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Docosahexaenoic acid biosynthesis via fatty acyl elongase and Δ4-desaturase and its modulation by dietary lipid level and fatty acid composition in a marine vertebrate

Sofia Morais[a,*], Gabriel Mourente[b], Almudena Martínez[a], Noélia Gras[a], Douglas R. Tocher[c]

[a] IRTA, Ctra. Poble Nou Km 5.5, 43540 Sant Carles de la Rápita, Spain.

[b] Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, 11510 Puerto Real, Cádiz, Spain.

[c] Institute of Aquaculture, School of Natural Sciences, University of Stirling, Stirling FK9 4LA, UK.

Email addresses: sofia.morais@irta.cat (S. Morais), gabriel.mourente@uca.es (G. Mourente), almudena.martinez@irta.cat (A. Martinez), noelia.gras@irta.cat (N. Gras), d.r.tocher@stir.ac.uk (D.R. Tocher)

*Corresponding author: Dr Sofia Morais, IRTA, Centre de Sant Carles de la Rápita, Ctra. Poble Nou km 5.5, 43540 Tarragona, Spain. Tel: +34 977745427; fax +34 977744138.

E-mail address: sofia.morais@irta.cat (S. Morais)

Abbreviations: ALA: α-linolenic acid; ARA: arachidonic acid; DHA: docosahexaenoic acid; DPA: docosapentaenoic acid; EPA: eicosapentaenoic acid; FA: fatty acid; FAME: fatty acid methyl esters; FM: fish meal; FO: fish oil; HL: high lipid; LC-PUFA: long-chain polyunsaturated fatty acids; LL: low lipid; LOA: linoleic acid; PUFA: polyunsaturated fatty acids; VO: vegetable oil.

Abstract

The present study presents the first "*in vivo*" evidence of enzymatic activity and nutritional regulation of a Δ4-desaturase-dependent DHA synthesis pathway in the teleost *Solea senegalensis*. Juvenile fish were fed diets containing 2 lipid levels (8 and 18%, LL and HL) with either 100 % fish oil (FO) or 75 % of the FO replaced by vegetable oils (VO). Fatty acyl elongation (Elovl5) and desaturation (Δ4Fad) activities were measured in isolated enterocytes and hepatocytes incubated with radiolabelled α-linolenic acid (ALA; 18:3n-3) and eicosapentaenoic acid (EPA; 20:5n-3). Tissue distributions of *elovl5* and *Δ4fad* transcripts were also determined, and the transcriptional regulation of these genes in liver and intestine was assessed at fasting and postprandially. DHA biosynthesis from EPA occurred in both cell types, although Elovl5 and Δ4Fad activities tended to be higher in hepatocytes. In contrast, no Δ6Fad activity was detected on ^{14}C-ALA, which was only elongated to 20:3n-3. Enzymatic activities and gene transcription were modulated by dietary lipid level (LL > HL) and fatty acid (FA) composition (VO > FO), more significantly in liver than in intestine, which was reflected in tissue FA compositions. Dietary VO induced a significant up-regulation of *Δ4fad* transcripts in liver 6 h after feeding, whereas in fasting conditions the effect of lipid level possibly prevailed over or interacted with FA composition in regulating the expression of *elovl5* and *Δ4fad*, which were down-regulated in liver of fish fed the HL diets. Results indicated functionality and biological relevance of the Δ4 LC-PUFA biosynthesis pathway in *S. senegalensis*.

Keywords: DHA; polyunsaturated fatty acid synthesis; desaturation and elongation activity; nutritional regulation; dietary lipid level; fatty acid composition

1. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFA) are essential nutrients with a variety of important structural, functional and signaling roles. They are major components of biological membranes, particularly of neural tissue and immune cells [1-3], and are implicated in a vast range of metabolic and immune pathways, either via direct activation of transcription of multiple genes, by functioning as secondary messengers, or acting as potent bioactive molecules and precursors of eicosanoids with pro- or anti-inflammatory properties [4,5]. These roles imply that LC-PUFA are critically important in normal development and health and, conversely, they are implicated in several disease processes [2,3,6]. Therefore, not surprisingly, the pathway of LC-PUFA biosynthesis has been an important topic of research in many organisms, from lower eukaryotes to higher vertebrates, for several decades now.

Polyunsaturated fatty acids (PUFA), such as α-linolenic acid (ALA; 18:3n-3) and linoleic acid (LOA; 18:2n-6), are essential dietary nutrients in all vertebrates since they cannot be synthesized *de novo* and hence must be obtained from the diet. Subsequent biosynthesis of LC-PUFA such as arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) involves sequential desaturation and elongation of precursor PUFA [7]. Within vertebrates, the extent to which a species can produce LC-PUFA from C_{18} PUFA precursors varies greatly, depending on their repertoire of fatty acyl elongase (Elovl) and desaturase (Fad) enzymes. With respect to the final steps of DHA synthesis, until recently the classic and only demonstrated pathway of LC-PUFA biosynthesis in vertebrates was the "Sprecher" pathway that involves two sequential elongations of EPA to 24:5n-3 followed by Δ6 desaturation and one round of peroxisomal β-oxidation [8]. A theoretically simpler and more direct pathway for biosynthesis of DHA from EPA would be via one elongation

step followed by Δ4 desaturation. However, for many years a Δ4Fad could only be found in lower eukaryotes [e.g., 9-12]. This changed recently, when a *fad* transcript was reported in the marine herbivorous fish *Siganus canaliculatus* that, when functionally characterized in yeast, was found to have Δ4 desaturation activity [13]. Shortly after, other teleosts such as *Solea senegalensis*, a marine carnivore, and *Chirostoma estor*, a freshwater carnivore (mostly feeding on zooplankton), were found to have a similar gene encoding a protein with Δ4Fad activity in *in vitro* heterologous expression assays [14,15]. However, the *in vivo* activity and biological relevance of the Δ4 pathway in vertebrates remained to be established.

Senegalese sole, *Solea senegalensis*, is a species with high aquaculture interest whose production has been intensifying in recent years in Southern Europe [16]. One of the early identified advantages of this species was its apparently low LC-PUFA (particularly DHA) requirements, for a marine teleost, during early larval stages [16]. Therefore, the LC-PUFA biosynthesis ability of this species and the degree to which it can perform well on diets containing low levels of these nutrients are highly relevant issues of academic and commercial interest, and have started being investigated. Previous studies on the transcriptional regulation of *elovl5* and *Δ4fad* by dietary DHA levels during the larval stage and changes in transcript levels during early ontogeny [14,17], in addition to effects of maternal diet on *elovl5* and *Δ4fad* transcription in eggs and newly hatched larvae [18], indicated a high degree of regulation of these genes. Further interest in this subject is driven by the lack of sufficient and affordable supplies of fishmeal (FM) and fish oil (FO) originating from marine fisheries, which were classically used to produce fish feeds, to maintain current rates of aquaculture production growth, which already provides almost 50 % of global fish supply for human consumption [19]. Therefore, replacement of marine ingredients in aquafeed

formulations with ingredients from more available plant sources is considered a major necessity and one of the factors currently limiting aquaculture sustainability [20]. However, although many fish species can perform well on diets with variable inclusions of plant ingredients, a major drawback is decreased levels in farmed fish of the health-beneficial n-3 LC-PUFA, which are not present in vegetable oils (VO) and concurrent increased levels of C_{18} PUFA, ALA and LOA [21]. Recent studies in *S. senegalensis* have shown that considerable levels of FM and FO can be replaced in the diets of this species with only a slight reduction in the flesh content of DHA [22-24]. These studies suggested that the Δ4Fad pathway was active *in vivo* and that its activity is transcriptionally regulated and possibly sufficient to maintain levels of DHA in the muscle when dietary levels are low, although this remained to be proved experimentally.

The primary objective of this study is to test the hypothesis that the Δ4 biosynthetic pathway is functionally active in *S. senegalensis*, producing biologically relevant amounts of DHA, and is under nutritional control by dietary lipid content and FA composition. To this aim, Senegalese sole juveniles were fed diets containing either FO or a VO blend replacing 75% of FO, at two different lipid levels (8 % and 18 %). The elongation and desaturation activities were assessed in enterocytes and hepatocytes by incubation with radiolabelled FA substrates (ALA and EPA) and determining the radioactivity recovered in FA products. The transcriptional regulation of the pathway was investigated by determining changes in the levels of the key fatty acyl elongase and desaturase genes (*elovl5* and *Δ4fad*, respectively) in the intestine and liver under the different dietary conditions. The effect of diet-induced changes in gene expression and enzymatic activities was assessed in the FA profile of tissues at the end of the 13-week

100 experimental feeding period. Finally, the tissue expression profile of the two genes is
101 also reported.
102
103 **2. Materials and methods**
104
105 *2.1. Tissue distribution of genes of LC-PUFA biosynthesis*
106 Tissues were collected from juvenile Senegalese sole (average weight: 251 g) held in
107 the experimental culture facilities of IRTA, Center of Sant Carles de la Ràpita (Spain) in
108 16 m^3 tanks, with natural thermo-photoperiod, a salinity of 36 ppt and fed a standard
109 commercial feed (LE-3, Skretting, Burgos, Spain) supplemented twice a week with
110 natural feeds (mussels and polychaetes). Fish were fasted for 24 h prior to sampling and
111 tissue samples were dissected and immediately frozen in dry ice and stored at -80 ºC. A
112 homogeneous sample of about 100 mg of tissue, from the same relative position in all
113 animals, was collected from: stomach (Sto), anterior intestine (AI), posterior intestine
114 (PL), liver (L), spleen (Spl), anterior kidney (K), heart (H), ventral skin (VS), dorsal
115 skin (DS), and ovaries (O). Other tissues including eye (E, closest to mouth), brain (B),
116 olfactory rosettes (OR) and one testis (T), were sampled whole, and for gills (G) one gill
117 arch was taken from the middle region.
118
119 *2.2. Dietary experiment and sampling*
120 *Solea senegalensis* (Kaup, 1858) with an average body weight (BW) of 5.0 ± 0.1 g
121 were distributed into twelve rectangular flat bottom 20 l tanks (containing 50 fish each)
122 and cultured in a recirculation system at CCMAR, University of Faro, Portugal, at a
123 temperature of 19.3 ± 1.2, salinity of 32, and under a 12-h light/12-h dark photoperiod
124 for 13 weeks, to an average final weight of 22.3 ± 2.1 g. Fish were fed the experimental

diets using automatic feeders (22 h/day). Given the passive feeding behavior of sole, the daily feed ration was reduced by 10% in the case of excess uneaten feed and increased by 10% in the absence of uneaten feed. The fish were fed 4 isoproteic 2 mm extruded diets (to triplicate tanks) which differed in total lipid level (either low, LL ~8 % or high, HL ~18 %) and fatty acid composition. The FLL and FHL diets had 100 % of the lipid supplied by FO, while 75 % of the FO in diets VLL and VHL was replaced by a VO blend (Table 1). These diets were formulated to meet the nutritional requirements of Senegalese sole and were formulated and manufactured by Sparos Lda. (Portugal).

At the end of the experiment fish were fasted for 24 h (t0) and three individual fish per tank were sacrificed with a lethal dose of tricaine methanesulfomnate (MS222; Sigma, Sintra, Portugal). Samples of anterior intestine, liver and flesh (muscle) were taken, quickly frozen on dry ice and stored at -80 ºC pending FA and gene expression analysis. In addition, whole intestines and livers from two fish per tank were collected and pooled to immediately perform the fatty acyl elongation and desaturation activity assays (see below). Three fish per tank were then force fed 0.15% average BW (10 pellets) of their respective diets and 6 h after feeding (t6) were sacrificed and samples of anterior intestine and liver were excised for gene expression analysis.

This study was directed by trained researchers (following FELASA category C recommendations) and conducted according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE).

2.3. Determination of enterocyte and hepatocyte fatty acyl elongation/desaturation activities

For assay of LC-PUFA biosynthesis, livers and intestines were carefully dissected from six fish (3 pools of 2 fish) to produce three hepatocyte and three enterocyte

preparations per treatment. Each pool of tissues was chopped, incubated with 1 % collagenase and cells sieved through 100 μm nylon gauze as described in detail previously [25]. One hundred μL of each cell preparation was taken for protein determination by the method of Lowry et al. [26], following incubation with 1 M NaOH/0.25 % (w/v) SDS for 1 h at 60 °C. For each cell preparation, two 5 ml portions were dispensed into 25 cm^2 tissue culture flasks and incubated at 20 °C for 1 h with 0.25 μCi (final fatty acid concentration, 2 μM) of either [1-^{14}C]18:3n-3 or [1-^{14}C]20:5n-3, added as complexes with fatty acid free-bovine serum albumin (BSA) [27]. After

Table 1

Formulation and proximate composition of the experimental diets.

	Experimental diets			
	FLL	VLL	FHL	VHL
Ingredients (%)				
Fishmeal 70 LT[1]	22.00	22.00	22.00	22.00
Fishmeal 60[2]	15.00	15.00	15.00	15.00
Fish protein hydrolisate[3]	5.00	5.00	5.00	5.00
Squid meal[4]	5.00	5.00	5.00	5.00
Pea protein concentrate[5]	4.00	4.00	4.00	4.00
Soy protein concentrate[6]	2.00	2.00	2.00	2.00
Soybean meal 48[7]	9.80	9.80	10.00	10.00
Wheat gluten[8]	7.00	7.00	10.10	10.10
Corn gluten meal[9]	5.00	5.00	4.50	4.50
Pea grits[10]	11.10	11.10	2.50	2.50
Wheat meal	9.00	9.00	4.80	4.80
Fish oil[11]	2.60	0.65	12.60	3.15
Rapeseed oil[12]		0.65		3.15
Soybean oil[12]		0.65		3.15
Linseed oil[12]		0.65		3.15
Vitamin & Mineral Premix[13]	1.00	1.00	1.00	1.00
Binder (guar gum)[14]	1.00	1.00	1.00	1.00
Proximate composition				
Moisture (%)	5.5	4.6	4.3	4.4
Crude Protein (% DM)	56.0	56.9	58.0	57.2
Crude Fat (% DM)	7.9	7.4	17.6	17.4
Ash (% DM)	10.5	10.7	10.4	10.3

[1] Peruvian fishmeal LT: 71% crude protein (CP), 11% crude fat (CF), EXALMAR, Peru.
[2] Fair Average Quality (FAQ) fishmeal: 62% CP, 12%CF, COFACO, Portugal.
[3] CPSP 90: 84% CP, 12% CF, Sopropêche, France.
[4] Super prime squid meal: 80% CP, 3.5% CF, Sopropêche, France.
[5] Lysamine GP: 78% CP, 8% CF, ROQUETTE, France.
[6] Soycomil P: 65% CP, 0.8% CF, ADM, The Netherlands.
[7] Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL SA, Portugal.
[8] VITEN: 85.7% CP, 1.3% CF, ROQUETTE, France.
[9] Corn gluten feed: 61% CP, 6% CF, COPAM, Portugal.
[10] Aquatex G2000: 24% CP, 0.4% CF, SOTEXPRO, France.
[11] COPPENS International, The Netherlands.
[12] Henry Lamotte Oils GmbH, Germany.
[13] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine, 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 1000mg; inositol, 500mg; biotin, 3mg; calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65mg; copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc sulphate,7.5mg; sodium chloride, 400mg; calcium carbonate, 1.86g; excipient wheat middlings.
[14] Guar gum 101 HV- E412, Seah International, France.

incubation, cells were harvested, washed and lipid extracted as described previously [25]. Total lipid was transmethylated, fatty acid methyl esters (FAME) prepared and separated by argentation (silver nitrate) TLC as described previously [28]. Radiolabelled FAME were located on TLC plate by autoradiography, and quantified by liquid scintillation after scraping from the TLC plates [29].

2.4. Fatty acid composition analysis

Total lipids of the experimental diets (Table 2) and intestine, liver and muscle from a pool of 3 fish per tank (n = 3 per treatment) were extracted by chloroform/methanol (2:1, v/v) according to Folch et al. [30] and quantified gravimetrically after evaporation of the solvent under nitrogen flow, followed by vacuum desiccation overnight. Total lipids were resuspended at 20 mg/ml in chloroform/methanol (2:1) containing 0.01 % BHT and 100 µl subjected to acid-catalyzed transesterification with 21:0 internal standard [31]. FAME were extracted using isohexane/diethyl ether (1:1, v/v), purified by TLC (Silica gel 60, VWR, Lutterworth, UK) and analyzed by gas–liquid chromatography on a Thermo Electron-TraceGC (Winsford, UK) instrument fitted with a BPX70 capillary column (30 m × 0.25 mm id; SGE, UK), using a two-stage thermal gradient initially at 40 °C/min from 50 °C (injection temperature) to 150 °C and then to 250 °C at 2 ºC/min. Helium (1.2 ml/min constant flow rate) was used as the carrier gas and on-column injection and flame ionization detection was performed at 250 °C. Fatty acid were identified by comparison with known standards (Supelco Inc., Spain) and a well-characterized fish oil (Marinol, Stepan Specialty Products, LLC, USA) and quantified using Chrom-card for Windows (TraceGC, Thermo Finnigan, Italy).

Table 2

Fatty acid composition, expressed as % total FA or μg/mg DW (in brackets), of experimental diets (n = 3).

	Experimental diets			
	FLL	VLL	FHL	VHL
Total SFA	23.1 (11.1)	20.4 (11.2)	26.1 (26.1)	17.3 (19.9)
Total MUFA	27.1 (13.0)	29.8 (16.3)	24.8 (24.8)	32.7 (37.5)
18:2n-6	12.1 (5.8)	17.4 (9.5)	6.7 (6.7)	20.4 (23.4)
18:3n-3	1.7 (0.8)	5.5 (3.0)	1.3 (1.3)	11.9 (13.7)
18:4n-3	1.9 (0.9)	1.5 (0.8)	2.3 (2.4)	1.1 (1.3)
20:4n-6	1.1 (0.5)	0.7 (0.4)	1.2 (1.3)	0.4 (0.5)
20:4n-3	0.6 (0.3)	0.4 (0.2)	0.8 (0.8)	0.3 (0.3)
20:5n-3	15.5 (7.5)	11.1 (6.1)	18.3 (18.3)	7.5 (8.6)
22:5n-3	1.1 (0.5)	0.8 (0.5)	1.5 (1.5)	0.6 (0.7)
22:6n-3	12.8 (6.2)	9.8 (5.4)	13.0 (13.1)	6.1 (7.0)
Total PUFA	48.0 (23.1)	48.0 (26.3)	46.7 (46.7)	48.9 (56.1)
Total n-3 PUFA	34.0 (16.3)	29.3 (16.0)	37.8 (37.8)	27.6 (31.6)
Total n-6 PUFA	14.0 (6.7)	18.7 (10.2)	8.9 (8.9)	21.3 (24.5)
n-3/n-6	2.4	1.6	4.2	1.3
DHA/EPA	0.8	0.9	0.7	0.8

2.5. RNA extraction and real time quantitative PCR (qPCR)

Total RNA was isolated from anterior intestine and liver of 2 individuals per tank (n = 6 per dietary treatment) at t0 and t6, and from a range of tissues from three individuals (n = 3). For RNA extraction, samples were homogenized in 1ml of TRIzol (Ambion, Life Technologies, Madrid, Spain) with 50 mg of 1mm diameter zirconium glass beads (Mini-Beadbeater, Biospec Products Inc., U.S.A.). Solvent extraction was performed following manufacturer's instructions and RNA quality and quantity assessed by gel electrophoresis and spectrophotometry (NanoDrop2000, Thermo Fisher Scientific, Madrid, Spain). Two micrograms of total RNA per sample were reverse transcribed into cDNA using the High-Capacity cDNA RT kit (Applied Biosystems, Life Technologies, U.S.A.), following manufacturer's instructions, but using a mixture of random primers

(1.5 µl as supplied) and anchored oligo-dT (0.5 µl at 400 ng/µl, Eurogentec, Cultek, S.L., Madrid, Spain). Negative controls (containing no enzyme) were performed to check for genomic DNA contamination. A similar amount of cDNA was pooled from all samples from the dietary experiment and the remaining cDNA was diluted 60-fold with water. The cDNA used for the tissue expression profile was diluted 20-fold.

Expression of fatty acyl elongase (*elovl5*) and Δ4-desaturase (*Δ4fad*) was quantified using primers reported previously [14]. Ubiquitin (*ubq*), 40S ribosomal protein S4 (*rps4*) and elongation factor 1 alpha (*ef1a1*) were used as reference genes to study nutritional regulation, and 18S rRNA (*18s*) to characterize tissue distribution of *elovl5* and *Δ4fad* transcripts [32] (Table 3). Amplifications were carried out in duplicate on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Alcobendas, Spain) in a final volume of 20 µl containing 5 µl of diluted cDNA (except for *18s*, for which 1 µl was used), 0.5 µM of each primer and 10 µl of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and included a systematic negative control (NTC-non template control). The qPCR profiles contained an initial activation step at 95 °C for 2 min, followed by 35 cycles: 15 s at 95 °C, 1 min at 60 ºC (target genes) or 15 s at 95 °C, 1 min at 70 °C (reference genes). After the amplification phase, a melt curve was performed enabling confirmation of the amplification of a single product in each reaction. Non-occurrence of primer-dimer formation in the NTC was also confirmed. The amplification efficiency of the primer pairs was assessed by serial dilutions of the cDNA pool.

2.6. Statistical analysis

Elongation and desaturation activities and arcsin-transformed FA percentage composition data were analyzed by two-way ANOVA in SPSS v20 (SPSS Inc.,

Chicago, IL, U.S.A.), to assess significant effects of the factors "lipid level" and "lipid source" and their respective interaction, at a significance level of 0.05. Gene expression results from the dietary experiment were imported into the software qBase+ (Biogazelle, Zwijnaarde, Belgium), and normalized relative quantities calculated employing target and run-specific amplification efficiencies and using the geometric mean of the three reference genes (M values 0.136-0.271 and coefficient of variance, CV, 0.055-0.107 depending on tissue and time point) [33]. Furthermore, inter-run calibrators were included in all runs to offset differences in expression between time points (t0 and t6, which were analyzed in separate runs). The final values obtained (calibrated normalized relative quantities, CNRQ) [34] were exported and analyzed by two-way ANOVA in SPSS v20. In addition, the expression levels of *elovl5* and *Δ4fad* in different tissues were determined using the delta-delta C_T method ($2^{-\Delta\Delta CT}$) describing the normalized (by *18s*) relative expression (RE) of the target genes in each tissue in relation to the average across all tissues [35]. The differential tissue expression of each gene was assessed in SPSS v20 using the Welch test, followed by the Games-Howell test (both tests not assuming homogeneity of variances) to perform multiple comparisons of the RE values across tissues.

Table 3

Primers used for real-time quantitative PCR (qPCR). Shown are sequence and annealing temperature (Ta) of the primer pairs, size of the fragment produced, reaction efficiency and accession number of the target and reference genes.

Transcript	Primer sequence	Fragment	Ta	Efficiency* (%)	Accession No.
Δ4fad	AAGCCTCTGCTGATTGGAGA GGCTGAGCTTGAAACAGACC	131 bp	60 ºC	99.9[1]/102.1[2]	JN673546
elovl5	TTTCATGTTTTTGCACACTGC GACACCTTTAGGCTCGGTTTT	161 bp	60 ºC	100.7[1]/100.4[2]	JN793448
ubq[a]	AGCTGGCCCAGAAATATAACTGCGACA ACTTCTTCTTGCGGCAGTTGACAGCAC	93 bp	70 ºC	100.6[1]/98.8[2]	AB291588

rps4[a]	GTGAAGAAGCTCCTTGTCGGCACCA AGGGGGTCGGGGTAGCGGATG	83 bp	70 ºC	99.7[1]/100.1[2]	AB291557
ef1a1[a]	GATTGACCGTCGTTCTGGCAAGAAGC GGCAAAGCGACCAAGGGGAGCAT	142 bp	70 ºC	99.6[1]/100.1	AB326302
18s[b]	GAATTGACGGAAGGGCACCACCAG ACTAAGAACGGCCATGCACCACCAC	148 bp	70 ºC	-	AM882675

[a] Dietary trial; [b] Tissue distribution.

* Average efficiency from 2 qPCR runs (T0 and T6) done in [1]anterior intestine and [2]liver. $R^2 > 0.993$ in all runs.

3. Results

3.1. Tissue distribution of genes of LC-PUFA biosynthesis

The tissue expression profile was determined by qPCR for both *elovl5* and *Δ4fad*, which showed significant differences between tissues ($p<0.001$ for both genes), with a similar pattern of tissue distribution. Both genes showed a predominant expression in liver and intestine (equally in the anterior and posterior sections), although the individual variation in these organs tended to be high (affecting the statistical analysis results), followed by brain (showing much lower individual variability), eye and the olfactory rosettes (at least one order of magnitude lower, except for *Δ4fad* in brain) (Fig. 1). What differed between the two genes was that *elovl5* was also expressed in kidney and skin (dorsal and ventral), whereas *Δ4fad* expression was also found in stomach, testis and ovaries.

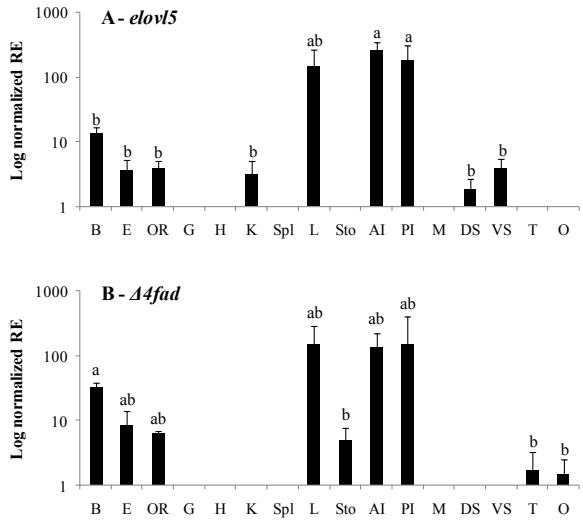


Fig. 1. Tissue distribution of *elovl5* (A) and *Δ4fad* (B) transcripts. Values are represented in logarithmic scale and correspond to the normalized (by *18s*) relative expression (RE) of the target genes in each tissue in relation to the average across all tissues, calculated using the delta-delta C_T method ($2^{-\Delta\Delta CT}$). Values are an average of 3 individuals (n = 3) with standard deviation (SD). B- brain, E- eye, OR- olfactory rosettes, G- gills, H- heart, K- kidney, Spl- spleen, L- liver, Sto- stomach, AI- anterior intestine, PI- posterior intestine, M- muscle, DS- dorsal skin, VS- ventral skin, T- testis, O- ovary. Different letters indicate significant differences between tissues ($p<0.05$), determined by the Games-Howell test (SPSS v20), for each one of the genes.

3.2. Elongation and desaturation activities in enterocytes and hepatocytes

Assay of fatty acyl elongation and desaturation activities in enterocytes and hepatocytes of Senegalese sole showed no apparent Δ6 desaturation of ^{14}C-ALA in either cell type with only elongation to 20:3n-3 observed (Table 4). In enterocytes, both lipid level and lipid source, as well as the interaction between the two factors, significantly affected the elongation activity, which was higher in fish fed the VO and HL diets, with a clear synergistic effect. In hepatocytes, on the other hand, only dietary lipid level had a significant effect, with higher elongation activity being measured in

fish fed the LL diets. Nevertheless, p-values of both lipid source and interaction were very close to being significant, which means that with a higher replicate number this result could change. However, the elongation activity in fish fed the VHL diet appeared to be much lower in the hepatocytes compared to the enterocytes and hence the increase in elongation activity in fish fed the VO diets compared to the FO diets was only noticeable at a LL level.

In contrast, substantial amounts of radioactivity from ^{14}C-EPA were recovered in 22:5n-3, 24:5n-3 and DHA, indicating both elongation and Δ4-desaturation activities in enterocytes and hepatocytes, that were significantly affected by both lipid level and source, and also showed significant interaction (Table 5). These activities were higher in fish fed the VO and LL diets except elongation of EPA to 22:5n-3 in enterocytes, which was not affected by dietary lipid level. However, effects of dietary treatments were more subtle in the enterocytes compared to the hepatocytes, where a synergistic effect was clearly observed in fish fed the VLL diet.

3.3. Nutritional regulation of gene transcription

The transcriptional regulation of *elovl5* and *Δ4fad* expression in response to dietary lipid level and FA profile was investigated by qPCR. Results showed that neither dietary factor significantly influenced basal (t0) or postprandial (t6) levels of *elovl5* or

Table 4

Elongation of ^{14}C-ALA (pmol/mg protein/h) in *Solea senegalensis* hepatocytes and enterocytes.

	Experimental diets				P-value (two-way ANOVA)		
	FLL	VLL	FHL	VHL	Lipid level	Lipid source	Interaction
Enterocyte							
20:3n-3	0.3 ± 0.0 (0.9 %)	1.1 ± 0.1 (2.6 %)	0.4 ± 0.0 (1.1 %)	2.8 ± 0.2 (2.4 %)	<0.0001	<0.0001	<0.0001
Hepatocyte							
20:3n-3	0.5 ± 0.2 (1.2 %)	0.8 ± 0.1 (1.8 %)	0.2 ± 0.0 (1.3 %)	0.2 ± 0.0 (0.7 %)	0.0001	0.0543	0.0543

Results are means ± SD (n = 3). Values in brackets represent the percentage of ^{14}C-ALA elongated. No desaturated products of ^{14}C-ALA were observed.

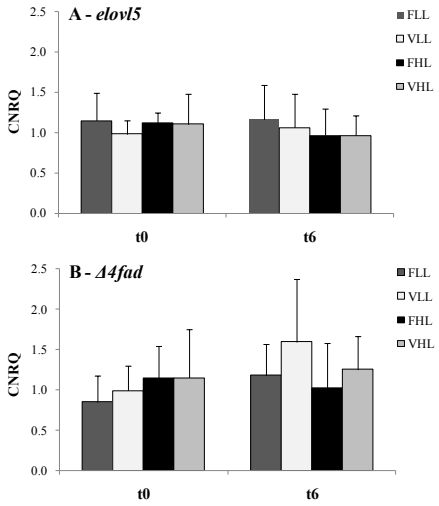
Table 5

Elongation and desaturation (pmol/mg protein/h) of ^{14}C-EPA in *Solea senegalensis* hepatocytes and enterocytes.

	Experimental diets				P-value (two-way ANOVA)		
	FLL	VLL	FHL	VHL	Lipid level	Lipid source	Interaction
Enterocyte							
22:5n-3	6.8 ± 0.7 (11.7%)	10.5 ± 0.8 (15.7%)	5.7 ± 0.1 (9.0%)	14.6 ± 0.4 (18.1%)	0.002	<0.001	<0.001
24:5n-3	0.9 ± 0.0 (1.5%)	0.8 ± 0.1 (1.3 %)	0.6 ± 0.0 (0.9%)	0.9 ± 0.0 (1.1%)	0.011	0.011	<0.001
22:6n-3	1.4 ± 0.1 (2.5%)	1.8 ± 0.1 (3.0%)	1.2 ± 0.1 (1.9%)	1.3 ± 0.1 (1.7%)	<0.001	0.003	0.032
Hepatocyte							
22:5n-3	11.1 ± 0.2 (11.7%)	29.3 ± 0.2 (29.0%)	5.4 ± 0.3 (7.1%)	6.4 ± 0.2 (8.2%)	<0.001	<0.001	<0.001
24:5n-3	1.2 ± 0.0 (1.3%)	2.1 ± 0.0 (2.0%)	0.6 ± 0.0 (0.9%)	0.8 ± 0.0 (1.1%)	<0.001	<0.001	<0.001
22:6n-3	2.9 ± 0.1 (3.0%)	5.0 ± 0.3 (4.6%)	0.9 ± 0.0 (1.3%)	1.7 ± 0.1 (2.3%)	<0.001	<0.001	0.002

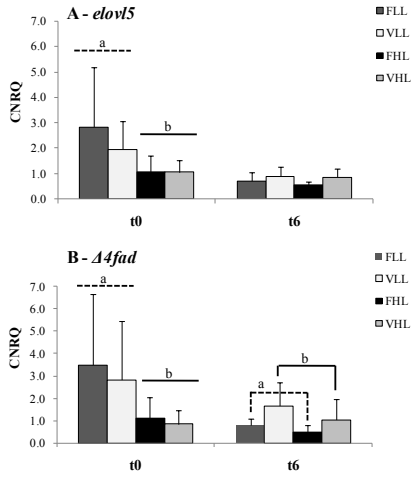
Results are means ± SD (n = 3). Values in brackets represent the percentage of ^{14}C-EPA desaturated or elongated.

328 *Δ4fad* transcripts in the intestine (Fig. 2). However, in liver, a significant effect of
329 lipid level was observed in the basal (t0) expression of both *elovl5* and *Δ4fad*, with
330 significantly higher transcript levels in fish fed the LL diets (Fig. 3). On the other hand,
331 6 h after feeding the transcription of *Δ4fad* was significantly affected by dietary lipid
332 source, being up-regulated in fish fed the VO diets, and a similar but non-significant
333 trend was observed in *elovl5*.
334



335

336 **Fig. 2.** Nutritional regulation of *elovl5* (A) and *Δ4fad* (B) gene transcription in intestine
337 of *Solea senegalensis* juveniles after 24h-fasting (t0) and 6h after refeeding (t6). Values
338 are calibrated normalized relative quantities (CNRQ) obtained from qBASE+,
339 corresponding to an average of 6 individuals (n = 6) with standard deviation (SD). None
340 of the observed differences were statistically significant.
341



342

343 **Fig. 3.** Nutritional regulation of *elovl5* (A) and *Δ4fad* (B) gene transcription in liver of
344 *Solea senegalensis* juveniles after 24h-fasting (t0) and 6h after refeeding (t6). Values
345 are calibrated normalized relative quantities (CNRQ) obtained from qBASE+,
346 corresponding to an average of 6 individuals (n = 6) with standard deviation (SD).
347 Columns (representing dietary treatments) with different letters within each time point
348 are significantly affected by dietary 'lipid level' or 'lipid source' ($P<0.05$).

349

350 *3.4. Lipid composition*

351 The FA composition of the experimental diets reflected the main lipid source used in
352 their formulations, with the FLL and FHL diets being richer sources of saturated fatty
353 acids (SFA) and LC-PUFA, particularly EPA and DHA, characteristic of FO, while the
354 VLL and VHL diets presented higher levels of monounsaturated fatty acids (MUFA),
355 LOA and ALA, characteristic of VO (Table 1). In contrast, dietary effects on fish FA
356 profiles were diverse and dependent on tissue (Tables 6 - 8). In terms of total lipid
357 contents of the tissues, differences were more marked between fish fed HL or LL diets
358 than between those fed FO and VO-based diets, but a significant difference related to
359 lipid level was only measured in the intestine.

Table 6

Total lipid (% of DW) and fatty acid composition (% total FA) of intestinal tissue.

	Experimental diets				P-value (two-way ANOVA)		
	FLL	VLL	FHL	VHL	Lipid level	Lipid source	Interaction
Total lipids	8.8 ± 1.5	8.7 ± 3.7	12.7 ± 2.7	12.8 ± 1.2	0.026	0.991	0.953
Total SFA[1]	27.7 ± 1.3	26.6 ± 3.2	25.1 ± 3.7	19.2 ± 2.0	0.013	0.058	0.171
Total MUFA[2]	24.5 ± 2.6	24.6 ± 3.7	35.7 ± 2.1	39.1 ± 1.9	<0.001	0.284	0.312
18:2n-6	10.7 ± 0.5	13.7 ± 1.7	7.5 ± 0.3	21.3 ± 1.3	0.009	<0.001	<0.001
18:3n-3	0.7 ± 0.0	1.6 ± 0.3	1.2 ± 0.2	7.5 ± 0.7	<0.001	<0.001	<0.001
20:5n-3	1.3 ± 0.2	0.8 ± 0.2	3.1 ± 1.5	1.3 ± 1.3	0.089	0.097	0.275
22:5n-3	4.9 ± 0.3	3.5 ± 0.7	5.8 ± 1.8	1.5 ± 0.1	0.368	0.001	0.034
22:6n-3	21.5 ± 1.6	21.0 ± 1.8	14.4 ± 1.1	5.8 ± 0.3	<0.001	<0.001	0.001
Total n-6 PUFA[3]	16.2 ± 0.6	18.8 ± 2.2	11.6 ± 0.3	24.0 ± 1.5	0.691	<0.001	<0.001
Total n-3 PUFA[4]	29.0 ± 2.3	27.6 ± 1.9	26.3 ± 5.0	16.5 ± 1.0	0.004	0.011	0.041

Results are means ± SD (n = 3). [1]Includes 14:0, 16:0 and 18:0; [2]Includes 16:1, 18:1n-9, 18:1n-7, 20:1 and 22:1; [3]Includes 18:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6; [4]Includes 18:4n-3, 20:3n-3, 20:4n-3, 21:5n-3 and 22:4n-3.

Table 7

Total lipid (% of DW) and fatty acid composition (% total FA) of liver.

	Experimental diets				P-value (two-way ANOVA)		
	FLL	VLL	FHL	VHL	Lipid level	Lipid source	Interaction
Total lipids	13.1 ± 2.6	12.5 ± 3.5	15.2 ± 1.8	15.6 ± 5.5	0.279	0.967	0.818
Total SFA[1]	24.7 ± 3.9	22.5 ± 5.7	23.0 ± 1.4	20.0 ± 3.2	0.372	0.278	0.869
Total MUFA[2]	26.2 ± 1.1	28.9 ± 3.0	28.0 ± 1.4	29.0 ± 2.1	0.468	0.152	0.501
18:2n-6	7.2 ± 1.1	15.3 ± 8.9	12.8 ± 2.1	19.0 ± 3.3	0.138	0.034	0.745
18:3n-3	1.1 ± 0.0	5.3 ± 4.0	1.4 ± 0.3	5.8 ± 3.6	0.835	0.025	0.935
20:5n-3	9.1 ± 6.2	6.1 ± 8.0	2.2 ± 0.8	1.8 ± 0.3	0.092	0.584	0.670
22:5n-3	5.6 ± 3.5	2.7 ± 0.9	4.6 ± 0.7	3.6 ± 0.1	0.954	0.100	0.392
22:6n-3	18.7 ± 4.3	13.6 ± 3.9	21.3 ± 4.5	14.4 ± 4.3	0.502	0.041	0.723
Total n-6 PUFA[3]	10.5 ± 2.0	18.1 ± 9.2	16.8 ± 1.5	23.1 ± 2.5	0.083	0.040	0.832
Total n-3 PUFA[4]	36.9 ± 1.3	29.3 ± 5.5	30.7 ± 3.7	26.6 ± 1.6	0.057	0.019	0.399

Results are means ± SD (n = 3). [1]Includes 14:0, 16:0 and 18:0; [2]Includes 16:1, 18:1n-9, 18:1n-7, 20:1 and 22:1; [3]Includes 18:3n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6; [4]Include 18:4n-3, 20:4n-3, 21:5n-3 and 22:4n-3.

Table 8

Total lipid (% of DW) and fatty acid composition (% total FA) of muscle.

	Experimental diets				P-value (two-way ANOVA)		
	FLL	VLL	FHL	VHL	Lipid level	Lipid source	Interaction
Total lipids	1.7 ± 0.3	2.3 ± 1.1	2.2 ± 0.4	2.3 ± 0.2	0.461	0.333	0.415
Total SFA[1]	25.6 ± 1.0	23.7 ± 1.1	25.6 ± 1.8	20.3 ± 0.4	0.039	0.001	0.038
Total MUFA[2]	22.4 ± 1.3	26.6 ± 5.1	25.7 ± 2.3	24.0 ± 0.4	0.852	0.458	0.112
18:2n-6	9.6 ± 0.6	13.3 ± 1.6	6.4 ± 0.4	16.2 ± 0.2	0.743	<0.001	<0.001
18:3n-3	0.8 ± 0.2	2.8 ± 1.3	0.9 ± 0.3	5.9 ± 0.1	0.004	<0.001	0.005
20:5n-3	2.8 ± 1.3	2.7 ± 0.2	6.7 ± 0.6	2.5 ± 0.1	0.002	0.001	0.001
22:5n-3	5.3 ± 0.3	4.0 ± 0.4	6.3 ± 0.3	4.0 ± 0.2	0.013	<0.001	0.024
22:6n-3	27.0 ± 1.4	21.3 ± 5.9	21.6 ± 2.2	21.3 ± 0.8	0.191	0.150	0.183
Total n-6 PUFA[3]	13.2 ± 0.3	16.2 ± 1.1	9.6 ± 0.2	19.3 ± 0.4	0.485	<0.001	<0.001
Total n-3 PUFA[4]	36.9 ± 1.5	31.8 ± 4.7	37.4 ± 1.7	34.6 ± 1.0	0.305	0.032	0.474

Results are means ± SD (n = 3). [1]Includes 14:0, 16:0 and 18:0; [2]Includes 16:1, 18:1n-9, 18:1n-7, 20:1 and 22:1; [3]Includes 18:3n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6; [4]Includes 18:4n-3, 20:3n-3, 20:4n-3, 21:5n-3 and 22:4n-3.

In the intestine there were significant effects of dietary lipid level and source, and significant interaction between the two factors, on the main FA with the exception of EPA (likely due to the high variability in the content of this FA) (Table 6). In addition, total SFA and MUFA were only significantly affected by dietary lipid level, while for 22:5n-3 and total n-6 PUFA there was a significant effect of lipid source and significant interaction. Also noteworthy in this tissue was the fact that similar high levels of DHA were measured in fish fed the FLL and VLL diets. In liver the FA profile showed less significant differences and only the lipid source caused significant changes, with higher relative levels of LOA, ALA (and hence total n-6 and n-3 PUFA) and lower contents of DHA in fish fed the VO-based diets (Table 7). However, the FA contents in liver, particularly of the LC-PUFA, tended to show higher variability, which might have contributed to lower differences being found in this organ. In muscle, similar to intestine, there were significant effects of either one or both factors as well as significant interactions in most of the main FA except for total MUFA, total n-3 PUFA and, strikingly, DHA (Table 8).

4. Discussion

Although the most direct route of DHA biosynthesis involves elongation of EPA to 22:5n-3 (DPA; docosapentaenoic acid) followed by Δ4 desaturation to DHA, for many decades Δ4Fad could only be found in lower eukaryotes [e.g., 9-12] and the only known pathway of DHA biosynthesis in vertebrates was the "Sprecher" pathway [8]. However, genes with a putative Δ4Fad activity were recently revealed in three teleost species with habitats ranging from freshwater to marine and dietary habits from herbivore to carnivore [13-15]. In these studies the activity of the *Δ4fad* transcript was assessed using an *in vitro* heterologous yeast expression assay, and further work was necessary to

unequivocally establish that this pathway is active and had physiological relevance *in vivo*, meaning that the species possessing this gene are able to synthesize DHA via a Δ4Fad-dependent pathway.

Previous strong circumstantial evidence of the possible existence of an active LC-PUFA biosynthesis pathway in Senegalese sole has been unequivocally confirmed in the present study in which both enterocytes and hepatocytes were able to produce labeled DPA and DHA from ^{14}C-EPA. Although labeled 24:5n-3 was also produced, the lack of Δ6 desaturase activity suggested this could not be subsequently desaturated in Senegalese sole and that the DHA must have arisen from Δ4 desaturation of DPA. In general, activity appeared higher in hepatocytes than in enterocytes, but we can conclude that DHA biosynthesis from EPA can occur both in the intestine and in the liver. This is not surprising considering that the intestine is not simply a site of absorption but also of lipid metabolism, including reacylation and packaging of dietary lipids and LC-PUFA biosynthesis activity, as described in salmonid species [36,37]. In addition, the results showed that the desaturation and elongation activities in the two cell types were influenced by both the FA composition and lipid content of the diet.

When *S. senegalensis* were fed VO-based diets containing lower levels of LC-PUFA there were significantly higher activities of elongation and desaturation from EPA in the enterocytes and hepatocytes. It was shown previously that elongation and desaturation (Δ6Fad and Δ5Fad) activities were increased in both enterocytes and hepatocytes of salmonid species when VO replaced FO in the diet [27,38]. Furthermore, increased DHA production in hepatocytes was associated with a significant up-regulation of *Δ4fad* expression in liver of Senegalese sole that were fed the VO-based diets at 6h after feeding. A similar trend was observed with *elovl5* expression in the liver postprandially but, in this case, changes were non-significant, which is consistent with previous data

457 from sole larvae showing a lower responsiveness of *elovl5* than *Δ4fad* transcription to
458 dietary LC-PUFA levels [14,34] and from Atlantic salmon showing lower nutritional
459 regulation of fatty acyl elongases compared to desaturases [38,39]. However, in these
460 and in most other studies investigating nutritional regulation of *elovl*s and *fads* in
461 response to dietary LC-PUFA contents, samples were generally from unfed or fasted
462 fish, whereas in the present study no significant effect of FA composition was observed
463 in the liver when juveniles were fasting. This result was therefore unexpected and might
464 be explained by the fact that in basal conditions dietary lipid level exerted a strong and
465 significant effect, which prevailed over, or interacted with, FA composition. On the
466 other hand, the present results suggested that dietary FA composition exerted an
467 immediate postprandial effect in the transcriptional regulation of these genes,
468 independent of their basal expression levels.

469 In enterocytes there was no significant transcriptional regulation of the expression of
470 either gene at fasting or postprandially, which was unexpected given the observed
471 differences in enzyme activity. Nevertheless, the pattern of expression of *Δ4fad* at t6
472 was comparable to that observed in liver, and therefore the absence of significant
473 differences may be due to higher variability and lack of statistical power.

474 Dietary lipid level had clear effects on fatty acyl elongase and desaturase activities,
475 which were significantly lower in hepatocytes of fish fed HL diets. This correlated with
476 the basal expression of both *elovl5* and *Δ4fad* in liver showing a significant down-
477 regulation in fish fed HL diets. Research in mammals has firmly established that FA
478 have key roles in regulating expression of genes involved in lipid metabolism and
479 energy homeostasis through activation of nuclear receptors and transcription factors,
480 and that not all FA have the same effect. In contrast to PUFA, SFA and MUFA have
481 little effect, and within PUFA, LC-PUFA are more potent than C_{18} PUFA [4]. However,

few studies exist on the effect of dietary lipid level on the expression of fatty acyl desaturase or elongase genes and they tend to be flawed by an experimental design that does not enable discriminating effects of total lipid from FA composition. For instance, Cho et al. [40], looking at the nutritional regulation of *Δ5fad* and *Δ6fad* in rat liver showed that, even though these genes were down-regulated by diets rich in 18:2n-6 (safflower oil) or n-3 LC-PUFA (FO) compared to rats fed a fat-free diet, no differences were found between the latter and those fed triolein (containing 18:1n−9). Hence, the authors concluded that it was the FA composition rather than lipid content regulating the expression of these genes. Another example was a previous study on rainbow trout showing a down-regulation of *Δ6fad* transcription in liver of fish fed HL diets [41]. In this case, the increase in lipid level was achieved by adding FO to the diet, hence raising the LC-PUFA content, which would explain the results. On the other hand, Martinez et al. [42] also reported a down-regulation of *Δ5fad* and *Δ6fad* in salmon liver fed a HL diet compared to a LL diet with a similar relative FA composition, which supports the results from the present study of an effect caused by changes in dietary lipid content. It is however noteworthy that salmon fed the HL diet ingested and accumulated higher levels of lipids in the liver, implying that the absolute levels of LC-PUFA were also higher in this treatment [42].

In a study looking at the hepatic transcriptome of lean and fat Atlantic salmon families which accumulated higher or lower amounts of LC-PUFA in the muscle when fed a similar VO-based diet, an interaction was found between flesh adiposity and n-3 LC-PUFA levels in the regulation of several lipid metabolism genes, particularly of cholesterol metabolism, which are regulated by LC-PUFA levels via *srebp2* [43]. These were down-regulated by higher LC-PUFA levels but only in the lean family. This had also been observed in genes of the LC-PUFA biosynthesis pathway, where a significant

up-regulation of *Δ5fad*, *Δ6fad* and *elovl2* when VO replaced FO in the diet was only measured in lean Atlantic salmon families [38]. Based on these results, it was suggested that absolute, rather than relative, levels of n-3 LC-PUFA may be the determinant factor affecting gene transcription [43]. In the present study, this hypothesis could only partially explain the results when animals were fasting given that the HL diets provided the highest absolute amounts (as µg/mg DW of diet) of EPA and DHA, with levels in the VHL diet being slightly higher than in the FLL diet. However, the fish responded in a classic way postprandially, with down-regulation of *Δ4fad* expression in fish fed both diets containing FO. Still, the above-mentioned hypothesis refers to regulation driven by the FA deposited in the tissues (liver being the main lipid-containing organ in sole) rather than a direct dietary influence, which is also less likely in fasting conditions. Therefore, it is also important to consider absolute amounts of FA (presented in supplementary files S1-3). Levels of LC-PUFA in the liver, expressed as µg FA/mg DW, tended to be higher in both HL diets compared to VLL but not compared to FLL, but differences were not significant due to large variability of the FA composition data, as was already seen for the relative (%) results (supplementary file S2). This high variability affected particularly the liver, where there is higher lipid accumulation, and does not enable us to verify this hypothesis at present. Nevertheless, if we consider the effect at the level of enzyme activity, which was also measured at fasting, results showed that elongation and desaturation of EPA were significantly affected by both factors, with significant interaction. Therefore, the present study suggests that there is a possible effect of dietary lipid level, independent but interrelated with fatty acid composition, in regulating the expression and activity of the LC-PUFA synthesis pathway. Further studies are required to uncover the mechanisms explaining these results, and future experimental designs should consider possible influences from both

dietary and body lipid stores origin, and include a higher number of individuals to overcome the limitations of an apparently high individual variation in LC-PUFA biosynthesis efficiency and possibly also mobilization/transport and deposition of LC-PUFA in body tissues.

In enterocytes a similar effect of dietary lipid level was observed as in hepatocytes in terms of desaturase activity, which was also significantly reduced in fish fed the HL diets. However, elongation activities from EPA to DPA and then to 24:5n-3 showed interaction, given that in fish fed the VO-based diets the elongation activity was similar or higher in the VHL compared to the VLL treatment. These results, combined with gene expression data, might partly explain the FA compositions of intestine, where significant interactions were observed in the levels of several FA (including DHA). Muscle tissue was also analyzed given that it is the edible portion of the fish and, therefore, its composition is of interest to consumers. It was noteworthy that there were no significant differences between fish fed the different diets in terms of flesh DHA, as previously reported [22], even if the levels of EPA were affected by both lipid level and source.

A question that remained uncertain until now was the possible existence of a separate gene with Δ6/Δ5Fad activity, which could not be found in sole, or whether the characterized Δ4Fad might also possess residual Δ6/Δ5-desaturation activity [34]. In the present study, hepatocytes and enterocytes isolated from *S. senegalensis* and incubated with [1-^{14}C]ALA did not show any Δ6-desaturation activity with only elongation to 20:3n-3 apparent. This suggests that *S. senegalensis* may be unique amongst teleosts in which *fads* have been cloned and functionally characterized so far, where at least one *Δ6fad* has been found [44,45]. In the case of Atlantic salmon (*Salmo salar*) two separate Δ5 and Δ6 genes exist [46,47], while zebrafish (*Danio rerio*) has a single bifunctional

desaturase with both Δ5 and Δ6 activities [48]. Finally, in the only other two vertebrate species where a Δ4Fad has been described until now, a second gene was functionally characterized and shown to have Δ6/Δ5 activity *in vitro* [13,15]. Although it remains to be shown that the activity of the two *fads* transcripts that have been functionally characterized in *S. canaliculatus* and *C. estor* are indeed of physiological relevance *in vivo*, data suggests that, contrary to *S. senegalensis*, these two species could have all the enzymatic abilities required for DHA biosynthesis from C_{18} PUFA via Δ4-desaturation as well as the "Sprecher" pathway. These results are interesting as they point to the high evolutionary plasticity and functional diversification of the LC-PUFA synthesis pathway in teleosts, most likely linked to habitat-specific food web structures in different environments [49]. As previously noted [14], the unique characteristics of the *S. senegalensis* LC-PUFA synthesis pathway might be related to its natural dietary regime, associated to its benthic lifestyle, which differs from other species most commonly studied so far, having a diet generally poor in lipid and proportionally high in EPA.

In conclusion, results from the present study confirmed the existence of a biologically relevant capacity to synthesize DHA from EPA in Senegalese sole, consistent with the previously reported substrate specificities of the LC-PUFA biosynthesis enzymes characterized *in vitro*. Furthermore, results appeared to confirm the lack of Δ6Fad activity in sole, demonstrating the high plasticity and functional variability of this pathway in teleosts. Both *elovl5* and *Δ4fad* had a similar pattern of tissue distribution, with a main expression in nutrition-related tissues (liver and intestine), followed by tissues with a neural and sensorial function (mainly brain but also eye and olfactory rosettes). Both enterocytes and hepatocytes have the capacity to biosynthesize DHA, although fatty acyl elongation and desaturation activities tended to

582 be higher in hepatocytes than in enterocytes. In addition, both enzymatic activities and
583 gene transcription rates were modulated by dietary lipid level and FA composition,
584 particularly in liver. These data confirm previous studies in which dietary LC-PUFA
585 levels, associated with replacement of FO by VO, affected activity and transcriptional
586 regulation of this pathway, but further demonstrate that transcriptional regulation also
587 occurs postprandially. An effect of dietary lipid level was also observed particularly in
588 liver, with HL diets significantly decreasing enzymatic activities and gene expression
589 levels in fasting fish. Although the mechanisms are unclear data showed that in basal
590 conditions dietary lipid level possibly prevailed over or interacted with FA composition
591 in regulating the expression of *elovl5* and *Δ4fad*. Finally, the results showed tissue-
592 specific differences in the activity and regulation of this pathway, which were reflected
593 in the FA compositions of the tissues, indicating both functionality and biological
594 relevance of the pathway in *S. senegalensis*. Independent to this, flesh DHA levels were
595 unaffected by diet composition which, with regard to the need to replace FO by VO in
596 aquafeeds, highlights the important advantage of this species for aquaculture.
597

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607

608 Supplementary files

609 S1. Absolute levels of FA (μg FA/mg DW) in intestinal tissue.

610 S2. Absolute levels of FA (μg FA/mg DW) in liver.

611 S3. Absolute levels of FA (μg FA/mg DW) in muscle.

612

613

614 **References**

615 [1] M.V. Bell, D.R. Tocher, Molecular species composition of the major phospholipids
616 in brain and retina from rainbow trout (*Salmo gairdneri*). Occurrence of high levels
617 of di-(n-3)polyunsaturated fatty acid species, Biochem. J. 264 (1989) 909-915.

618 [2] A. Gil, M. Ramirez, M. Gil, Role of long-chain polyunsaturated fatty acids in infant
619 nutrition, Eur. J. Clin. Nutr. 57 Suppl 1 (2003) S31-34.

620 [3] B. Koletzko, E. Lien, C. Agostoni, H. Böhles, C. Campoy, I. Cetin, T. Decsi, J.W.
621 Dudenhausen, C. Dupont, S. Forsyth, I. Hoesli, W. Holzgreve, A. Lapillonne, G.
622 Putet, N.J. Secher, M. Symonds, H. Szajewska, P. Willatts, R. Uauy, World
623 Association of Perinatal Medicine Dietary Guidelines Working Group, The roles of
624 long-chain polyunsaturated fatty acids in pregnancy, lactation and infancy: review of
625 current knowledge and consensus recommendations, J. Perinat. Med. 36 (2008) 5-14.

626 [4] D.B. Jump, S.D. Clarke, A. Thelen, M. Liimatta, B. Ren, M. Badin, Dietary
627 polyunsaturated fatty acid regulation of gene transcription, Prog. Lipid Res. 35
628 (1996) 227-241.

629 [5] P.C. Calder, Dietary modification of inflammation with lipids, Proc. Nutr. Soc. 61
630 (2002) 345-58.

631 [6] N.D. Riediger, R.A. Othman, M. Suh, M.H. Moghadasian, A systemic review of the
632 roles of n-3 fatty acids in health and disease, J. Am. Diet. Assoc. 109 (2009) 668-
633 679.
634 [7] H.W. Cook, Fatty acid desaturation and chain elongation in eukaryotes, In: D.E.
635 Vance, J.E. Vance (Eds), Biochemistry of Lipids, Lipoproteins and Membranes,
636 Elsevier, Amsterdam, 1996, pp. 129-152.
637 [8] A. Voss, M. Reinhart, S. Sankarappa, H. Sprecher, The metabolism of
638 7,10,13,16,19-docosapentaenoic acid to 4,7,10,13,16,19-docosahexaenoic acid in rat
639 liver is independent of a 4-desaturase, J. Biol. Chem. 266 (1991) 19995-20000.
640 [9] X. Qiu, H. Hong, S.L. MacKenzie, Identification of a Delta 4 fatty acid desaturase
641 from *Thraustochytrium* sp. involved in the biosynthesis of docosahexanoic acid by
642 heterologous expression in *Saccharomyces cerevisiae* and *Brassica juncea*, J. Biol.
643 Chem. 276 (2001) 31561-31566.
644 [10] A. Meyer, P. Cirpus, C. Ott, R. Schlecker, U. Zähringer, E. Heinz, Biosynthesis of
645 docosahexaenoic acid in *Euglena gracilis*: biochemical and molecular evidence for
646 the involvement of a Delta4-fatty acyl group desaturase, Biochemistry 42 (2003)
647 9779-9788.
648 [11] T. Tonon, D. Harvey, T.R. Larson, I.A. Graham, Identification of a very long chain
649 polyunsaturated fatty acid Delta4-desaturase from the microalga *Pavlova lutheri*,
650 FEBS Lett. 553 (2003) 440-444.
651 [12] S.L. Pereira, A.E. Leonard, Y.S. Huang, L.T. Chuang, P. Mukerji, Identification of
652 two novel microalgal enzymes involved in the conversion of the omega3-fatty acid,
653 eicosapentaenoic acid, into docosahexaenoic acid, Biochem. J. 384 (2004) 357-366.

654 [13] Y. Li, O. Monroig, L. Zhang, S. Wang, X. Zheng, J.R. Dick, C. You, D.R. Tocher,
655 Vertebrate fatty acyl desaturase with Δ4 activity, Proc. Natl. Acad. Sci. U.S.A. 107
656 (2010) 16840-16845.
657 [14] S. Morais, F. Castanheira, L. Martinez-Rubio, L.E.C. Conceição, D.R. Tocher,
658 Long chain polyunsaturated fatty acid synthesis in a marine vertebrate: ontogenetic
659 and nutritional regulation of a fatty acyl desaturase with Δ4 activity, Biochim.
660 Biophys. Acta 1821(2012) 660-671.
661 [15] J. Fonseca-Madrigal, J.C. Navarro, F. Hontoria, D.R. Tocher, C.A. Martínez-
662 Palacios, O. Monroig, Diversification of substrate specificities in teleostei Fads2:
663 characterization of Δ4 and Δ6Δ5 desaturases of *Chirostoma estor*, J. Lipid Res. 55
664 (2014) 1408-1419.
665 [16] S. Morais, C. Aragão, E. Cabrita, L.E.C. Conceição, M. Constenla, B. Costas, J.
666 Dias, N. Duncan, S. Engrola, A. Estevez, E. Gisbert, E. Mañanós, L.M.P. Valente,
667 M. Yúfera, M.T. Dinis, New developments and biological insights into the farming
668 of *Solea senegalensis* reinforcing its aquaculture potential, Rev. Aquaculture (*in*
669 *press*) doi: 10.1111/raq.12091
670 [17] C. Navarro-Guillén, S. Engrola, F. Castanheira, N. Bandarra, I. Hachero-Cruzado,
671 D.R. Tocher, L.E.C. Conceição, S. Morais, Effect of varying dietary levels of LC-
672 PUFA and vegetable oil sources on performance and fatty acids of Senegalese sole
673 post larvae: Puzzling results suggest complete biosynthesis pathway from C18 PUFA
674 to DHA, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 167 (2014) 51-58.
675 [18] S. Morais, A. Candeias-Mendes, M.F. Castanheira, J. Coutinho, N. Bandarra, J.
676 Dias, L.E.C. Conceição, P. Pousão-Ferreira, New formulated diets for *Solea*
677 *senegalensis* broodstock: effects of parental nutrition on biosynthesis of long-chain

678 polyunsaturated fatty acids and performance of early larval stages and juvenile fish,
679 Aquaculture 432 (2014) 374–382.
680 [19] FAO, The State of World Fisheries and Aquaculture, Food and Agriculture
681 Organization of the United Nations, Rome, 2014.
682 [20] A.G.J. Tacon, M. Metian, Global overview on the use of fish meal and fish oil in
683 industrially compounded aquafeeds: Trends and future prospects, Aquaculture 285
684 (2008) 146-158.
685 [21] B.E. Torstensen, D.R. Tocher, The Effects of fish oil replacement on lipid
686 metabolism of fish, In: G.M. Turchini, W.-K. Ng, D.R. Tocher (Eds.), Fish Oil
687 Replacement and Alternative Lipid Sources in Aquaculture Feeds, Taylor & Francis,
688 CRC Press, Boca Raton, 2010, pp. 405-437.
689 [22] L.M.P. Valente, F. Linares, J.L.R. Villanueva, J.M.G. Silva, M. Espe, C. Escórcio,
690 M.A. Pires, M.J. Saavedra, P. Borges, F. Medale, B. Alvárez-Blázquez, J.B.
691 Peleteiro, Dietary protein source or energy levels have no major impact on growth
692 performance, nutrient utilisation or flesh fatty acids composition of market-sized
693 Senegalese sole, Aquaculture 318 (2011) 128-137.
694 [23] E.M. Cabral, T.J.R. Fernandes, S.D. Campos, M. Castro-Cunha, M.B.P.P. Oliveira,
695 L.M. Cunha, L.M.P. Valente, Replacement of fish meal by plant protein sources up
696 to 75% induces good growth performance without affecting flesh quality in
697 ongrowing Senegalese sole, Aquaculture 380–383 (2013) 130-138.
698 [24] P. Borges, B. Reis, T.J.R. Fernandes, Â. Palmas, M. Castro-Cunha, F. Médale,
699 M.B.P.P. Oliveira, L.M.P. Valente, Senegalese sole juveniles can cope with diets
700 devoid of supplemental fish oil while preserving flesh nutritional value, Aquaculture
701 418–419 (2014) 116-125.

[25] G. Mourente, J.R. Dick, J.G. Bell, D.R. Tocher, Effect of partial substitution of dietary fish oil by vegetable oil on desaturation and oxidation of [1-^{14}C]18:3n-3 and [1-^{14}C]20:5n-3 in hepatocytes and enterocytes of European seabass (*Dicentrarchus labrax* L.). Aquaculture 248 (2005) 173-186.

[26] O.H. Lowry, N.J. Rosenbourg, A.L. Farr, R.J. Randall, Protein measurement with folin phenol reagent, J. Biol. Chem. 193 (1951) 265-275.

[27] C. Ghioni, D.R. Tocher, J.R. Sargent, The effect of culture on morphology, lipid and fatty acid composition, and polyunsaturated fatty acid metabolism of rainbow trout (*Oncorhynchus mykiss*) skin cells, Fish Physiol. Biochem. 16 (1997) 499-513.

[28] R. Wilson, J.R. Sargent, High-resolution separation of polyunsaturated fatty acids by argentation thin-layer chromatography. J. Chromatogr. 623 (1992) 403–407.

[29] I. Stubhaug, D.R. Tocher, J.G. Bell, J.R. Dick, B.E. Torstensen, Fatty acid metabolism in Atlantic salmon (*Salmo salar* L.) hepatocytes and influence of dietary vegetable oil, Biochim. Biophys. Acta 1734 (2005) 277-288.

[30] J. Folch, M. Lees, G.H. Sloane-Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem. 226 (1957) 497-509.

[31] W.W. Christie, Lipid Analysis, 2nd edR, Pergamon press, Oxford, 1982.

[32] C. Infante, M.P. Matsuoka, E. Asensio, J.P. Cañavate, M. Reith, M. Manchado, Selection of housekeeping genes for gene expression studies in larvae from flatfish using real-time PCR, BMC Mol. Biol. 9 (2008) 28.

[33] J. Vandesompele, K.De Preter, F. Pattyn, B. Poppe,N. Van Roy, A.De Paepe, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (2002) 34.1–34.11.

726 [34] J. Hellemans, G. Mortier, A. De Paepe, F. Speleman, J. Vandesompele, qBase
727 relative quantification framework and software for management and automated
728 analysis of real-time quantitative PCR data, Genome Biol. 8 (2007) R19.
729 [35] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-
730 time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25 (2001) 402-408.
731 [36] M.V. Bell, J.R. Dick, A.E.A. Porter, Pyloric ceca are a major site of 22:6n-3
732 synthesis in rainbow trout (*Oncorhynchus mykiss*). Lipids 39 (2003) 39-44.
733 [37] J. Fonseca-Madrigal, J.G. Bell, D.R. Tocher, Nutritional and environmental
734 regulation of the synthesis of highly unsaturated fatty acids and of fatty-acid
735 oxidation in Atlantic salmon (*Salmo salar* L.) enterocytes and hepatocytes. Fish
736 Physiol. Biochem. 32 (2006) 317–328.
737 [38] S. Morais, J. Pratoomyot, J.B. Taggart, J.E. Bron, D.R. Guy, J.G. Bell, D.R.
738 Tocher, Genotype-specific responses in Atlantic salmon (*Salmo salar*) subject to
739 dietary fish oil replacement by vegetable oil: a liver transcriptomic analysis. BMC
740 Genomics 12 (2011) 255.
741 [39] M.J. Leaver, L.A.N. Villeneuve, A. Obach, L. Jensen, J.E. Bron, D.R. Tocher, J.B
742 Taggart, Functional genomics reveals increases in cholesterol biosynthetic genes and
743 highly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with
744 vegetable oils in Atlantic salmon (*Salmo salar*). BMC Genomics 9 (2008) 299.
745 [40] H.P. Cho, M.T. Nakamura, S.D. Clarke, Cloning, expression, and fatty acid
746 regulation of the human Δ-5 desaturase. J. Biol. Chem. 274 (1999) 37335–37339.
747 [41] C. Kolditz, G. Paboeuf, M. Borthaire, D. Esquerre, M. San Cristobal, F. Lefevre, F.
748 Médale, Changes induced by dietary energy intake and divergent selection for
749 muscle fat content in rainbow trout (*Oncorhynchus mykiss*), assessed by
750 transcriptome and proteome analysis of the liver. BMC Genomics 9 (2008) 506.

751 [42] L. Martinez-Rubio, S. Wadsworth, J.L. González Vecino, J.G. Bell, D.R. Tocher,
752 Effect of dietary digestible energy content on expression of genes of lipid
753 metabolism and LC-PUFA biosynthesis in liver of Atlantic salmon (*Salmo salar* L.),
754 Aquaculture 384–387 (2013) 94–103.
755 [43] S. Morais, J.B. Taggart, D.R. Guy, J.G. Bell, D.R. Tocher, Hepatic transcriptome
756 analysis of inter-family variability in flesh n-3 long-chain polyunsaturated fatty acid
757 content in Atlantic salmon, BMC Genomics (2012) 13:410.
758 [44] I. Seiliez, S. Panserat, G. Corraze, S. Kaushik, P. Bergot, Cloning and nutritional
759 regulation of a Δ6-desaturase-like enzyme in the marine teleost gilthead seabream
760 (*Sparus aurata*). Comp. Biochem. Physiol. B 135 (2003) 449–460.
761 [45] X. Zheng, I. Seiliez, N. Hastings, D.R. Tocher, S. Panserat, C.A. Dickson, P.
762 Bergot, A.J. Teale, Characterization and comparison of fatty acyl Δ6 desaturase
763 cDNAs from freshwater and marine teleost fish species. Comp. Biochem. Physiol. B
764 139 (2004) 269–279.
765 [46] N. Hastings, M.K. Agaba, D.R. Tocher, X. Zheng, C.A. Dickson, J.R. Dick, A.J.
766 Teale, Molecular cloning and functional characterization of fatty acyl desaturase and
767 elongase cDNAs involved in the production of eicosapentaenoic and
768 docosahexaenoic acids from alpha-linolenic acid in Atlantic salmon (*Salmo salar*).
769 Mar. Biotechnol. 6 (2004) 463–474.
770 [47] X. Zheng, D.R. Tocher, C.A. Dickson, J.G. Bell, A.J. Teale, Highly unsaturated
771 fatty acid synthesis in vertebrates: new insights with the cloning and characterization
772 of a delta6 desaturase of Atlantic salmon. Lipids 40 (2005) 13–24.
773 [48] N. Hastings, M. Agaba, D.R. Tocher, M.J. Leaver, J.R. Dick, J.R. Sargent, A.J.
774 Teale, A vertebrate fatty acid desaturase with delta 5 and delta 6 activities. Proc.
775 Natl. Acad. Sci. U. S. A. 98 (2001) 14304–14309.

776 [49] L.F.C. Castro, Ó. Monroig, M.J. Leaver, J. Wilson, I. Cunha, D.R. Tocher,
777 Functional desaturase *Fads1* (Δ5) and *Fads2* (Δ6) orthologues evolved before the
778 origin of jawed vertebrates. PLoS One 7 (2012) e31950.
779
780
781