Effect of varying dietary levels of LC-PUFA and vegetable oil sources on performance and fatty acids of Senegalese sole post larvae: Puzzling results suggest complete biosynthesis pathway from C18 PUFA to DHA

Carmen Navarro-Guillén[1], Sofia Engrola[2], Filipa Castanheira[2], Narcisa Bandarra[3], Ismael Hachero-Cruzado[4], Douglas R. Tocher[5], Luís E.C. Conceição[2,6], Sofia Morais[7*]

[1]ICMAN-CSIC, Campus Río San Pedro, 11519 Puerto Real, Cádiz, Spain.
[2]CCMAR/CIMAR LA, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal.
[3]IPMA, Av. Brasilia, 1449-006 Lisboa, Portugal.
[4]IFAPA "El Toruño", Ctra. N. IV Km. 654ª, 11500 El Puerto de Santa María, Cádiz, Spain.
[5]Institute of Aquaculture, School of Natural Sciences, University of Stirling, Stirling FK9 4LA, U.K.
[6]Sparos Lda-CRIA, Universidade do Algarve, 8005-139 Faro, Portugal.
[7]IRTA, Ctra. Poble Nou Km 5.5, 43540 Sant Carles de la Rápita, Spain.

* Corresponding author: Sofia Morais, tel +34 977745427, fax +34 977744138, email sofia.morais@irta.cat

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Abstract

Lipid nutrition of marine fish larvae has focused on supplying essential fatty acids (EFA) at high levels to meet requirements for growth and development. However, some deleterious effects have been reported suggesting that excessive supply of EFA might result in insufficient supply of energy substrates, particularly in species with lower EFA requirements such as Senegalese sole. This study addressed how the balance between EFA and non-EFA (better energy sources) affects larval performance, body composition and metabolism and retention of DHA, by formulating enrichment emulsions containing two different vegetable oil sources (olive oil or soybean oil) and three DHA levels. DHA positively affected growth and survival, independent of oil source, confirming that for sole post-larvae it is advantageous to base enrichments on vegetable oils supplying higher levels of energy, and supplement these with a DHA-rich oil. In addition, body DHA levels were generally comparable considering the large differences in their dietary supply, demonstrating that the previously reported Δ4 fatty acyl desaturase operates *in vivo* and that DHA was synthesized at physiologically significant rates through a mechanism involving transcriptional up-regulation of Δ*4fad*, which was significantly up-regulated in the low DHA treatments Furthermore, data suggested that DHA biosynthesis may be regulated by an interaction between dietary *n*-3 and *n*-6 PUFA, as well as by levels of LC-PUFA, and this may, under certain nutritional conditions, lead to DHA production from C18 precursors. The molecular basis of putative fatty acyl Δ5 and Δ6 desaturation activities remains to be fully determined as thorough searches have found only a single (Δ4)*Fads2*-type transcript. Therefore, further studies are required but this might represent a unique activity described within vertebrate fads.

Introduction

Until now, the major focus in lipid nutrition of marine fish larvae has been to study requirements for essential fatty acids (EFA), particularly for the long-chain polyunsaturated fatty acids (LC-PUFA), docosahexaenoic (DHA), eicosapentaenoic (EPA) and arachidonic (ARA) acids. These LC-PUFA are important components of biomembranes and dietary levels, particularly of DHA, have been associated with increased visual acuity and the capacity of larvae to capture prey[1], higher growth and survival, and reduced pigmentation abnormalities, abnormal behavior and susceptibility to disease and stress (increased immunity) in several species of marine fish larvae[2-5]. In most cases, providing high dietary levels of LC-PUFA, achieved through enrichment of live prey with specialist oils and dried single cell products, is crucial to cultivate marine fish species as it promotes larval growth and increases survival[6,7]. Nonetheless, in some instances, deleterious effects of dietary LC-PUFA have also been reported, including reduced growth of sole, *Solea spp.*[8-12]. A hypothesis put forward to explain this negative effect was the possibility that excessive levels of dietary LC-PUFA, which have higher susceptibility to peroxidation, would result in oxidative stress[13,14] and/or in an insufficient supply of energy substrates, given that LC-PUFA, and especially DHA, are relatively poorly oxidized[15].

Fish larval stages are characterized by extremely high growth rates (10-100% per day;[16]) and intense organogenesis, which both imply high metabolic and membrane synthesis demands. Therefore, it has become clear that increased attention should be given to the balance between EFA and other dietary fatty acids, which are the main source of metabolic energy, and to determine suitable ratios leading to optimized utilization (absorption and retention) of EFA, while covering the energetic needs of fast growing and developing fish larvae. Senegalese sole (*Solea senegalensis*) larvae and post-larvae are an interesting biological model in which to study interactions between dietary EFA (LC-PUFA) and non-essential fatty acids. Besides the high commercial interest of this species for aquaculture diversification in the South of Europe[17,18], this species is also unique amongst cultivated carnivorous marine fish species, given the particularly low LC-PUFA requirements observed during the larval and post-larval stages[9,10,19]. This was recently explained at a molecular level by the cloning and functional characterization of a fatty acyl desaturase with Δ4 activity (Δ*4fad*) and a fatty acyl elongase (*elovl5*), which together provide Senegalese sole with the enzymatic machinery required for DHA synthesis from EPA[19]. This discovery shortly followed the first description of a fatty acyl desaturase presenting Δ4 activity in a vertebrate species, also in a marine fish, the herbivorous rabbitfish *Siganus canaliculatus*[20]. However, neither of the previous studies could demonstrate whether the pathway is active *in vivo*[19,20].

In the present study, the primary aim was to investigate the appropriate balance between the dietary supply of LC-PUFA as structural components of membranes and other fatty acids as energetic fuel in Senegalese sole. This was addressed by determining the effects of *Artemia* enrichment emulsions containing different DHA levels (low, medium and high), in combination with vegetable oil sources including olive oil, rich in the monounsaturated fatty acid oleic acid (OA-18:1*n*-9), and soybean oil, rich in the short-chain PUFA linoleic acid (LOA-18:2*n*-6), on body fatty acid composition and DHA metabolism (absorption and catabolic oxidation) of post-larvae.

Materials and methods

Larval rearing and experimental diets

Larvae were obtained from IPMA Aquaculture Research Centre (Olhão, Portugal) at 19 days post hatching (dph), with an average dry weight of 0.56 ± 0.25 mg. Until this age larvae were fed rotifers enriched with a mixture of microalgae (*Nannochloropsis sp*. and *Isochrysis sp*.), up to 5dph, *Artemia* AF nauplii up to 10dph and *Artemia* EG enriched with *Red Pepper* (BernAqua NV, Belgium) from 11dph onwards. At 19dph larvae were transferred to a recirculation system in the Centre of Marine Sciences (University of Algarve, Faro, Portugal) consisting of eighteen 3-litre flat bottom trays with 240 larvae each. Photoperiod was 14h light:10h dark, salinity was around 35 and temperature 18.5± 0.9 ºC.

Larvae were fed one of six experimental treatments, consisting of *Artemia* metanauplii enriched with different oil emulsions, in triplicate trays. The emulsions were formulated with 5 g/100g soybean lecithin (MP Biomedicals, LLC, Illkirch, France), 3 g/100g Tween 80 (Panreac Quimica S.A., Castellar de Vallès, Spain), 2 g/100g alginic acid (MP Biomedicals), 1 g/100g vitamin E (MP Biomedicals) and 0.7 g/100g vitamin C (Rovimix STAY-C-35, DSM Nutritional Products Inc., Basel, Switzerland) as constant ingredients, and differed in the oil base that was used (olive oil or soybean oil – from 64 to 80 g/100g), and on the level of DHA supplemented in the form of Algatrium® (Brudy Technology, Barcelona, Spain; 5 to 24 g/100g), a specialist tuna oil providing high levels of LC-PUFA, mainly DHA triacylglycerols (>70% DHA; 6-8% EPA and 5-8% DPA, 22:5n-3). Enrichments were conducted at a density of 200 nauplii/ml, over 16h, and with 0.6 g emulsion/l. A single batch of enriched *Artemia* was produced for each treatment and kept frozen at -20ºC for the duration of the trial. Larvae were fed the *Artemia*, after thawing in seawater, in excess four times daily. At 19dph and 31dph, twenty larvae were collected from each tray for the

115 determination of individual dry weight. Samples were rinsed in distilled water, frozen in liquid
116 nitrogen and freeze-dried.

117 Animal manipulations were carried out in compliance with the Guidelines of the European Union
118 Council (86/609/EU) and Portuguese legislation for the use of laboratory animals. Protocols were
119 performed under license of Group-1 from the General Directorate of Veterinary (Ministry of
120 Agriculture, Rural Development and Fisheries, Portugal).

121 *Fatty acid analysis*

122 Triplicate samples of *Artemia* from each treatment were thoroughly washed, flash-frozen and kept
123 in liquid nitrogen pending fatty acid (FA) analysis. Similarly, twenty larvae were collected from
124 each tray at 31dph. Total lipids were extracted in chloroform/methanol (2:1, v/v) containing 0.01%
125 BHT[21,22]. Subsequently, total lipids were subjected to acid-catalyzed transmethylation at 50 ºC for
126 16-20 h. The fatty acid methyl esters (FAME) obtained were purified by thin-layer chromatography
127 (TLC) and visualized with iodine in chloroform (1%, v/v)[22]. FAME were separated and quantified
128 using a gas chromatograph (Shimadzu GC 2010) equipped with a flame ionization detector (280 ºC)
129 and a silica glass capillary column (SupraWax-280; 15m x 0.1 mm I.D.). The initial oven
130 temperature was 100°C, raised to 250°C (at a rate of 20ºC min^{-1}) and maintained at this temperature
131 for 8 min. FAME were identified using standard mixtures (C4C24 and Mehaden oil by Supelco,
132 Sigma-Aldrich, U.S.A.) as reference.

133 *Tube feeding procedure and metabolic trial*

134 To examine the absorption and metabolism of DHA, a tube feeding trial was conducted with
135 larvae at 30dph using [1-^{14}C] DHA (1.48-2.22GBq/mmol in ethanol, $37x10^{-6}$ GBq/ml, American
136 Radiolabelled Chemicals Inc., St Louis, MO, USA), following the methodology and experimental
137 procedures described previously[23,24]. Briefly, an oil mixture was prepared containing 20µl of
138 soybean oil to which $74x10^{-6}$ GBq of the radioactive tracer was added and the excess solvent
139 evaporated under a stream of oxygen-free nitrogen. On the day preceding the metabolic trial, 5
140 larvae from each triplicate tank were removed to smaller trays in the nutrient flux laboratory, where
141 larvae were acclimated and kept unfed overnight. Before tube feeding, enriched *Artemia* from each
142 treatment were added to the corresponding tray and larvae were allowed to feed for 1h. Ten larvae
143 from each treatment were first sedated with tricaine methanesulfonate (MS-222, Sigma-Aldrich,
144 U.S.A.) and then tube fed 18.4nl ^{14}C-DHA mixture. Each larva was then individually incubated for
145 24 h in vials containing 5ml of seawater in a sealed system, linked up by a capillary to a CO_2
146 metabolic trap (5 ml 0.5 mol/1 KOH) [23]. In order to determine body retention of the label, whole

larvae were sampled and dissolved in 0.5 ml of aqueous based solubilizer (Solvable™, PerkinElmer, U.S.A.) at 40°C for 24h. After acidification (with 1 ml 0.1 M HCl) of the incubation water, the fraction of the label that was catabolized by the larvae and became entrapped in seawater by conversion to HCO^-_3, was recovered in the metabolic trap as $^{14}CO_2$ that diffused out of the water. Finally, the label remaining in the water corresponds to label that was evacuated unabsorbed. The larval dissolved tissues were prepared for scintillation counting by adding 5 ml of scintillation cocktail (Ultima Gold XR, PerkinElmer, USA), and the incubation water and metabolic trap by adding 15 ml. The samples were counted in a liquid scintillation counter (Tri-Carb 2910TR, PerkinElmer, U.S.A.) and the results presented as a percentage of disintegrations per minute (dpm) in each fraction (retained in body and catabolized) in relation to the total absorbed radiolabel (total tube fed minus evacuated).

Expression of fatty acyl desaturase and elongase genes by real time quantitative PCR (qPCR)

In order to analyze the expression of genes involved in the LC-PUFA biosynthesis pathway, samples of 10 post-larvae per tray were collected into RNALater (Sigma-Aldrich, USA) at 31dph. For RNA extraction, samples were transferred into 2-ml screw-cap tubes containing 1ml of TRIzol (Ambion, Life Technologies, Madrid, Spain) and approximately 50 mg of 1mm diameter zirconium glass beads and homogenized (Mini-Beadbeater, Biospec Products Inc., U.S.A.). Solvent extraction was performed following manufacturer's instructions and RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (GeneQuant Pro, GE Healthcare, U.K.) using a nanovette microliter cell (Beckman Coulter Inc., U.S.A.). For RT-qPCR, 2 µg 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 and the remaining cDNA was diluted 60-fold with water. Quantification of the expression of fatty acyl desaturase *(Δ4fad*) and elongase (*elovl5*) was performed using primers reported previously[19] and three reference genes (ubiquitin - *ubq*; 40S ribosomal protein S4 - *rpsa*; and elongation factor 1 alpha – *ef1a1*) previously validated in studies with larval Senegalese sole[25]. Amplifications were carried out in duplicate (7300 Real time PCR System, Applied Biosystems, U.S.A.) in a final volume of 20 µl containing 5 µl (target genes) or 2 µl (reference genes) of diluted (1/60) cDNA, 0.5 µM of each primer and 10 µl SYBR GREEN qPCR Master Mix (Applied Biosystems) and included a systematic negative control (NTC-non template control). The qPCR profiles contained an initial activation step at 95 °C for 10 min, followed by 35 cycles: 15 s at 95 °C, 15 s at 60ºC and 15 s at 72

°C (3-step PCR for target genes) or 15 s at 95 °C and 30 s at 70 °C (2-step PCR for 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, which also allowed conversion of threshold cycle (Ct) values to arbitrary copy numbers. The reference genes showing the most stable expression were *rpsa* and *ef1a1* and hence expression of the target genes was normalized using a factor calculated by geNorm for the average expression of these two genes[26].

Statistical analysis

In order to examine the effects of "lipid source" and "DHA level" results were analyzed by two-way ANOVA. Whenever an interaction was detected between the two factors, or if the "DHA level" was found to significantly affect the results ($P<0.05$), a Tukey's multiple comparisons test was performed. All statistical analyses were performed with SPSS 15.0 software (IBM, New York, USA). Data are given as means and standard deviations (SD).

Results

The different dietary treatments led to significant differences in terms of growth and survival (Figs. 1 and 2). With regards to growth, both lipid source and DHA level induced significant differences ($P < 0.001$), with dry weight being higher when larvae were fed olive oil-based diets and also increasing significantly with DHA level. Hence, the significantly highest growth was achieved in larvae fed olive oil/high DHA, and the lowest was in larvae fed soybean oil/low DHA, while no significant differences were found between the remaining treatments. In terms of mortality, only DHA level had a significant effect ($P < 0.001$) and the lowest mortalities were obtained with the two high DHA treatments, irrespective of lipid source.

The FA compositions of the dietary treatments (enriched *Artemia*; Table 1) were as expected considering the formulation of the enrichment emulsions. *Artemia* enriched with the olive oil treatments presented higher levels of monounsaturated FA (due to the OA content), lower levels of *n*-6 PUFA (mainly related to the LOA content) and increasing percentages of LC-PUFA, particularly DHA, from low to high DHA supplements (0.3, 0.8 and 4.0% of total FA). In contrast, the soybean oil treatments showed lower levels of OA and higher levels LOA, but also presented increasing levels of DHA from low to high DHA treatments (0.1, 0.6 and 2.6%). Irrespective of the enricher, *Artemia* showed high levels of α-linolenic acid (ALA) that decreased as DHA level increased. In addition, in comparison with the low and medium DHA treatments, the high DHA

213 treatments presented generally lower levels of saturated FA and stearidonic acid (18:4*n*-3, SDA),
214 and higher levels of OA, LOA, and LC-PUFA including ARA, EPA, DPA, 22:5*n*-6 and DHA.

215 The FA compositions of the post-larvae generally reflected their diet, but there were some
216 interesting deviations (Table 2). Larval DHA levels showed significant differences between
217 treatments but, overall, were higher than would be expected based on diet composition. In larvae
218 fed the olive oil treatments, the DHA level was significantly higher in the high DHA treatment but
219 the highest level of larval DHA was obtained in the soybean oil/low DHA treatment. The EPA
220 content showed a similar tendency but was less marked with fewer significant differences. The
221 soybean oil/low DHA treatment was the one that least reflected the diet composition, showing much
222 lower levels than expected of ALA and SDA and higher than expected levels of all LC-PUFA
223 including EPA, DPA and DHA, as well as of ARA and 22:5*n*-6.

224 The tube feeding trial revealed no significant differences between individual treatments in
225 absorption, retention and catabolism of the DHA radiotracer (Fig. 3). However, two-way ANOVA
226 indicated a significant effect of oil source in DHA retention, which was generally higher in larvae
227 fed the soybean oil treatments (P=0.029).
228 A significant effect of DHA was observed in the expression of Δ*4fad* ($P < 0.001$), which was down-
229 regulated by increasing levels of dietary DHA, irrespective of the oil base (Fig. 4). In contrast, no
230 significant differences between treatments were observed in the expression of *elovl5*, despite a
231 significant interaction between the two factors (P=0.040) with a trend for higher expression in
232 larvae fed the olive oil/high DHA and soybean oil/low DHA treatments.
233
234 **Discussion**
235
236 Lipids have multiple key roles including being major sources of metabolic energy, critical
237 components maintaining the structural and functional integrity of cell membranes, and precursors of
238 important metabolites such as eicosanoids[27]. However, in the context of larval fish nutrition, and
239 given that the main bottleneck in rearing marine fish is the poor nutritional quality of live prey
240 commonly used as feeds in hatcheries[28], most research has focused on increasing dietary levels of
241 EFA, particularly DHA, and many commercial products are available for this purpose. However,
242 the use of different enrichment products, which differ in physical form, ingredients and nutrient
243 composition, has led to variable results both between related species as well as within the same
244 species[29-32]. It has long been suggested that not only total levels of EFA, but also the ratio between
245 them should be considered and, furthermore, more attention should be given to the balance between
246 levels of LC-PUFA and energy-yielding FA[4]. This area of research is challenging and generally

247 little progress has been made due to the fact that marine fish larvae do not accept or perform well on
248 formulated microparticulate diets. Therefore, studies require the manipulation of the biochemical
249 composition of live prey, part of which is fixed, with the variable portion subject to alteration by
250 their metabolism or affected by culture conditions and variability within the population[28]. In the
251 present study we addressed the question of how the balance between EFA and non-EFA (as better
252 sources of metabolic energy) affected larval performance, body composition and the metabolism
253 and retention of DHA, by formulating enrichment emulsions containing two different vegetable oil
254 sources (olive oil, supplying mostly OA; and soybean oil, supplying mostly LOA) and three
255 different DHA levels. It is generally well established that saturated and monounsaturated FA
256 (including OA) are preferential substrates for mitochondrial and peroxisomal β-oxidation in marine
257 and freshwater fish[33,34]. In rainbow trout, PUFA are also readily oxidized[35] but LOA and ALA,
258 which are EFA in freshwater fish and can be elongated and desaturated to LC-PUFA, have a slower
259 oxidation rate[34]. In the marine teleost *Myoxocephalus octodecimspinosus*, mitochondrial selectivity
260 for PUFA was less than 10% that of palmitoyl CoA (16:0) and the presence of polyunsaturated acyl
261 CoA esters inhibited the oxidation of palmitoyl CoA by intact peroxisomes by up to 70 %[33]. In
262 Senegalese sole post-larvae, catabolic oxidation of a tube fed DHA radiotracer was found to be
263 minimal, while OA was mostly oxidized, at similar or higher levels than stearic acid (18:0)[24]. In
264 the present study, the oil base of the diets appeared to affect growth as larval weights were higher in
265 fish fed the olive oil treatments. However, this study was hindered by the common difficulties in
266 trying to manipulate precisely the biochemical composition of live preys as mentioned above and,
267 unfortunately, these treatments also provided higher levels of DHA than the equivalent soybean oil-
268 based treatments. Hence, it cannot be unequivocally determined whether the effect was due to the
269 higher OA of the olive oil diets that might be a better energy source that LOA. In contrast, it was
270 clear that dietary DHA level significantly and positively affected growth and survival, independent
271 of the base oil used in the enrichers. In previous studies, high dietary DHA levels were not always
272 beneficial for growth and survival of Senegalese sole larvae and it was hypothesized that this may
273 be due to an excessive supply of LC-PUFA, relative to the low requirements of the species, that
274 reduced dietary space for other FA with higher energy availability[9,10]. The present experiment
275 confirms that for rearing Senegalese sole larvae it is advantageous to base enrichment emulsions on
276 vegetable oils, which supply higher levels of energy substrates, and to supplement these with a
277 DHA-rich oil, to achieve a correct balance between dietary energy and EFA.
278 Recently, we cloned and functionally characterized two enzymes of the LC-PUFA biosynthesis
279 pathway, including a fatty acyl desaturase (Fad) with Δ4 activity[19]. Although Δ4 desaturation
280 represents the simplest and most direct route for biosynthesis of DHA from EPA, for several
281 decades the presence of this pathway could not be demonstrated, other than in lower

eukaryotes[36,37]. Furthermore, Sprecher and co-workers in the early 1990s revealed a Δ4-independent pathway for DHA synthesis, involving two sequential elongations of EPA to 24:5*n*−3 followed by Δ6 desaturation and limited peroxisomal β-oxidation[38,39],which for long remained the only accepted mechanism for DHA biosynthesis in vertebrates (Fig. 5). This view changed recently, when Li et al. reported a gene coding for Δ4 Fad in the marine herbivorous rabbitfish *Siganus canaliculatus*[20], shortly followed by the discovery of a similar gene in Senegalese sole[19]. However, both studies used a heterologous (yeast) expression system to assay function and thus it was not possible to determine whether such activity is present and operates *in vivo*. In the sole study, a nutritional trial with larvae and post larvae tested extreme diets with either very high (*Artemia* enriched with a commercial product) or very low (non-enriched *Artemia*) LC-PUFA contents[19] but the body composition (DHA content) still reflected the dietary FA composition, with significantly lower levels of DHA being found in larvae and post-larvae fed the non-enriched *Artemia*, in spite of an up-regulation of Δ*4fad* expression in fish fed this treatment. In the present study, we provide for the first time evidence that the LC-PUFA pathway is indeed active *in vivo* in sole larvae and that DHA is synthesized from EPA at physiologically significant rates through a mechanism involving the transcriptional up-regulation of Δ*4fad* when dietary DHA is limiting. This is evidenced by the fact that, even although larval DHA contents showed significant differences between treatments, these levels were generally higher than would be expected based on diet compositions. In addition, the present results suggest that sole larvae also appear to be capable of biosynthesizing DHA from ALA, particularly under dietary conditions of low supply of DHA and high availability of the C18 precursor. Thus, although DHA levels in larvae fed the olive oil treatments were higher in larvae fed the high DHA diet, it was quite unexpected that the highest DHA level was obtained in larvae fed the soybean oil/low DHA treatment. This cannot be explained simply by higher retention of DHA supplied by the diet, as the tube feeding trial showed that, even though ^{14}C-DHA retention was generally higher in larvae fed the soybean oil treatments, this was not particularly accentuated in the soybean oil/low DHA treatment. Additionally, larvae fed this treatment did not directly reflect diet composition, showing higher than expected levels EPA, DPA, DHA, ARA and 22:5*n*-6 and much lower than expected levels of ALA, LOA and SDA. The biosynthesis of ARA and EPA from LOA and ALA, respectively, involves an initial Δ6 desaturation, followed by chain elongation, and a further Δ5 desaturation[7]. Therefore, the FA composition of larvae fed the soybean oil/low DHA treatment shows higher levels than expected of LC-PUFA products of not just Δ4-desaturation activity (DHA and 22:5*n*-6), but also of Δ5 desaturation activity (EPA and ARA) and lower levels of substrates of Δ6 desaturase (ALA and LOA).

316 The molecular basis of putative fatty acyl Δ5 and Δ6 desaturation activities in Senegalese sole
317 remains to be fully determined. Previously, functional characterization of the sole Δ4 Fad showed
318 that the enzyme also displayed Δ5 activity at a level around 15 % of the Δ4 activity for n-3
319 substrates[19]. This may be biologically relevant but only trace levels (0.3-0.6 % conversion) of Δ6
320 activity were reported to be associated with the Δ4 Fad. However, as vertebrate Δ4 activity was
321 highly novel, the yeast functional assay was repeated using a different ORF clone and, in this assay,
322 all activities were slightly higher and Δ6 activity (1.0-4.7% conversion) was clearly present
323 (unpublished). However, a single enzyme protein expressing all three (Δ6, Δ5 and Δ4) fatty acyl
324 desaturase activities is unprecedented. To put this in context, in the only other vertebrate where Δ4
325 fatty acyl desaturase has been reported so far, the rabbitfish, two separate *Fads2*-related genes have
326 been characterized, one having Δ6/Δ5 activity and the other with Δ4 activity[20]. Although the *Fads1*
327 (Δ5) gene is believed to have been lost from the teleost lineage, at least one Δ*6fad* has been found in
328 all teleost species examined so far[40]. Atlantic salmon (*Salmo salar*) have separate Δ5 and Δ6
329 genes[41,42], being both of the *Fads2*-type[40]. In contrast, the freshwater teleost, zebrafish (*Danio*
330 *rerio*), has a single bifunctional desaturase with both Δ5 and Δ6 activities[43]. In both these cases, Δ5
331 activity is believed to have evolved subsequent to the loss of the *Fads1*-Δ*5* gene, through mutations
332 (duplication/diversification) of *Fads2*-Δ*6*-type genes[40]. Despite serious efforts to find another Fad
333 with predominant Δ6 activity in Senegalese sole, both through cloning using degenerated primers
334 for Δ*6fad* genes, and *in silico* searches of a *Solea* transcriptome next-generation 454 sequencing
335 database (generated from different tissues, developmental stages or stimuli treatments) with a global
336 assembly containing >4 million reads and >250K UniGenes (SoleaDB,
337 http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/home_page;[44]), only the
338 single previously characterized (Δ4)*Fads2*-type transcript has been found[19].
339 Castro et al. [40] suggested that the evolution and variability found in teleost *Fads2*-type genes might
340 be linked to habitat-specific food web structures in different environments, and we had previously
341 commented on the natural dietary regime of *S. senegalensis* that, due to its benthic lifestyle, differs
342 from other commonly cultivated carnivorous fish species, having a diet generally poor in lipid and
343 with substantially higher levels of EPA than DHA[19]. Further studies are required for confirmation
344 but, at present, our results suggest that in this species there is a single *Fads2*-type gene which has a
345 predominant Δ4 activity but that, under particular dietary conditions of low DHA levels combined
346 with high levels of C_{18}PUFA, may act also on Δ6 and Δ5 substrates to biosynthesize DHA from C_{18}
347 precursors. This appears to be tightly regulated given that biochemical signs of desaturation and
348 elongation of C_{18}PUFA, associated with the highest up-regulation of Δ4fad transcription, were only
349 observed in post-larvae fed the dietary treatment (soybean oil/low DHA) with lowest DHA, highest
350 ALA, and also high LOA.

351 It should be noted that the other enzyme involved in the LC-PUFA biosynthesis pathway and
352 cloned from *S. senegalensis*, *elovl5*, has wide substrate specificity and can elongate C_{18} up to C_{22}[19]
353 and, hence, this enzyme would be capable of performing all of the elongations necessary in the
354 biosynthesis pathway of DHA from ALA, via a Δ4Fad. As shown previously, the expression of
355 *elovl5* does not appear to be significantly regulated by diet, although a trend of higher expression in
356 the soybean oil/low DHA treatment, resulting in significant interaction between the two factors,
357 lipid source and DHA level, was observed.

358 In conclusion, DHA positively affected growth and survival in this study, independently of the oil
359 source used. The difficulty in manipulating precisely the biochemical composition of live preys,
360 which unfortunately is quite common in larval nutrition studies, did not enable ascertaining whether
361 one oil type improved growth relative to the other. Nonetheless, results show that for sole post-
362 larvae it is advantageous to base enrichments on vegetable oils supplying higher levels of energy,
363 and supplement these with a DHA-rich oil, at least at the inclusion levels tested here. Finally, an
364 unexpected outcome of the results was to point out that this marine teleost may be capable of DHA
365 synthesis from ALA. This hypothesis requires to be fully tested but, if proved, would be highly
366 novel and would establish Senegalese sole as an interesting model in which to study this important
367 pathway and its regulation by dietary composition in lower vertebrates.

368

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374 interest to disclose. The authors' contributions were as follows: S.M. and L.E.C. designed and
375 coordinated the study; C.N.G. conducted the experiment, assisted by S.E. and F.C. for the tube
376 feeding assays and preparation of the diets; I.H-C. and N.B. performed the lipid analysis of the
377 larvae and enriched *Artemia*, respectively; C.N.G., S.M. and D.R.T. had primary responsibility in
378 writting the manuscript, which was read and approved by all other authors.

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Table 1. Fatty acid composition (% total FA) of *Artemia* metanauplii enriched with the different experimental emulsions. Represented are also p-values of the 2-way ANOVA analysis and, whenever a significant interaction was found, a Tukey test was performed (numbers with different letters within the same row are significantly different, at P<0.05).

	Olive oil									Soybean oil									2-way ANOVA		
	Low DHA			Medium DHA			High DHA			Low DHA			Medium DHA			High DHA			Oil base	DHA level	Oil*DHA
Fatty acids (%)	Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD				
14:0	0.6	0.0	b	0.6	0.0	c	0.4	0.0	e	0.7	0.0	a	0.5	0.0	d	0.4	0.0	e	n.s.	<0.0001	0.0001
16:0	11.8	0.3	ab	11.9	0.4	a	9.8	0.1	d	11.9	0.3	a	11.0	0.1	c	11.1	0.1	bc	n.s.	<0.0001	<0.0001
18:0	5.1	0.1	b	5.1	0.1	b	4.5	0.1	c	5.4	0.2	a	5.1	0.0	b	5.0	0.1	b	<0.0001	<0.0001	0.0063
Other SFA	2.6	0.0		2.5	0.1		1.9	0.0		3.0	0.1		2.7	0.2		2.0	0.0		0.0004	<0.0001	n.s.
Total - SFA	20.0	0.4	ab	20.0	0.6	ab	16.5	0.2	d	21.0	0.5	a	19.4	0.3	bc	18.5	0.2	c	0.0028	<0.0001	0.0005
16:1	2.9	0.0	b	2.7	0.0	c	2.3	0.0	e	3.0	0.0	a	2.5	0.0	d	1.6	0.0	f	<0.0001	<0.0001	<0.0001
18:1	31.2	0.3	c	31.9	0.3	b	45.7	0.2	a	26.8	0.1	e	26.9	0.2	e	29.5	0.1	d	<0.0001	<0.0001	<0.0001
20:1	0.6	0.0		0.6	0.0		0.5	0.0		0.7	0.2		0.6	0.1		0.6	0.0		0.0194	n.s.	n.s.
Other MUFA	0.9	0.1		0.9	0.1		0.5	0.0		0.9	0.2		1.0	0.0		0.5	0.1		n.s.	<0.0001	n.s.
Total - MUFA	35.6	0.3	b	36.1	0.3	b	49.0	0.2	a	31.4	0.2	d	31.1	0.4	d	32.2	0.1	c	<0.0001	<0.0001	<0.0001
18:2*n* - 6	5.9	0.1	de	5.7	0.1	e	6.3	0.1	d	8.0	0.1	c	13.4	0.2	b	24.4	0.2	a	<0.0001	<0.0001	<0.0001
18:3*n* - 6	0.1	0.2		0.1	0.2		0.1	0.1		0.1	0.2		0.1	0.2		0.1	0.1		n.s.	n.s.	n.s.
20:3*n* - 6	0.1	0.0		0.0	0.0		0.0	0.0		0.0	0.0		0.0	0.0		0.0	0.0		n.s.	n.s.	n.s.
20:2*n* - 6	0.2	0.0		0.2	0.0		0.2	0.0		0.2	0.0		0.2	0.0		0.2	0.0		<0.0001	0.0015	n.s.
20:4*n* - 6	0.2	0.0	b	0.2	0.0	b	0.3	0.0	a	0.2	0.0	b	0.2	0.0	b	0.2	0.0	b	<0.0001	<0.0001	<0.0001
22:5*n* - 6	0.0	0.0	c	0.0	0.0	c	0.2	0.0	a	0.0	0.0	c	0.0	0.0	c	0.1	0.0	b	<0.0001	<0.0001	<0.0001
Total *n* - 6 PUFA	6.5	0.1	e	6.2	0.1	e	7.0	0.1	d	8.6	0.2	c	14.0	0.0	b	25.0	0.1	a	<0.0001	<0.0001	<0.0001
18:3*n* - 3	26.1	0.7	ab	25.4	0.7	b	15.2	0.2	c	27.5	0.5	a	24.7	0.5	b	14.3	0.2	c	n.s.	<0.0001	0.0059
18:4*n* - 3	3.8	0.2		3.8	0.3		1.8	0.0		3.8	0.2		3.5	0.1		1.6	0.0		0.0413	<0.0001	n.s.
20:3*n* - 3	0.8	0.0	a	0.8	0.0	a	0.5	0.0	b	0.8	0.0	a	0.8	0.0	a	0.4	0.0	c	n.s.	<0.0001	0.0002
20:4*n* - 3	0.7	0.0		0.7	0.0		0.4	0.0		0.7	0.0		0.7	0.0		0.5	0.0		n.s.	<0.0001	n.s.
20:5*n* - 3	0.7	0.1	c	0.8	0.1	c	1.8	0.1	a	0.7	0.1	c	0.8	0.0	c	1.3	0.0	b	0.0001	<0.0001	<0,0001
22:5*n* - 3	0.0	0.0	d	0.1	0.0	c	0.5	0.0	a	0.0	0.0	d	0.0	0.0	d	0.3	0.0	b	<0.0001	<0.0001	<0.0001
22:6*n* - 3	0.3	0.0	d	0.8	0.0	c	4.0	0.2	a	0.1	0.1	d	0.6	0.0	c	2.6	0.1	b	<0.0001	<0.0001	<0.0001
Total *n* - 3 PUFA	32.9	1.0	ab	32.8	1.2	ab	24.6	0.5	c	34.2	0.8	a	31.6	0.7	b	21.2	0.3	d	0.0123	<0.0001	0.0009
DHA/EPA	0.4	0.1		0.9	0.1		2.3	0.0		0.2	0.1		0.7	0.0		2.1	0.1		0.0004	<0.0001	n.s.
Total PUFA	39.9	0.6	c	39.6	0.8	c	31.9	0.5	d	43.1	0.6	b	45.8	0.7	a	46.5	0.3	a	<0.0001	<0.0001	<0.0001

Table 2. Fatty acid composition (% total FA) of *Solea senegalensis* post-larvae (31dph) fed the different experimental treatments. Represented are also p-values of the 2-way ANOVA analysis and, whenever a significant interaction was found, a Tukey test was performed (numbers with different letters within the same row are significantly different, at P<0.05).

	Olive oil								Soybean oil								2-way ANOVA		
	Low DHA			Medium DHA			High DHA			Low DHA			Medium DHA			High DHA					
Fatty acids	Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Oil base	DHA level	Oil*DHA
14:0	0.6	0.1	ab	0.6	0.0	ab	0.5	0.1	b	0.8	0.1	a	0.6	0.1	ab	0.6	0.2	ab	0.0119	n.s.	n.s.
16:0	12.8	0.9	b	12.8	0.7	b	12.5	0.7	b	15.7	0.9	a	13.2	0.7	b	13.6	1.0	b	<0.0001	0.0026	0.0076
18:0	7.8	0.8	b	8.0	0.6	b	7.4	0.7	b	10.3	0.8	a	8.4	0.7	b	8.0	0.3	b	<0.0001	0.0003	0.0026
Other SFA	3.4	0.6	bc	3.6	0.3	bc	2.9	0.5	c	4.6	1.1	a	3.8	0.7	b	3.1	0.9	c	0.0010	<0.0001	0.0031
Total - SFA	24.7	2.2	b	25.0	1.5	b	23.4	1.4	b	31.3	1.8	a	25.9	1.7	b	25.4	1.7	b	<0.0001	0.0002	0.0018
16:1	2.8	0.2	ab	2.9	0.3	a	2.5	0.2	ab	2.4	0.4	ab	2.5	0.2	ab	1.9	0.2	c	<0.0001	<0.0001	n.s.
18:1	28.4	1.1	b	26.0	1.3	c	34.8	0.8	a	22.6	1.0	d	23.1	1.5	d	24.2	2.1	cd	<0.0001	<0.0001	<0.0001
20:1	1.0	0.0	a	0.9	0.1	ab	0.8	0.1	ab	0.8	0.2	ab	0.8	0.1	ab	0.7	0.2	b	0.0166	n.s.	n.s.
Other MUFA	0.5	0.2	abc	0.7	0.2	a	0.3	0.1	c	0.4	0.2	abc	0.5	0.1	ab	0.4	0.2	bc	n.s.	0.0001	0.0703
Total - MUFA	32.6	1.3	b	30.5	1.5	b	38.3	1.0	a	26.3	1.1	c	26.9	1.5	c	27.2	0.7	c	<0.0001	<0.0001	<0.0001
18:2*n*-6	7.3	0.4	b	5.9	0.2	b	6.4	0.3	b	6.7	0.9	b	11.1	0.7	ab	14.1	6.8	a	0.0005	0.0496	0.0101
18:3*n*-6	0.0	0.0		0.0	0.0		0.0	0.0		0.2	0.3		0.1	0.2		0.0	0.0		0.0462	n.s.	n.s.
20:3*n*-6	0.2	0.0	ab	0.2	0.0	ab	0.0	0.1	c	0.3	0.0	a	0.2	0.0	ab	0.1	0.1	bc	0.0088	0.0001	n.s.
20:2*n*-6	0.3	0.0	bc	0.3	0.1	c	0.3	0.0	c	0.3	0.1	bc	0.5	0.1	a	0.4	0.1	ab	0.0002	n.s.	0.0145
20:4*n*-6	1.8	0.3	bc	1.9	0.2	bc	1.5	0.1	c	2.8	0.3	a	2.0	0.1	b	1.9	0.3	bc	<0.0001	<0.0001	0.0003
22:5*n*-6	1.1	0.2	bc	1.1	0.1	bc	0.9	0.0	c	1.7	0.2	a	1.2	0.1	bc	1.3	0.4	b	<0.0001	0.0024	0.0185
Total *n* - 6 PUFA	10.7	0.4	bc	9.4	0.3	c	9.2	0.4	c	12.1	1.2	bc	15.1	0.5	ab	17.8	6.2	a	<0.0001	n.s.	0.0143
18:3*n*-3	15.5	1.8	ab	17.4	0.8	a	12.7	0.5	bc	11.5	1.6	c	15.0	1.0	abc	13.4	4.0	bc	0.0115	0.0021	0.0313
18:4*n*-3	2.6	0.4	a	2.9	0.2	a	1.7	0.1	c	1.2	0.3	c	2.4	0.2	ab	1.8	0.7	bc	0.0001	<0.0001	0.0002
20:3*n*-3	1.4	0.1		1.4	0.1		1.1	0.0		1.3	0.1		1.4	0.1		1.2	0.4		n.s.	0.0121	n.s.
20:4*n*-3	0.9	0.1	ab	1.0	0.1	a	0.6	0.0	c	0.7	0.1	abc	0.9	0.1	abc	0.7	0.3	bc	n.s.	0.0004	n.s.
20:5*n*-3	1.5	0.2	c	1.7	0.2	abc	2.1	0.2	a	2.0	0.3	ab	1.6	0.2	bc	1.8	0.2	abc	n.s.	0.0158	0.0057
22:5*n*-3	1.0	0.1	d	1.0	0.1	d	1.5	0.1	a	1.3	0.1	b	1.1	0.1	cd	1.2	0.1	bc	n.s.	<0.0001	<0.0001
22:6*n*-3	5.6	0.9	c	5.6	0.5	c	7.0	0.3	ab	7.5	0.7	a	6.0	0.4	bc	6.4	0.5	bc	0.0050	0.0013	0.0001
Total *n* - 3 PUFA	29.1	2.0	ab	31.6	1.4	a	27.1	1.2	ab	26.3	1.3	b	28.9	1.0	ab	27.0	5.5	ab	n.s.	0.0236	n.s.
DHA/EPA	3.6	0.4		3.3	0.4		3.4	0.2		3.9	0.5		3.7	0.4		3.6	0.7		n.s.	n.s.	n.s.
Total PUFA	40.4	2.0	b	41.4	1.5	b	36.3	1.1	c	39.1	0.9	b	44.6	1.3	a	45.3	1.5	a	<0.0001	0.0001	<0.0001

Figure legends

Fig. 1. Dry weight (mg/larva) of Senegalese sole post-larvae fed each dietary treatment at the end of experimental period (31dph). Different letters represent significant differences between treatments (Tukey test; $P<0.05$). Results are means (n=60) with SD.

Fig. 2. Larval mortality (%) of Senegalese sole larvae fed each dietary treatment at the end of experimental period (31dph). Different letters represent significant differences between treatments (Tukey test; $P<0.05$). Results are means (n=3) with SD.

Fig. 3. Absorption (black), retention (dark grey) and catabolism (light grey) of DHA in Senegalese sole larvae tube-fed ^{14}C-DHA at 30dph. Absorption is expressed as % of total label that was tube fed, and retention and catabolism correspond to the percentage of label found in the body and metabolic trap compartments, respectively, in relation to total absorption. Soybean oil lead to a significantly higher DHA body retention than olive oil (two-way ANOVA, $P= 0.029$). Results are means (n=10) with SD.

Fig. 4. Nutritional regulation of fatty acyl desaturase (Δ*4fad*; black) and elongase (*elovl5*; grey) expression in whole Senegalese sole post-larvae at 31dph, determined by qPCR. Results were normalized by a normalizing factor representing average expression of *rpsa* and *ef1a1*. Different letters represent significant differences between treatments for Δ*4fad* (Tukey test; $P<0.05$); no significant differences were found for *elovl5*. Results are means (n=3) with SD.

Fig. 5. Schematic representation of the LC-PUFA biosynthesis pathway, including the complement of enzymes intervening in the different steps (not all are necessarily present in a same species).

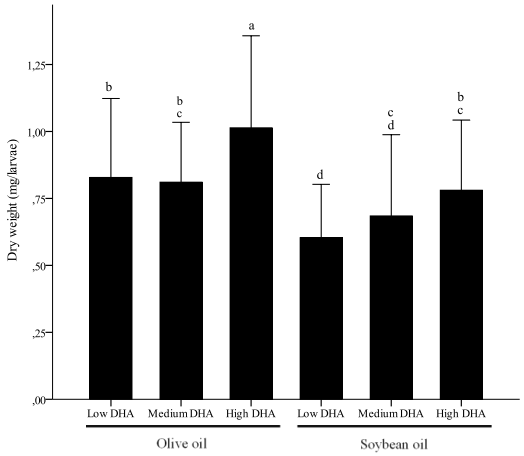

Fig1. Dry weight (mg/larva) of Senegalese sole larvae fed olive oil (a) and soybean (b) treatments at the end of experimental period (31 DAH). Letters mean statistical differences due to treatments (one-way ANOVA, Tukey test; $P<0.05$).

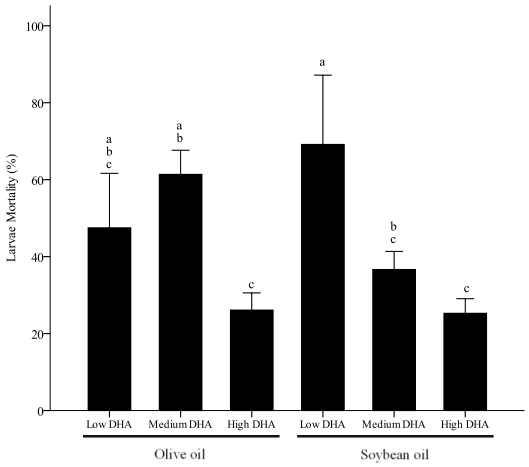

Fig2. Larvae mortality (%) of Senegalese sole larvae fed each treatment at the end of experimental period (31 DAH). Letters mean statistical differences due to treatments (one-way ANOVA; Tukey test; $P<0.05$).

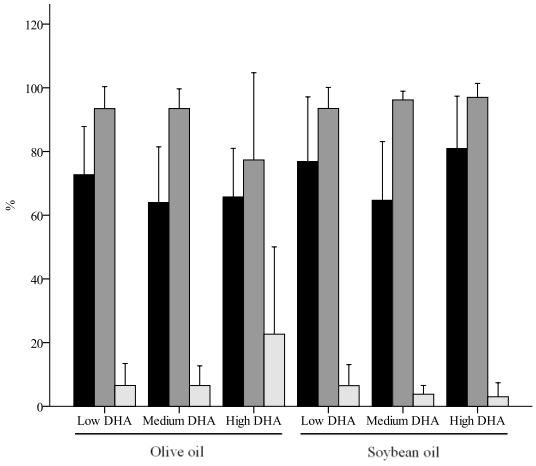

Fig4. Absorption (dark), retention (dark grey) and catabolism (light grey) of DHA in Senegalese sole larvae tube-fed ^{14}C-DHA at 30 DAH. Soybean oil lead to a significantly higher DHA retention than olive oil (two-way ANOVA, P= 0.029).

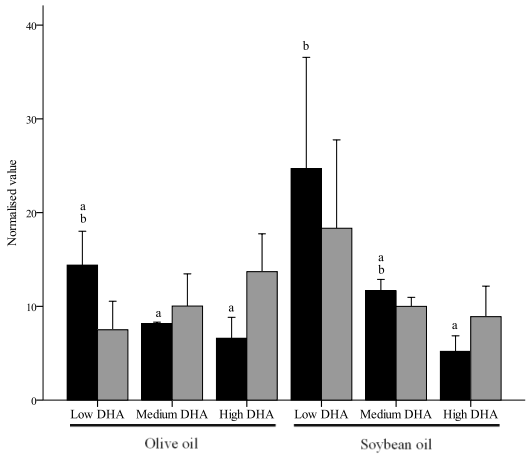

Fig5. qPCR results for elongase *elovl5* (grey) and desaturase *d4fad* (black) enzymes in larvae fed olive oil (a) and soybean oil (b) treatments. Results were normalized using two genes; ELF1A1+RPSA, with D-T4. Letters mean statistical differences in *d4fad* values due to treatments, *elovl5* did not show significant differences (one-way ANOVA; Tukey test; P<0.05).

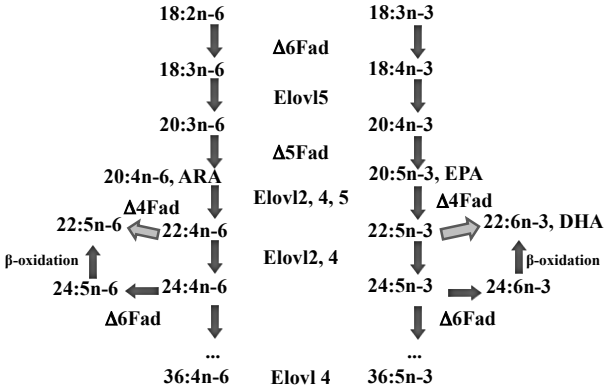

Fig6. Schematic representation of the LC-PUFA biosynthesis pathway, including the complement of enzymes intervening at the different steps (note: not all are necessarily present in a same species).