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Docosahexaenoic acid biosynthesis via fatty acyl elongase and Δ 4-desaturase and its modulation by dietary lipid level and fatty acid composition in a marine vertebrate

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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 $\Delta 4$ -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 ($\Delta 4$ Fad) 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 *$\Delta 4$ fad* 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 $\Delta 4$ Fad activities tended to be higher in hepatocytes. In contrast, no $\Delta 6$ Fad 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 *$\Delta 4$ fad* 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 *$\Delta 4$ fad*, which were down-regulated in liver of fish fed the HL diets. Results indicated functionality and biological relevance of the $\Delta 4$ 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 C₁₈ 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 Δ 6 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 $\Delta 4$ desaturation. However, for many years a $\Delta 4$ Fad 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 $\Delta 4$ 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 $\Delta 4$ Fad activity in *in vitro* heterologous expression assays [14,15]. However, the *in vivo* activity and biological relevance of the $\Delta 4$ 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 *$\Delta 4$ fad* 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 *$\Delta 4$ fad* 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 C₁₈ 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 $\Delta 4$ Fad 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 $\Delta 4$ 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 *$\Delta 4$ fad*, 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

experimental feeding period. Finally, the tissue expression profile of the two genes is also reported.

2. Materials and methods

2.1. Tissue distribution of genes of LC-PUFA biosynthesis

Tissues were collected from juvenile Senegalese sole (average weight: 251 g) held in the experimental culture facilities of IRTA, Center of Sant Carles de la Ràpita (Spain) in 16 m³ tanks, with natural thermo-photoperiod, a salinity of 36 ppt and fed a standard commercial feed (LE-3, Skretting, Burgos, Spain) supplemented twice a week with natural feeds (mussels and polychaetes). Fish were fasted for 24 h prior to sampling and tissue samples were dissected and immediately frozen in dry ice and stored at -80 °C. A homogeneous sample of about 100 mg of tissue, from the same relative position in all animals, was collected from: stomach (Sto), anterior intestine (AI), posterior intestine (PL), liver (L), spleen (Spl), anterior kidney (K), heart (H), ventral skin (VS), dorsal skin (DS), and ovaries (O). Other tissues including eye (E, closest to mouth), brain (B), olfactory rosettes (OR) and one testis (T), were sampled whole, and for gills (G) one gill arch was taken from the middle region.

2.2. Dietary experiment and sampling

Solea senegalensis (Kaup, 1858) with an average body weight (BW) of 5.0 ± 0.1 g were distributed into twelve rectangular flat bottom 20 l tanks (containing 50 fish each) and cultured in a recirculation system at CCMAR, University of Faro, Portugal, at a temperature of 19.3 ± 1.2 , salinity of 32, and under a 12-h light/12-h dark photoperiod 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 methanesulfonate (MS222; Sigma, Sintra, Portugal). Samples of anterior intestine, liver and flesh (muscle) were taken, quickly frozen on dry ice and stored at -80 °C 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 cm² tissue culture flasks and incubated at 20 °C for 1 h with 0.25 µCi (final fatty acid concentration, 2 µM) of either [1-¹⁴C]18:3n-3 or [1-¹⁴C]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 hydrolysate 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

160 ¹ Peruvian fishmeal LT: 71% crude protein (CP), 11% crude fat (CF), EXALMAR, Peru.
161 ² Fair Average Quality (FAQ) fishmeal: 62% CP, 12%CF, COFACO, Portugal.
162 ³ CPSP 90: 84% CP, 12% CF, Sopropêche, France.
163 ⁴ Super prime squid meal: 80% CP, 3.5% CF, Sopropêche, France.
164 ⁵ Lysamine GP: 78% CP, 8% CF, ROQUETTE, France.
165 ⁶ Soycomil P: 65% CP, 0.8% CF, ADM, The Netherlands.
166 ⁷ Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL SA, Portugal.
167 ⁸ VITEN: 85.7% CP, 1.3% CF, ROQUETTE, France.
168 ⁹ Corn gluten feed: 61% CP, 6% CF, COPAM, Portugal.
169 ¹⁰ Aquatex G2000: 24% CP, 0.4% CF, SOTEXPRO, France.
170 ¹¹ COPPENS International, The Netherlands.
171 ¹² Henry Lamotte Oils GmbH, Germany.
172 ¹³ Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium
173 menadione bisulphate, 25mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30mg; riboflavin, 30mg; pyridoxine,
174 20mg; cyanocobalamin, 0.1mg; nicotinic acid, 200mg; folic acid, 15mg; ascorbic acid, 1000mg; inositol, 500mg; biotin, 3mg;
175 calcium panthotenate, 100mg; choline chloride, 1000mg, betaine, 500mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65mg;
176 copper sulphate, 9mg; ferric sulphate, 6mg; potassium iodide, 0.5mg; manganese oxide, 9.6mg; sodium selenite, 0.01mg; zinc
177 sulphate, 7.5mg; sodium chloride, 400mg; calcium carbonate, 1.86g; excipient wheat middlings.
178 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 °C/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 $\Delta 4$ -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 °C (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 ($2^{-\Delta\Delta C_T}$) 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.
<i>Δ4fad</i>	AAGCCTCTGCTGATTGGAGA GGCTGAGCTTGAAACAGACC	131 bp	60 °C	99.9 ¹ /102.1 ²	JN673546
<i>elovl5</i>	TTTCATGTTTTGTCACACTGC GACACCTTTAGGCTCGGTTTT	161 bp	60 °C	100.7 ¹ /100.4 ²	JN793448
<i>ubq</i> ^a	AGCTGGCCCAGAAATATAACTGCGACA ACTTCTTCTTGCGGCAGTTGACAGCAC	93 bp	70 °C	100.6 ¹ /98.8 ²	AB291588

<i>rps4</i> ^a	GTGAAGAAGCTCCTTGTCGGCACCA AGGGGGTCGGGGTAGCGGATG	83 bp	70 °C	99.7 ¹ /100.1 ²	AB291557
<i>efla1</i> ^a	GATTGACCGTCGTTCTGGCAAGAAGC GGCAAAGCGACCAAGGGGAGCAT	142 bp	70 °C	99.6 ¹ /100.1	AB326302
<i>18s</i> ^b	GAATTGACGGAAGGGCACCACCAG ACTAAGAACGGCCATGCACCACCAC	148 bp	70 °C	-	AM882675

^a Dietary trial; ^b Tissue distribution.

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

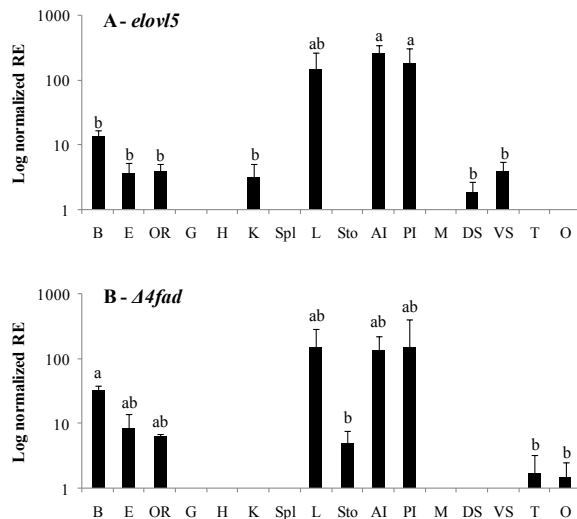


Fig. 1. Tissue distribution of *elovl5* (A) and *Δ4fad* (B) transcripts. Values are represented in logarithmic scale and correspond to the normalized (by *18s*) relative expression (RE) of the target genes in each tissue in relation to the average across all tissues, calculated using the delta-delta C_T method ($2^{-\Delta\Delta C_T}$). 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 $\Delta 6$ 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 $\Delta 4$ -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

320 **Table 4**

321 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

322 Results are means ± SD (n = 3). Values in brackets represent the percentage of ^{14}C -ALA elongated. No desaturated products of
 323 ^{14}C -ALA were observed.
 324

325 **Table 5**

326 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

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

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

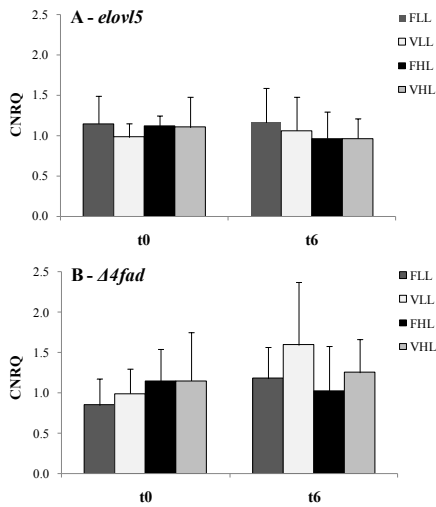


Fig. 2. Nutritional regulation of *elovl5* (A) and *Δ4fad* (B) gene transcription in intestine of *Solea senegalensis* juveniles after 24h-fasting (t0) and 6h after refeeding (t6). Values are calibrated normalized relative quantities (CNRQ) obtained from qBASE+, corresponding to an average of 6 individuals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

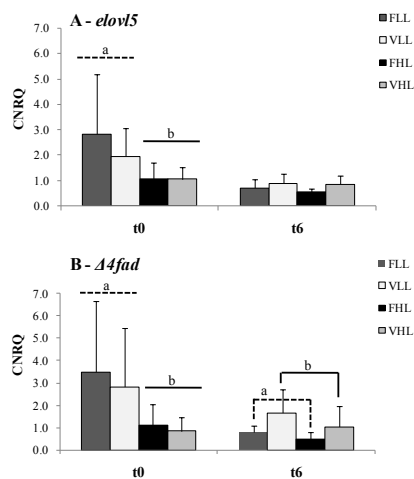


Fig. 3. Nutritional regulation of *elovl5* (A) and *Δ4fad* (B) gene transcription in liver of *Solea senegalensis* juveniles after 24h-fasting (t0) and 6h after refeeding (t6). Values are calibrated normalized relative quantities (CNRQ) obtained from qBASE+, corresponding to an average of 6 individuals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

3.4. Lipid composition

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

360 **Table 6**

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

362		Experimental diets				P-value (two-way ANOVA)		
		FLL	VLL	FHL	VHL	Lipid level	Lipid source	Interaction
363	Total lipids	8.8 ± 1.5	8.7 ± 3.7	12.7 ± 2.7	12.8 ± 1.2	0.026	0.991	0.953
364	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
365	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
366	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
367	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
368	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

369 Results are means ± SD (n = 3). ¹Includes 14:0, 16:0 and 18:0; ²Includes 16:1, 18:1n-9, 18:1n-7, 20:1 and 22:1; ³Includes 18:3n-6, 20:4n-6,
 370 22:4n-6 and 22:5n-6; ⁴Includes 18:4n-3, 20:3n-3, 20:4n-3, 21:5n-3 and 22:4n-3.

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377 **Table 7**

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

379		Experimental diets				P-value (two-way ANOVA)		
		FLL	VLL	FHL	VHL	Lipid level	Lipid source	Interaction
380	Total lipids	13.1 ± 2.6	12.5 ± 3.5	15.2 ± 1.8	15.6 ± 5.5	0.279	0.967	0.818
381	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
382	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
383	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
384	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
385	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

386 Results are means ± SD (n = 3). ¹Includes 14:0, 16:0 and 18:0; ²Includes 16:1, 18:1n-9, 18:1n-7, 20:1 and 22:1; ³Includes 18:3n-6, 20:3n-6,
 387 20:4n-6, 22:4n-6 and 22:5n-6; ⁴Include 18:4n-3, 20:4n-3, 21:5n-3 and 22:4n-3.

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394 **Table 8**

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

396		Experimental diets				P-value (two-way ANOVA)		
		FLL	VLL	FHL	VHL	Lipid level	Lipid source	Interaction
397	Total lipids	1.7 ± 0.3	2.3 ± 1.1	2.2 ± 0.4	2.3 ± 0.2	0.461	0.333	0.415
398	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
399	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
400	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
401	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
402	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

403 Results are means ± SD (n = 3). ¹Includes 14:0, 16:0 and 18:0; ²Includes 16:1, 18:1n-9, 18:1n-7, 20:1 and 22:1; ³Includes 18:3n-6, 20:3n-6,
 404 20:4n-6, 22:4n-6 and 22:5n-6; ⁴Includes 18:4n-3, 20:3n-3, 20:4n-3, 21:5n-3 and 22:4n-3.

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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 $\Delta 4$ desaturation to DHA, for many decades $\Delta 4$ Fad 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 $\Delta 4$ Fad 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 *$\Delta 4$ fad* 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 $\Delta 4$ Fad-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 $\Delta 6$ desaturase activity suggested this could not be subsequently desaturated in Senegalese sole and that the DHA must have arisen from $\Delta 4$ 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 ($\Delta 6$ Fad and $\Delta 5$ Fad) 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 $\Delta 4$ fad 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

from sole larvae showing a lower responsiveness of *elovl5* than $\Delta 4fad$ transcription to dietary LC-PUFA levels [14,34] and from Atlantic salmon showing lower nutritional regulation of fatty acyl elongases compared to desaturases [38,39]. However, in these and in most other studies investigating nutritional regulation of *elovls* and *fads* in response to dietary LC-PUFA contents, samples were generally from unfed or fasted fish, whereas in the present study no significant effect of FA composition was observed in the liver when juveniles were fasting. This result was therefore unexpected and might be explained by the fact that in basal conditions dietary lipid level exerted a strong and significant effect, which prevailed over, or interacted with, FA composition. On the other hand, the present results suggested that dietary FA composition exerted an immediate postprandial effect in the transcriptional regulation of these genes, independent of their basal expression levels.

In enterocytes there was no significant transcriptional regulation of the expression of either gene at fasting or postprandially, which was unexpected given the observed differences in enzyme activity. Nevertheless, the pattern of expression of $\Delta 4fad$ at t6 was comparable to that observed in liver, and therefore the absence of significant differences may be due to higher variability and lack of statistical power.

Dietary lipid level had clear effects on fatty acyl elongase and desaturase activities, which were significantly lower in hepatocytes of fish fed HL diets. This correlated with the basal expression of both *elovl5* and $\Delta 4fad$ in liver showing a significant down-regulation in fish fed HL diets. Research in mammals has firmly established that FA have key roles in regulating expression of genes involved in lipid metabolism and energy homeostasis through activation of nuclear receptors and transcription factors, and that not all FA have the same effect. In contrast to PUFA, SFA and MUFA have little effect, and within PUFA, LC-PUFA are more potent than C₁₈ 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 $\Delta 6/\Delta 5$ Fad activity, which could not be found in sole, or whether the characterized $\Delta 4$ Fad might also possess residual $\Delta 6/\Delta 5$ -desaturation activity [34]. In the present study, hepatocytes and enterocytes isolated from *S. senegalensis* and incubated with [1-¹⁴C]ALA did not show any $\Delta 6$ -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 $\Delta 5$ and $\Delta 6$ genes exist [46,47], while zebrafish (*Danio rerio*) has a single bifunctional

desaturase with both $\Delta 5$ and $\Delta 6$ activities [48]. Finally, in the only other two vertebrate species where a $\Delta 4$ Fad has been described until now, a second gene was functionally characterized and shown to have $\Delta 6/\Delta 5$ 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 C₁₈ PUFA via $\Delta 4$ -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 $\Delta 6$ Fad 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

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

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Supplementary files

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

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

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

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