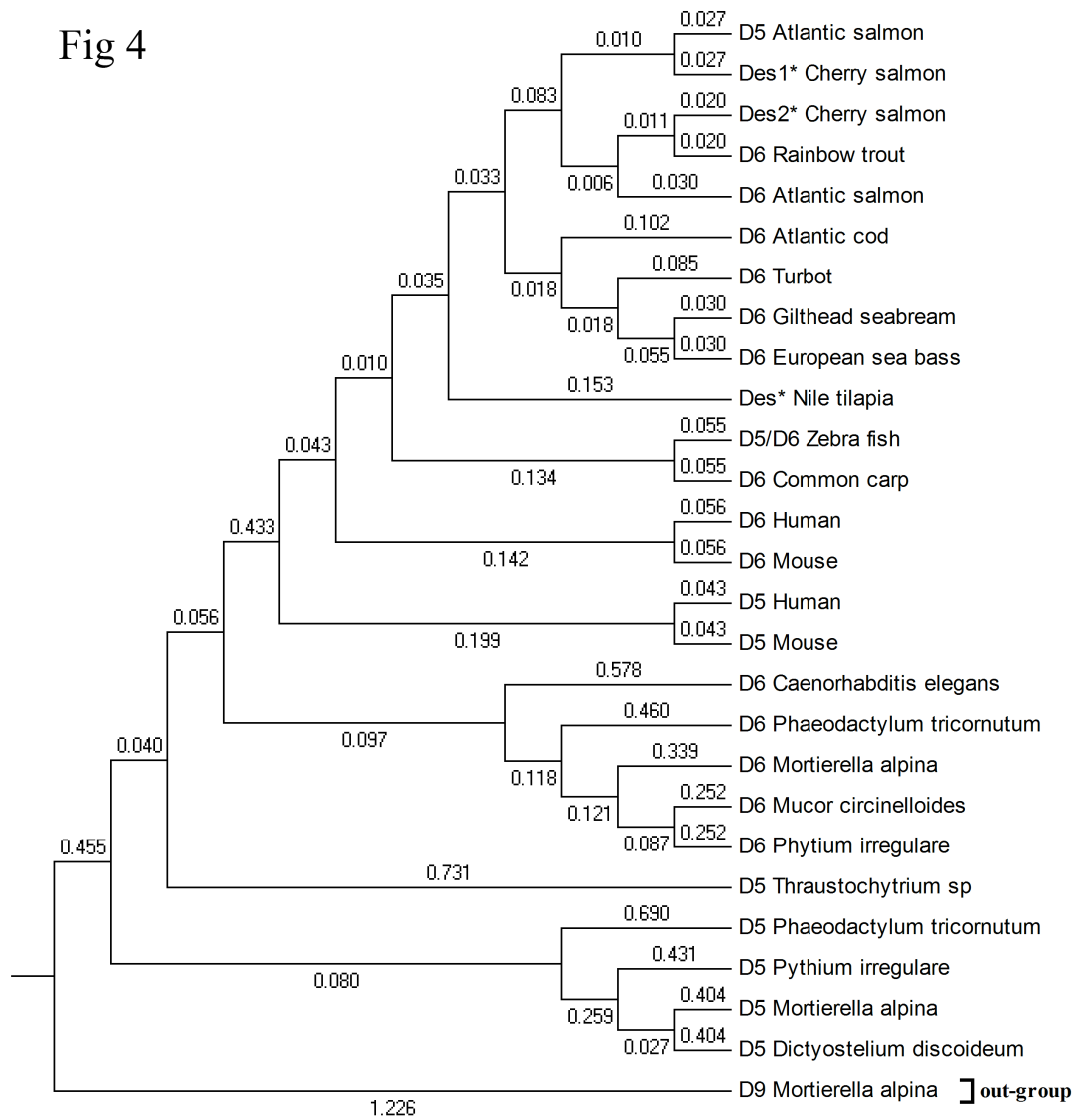


FIG 3



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Fig 4



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FIG. 5

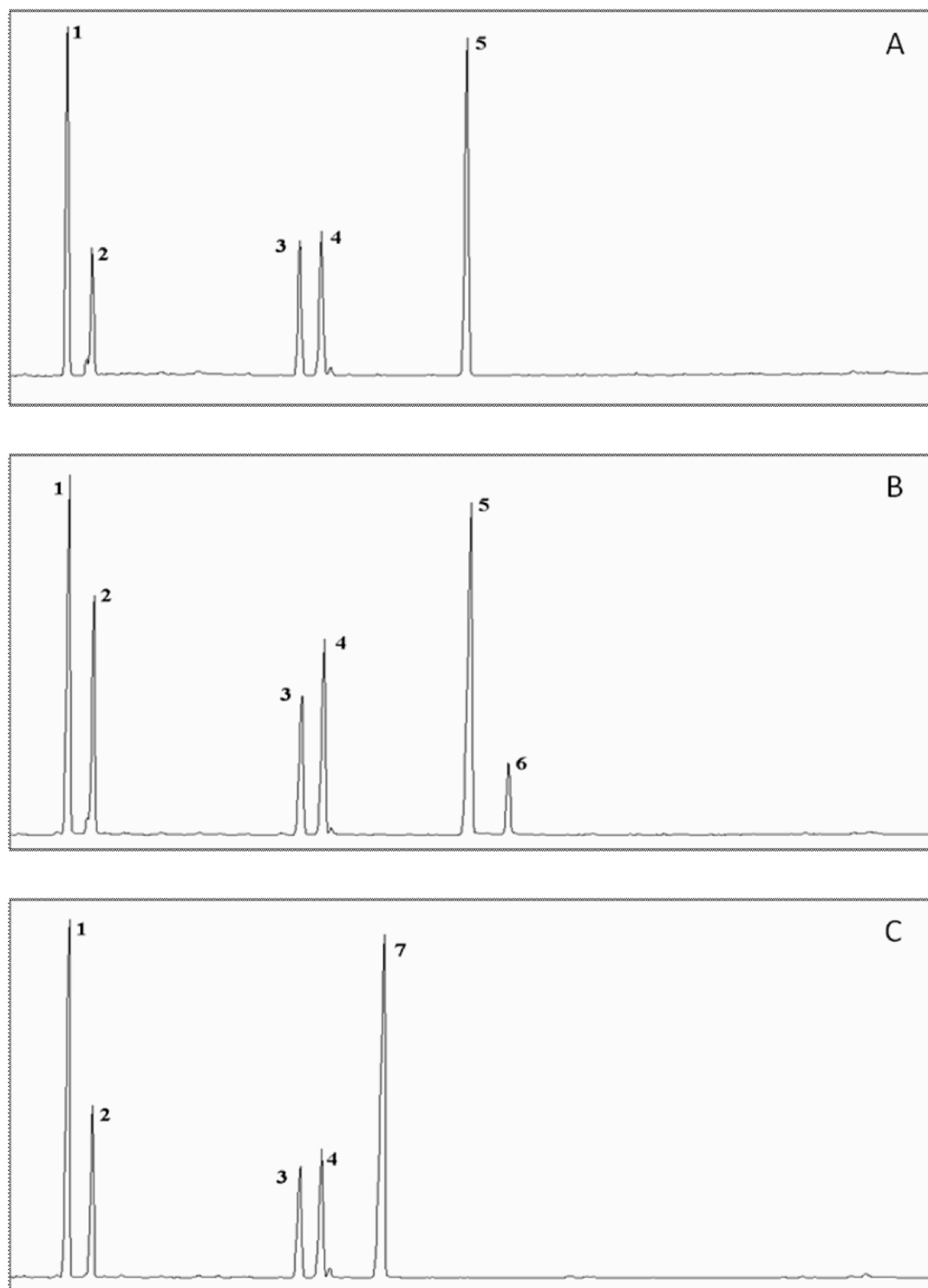


FIG. 5

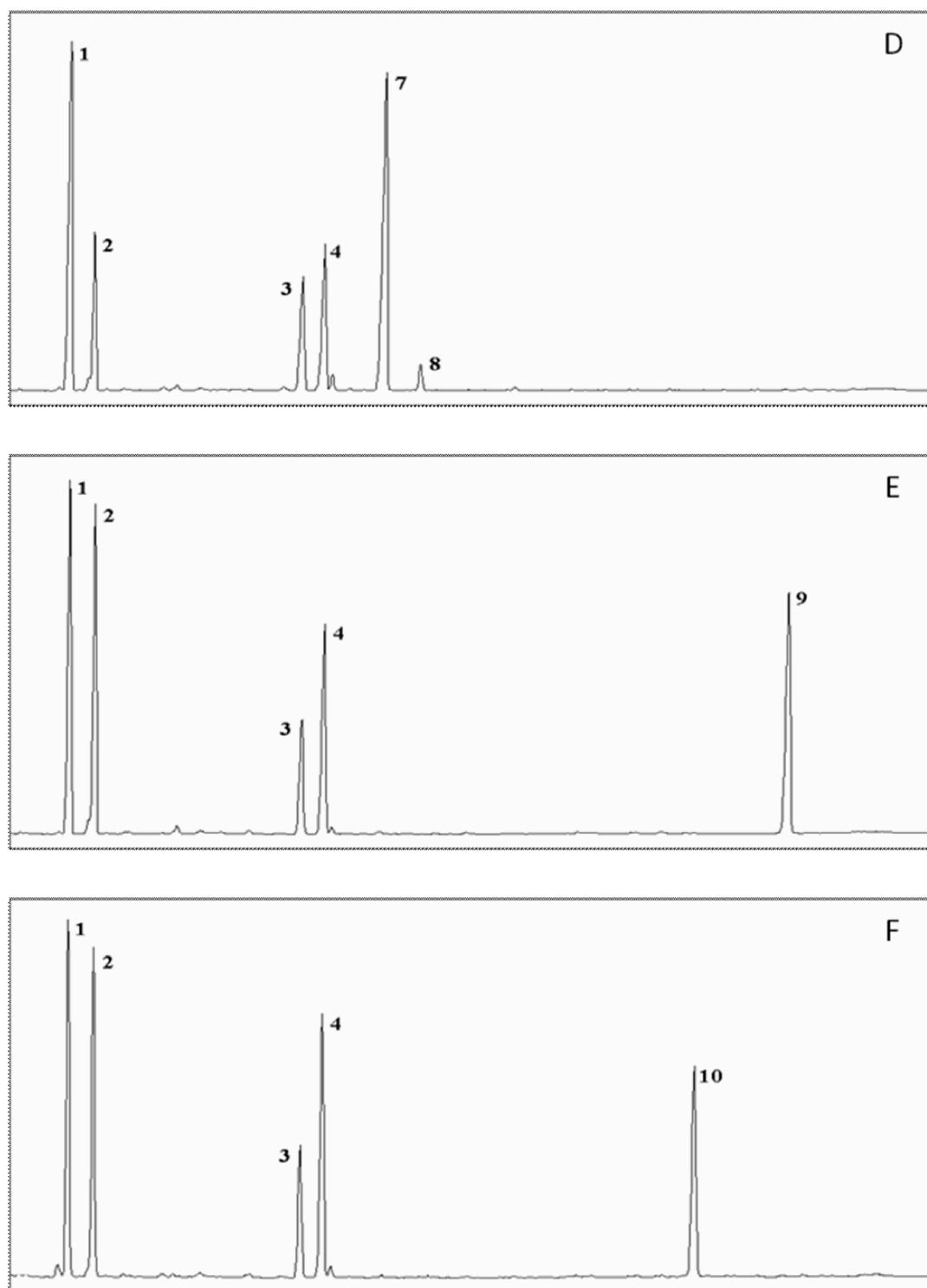
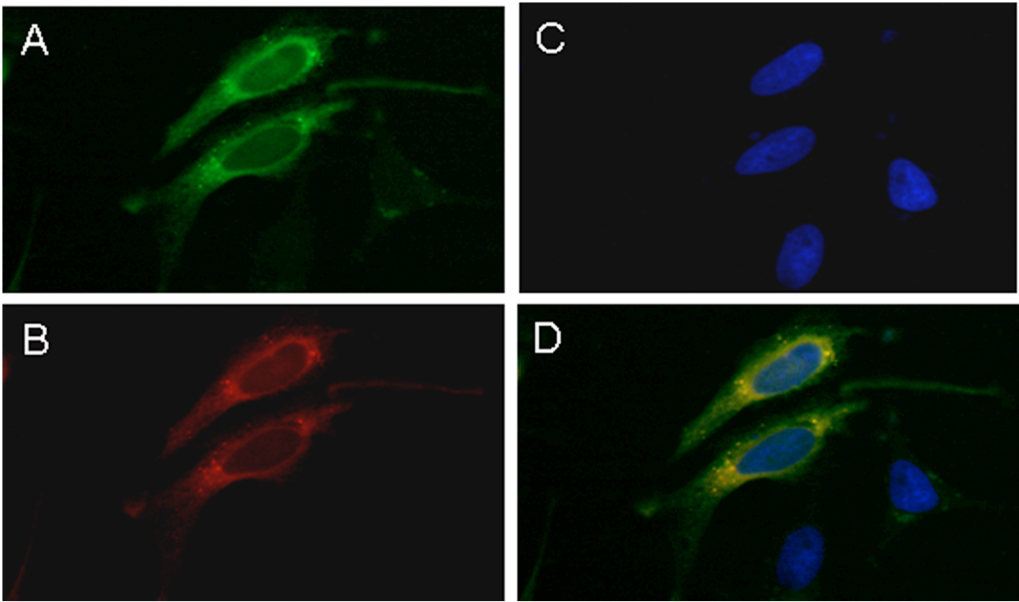


FIG. 6



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

Tissue distribution of $\Delta 6$ fad genes in sea bass (*D. labrax*)

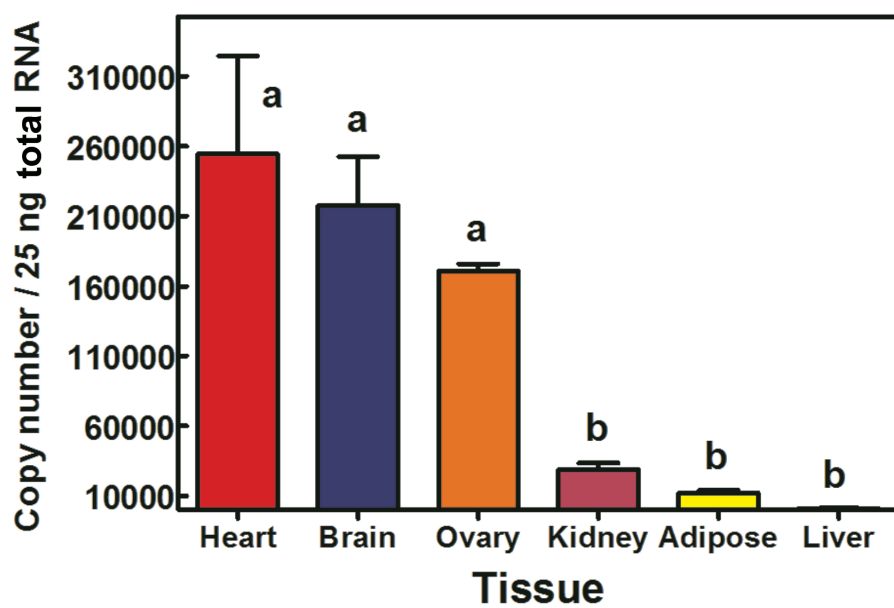
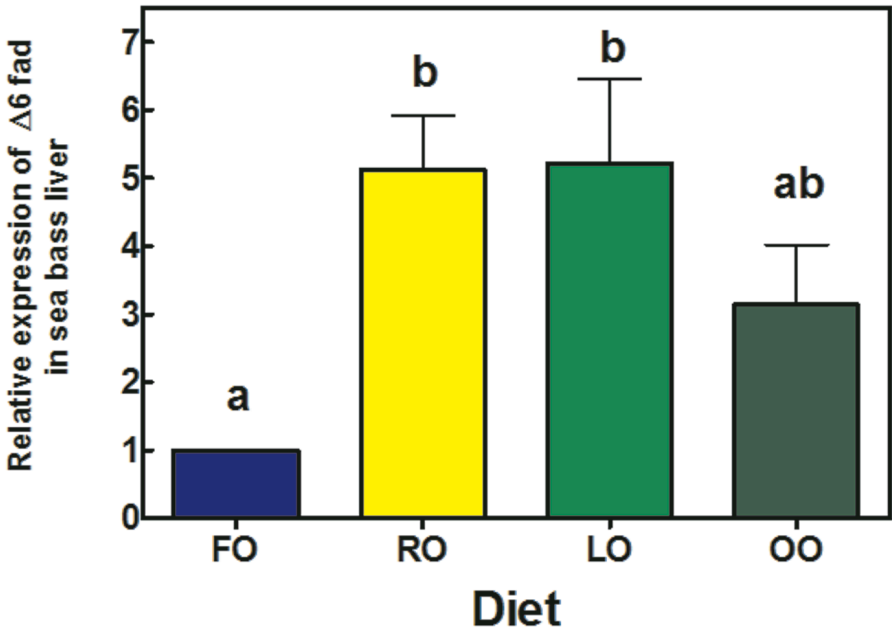


Fig. 8

A Nutritional regulation by 60% substitution of dietary FO with single vegetable oils (RO, LO, OO)



B Nutritional regulation by 60% substitution of dietary FO with blends of vegetable oils (RO, LO, PO)

