Morais S, Castanheira F, Martinez-Rubio L, Conceicao LEC & Tocher DR (2012) Long chain polyunsaturated fatty acid synthesis in a marine vertebrate: Ontogenetic and nutritional regulation of a fatty acyl desaturase with Δ4 activity, *Biochimica et Biophysica Acta (BBA)- Molecular and Cell Biology of Lipids*, 1821 (4), pp. 660-671

This is the peer reviewed version of this article.

NOTICE: this is the author's version of a work that was accepted for publication in Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, [1821, 4, 04/2012] DOI 10.1016/j.bbalip.2011.12.011

Elsevier Editorial System(tm) for BBA - Molecular and Cell Biology of Lipids
Manuscript Draft

Manuscript Number:

Title: Long chain polyunsaturated fatty acid synthesis in a marine vertebrate: ontogenetic and nutritional regulation of a fatty acyl desaturase with Delta4 activity

Article Type: Regular Paper

Keywords: 4 desaturase; polyunsaturated fatty acid synthesis; ontogenesis; transcriptional regulation; Solea senegalensis; marine teleost

Corresponding Author: Dr Sofia Morais, Ph.D.

Corresponding Author's Institution: IRTA

First Author: Sofia Morais, Ph.D.

Order of Authors: Sofia Morais, Ph.D.; Filipa Castanheira; Laura Martinez-Rubio; Luis Conceição; Douglas R Tocher

Abstract: Solea senegalensis is an unusual marine teleost with very low dietary requirement for long-chain polyunsaturated fatty acids (LC-PUFA) during early development. Aquaculture is rapidly becoming the main source of health-beneficial fish products for human consumption. This, associated with limited supply of LC-PUFA-rich ingredients for fish feeds, render S. senegalensis a highly interesting species in which to study the LC-PUFA biosynthesis pathway. We have cloned and functionally characterized fatty acyl desaturase and elongase cDNAs corresponding to 4fad (with some 5 activity for the n-3 series) and elovl5 with the potential to catalyze docosahexaenoic acid (DHA) biosynthesis from eicosapentaenoic acid (EPA). Changes in expression of both transcripts were determined during embryonic and early larval development, and transcriptional regulation in response to dietary n-3 LC-PUFA was assessed during larval and post-larval stages. There was a marked pattern of regulation during early ontogenesis, with transcripts showing peak expression coinciding with the start of exogenous feeding. Although elovl5 transcripts were present in fertilized eggs, 4fad only appeared at hatching. However, eggs have high proportions of DHA (~ 20%) and high DHA/EPA ratio (~ 11) to meet the high demands for early embryonic development. Fatty acid profile of larvae after the start of exogenous feeding closely reflected dietary composition. Nonetheless, 4fad was significantly up-regulated in response to LC-PUFA-poor diets, which may suggest biological relevance of this pathway in reducing LC-PUFA dietary requirements in this species, compared to other marine teleosts. These results indicate that sole is capable of synthesizing DHA from EPA through a Sprecher-independent pathway.

Suggested Reviewers: Xiaozhong Zheng
xzheng@staffmail.ed.ac.uk
Dr Zheng has done considerable work involving cloning and functional characterization of Fads and Elovls in fish and is therefore a very specific expert in this field.

Johnathan A. Napier
jon.napier@bbsrc.ac.uk
Prof. Napier is an expert in Fad molecular biology and has considerable expertise in pathways of DHA biosynthesis.

Chantal Cahu
Chantal.Cahu@ifremer.fr
Dr. Chantal Cahu is an expert in marine larvae nutrition including fatty acid metabolism. In addition, she has also been involved in molecular and genomic studies, including the cloning and Fad expression in fish.

Gabriel Mourente
gabriel.mourente@uca.es
Dr. Gabriel Mourente is an expert in marine larvae nutrition including fatty acid metabolism. He has additionally performed several larval nutrition studies in S. senegalensis and has also worked specifically with enzymes of the pathway of LC-PUFA biosynthesis.

Manuel Yúfera
manuel.yufera@icman.csic.es
Dr. Manuel Yúfera is an expert in marine larvae nutrition including fatty acid metabolism and has additionally performed several larval nutrition studies in S. senegalensis.

Cover Letter

IRTA - Sant Carles de la Ràpita
Spain

12 October 2011

Dear Sirs,

I am submitting electronically, to *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, a manuscript entitled "Long-chain polyunsaturated fatty acid synthesis in a marine vertebrate: ontogenetic and nutritional regulation of a fatty acyl desaturase with Δ4 activity", by Sofia Morais, Filipa Castanheira, Laura Martínez-Rubio, Luis E.C. Conceição and Douglas R. Tocher.

Fatty acyl desaturase and elongase cDNAs corresponding to a Δ4fad (with some Δ5 activity for PUFA of the n-3 series) and elovl5 were cloned from a marine teleost (*Solea senegalensis*) and functionally characterized by heterologous expression in yeast. Historically, it was believed that the pathway for DHA synthesis would involve a Δ4 desaturase, as this would be the most direct route, but the presence of Δ4Fad enzymes could not be established in vertebrates and a Δ4-independent pathway of DHA synthesis involving sequential elongations of EPA to 24:5n-3 followed by Δ6 desaturation and partial oxidation was identified in rats and rainbow trout. Later, Fads with Δ4 activity were demonstrated in several lower eukaryotes but the Δ6 pathway remained the only demonstrated mechanism for DHA biosynthesis in vertebrates. Very recently (PNAS 107 (2010) 16840-16845), a Fad enzyme with Δ4 activity was reported for the first time in a vertebrate species, a marine herbivorous fish. In the present study, characterization of another Δ4fad, this time in a marine carnivorous teleost, has established that production of DHA via Δ4-desaturation is likely more widespread among vertebrate species. From an evolutionary standpoint this finding is surprising as it was the established paradigm that in the marine environment, and particularly associated with a carnivorous diet, fish had adapted to a rich dietary supply of DHA, and therefore there would be no pressure for endogenous DHA biosynthesis. Therefore, the data are discussed in relation to the natural diet of *S. senegalensis* and support the alternative hypothesis that this pathway has high adaptative plasticity to trophic level. Furthermore, this is the first time that transcriptional regulation of Δ4fad has been demonstrated. Transcripts of Δ4fad and elovl5 showed a pattern of regulation during early ontogenesis indicating adaptation to ensure availability of DHA for neurogenesis independent of dietary supply during a short temporal window corresponding to the start of exogenous feeding. Furthermore, Δ4fad was up-regulated in response to LC-PUFA-poor diets, confirming biological relevance of this pathway in reducing LC-PUFA dietary requirements in this species, which were empirically known to be lower than other marine fish species. Therefore, this paper presents novel information on LC-PUFA biosynthesis and its transcriptional regulation, and evidence of the plasticity of its adaptation to trophic environment.

The work has not been published nor is under consideration for publication elsewhere, and its publication is approved by all authors. We hope the manuscript will be considered for publication in BBA and below append suggestions for appropriate reviewers.

Yours sincerely,

Sofia Morais

Suggested referees:

1. Dr. Xiaozhong Zheng
Centre for Inflammation Research, C2.18
Queen's Medical Research Institute
The University of Edinburgh
47 Little France Crescent
Edinburgh EH16 4SA
United Kingdom
Tel: 0131 2426593, Fax: 0131 2426578
email: xzheng@staffmail.ed.ac.uk

Dr Zheng has done considerable work involving cloning and functional characterization of Fads and Elovls in fish and is therefore a very specific expert in this field.

2. Professor Johnathan A. Napier,
Rothamsted Research,
Harpenden,
Herts AL5 2JQ, UK.
Tel: +441582763133
Fax: +441582780981
Email: jon.napier@bbsrc.ac.uk

Prof. Napier is an expert in Fad molecular biology and has considerable expertise in pathways of DHA biosynthesis.

3. Dr. Chantal Cahu
Unité Mixte de Nutrition des poissons IFREMER-INRA B.P. 70
29280 Plouzané
France
Tel: +33 02 98 22 44 03, Fax: +33 02 98 22 46 53
E-mail: Chantal.Cahu@ifremer.fr

4. Dr. Gabriel Mourente
Departmento de Biologia
Facultad de Ciencias del Mar
Universidad de Cadiz
Rio San Pedro
E-11510, Puerto real (Cadiz)
Spain
Tel: +34 956016013, Fax: +34 956016019
E-mail: gabriel.mourente@uca.es

5. Dr. Manuel Yúfera
Instituto de Ciencias Marinas de Andalucia - Consejo Superior de Investigaciones Cientificas
Apartado Oficial
11519 Puerto Real
Spain
Tel. +34 956 832612 ext 34, Fax +34 956 834701
manuel.yufera@icman.csic.es

Referees #3, 4 and 5 are all experts in marine larvae nutrition including fatty acid metabolism. Dr Manuel Yúfera and Dr. Gabriel Mourente additionally have performed several larval nutrition studies in *S. senegalensis.* Dr. Chantal Cahu has also been involved in molecular and genomic studies, including the cloning and Fad expression in fish and Dr. Gabriel Mourente has also worked specifically with enzymes of the pathway of LC-PUFA biosynthesis.

Highlights:

> We cloned and functionally characterized a fatty acyl desaturase and elongase in sole. > Fad has Δ4-activity and Elovl has substrate specificity typical of fish Elovl5. > Sole appears to be able to synthesize DHA from EPA via a Sprecher-independent pathway. > Δ4fad and elovl5 showed a marked pattern of regulation during early ontogenesis. > Δ4fad shows nutritional regulation, being up-regulated in response to LC-PUFA-poor diets.

*Manuscript
Click here to view linked References

Long chain polyunsaturated fatty acid synthesis in a marine vertebrate: ontogenetic and nutritional regulation of a fatty acyl desaturase with Δ4 activity

Sofia Morais[a,*], Filipa Castanheira[b], Laura Martinez-Rubio[c], Luis E.C. Conceição[b], Douglas R. Tocher[c]

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

[b] Centro de Ciências do Mar do Algarve, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

[c] Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK

Email addresses: sofia.morais@irta.cat (S. Morais), mcastanheira@ualg.pt (F. Castanheira), laura.martinez@stir.ac.uk (L. Martínez-Rubio), lconcei@ualg.pt (L.E.C. Conceição), d.r.tocher@stir.ac.uk (D.R. Tocher)

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

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

Abbreviations: ALA: α-linolenic acid; ARA: arachidonic acid; DAH: days after hatching; DHA: docosahexaenoic acid; DPA: docosapentaenoic acid; EFA: essential fatty acid; EPA: eicosapentaenoic acid; FA: fatty acid; FAME: Fatty acid methyl esters; LC-PUFA: long-chain polyunsaturated fatty acids; LOA: linoleic acid; ORF: open reading frame; PCR: polymerase chain reaction; RACE: rapid amplification of cDNA ends.

Abstract

Solea senegalensis is an unusual marine teleost as it has very low dietary requirement for long-chain polyunsaturated fatty acids (LC-PUFA) during early development. Aquaculture is rapidly becoming the main source of health-beneficial fish products for human consumption. This, associated with limited supply of LC-PUFA-rich ingredients for fish feeds, render *S. senegalensis* a highly interesting species in which to study the LC-PUFA biosynthesis pathway. We have cloned and functionally characterized fatty acyl desaturase and elongase cDNAs corresponding to *Δ4fad* (with some Δ5 activity for the n-3 series) and *elovl5* with the potential to catalyze docosahexaenoic acid (DHA) biosynthesis from eicosapentaenoic acid (EPA). Changes in expression of both transcripts were determined during embryonic and early larval development, and transcriptional regulation in response to higher or lower dietary n-3 LC-PUFA was assessed during larval and post-larval stages. There was a marked pattern of regulation during early ontogenesis, with both transcripts showing peak expression coinciding with the start of exogenous feeding. Although *elovl5* transcripts were present in fertilized eggs, *Δ4fad* only appeared at hatching. However, eggs have high proportions of DHA (~ 20%) and high DHA/EPA ratio (~ 11) to meet the high demands for early embryonic development. The fatty acid profile of larvae after the start of exogenous feeding closely reflected dietary composition. Nonetheless, *Δ4fad* was significantly up-regulated in response to LC-PUFA-poor diets, which may suggest biological relevance of this pathway in reducing LC-PUFA dietary requirements in this species, compared to other marine teleosts. These results indicate that sole is capable of synthesizing DHA from EPA through a Sprecher-independent pathway.

Keywords: Δ4 desaturase; polyunsaturated fatty acid synthesis; ontogenesis; transcriptional regulation; *Solea senegalensis*; marine teleost

1. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFA) are essential nutrients in nutrition and health. They are major components of biological membranes, particularly of immune cells and neural tissue, being vital for visual and cognitive development during early ontogeny, important in normal growth and development, as well as for tissue repair during injury [1-4]. The LC-PUFA are implicated in the regulation of a multitude of metabolic and immune pathways through their roles as secondary messengers and transcription factor ligands, and are potent bioactive molecules and precursors of eicosanoids with pro- or anti-inflammatory properties, hence intervening in a number of prevalent inflammatory disorders, including cardiovascular diseases, cancer, asthma, and several mental disorders [5-7].

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 diet. However, within vertebrates, the extent to which a species can produce LC-PUFA such as arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) from C18 PUFA precursors varies greatly, depending on their repertoire of fatty acyl elongase (Elovl) and desaturase (Fad) enzymes. In teleosts a principle paradigm has been that species diverge according to the environment and/or trophic level they occupy, with essential fatty acid (EFA) requirements being satisfied by C18 PUFA in freshwater/diadromous species, whereas marine fish have a strict requirement for LC-PUFA [8]. Accordingly, in all freshwater and marine fish species

studied so far a cDNA has been isolated for Δ6Fad, required for the initial desaturation of LOA and ALA [9,10], but unifunctional or bifunctional Fad enzymes possessing the Δ5 activity necessary to desaturate 20:3n-6 and 20:4n-3 to ARA and EPA, respectively, have only been found in the diadromous/freshwater species Atlantic salmon and zebrafish [11,12]. Thus, the inability of marine fishes to produce LC-PUFA has been explained by the loss of a gene encoding Δ5 activity, as an evolutionary adaptation to a DHA-rich marine ecosystem [8]. This paradigm was recently revised with the discovery of the first vertebrate Fad enzyme showing Δ4 activity and a bifunctional Δ6/Δ5 Fad, in a herbivorous marine fish species, *Siganus canaliculatus* [13]. This study also revealed the possibility, in some vertebrate species at least, for another pathway for synthesis of DHA from EPA involving direct Δ4 desaturation of docosapentaenoic acid (DPA; 22:5n-3), as an alternative to the "Sprecher" pathway that involves two sequential elongations of EPA to 24:5n-3 followed by Δ6 desaturation and one round of peroxisomal β-oxidation [14].

Fish are highly nutritious components of the human diet and the main source of essential n-3 LC-PUFA. With the decrease in wild fisheries worldwide, aquaculture is supplying an increasing proportion of fish for human consumption, estimated at around 50% of total supply in 2008 [15]. However, the health-beneficial fatty acid (FA) profile of farmed fish is achieved by the use of dietary fish oil and fishmeal, derived paradoxically from marine commercial fisheries, which is an unsustainable practice. Therefore, over the last few years, intensive research has been conducted to characterize LC-PUFA biosynthesis in species of aquaculture interest, including how this pathway might be modulated by changes in diet formulation [16]. Another complementary strategy to improve aquaculture sustainability is to identify fish species with lower

dependence on dietary n-3 LC-PUFA and that could thrive on diets containing higher levels of vegetable ingredients.

Senegalese sole (*Solea senegalensis* Kaup, 1858) has generated great interest in Southern European aquaculture for two decades, based on the need to diversify production centered on two main species, gilthead seabream (*Sparus aurata* Linnaeus, 1758) and European seabass (*Dicentrarchus labrax* Linnaeus, 1758), along with its high market value, high growth rate and ease of larval culture [17,18]. The replacement of fish oil by vegetable oil in on-growing feeds has not been investigated, possibly as this species does not perform well on diets with high lipid contents [19]. In contrast, replacement of fishmeal by plant-protein has been accomplished without loss in fish performance [20]. However, the most striking aspect in this species is the apparently low requirements for LC-PUFA in larval diets, which is highly unusual for a marine teleost. Marine fish larvae have a strict dietary requirement for high LC-PUFA and hence live prey commonly used in aquaculture to feed early larval stages requires enrichment with LC-PUFA, particularly DHA [21]. However, in contrast, Senegalese sole larvae can be grown on diets containing negligible DHA and low EPA levels, such as non-enriched live prey, without obvious detrimental effects [22,23].

Hence, the nutritional particularities of *S. senegalensis* make it an especially interesting model amongst marine teleosts to study LC-PUFA biosynthesis and its developmental and nutritional regulation. This was the aim of the present study, where cDNAs for two enzymes of the LC-PUFA biosynthesis pathway were cloned and shown to encode a Δ4Fad and Elovl5 with the potential to catalyze DHA biosynthesis from EPA. Expression of the respective transcripts, *Δ4fad* and *elovl5*, were determined during early embryonic and larval development, and their transcriptional regulation in response to dietary n-3 LC-PUFA (EPA and DHA) content was assessed. Results are

discussed in relation to the apparently lower n-3 LC-PUFA larval requirements of this species, compared to other marine fish species.

2. Materials and methods

2.1. Cloning, sequence and phylogenetic analysis

Sequences corresponding to the open reading frame (ORF) of fatty acyl desaturases (*fads*) and elongases (*elovls*) from several fish species were aligned and primers designed on conserved regions, as described in Morais et al. [24]. Sense and antisense primers used were 5'-CCTGGGAGGAGGTGCAGA-3' and 5'-TCCGCTGAACCAGTCGTTGA-3', respectively, for *fad*, and 5'-GCTCTACAATCTGGGCCTC-3' and 5'- CCACCAAAGATACGGCCG-3' for *elovl.* Fragments were obtained by polymerase chain reaction (PCR) with GoTaq® Colorless Master Mix (Promega, Southampton, U.K.) following manufacturer's instructions (with 35 cycles; 55 ºC annealing and 1 min extension) on cDNA synthesized (as described below) from 2 μg of total RNA from liver tissue of a Senegalese sole juvenile. After sequencing (CEQ-8800 Beckman Coulter Inc., Fullerton, U.S.A.), the *elovl* ORF fragment was further extended by 3' rapid amplification of cDNA ends (RACE) PCR (FirstChoice® RLM-RACE kit, Ambion, Applied Biosystems, Warrington, U.K.) using sole-specific sense primers located in the ORF (5'-GCGCCTGAAGTGGATAAGAA-3' and 5'-AGCATGCTGAACATCTGGTG-3') for nested PCR with the kit's antisense primers, to obtain the sequence of the 3' untranslated region (UTR). Additionally, the start of the ORF was obtained by PCR with sense primer 5'-AAGGTGACAAATGGAAACATTCA-3' (containing the sequence from the ATG start of the closely related flatfish *Psetta maxima*) and antisense primer 5'-

TGAGGCCCAGATTGTAGAGC-3'. In the case of *fad*, however, blasting of the fragment obtained initially by PCR with conserved ORF region primers in the *Solea senegalensis* Pleurogene EST database retrieved a clone (sequence kindly provided by J. Cerdá, IRTA, Spain) presenting the whole ORF as well as 127 bp and 456 bp of 5' and 3'UTR, respectively. The Senegalese sole sequences corresponding to the putative *fad* and *elovl* are available in the GenBank database under accession numbers JN673546 and JN793448, respectively.

The deduced amino acid (aa) sequence of the putative *fad* was aligned with that of *Salmo salar Δ5fad* and *Δ6fad* (GenBank ID: AAL82631 and AAR21624, respectively), *Psetta maxima Δ6fad* (AAS49163), *Siganus canaliculatus Δ6Δ5fad* and *Δ4fad* (ABR12315 and ADJ29913, respectively) and *Danio rerio Δ5Δ6fad* (AAG25710) using ClustalW2. Equally, the aa sequence of the putative *elovl* was aligned with that of other fish *elovl5*'s: *P. maxima* (AAL69984), *S. canaliculatus* (ADE34561) *S. salar* (*elovl5a*; NP_001117039 and *elovl5b*; ACI62499), and *D. rerio* (NP_956747). To compare sequences two by two, the EMBOSS Pairwise Alignment Algorithms tool (http://www.ebi.ac.uk/Tools/emboss/align/) was used. In addition, the deduced aa sequences of multiple Fad proteins from fish and other organisms were aligned using ClustalX and sequence phylogenies were reconstructed using the Neighbor Joining method [25] in MEGA version 5 [26]. Confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping through 1000 iterations.

2.2. Functional characterization by heterologous expression of ORFs

PCR fragments corresponding to the ORFs of the Senegalese sole putative Fad and Elovl were amplified from liver cDNA using sense and antisense primers containing a digestion site (underlined) - FadVF1 (CCCGAGCTCAGGATGAGAAACGGAGGT;

*Sac*I) and FadVR1 (CCGCTCGAGTCATTTATGGAGATATGCAT; *Xho*I); and ElovlVF1 (CCCGAGCTCAAAATGGAAACATTCAATCATAAACTGA; *Sac*I) and ElovlVR1 (CCGCTCGAGTTAGTTTCTTGTGTGCACTGTGC; *Xho*I). To obtain the Elovl ORF a nested PCR was performed with sense and antisense primers for the first-round PCR being 5'-AAGGTGACAAATGGAAACATTCA-3' and 5'-GGCTTATTTAATCTCAAGTCAACG-3', respectively. PCR was performed using the high fidelity PfuTurbo DNA Polymerase (Stratagene, Agilent Technologies, Cheshire, U.K.), following the manufacturer's protocol with 32 cycles, annealing at 60 ºC (or 58 ºC for Elovl first-round PCR) and 1 min extension. The DNA fragments were then purified (Illustra GFX™ PCR DNA and gel band purification kit, GE Healthcare Life Sciences, Buckinghamshire, U.K.), digested with the corresponding restriction endonucleases (New England BioLabs, Herts, U.K.) and ligated into a similarly restricted pYES2 yeast expression vector (Invitrogen, Paisley, U.K.). Ligation products were used to transform Top10F' *Escherichia coli* competent cells (Invitrogen) that were screened for the presence of recombinants. The purified plasmid constructs containing the ORFs (confirmed by sequencing) were then used to transform *Saccharomyces cerevisiae* competent cells (S.c. EasyComp Transformation Kit, Invitrogen). Transformation and selection of yeast with recombinant Fad and Elovl ORF-pYES2 plasmids, yeast culture and FA analysis was performed as described in detail previously [10,11]. Briefly, cultures of recombinant yeast were grown in *S. cerevisiae* minimal medium$^{-uracil}$ supplemented with one of the following FA substrates (>98-99% pure) sourced from either Cayman Chemical Co. (Ann Arbor, U.S.A.) or Sigma Chemical Co. Ltd. (Dorset, U.K.): 18:3n-3, 18:2n-6, 20:4n-3, 20:3n-6, 22:5n-3 or 22:4n-6 for Fad; 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3 or 22:4n-6 for Elovl. FAs were added to the yeast cultures at final concentrations of 0.5 (C18), 0.75 (C20) and 2.0 (C22) mM. After

2-days, yeast were harvested, washed, and lipid extracted. Fatty acid methyl esters (FAME) were analyzed by GC and the proportion of substrate FA converted to desaturated or elongated FA product was calculated as [product area/(product area +substrate area)] x 100. The identities of FA peaks were based on GC retention time and confirmed by GC-MS as described previously [13].

2.3. Larval ontogenetic development and nutritional experiments

Senegalese sole eggs were obtained from naturally spawning captive broodstock at INRB/IPIMAR EPPO (Olhão, Portugal) and reared at the facilities of the Centre of Marine Sciences (University of Algarve, Faro, Portugal). Details of the standard culture protocols for this species, including feeding regimes and rearing conditions, followed in these experiments can be found in Conceição et al. [27] and Engrola et al. [28]. Briefly, rearing includes a pelagic larval phase until around 19 days after hatching (DAH), when larvae metamorphose and acquire a benthic lifestyle. During the pelagic phase larvae were reared in a semi-closed recirculation system with 100 L cylindro-conical tanks at 80 larvae L^{-1}, temperature of 20.6 ± 0.9 ºC (mean ± standard deviation, SD), salinity of 35.2 and light/dark cycle of 14:10-h. From the start of exogenous feeding (2 DAH) until 4 DAH, larvae were fed rotifers (*Brachionus plicatilis*) enriched with Easy DHA Selco (INVE Aquaculture NV, Dendermonde, Belgium), followed by *Artemia* AF nauplii (INVE Aquaculture NV) until 9 DAH and then freshly enriched *Artemia* EG metanauplii (INVE Aquaculture NV), fed two times daily. The benthic rearing phase, from 19 DAH onwards, was performed in a closed recirculation system with rectangular tanks (44 x 70 cm), stocked with 300 settled post-larvae each, at a temperature of 19.6 ± 1.2 ºC, salinity of 36.8 and 12:12-h light/dark cycle. At this stage, post-larvae were fed frozen *Artemia* EG metanauplii four times daily.

In order to study changes in FA profile and in *fad* and *elovl* expression during early ontogeny, samples of eggs, newly hatched larvae (0 DAH) and larvae up to 6 DAH were collected daily. Three pools of eggs and larvae were sampled for FA (minimum 20 mg per pool) and molecular (100 mg per pool) analyses. Samples were immediately frozen in liquid nitrogen and kept at -20 ºC (FA) or -80 ºC (molecular) pending analysis.

In addition, a nutritional experiment was performed testing four different dietary regimes. During the pelagic stage, larvae were reared in 6 replicate tanks per dietary treatment consisting of either *Artemia* EG metanauplii freshly enriched (E) with a mixture of Easy DHA Selco and Microfeed (EWOS, Bathgate, Scotland) added to the enrichment tanks it two doses according to manufacturer's instructions; or non-enriched *Artemia* (NE) kept in the same conditions but without adding the enrichment product. From 19 to 40 DAH, post-larvae were reared in triplicate tanks and either continued on E or NE or their diet was swapped, giving rise to 2 further dietary treatments: E-NE and NE-E. Samples of the *Artemia* (E and NE) used to feed the larvae were taken for FA analysis. In addition, larvae at 19 DAH and post-larvae at 40 DAH were collected for FA (pool of 30 larvae per tank) and molecular (approx. 200 mg pool per tank) analysis, as indicated for the ontogenetic trial. Twenty fish per tank were also collected, freeze-dried and dry weight (DW) was measured. Survival was determined at the end of the larval and post-larval stages (19 and 40 DAH) by direct counting of individuals, relative to the initial number stocked, and excluding the individuals sampled. 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.

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

Total RNA was isolated from 3 pools of whole eggs, larvae at various stages of development (0-6 and 19 DAH) and post-larvae at 40 DAH by Ultra-Turrax homogenization (Fisher Scientific, Loughborough, U.K.) followed by organic solvent extraction, according to manufacturer's instructions (TRI Reagent; Ambion, Applied Biosystems, Warrington, U.K.). RNA quality and quantity was assessed by gel electrophoresis and spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, U.S.A.), respectively. Two µg of total RNA per sample pool was reverse transcribed into cDNA using the High-Capacity cDNA RT kit (Applied Biosystems, Paisley, U.K.), 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, Eurofins MWG Operon, Ebersberg, Germany). 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 then diluted 60-fold with water.

Primers for *fad* and *elovl* transcripts were designed using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Primer details can be found in Table 1. Quantification of the expression of these genes, as well as of three reference genes (ubiquitin - *ubq*; 40S ribosomal protein S4 - *rps4*; and elongation factor 1 alpha – *ef1a*), previously tested for Senegalese sole larval studies [29], was performed by real time quantitative PCR (qPCR) analysis using relative quantification. 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. Only *ef1a* showed a relatively stable expression across eggs and early larval stages while in the nutritional regulation experiment at later larval and post-larval stages, the most stable reference gene combination was achieved with *ubq* and *rps4* (geNorm M value = 0.305; [30]). Amplifications were carried out in duplicate (Quantica, Techne,

Cambridge, U.K.) in a final volume of 20 µl containing 5 µl diluted (1/60) cDNA, 0.5 µM of each primer and 10 µl AbsoluteTM QPCR SYBR® Green mix (ABgene) and included a systematic negative control (NTC-non template control). The qPCR profiles contained an initial activation step at 95 °C for 15 min, followed by 30 to 35 cycles: 15 s at 95 °C, 15 s at the specific primer pair annealing temperature (Ta; Table 1) and 15 s at 72 °C. After the amplification phase, a melt curve of 0.5 °C increments from 75 ºC to 90 °C 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 verified. Product sizes and presence of single bands were checked by agarose gel electrophoresis. Additionally, identities of *fad* and *elovl* amplicons were verified by sequencing.

Gene expression results were expressed as mean normalized values (±SD) corresponding to the ratio between copy numbers of *fad* and *elovl* transcripts and copy numbers of the reference gene *ef1a* or a normalization factor determined for the average expression of *ubq* and *rps4* using geNorm [30].

2.5. Fatty acid analysis

Total lipids were extracted and determined gravimetrically from 3 pools of eggs, larvae and post-larvae, as well as from diets (E and NE *Artemia*) used in the nutritional experiment (2 g wet weight of eggs and 0.2-1 g of remaining samples) by Ultra Turrax homogenization in 10-20 ml of chloroform/methanol (2:1 v/v) [31]. The FAME were prepared by acid-catalyzed transesterification of total lipids [32]. Following purification, FAME were separated and quantified by GC using a Thermo Fisher Trace GC 2000 (Thermo Fisher, Hemel Hempstead, UK) equipped with a fused silica capillary column (ZB wax, 30 m × 0.32 mm i.d.; Phenomenex, Macclesfield, UK) with hydrogen as carrier gas and using on-column injection. The temperature gradient was

from 50 to 150 °C at 40 °C/min and then to 195 °C at 1.5 °C/min and finally to 220 °C at 2 °C/min. Individual methyl esters were identified by comparison with known standards. Data were collected and processed using the Chromcard for Windows (version 2.00) computer package (Thermoquest Italia S.p.A., Milan, Italy).

2.6. Statistical analysis

Gene expression results, DW and arcsin-transformed survival and FA composition percentage data were checked for homogeneity of variances using a Levene's test and then analyzed by one-way analysis of variance (ANOVA) followed by a Tukey HSD post-hoc test or, in the case of the nutritional experiment at 19 DAH, by an independent samples t-test, at a significance level of $P<0.05$, using the statistical software SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Sequence and phylogenetic analysis of Solea senegalensis fad and elovl

The sequences characterized in the present paper, corresponding to a *Solea senegalensis fad* and *elovl* cDNA, contain 1338 bp (GenBank ID: JN673546) and 867 bp (JN793448) ORFs, respectively, which encode putative proteins with 445 aa and 288 aa. The deduced Fad protein contains characteristic features of microsomal fatty acyl desaturases, including three histidine boxes, two transmembrane regions, and N-terminal cytochrome b5 domains containing the heme-binding motif, HPGG (Fig. 1). The polypeptide sequence is 78 % identical to *Psetta maxima* Δ6Fad (AAS49163) and *Siganus canaliculatus* Δ4Fad (ADJ29913), 77 % identical to *S. canaliculatus* Δ6Δ5Fad (ABR12315) and presents slightly lower identity (72-73 %) with *Salmo salar* Δ6Fad

(AAR21624) and Δ5Fad (AAL82631) and 67 % with *Danio rerio* Δ5Δ6Fad (AAG25710). Phylogenetic analysis of the newly described Fad shows that it clusters with *S. canaliculatus* Δ4Fad and Δ6Δ5Fad, which are most closely related to marine teleost Δ6Fads and more distantly from lower eukaryotes Fads, including other Δ4Fads (Fig. 2). The deduced Elovl protein also presented characteristic structural features, namely, the diagnostic histidine box HXXHH motif and two lysine or arginine residuals at the carboxyl terminus, KXRXX, which are proposed to function as endoplasmic reticulum retrieval signals. However, the putative *S. senegalensis* Fad is shorter than other fish Elovl5's, missing 6 aa's between the predicted V putative membrane-spanning domain and the carboxyl terminus (Fig. 3). This is however an area of higher variation amongst Elovl's and hence it probably does not affect activity or substrate specificity. The polypeptide sequence is 83-84 % identical to Elovl5's of other marine carnivorous species, such as *P. maxima* and *Sparus aurata* (AAT81404), and has slightly lower identity to those of the marine herbivorous *S. canaliculatus* (78 %) and of freshwater species such as *S. salar* (78 %) and *D. rerio* (74 %). Comparison with polypeptide sequences from Elovl2's revealed relatively low identities: 57% with *S. salar* Elovl2 (FJ237532) and 54 % with *D. rerio* Elovl2 (NP_001035452).

3.2. Functional characterization

The fatty acid composition of untransformed yeast *S.cerevisiae* has four main fatty acids, namely 16:0, 16:1n−7, 18:0 and 18:1n−9, numbered 1-4 in Figs. 4 and 5, consistent with the well established lack of PUFA elongase and desaturase activity in *S. cerevisiae* [11]. When yeast, transformed with the *S. senegalensis* Fad cDNA insert, was grown in the presence of substrates for Δ6Fad (18:3n-3 and 18:2n-6), Δ5Fad (20:4n-3 and 20:3n-6) and Δ4Fad (22:5n-3 and 22:4n-6), additional peaks were

observed in the GC traces corresponding to the exogenously added FAs but no peaks were observed for desaturated products of Δ6Fad (Fig. 4A). However, an additional small peak (corresponding to 2 % conversion) was observed for 20:5n-3, the Δ5-desaturated product of 20:4n-3 (Fig. 4B; Table 2), and another peak (corresponding to 16 % conversion) was seen for 22:6n-3, resulting from Δ4-desaturation of exogenously added 22:5n-3 (Fig. 4C; Table 2). Parallel incubation of yeast with equivalent n-6 PUFA substrates only showed relevant activity for Δ4Fad (around 7 %) (Table 2). In addition, yeast was also transformed with sole Elovl cDNA insert and grown in the presence of different FA substrates and the GC traces show additional peaks for the exogenously added C18, C20 and C22 substrates, as well as for their respective products, indicating that all substrates were elongated, with order of activity being C20 > C18 > C22 (Fig. 5). In this heterologous expression system, sole Elovl showed similar activities towards C18 (32-37 %) and C22 (*circa* 4 %) of both n-3 and n-6 series but for C20 substrates a higher activity was measured for the n-3 substrate (79 % compared to 57 % for the n-6 substrate) (Table 2). Finally, the GC traces also showed a small peak for 18:1n−7 (peak 5; Fig. 5) indicating elongation of endogenous 16:1n-7.

3.3. Changes in FA profile and fad and elovl expression during ontogenesis

The FA profile and expression of *Δ4fad* and *elovl5* were determined in *S. senegalensis* eggs, newly hatched larvae and in larvae up to 6 DAH, in order to examine changes during early ontogeny. In terms of gene expression, sole eggs only presented transcripts in relevant amounts for *elovl5*, as expression of *Δ4fad* was negligible (Fig. 6). However, at hatching (0 DAH) there was a significant increase in *Δ4fad* transcript level showing a peak at 1-2 DAH followed by a return to the same level as that observed at hatching, from 3 to 6 DAH. In contrast, there was no change in expression

of *elovl5* from eggs to 1 DAH but then a significant peak in transcript levels occurred at 2-3 DAH, returning afterwards to previous levels of expression.

The eggs and early larval stages of Senegalese sole were characterized by high proportions of DHA, around 20 % of total FA, accompanied by EPA of under 2 %, which resulted in DHA/EPA ratios of 11 in eggs and newly hatched larvae (Table 3). There were also relatively high levels of DPA at over 2 %. DHA was one of the few FAs whose level did not change significantly during ontogenetic development, although there was a trend for a slight increase at 3 DAH. In contrast, EPA content significantly increased at 4 DAH and remained constant afterwards, which was reflected in the DHA/EPA ratio. Levels of DPA showed the opposite pattern to EPA. Another abundant n-3 PUFA in eggs was ALA, which showed a gradual reduction during ontogenetic development. For n-6 PUFA, LOA showed a gradual decrease after 2 DAH, while for ARA there was a gradual increase, which became more pronounced from 2 DAH onwards. There was a slight increase in the percentage of saturated FAs during ontogenetic development, a significant decrease in total monounsaturated FAs from 2 DAH onwards, and total PUFA broadly showed a peak at 3 DAH and decreased significantly by 5 DAH.

3.4. Nutritional regulation

Effects of n-3 PUFA supply during different windows of development (larvae *versus* post-larvae) were investigated using diets that contained comparable levels of n-3 PUFA but supplied primarily as C18 PUFA, particularly ALA (diet NE) or LC-PUFA, EPA and DHA (diet E) (Table 4). As a consequence, larvae presented significantly different compositions for most FAs (including EPA and DHA) at 19 DAH, that reflected diet composition. Surprisingly, differences were not significant for ALA,

although the trend was for a higher level in larvae fed NE. When larvae continued to be fed the same diets from 19 to 40 DAH, or their diets were switched, clear dietary influences were observed, with post-larvae fed the E diet at this stage presenting significantly higher levels of total PUFA and n-3 PUFA, including EPA, DPA and DHA, independent of previous dietary regime (i.e., no differences between E and NE-E). Similarly, all larvae fed NE diet during the post-larval stage (NE and E-NE) presented a similar FA profile (Table 4).

No differences were observed in either larvae or post-larvae survival (Table 5). However, the different dietary regimes induced significant differences in growth, assessed by DW, in both stages. At 19 DAH, larvae fed the E diet were heavier than those fed NE. However, when these larvae continued being fed the E diet during the post-larval stage their final weights were lower than post-larvae fed the E diet during only one of the developmental stages, E-NE or NE-E treatments. Larvae fed the NE diet throughout showed an intermediate weight not significantly different from any other treatment.

Clear differences were observed in the expression of *Δ4fad* but not of *elovl5*, with *Δ4fad* expression up-regulated at 19 and 40 DAH in fish fed diet NE (Fig. 6). Furthermore, at 40 DAH, levels of expression were similar in post-larvae being fed the same diet during the post-larval stage, independent of diet during the larval stage, i.e., higher expression in NE and E-NE and lower in E and NE-E.

4. Discussion

This paper presents novel findings concerning the LC-PUFA biosynthesis pathway in a marine carnivorous teleost, including a possible route for DHA synthesis from EPA via a Δ4Fad, and developmental and nutritional regulation of this transcript. The

simplest and most direct pathway for biosynthesis of DHA from EPA would contain a Δ4Fad. However, for many years the presence of Δ4Fad enzymes could not be established in vertebrates and in the 1990's work of Sprecher and co-workers revealed a Δ4-independent pathway of DHA synthesis [14,33]. However, a Fad with Δ4 activity was finally identified in *Thraustochytrium* [34], and subsequently other Δ4Fads were characterized in lower eukaryotes including the microalgae *Pavlova lutheri*, *Isochrysis* and *Thalassiosira pseudonana* [35-37], the protist *Euglena gracilis* [38] and the parasitic protozoans *Leishmania major*, *Trypanosoma brucei* and *T. cruzi* [39]. Additionally, an alternative pathway for DHA synthesis involving an anaerobic polyketide synthase was identified in the marine protist *Schizochytrium*, another family member of the Thraustochytriidae [40]. Despite this, the Sprecher pathway remained the only demonstrated mechanism for DHA biosynthesis in mammals and higher vertebrates. However very recently, a Fad enzyme with Δ4 activity was reported in a vertebrate species, the marine herbivorous fish *S. canaliculatus*, suggesting that, for at least some vertebrates, DHA could be produced from EPA via elongation followed by direct Δ4 desaturation [13]. *S. canaliculatus* also displayed a Δ6/Δ5Fad that, combined with the Δ4Fad, revised the view of an evolutionary dichotomy between freshwater (DHA-poor) and marine (DHA-rich) environments in the ability of fish species to produce LC-PUFA from C18 PUFA and, alternatively, indicated that trophic level is likely more important [13].

The characterization of another Fad presenting Δ4 activity in the present study indicates that that production of DHA via Δ4-desaturation is likely more widespread among vertebrate species and also brings further elements to the evolutionary discussion, suggesting a higher adaptative plasticity than previously thought. Contrary to *S. canaliculatus*, which is a strict herbivore, *S. senegalensis* is a carnivorous species

occupying a higher trophic level. This flatfish possesses planktonic larvae feeding on zooplankton but after metamorphosis adopts a benthic lifestyle, inhabiting sandy or muddy bottoms of coastal or brackish areas feeding on benthonic invertebrates, mainly polychaetes, small crustacean (such as amphipods) and bivalve molluscs [41,42]. In captivity, the feeding regime of broodstock fish is based on squid, *Loligo vulgaris*, supplemented with polychaetes, *Nereis (Hediste) diversicolor*, during final maturation, since this has proven to stimulate gonad maturation and spawning [18]. Generally, benthic invertebrates have relatively low lipid contents compared to herbivorous zooplankton and the primary source of n-3 LC-PUFA for juvenile and adult sole is most likely EPA rather than DHA [43,44]. In particular, the polychaete (*N. diversicolor*) that forms an important part of Senegalese sole's diet is a rich source of 16:0, 18:1n-9, LOA and EPA (5-10 mg/g DW), with only residual amounts of DHA (0.5-1 mg/g DW) [45]. Other natural prey items of *S. senegalensis*, such as the amphipod *Corophium* spp. and the bivalve *Scrobicularia plana* [42], also contain mainly EPA (26 % or 15 % in the amphipod and bivalve, respectively), with DHA at around 7 % [46,47].

Desaturases and elongases are enzymes of wide interest, not just for biotechnological applications such as the production of health-beneficial EPA and DHA [36,37], but also in environmental genomics. The desaturases required for LC-PUFA biosynthesis are all from the same phylogenetic branch of desaturases (one of four) known as the "front-end desaturases" [48]. These desaturases, including Δ4, Δ5 and Δ6 Fads, are very similar in sequence, share the same structural motifs, and cannot be functionally clustered [49]. After an initial divergence of desaturases into the four subfamilies, it is believed that a second diversification led to a variety of substrate specificities or regioselectivities within each subfamily leading to an important variability in the capacity of even closely related organisms to synthesize a range of unsaturated FA in response to their specific

individual environment [48]. The present study shows that categories such as "marine/freshwater" or "trophic level" may be too restrictive to distinguish between species with respect to requirements for C18 PUFA or LC-PUFA as EFA and, even if trophic ecology appears to have an important influence, the precise factors driving evolutionary pressures remain a question of great interest.

We have also cloned and functionally characterized an *elovl* cDNA with substrate specificity indicating a typical teleost *elovl5*, with the protein being mostly active on C20 followed by C18, with only low activity on C22 FA. It was suggested that an Elovl2-like enzyme, with higher substrate specificity towards C22, is required for efficient DHA synthesis from EPA via the Sprecher pathway but a gene coding for this enzyme in fish has, to date, only been shown in Atlantic salmon and zebrafish [50,51]. The Elovl5 characterized in the present study converted 75 % of 20:5n-3 and 54 % of 20:4n-6 to 22:5n-3 and 22:4n-6, respectively, the substrates for Δ4Fad.

Therefore, the present study presents molecular and biochemical data that suggest Senegalese sole possess genetic and enzymatic capabilities for DHA synthesis from EPA. This, in itself, does not prove that the EPA to DHA pathway is active *in vivo* or indicate the level to which it may operate. In fact, the FA composition of both larvae and post-larvae reflected the dietary FA composition, irrespective of regulation of *Δ4fad* expression, which was highly responsive to dietary n-3 LC-PUFA content being up-regulated when sole where fed lower levels of these FAs, even if this could not raise DHA contents to levels approaching the E and NE-E treatments at 40 DAH. However, the E diet, possessing high levels of total lipid and enriched in both EPA and DHA, probably does not fully reflect the natural feeds of sole post-settlement (possibly with a higher EPA/DHA ratio), which may explain why continuously feeding the E diet to larvae and post-larvae led to reduced growth at 40 DAH, as had been reported

previously [22,23]. It could be speculated that dietary supply of DHA well in excess of requirements may result in higher oxidative stress associated with the peroxidative susceptibility of LC-PUFA [52].

A higher DHA requirement is likely during the pelagic larval stage, which is a critical period of extremely fast growth and extensive neural development (fish larvae are visual hunters and their head/eyes constitute the major fraction of body mass), culminating in metamorphosis, which in flatfish involves dramatic functional and morphologic changes associated with extensive organogenesis and tissue remodeling [1,8,53]. However, at this stage it is probable that at least part of the DHA requirement is satisfied in nature by DHA-rich zooplanktonic diet [8]. Reduced growth at 19 DAH in sole fed the diet with lower n-3 LC-PUFA could be explained by insufficient DHA synthesis capacity, despite up-regulation of *Δ4fad*, to meet the demand required to support membrane synthesis and the high growth rate at this stage.

It had been shown previously that sole eggs have an unusually high proportion of DHA resulting in a very high DHA/EPA ratio compared to other marine fish species, and this was suggested to be necessary to satisfy the high requirements for growth and neural tissue development during early embryonic development [22]. In the present study, fertilized eggs and early larval stages up to the onset of exogenous feeding presented a DHA content of around 20 % of total FA and a DHA/EPA ratio of about 11. In addition, the influence of diet on larval FA composition was observed reflecting the introduction at 2 DAH of rotifers enriched with DHA [54], and their replacement at 4 DAH by *Artemia* AF nauplii, which are rich in EPA and practically devoid of DHA [55]. This explains the increase in larval EPA levels from 4 DAH onwards. In contrast, levels of DHA were not significantly altered during early larval development, which might indicate some degree of regulation of its level associated with the increased

expression of *Δ4fad* at 1-2 DAH and of *elovl5* at 2-3 DAH, coinciding with the start of endogenous feeding. Hence, there is possibly a developmentally fixed pattern of *Δ4fad* and *elovl5* expression during this critical period of development to ensure the availability of DHA for neurogenesis independent of dietary supply. The relatively high content of DPA in sole eggs may support the supply of DHA via Δ4-desaturation during a short temporal window in the event first feeding larvae do not immediately encounter their natural zooplanktonic prey rich in EPA and/or DHA. Hence, the decrease in DPA at 4 and 5 DAH, followed by an increase at 6 DAH, might reflect endogenous DHA synthesis after the introduction of a live prey (*Artemia* AF) with low levels of DPA and DHA but rich in EPA. Finally, an interesting difference was observed in sole eggs (fertilized oocytes) with respect to the start of transcription of these genes, with *elovl5* transcripts, but not *Δ4fad*, being already present at considerable levels before hatching. A previous study demonstrated maternal transfer of *elovl5*, *elovl2* and *Δ5/Δ6fad* mRNA in zebrafish zygotes and a coordinated regulation of *elovl2* and *fad* with the start of LC-PUFA biosynthesis in the embryo [51]. However, in the present study the higher levels of *elovl5* in the egg may not be related with DHA synthesis to support embryogenesis due to the lack of synchrony in *elovl5* and *Δ4fad* transcription. This difference is not necessarily surprising as the strategy in sole appears to be rather maternal provision of high relative levels of DHA in the egg yolk to cover the initial high requirements for embryonic development.

In conclusion, the present study is the first report showing developmental and nutritional regulation of a *Δ4fad* in a vertebrate species, which has only been found in one other vertebrate, also a teleost, and confirms the existence of DHA synthesis from EPA in at least some vertebrates, through a Sprecher-independent pathway. The existence of regulation at the transcriptional level and evidence for unusually low, for a

marine fish species, dietary DHA requirements during early development and post-larval stages indicates a likely relevant physiological role for this gene but the overall activity of the EPA to DHA biosynthetic pathway "*in vivo*" still requires to be assessed.

Acknowledgements

This study was funded by project EFARFish - "A New Method for the Study of Essential Fatty Acid Requirements in Fish Larvae" (PTDC/MAR/67017/2006), granted by "Fundação para a Ciência e a Tecnologia" (FCT), Portugal, with the support of FEDER. We are deeply thankful to Dr. Joan Cerdá for making available to us the sequence of the *Δ4fad* cDNA before GenBank submission.

References

[1] M.V. Bell, R.S. Batty, J.R. Dick, K. Fretwell, J.C. Navarro, J.R. Sargent, Dietary deficiency of docosahexaenoic acid impairs vision at low light intensities in juvenile herring (*Clupea harengus* L.), Lipids 30 (1995) 443-449.

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

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

[4] B. Koletzko, E. Lien, C. Agostoni, H. Böhles, C. Campoy, I. Cetin, T. Decsi, J.W. Dudenhausen, C. Dupont, S. Forsyth, I. Hoesli, W. Holzgreve, A. Lapillonne, G. Putet, N.J. Secher, M. Symonds, H. Szajewska, P. Willatts, R. Uauy, World Association of Perinatal Medicine Dietary Guidelines Working Group, The roles of

long-chain polyunsaturated fatty acids in pregnancy, lactation and infancy: review of current knowledge and consensus recommendations, J. Perinat. Med. 36 (2008) 5-14.

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

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

[7] N.D. Riediger, R.A. Othman, M. Suh, M.H. Moghadasian, A systemic review of the roles of n-3 fatty acids in health and disease, J. Am. Diet. Assoc. 109 (2009) 668-679.

[8] D.R. Tocher, Fatty acid requirements in ontogeny of marine and freshwater fish, Aquacult. Res. 41 (2010) 717-732.

[9] X. Zheng, I. Seiliez, N. Hastings, D.R. Tocher, S. Panserat, C.A. Dickson, P. Bergot, A.J. Teale, Characterisation and comparison of fatty acyl Δ6 desaturase cDNAs from freshwater and marine teleost fish species, Comp. Biochem. Physiol. 139B (2004) 269-279.

[10] X. Zheng, D.R. Tocher, C.A. Dickson, J.R. Dick, J.G. Bell, A.J. Teale, Highly unsaturated fatty acid synthesis in vertebrates: new insights with the cloning and characterisation of a Δ6 desaturase of Atlantic salmon, Lipids 40 (2005) 13-24.

[11] N. Hastings, M. Agaba, D.R. Tocher, M.J. Leaver, J.R. Dick, J.R. Sargent, A.J. Teale, A vertebrate fatty acid desaturase with Δ5 and Δ6 activities, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 14304-14309.

[12] N. Hastings, M.K. Agaba, D.R. Tocher, X. Zheng, C.A. Dickson, J.R. Dick, A.J. Teale, Molecular cloning and functional characterization of fatty acyl desaturase and elongase cDNAs involved in the production of eicosapentaenoic and

docosahexaenoic acids from α-linolenic acid in Atlantic salmon (*Salmo salar*), Mar. Biotechnol. 6 (2005) 463-474.

[13] Y. Li, O. Monroig, L. Zhang, S. Wang, X. Zheng, J.R. Dick, C. You, D.R. Tocher, Vertebrate fatty acyl desaturase with Δ4 activity, Proc. Natl. Acad. Sci. U.S.A. 107 (2010) 16840-16845.

[14] A. Voss, M. Reinhart, S. Sankarappa, H. Sprecher, The metabolism of 7,10,13,16,19-docosapentaenoic acid to 4,7,10,13,16,19-docosahexaenoic acid in rat liver is independent of a 4-desaturase, J. Biol. Chem. 266 (1991) 19995-20000.

[15] FAO, The State of World Fisheries and Aquaculture 2008, Food and Agriculture Organization of the United Nations, Rome, 2009.

[16] B.E. Torstensen, D.R. Tocher, The Effects of fish oil replacement on lipid metabolism of fish, In: G.M. Turchini, W.-K. Ng, D.R. Tocher (Eds.), Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds, Taylor & Francis, CRC Press, Boca Raton, 2010, pp. 405-437.

[17] M.T. Dinis, Aspects of the potential of *Solea senegalensis* Kaup for aquaculture: larval rearing and weaning to artificial diets, Aquacult. Fish. Manage. 23 (1992) 515-520.

[18] M.T. Dinis, L. Ribeiro, F. Soares, C. Sarasquete, A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal, Aquaculture 176 (1999) 27-38.

[19] P. Borges, B. Oliveira, S. Casal, J. Dias, L. Conceição, L.M. Valente, Dietary lipid level affects growth performance and nutrient utilisation of Senegalese sole (*Solea senegalensis*) juveniles, Br. J. Nutr. 102 (2009) 1007-1014.

[20] J.M.G. Silva, M. Espe, L.E.C. Conceição, J. Dias, L.M.P. Valente, Senegalese sole juveniles (*Solea senegalensis* Kaup, 1858) grow equally well on diets devoid of fish

meal provided the dietary amino acids are balanced, Aquaculture 296 (2009) 309-317.

[21] J.R. Sargent, L.A. McEvoy, J.G. Bell, Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds, Aquaculture 155 (1997) 117-128.

[22] S. Morais, L. Narciso, E. Dores, P. Pousão-Ferreira, Lipid enrichment for Senegalese sole (*Solea senegalensis*) larvae: effect on larval growth, survival and fatty acid profile, Aquacult. Int. 12 (2004) 281-298.

[23] M. Villalta, A. Estévez, M.P. Bransden, J.G. Bell, The effect of graded concentrations of dietary DHA on growth, survival and tissue fatty acid profile of Senegal sole (*Solea senegalensis*) larvae during the *Artemia* feeding period, Aquaculture 249 (2005) 353-365.

[24] S. Morais, G. Mourente, A. Ortega, J.A. Tocher, D.R. Tocher, Expression of fatty acyl desaturase and elongase genes, and evolution of DHA:EPA ratio during development of unfed larvae of Atlantic bluefin tuna (*Thunnus thynnus* L.), Aquaculture 313 (2010) 129-139.

[25] N. Saitou, M. Nei, The neighbor-joining method. A new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (1987) 406-425.

[26] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods, Mol. Biol. Evol. (2011) doi: 10.1093/molbev/msr121.

[27] L.E.C. Conceição, L. Ribeiro, S. Engrola, C. Aragão, S. Morais, M. Lacuisse, F. Soares, M.T. Dinis, Nutritional physiology during development of Senegalese sole (*Solea senegalensis*), Aquaculture 268 (2007) 64-81.

[28] S. Engrola, L. Figueira, L.E.C. Conceição, P.J. Gavaia, L. Ribeiro, M.T. Dinis, Co-feeding in Senegalese sole larvae with inert diet from mouth opening promotes growth at weaning, Aquaculture 288 (2009) 264-272.

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

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

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

[32] W.W. Christie, Lipid analysis, 3rd Edition, The Oily Press, Bridgewater, UK, 2003.

[33] H. Sprecher, D.L. Luthria, B.S. Mohammed, S.P. Baykousheva, Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids, J. Lipid Res. 36 (1995) 2471-2477.

[34] X. Qiu, H. Hong, S.L. MacKenzie, Identification of a Delta 4 fatty acid desaturase from *Thraustochytrium* sp. involved in the biosynthesis of docosahexanoic acid by heterologous expression in *Saccharomyces cerevisiae* and *Brassica juncea*, J. Biol. Chem. 276 (2001) 31561-31566.

[35] T. Tonon, D. Harvey, T.R. Larson, I.A. Graham, Identification of a very long chain polyunsaturated fatty acid Delta4-desaturase from the microalga *Pavlova lutheri*, FEBS Lett. 553 (2003) 440-444.

[36] T. Tonon, O. Sayanova, L.V. Michaelson, R. Qing, D. Harvey, T.R. Larson, Y. Li, J.A. Napier, I.A. Graham, Fatty acid desaturases from the microalga *Thalassiosira pseudonana*, FEBS J. 272 (2005) 3401-3412.

[37] S.L. Pereira, A.E. Leonard, Y.S. Huang, L.T. Chuang, P. Mukerji, Identification of two novel microalgal enzymes involved in the conversion of the omega3-fatty acid, eicosapentaenoic acid, into docosahexaenoic acid, Biochem. J. 384 (2004) 357-366.

[38] A. Meyer, P. Cirpus, C. Ott, R. Schlecker, U. Zähringer, E. Heinz, Biosynthesis of docosahexaenoic acid in *Euglena gracilis*: biochemical and molecular evidence for the involvement of a Delta4-fatty acyl group desaturase, Biochemistry 42 (2003) 9779-9788.

[39] K.E. Tripodi, L.V. Buttigliero, S.G. Altabe, A.D. Uttaro, Functional characterization of front-end desaturases from trypanosomatids depicts the first polyunsaturated fatty acid biosynthetic pathway from a parasitic protozoan, FEBS J. 273 (2006) 271-280.

[40] J.G. Metz, P. Roessler, D. Facciotti, C. Levering, F. Dittrich, M. Lassner, R. Valentine, K. Lardizabal, F. Domergue, A. Yamada, K. Yazawa, V. Knauf, J. Browse, Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes, Science 293 (2001) 290-293.

[41] E. Garcia-Franquesa, A. Molinero, J. Valero, R. Flos Influence of sex, age and season on the feeding habits of the flatfish *Solea senegalensis*, Environ. Biol. Fish. 47 (1996) 289-298.

[42] H.N. Cabral, Comparative feeding ecology of sympatric *Solea solea* and *S. senegalensis*, within the nursery areas of the Tagus estuary, Portugal, J. Fish Biol. 57 (2000) 1550-1562.

[43] M. Graeve, G. Kattner, D. Piepenburg, Lipids in Arctic benthos: does the fatty acid and alcohol composition reflect feeding and trophic interactions?, Polar Biol 18 (1997) 53-61.

[44] V.I. Kharlamenko, S.I. Kiyashko, S.A. Rodkina, A.B. Imbs, Determination of food sources of marine invertebrates from a subtidal sand community using analyses of fatty acids and stable isotopes, Russ. J. Mar. Biol. 34 (2008) 101-109.

[45] O.J. Luis, A.M. Passos, Seasonal changes in lipid content and composition of the polychaete *Nereis (Hediste) diversicolor*, Comp. Biochem. Physiol. 111B (1995) 579-586.

[46] R.G. Ackman, D.M. Nash, Lipids and fatty acids of *Corophium volutator* from Minas basin, Proc. N.S. Inst. Sci. 29 (1979) 501-516.

[47] S.A. Bradshaw, S.C.M. O'Hara, E.D.S. Corner, G. Eglinton, Effects on dietary lipids of the marine bivalve *Scrobicularia plana* feeding in different modes, J. Mar. Biol. Ass. U.K. 71 (1991) 635-653.

[48] K. Hashimoto, A.C. Yoshizawa, S. Okuda, K. Kuma, S. Goto, M. Kanehisa, The repertoire of desaturases and elongases reveals fatty acid variations in 56 eukaryotic genomes, J. Lipid Res. 49 (2008) 183-191.

[49] K. Hashimoto, A.C. Yoshizawa, K. Saito, T. Yamada, M. Kanehisa, The repertoire of desaturases for unsaturated fatty acid synthesis in 397 genomes, Genome Inform. 17 (2006) 173-183.

[50] S. Morais, O. Monroig, X. Zheng, M.J. Leaver, D.R. Tocher, Highly unsaturated fatty acid synthesis in Atlantic salmon: characterization of ELOVL5- and ELOVL2-like elongases, Mar. Biotechnol. 1 (2009) 627-639.

[51] O. Monroig, J. Rotllant, E. Sánchez, J.M. Cerdá-Reverter, D.R. Tocher, Expression of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis genes during

zebrafish *Danio rerio* early embryogenesis, Biochim. Biophys. Acta 1791 (2009) 1093-1101.

[52] M. Takahashi, N. Tsuboyama-Kasaoka, T. Nakatani, M. Ishii, S. Tsutsumi, H. Aburatani, O. Ezaki, Fish oil feeding alters liver gene expressions to defend against PPARα activation and ROS production, Am. J. Physiol. Gastrointest. Liver Physiol. 282 (2002) G338-348.

[53] C. Fernández-Díaz, M. Yúfera, J.P. Cañavate, F.J. Moyano, F.J. Alarcón, M. Díaz, Growth and physiological changes during metamorphosis of Senegal sole reared in the laboratory, J. Fish Biol. 58 (2001) 1-13.

[54] M. Naz, The changes in the biochemical compositions and enzymatic activities of rotifer (*Brachionus plicatilis*, Müller) and *Artemia* during the enrichment and starvation periods, Fish Physiol. Biochem. 34 (2008) 391-404.

[55] V.C. Nhu, K. Dierckens, T.H. Nguyen, M.T. Tran, P. Sorgeloos, Can umbrella-stage *Artemia franciscana* substitute enriched rotifers for Cobia (*Rachycentron canadum*) fish larvae?, Aquaculture 289 (2009) 64-69.

Table 1

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 sequence used for primer design.

Transcript	Primer sequence	Fragment	Ta	Efficiency	Accession No.
fad	AAGCCTCTGCTGATTGGAGA GGCTGAGCTTGAAACAGACC	131 bp	60ºC	0.989[1]/0.982[2]	JN673546
elovl	TTTCATGTTTTTGCACACTGC GACACCTTTAGGCTCGGTTTT	161 bp	60ºC	0.994[1]/0.999[2]	JN793448
Ubq[3]	AGCTGGCCCAGAAATATAACTGCGACA ACTTCTTCTTGCGGCAGTTGACAGCAC	93 bp	70ºC	0.838[2]	AB291588
rpsa[3]	GTGAAGAAGCTCCTTGTCGGCACCA AGGGGGTCGGGGTAGCGGATG	83 bp	70ºC	1.000[2]	AB291557
ef1a1[3]	GATTGACCGTCGTTCTGGCAAGAAGC GGCAAAGCGACCAAGGGGAGCAT	142 bp	70ºC	0.999[1]	AB326302

[1] Ontogenetic regulation experiment.

[2] Nutritional regulation experiment.

[3] Infante et al. [29].

Table 2

Functional characterization of *Solea senegalensis* proteins encoded by fatty acyl desaturase (*fad*) and elongase (*elovl*) genes. Results are expressed as a percentage of total fatty acid (FA) substrate converted to elongated/desaturated product. Percentage of stepwise conversion into intermediary products of the elongation pathway is also shown.

FA Substrate	Product	Conversion (%)	Activity
Fatty acyl desaturase (*fad*)			
18:3n-3	18:4n-3	0.3	Δ6
18:2n-6	18:3n-6	0.6	Δ6
20:4n-3	20:5n-3	2.2	Δ5
20:3n-6	20:4n-6	0.4	Δ5
22:5n-3	22:6n-3	16.1	Δ4
22:4n-6	22:5n-6	6.8	Δ4
Fatty acyl elongase (*elovl*)			
18:4n-3	20:4n-3	20.1	C18→20
	22:4n-3	11.8	C20→22
	24:4n-3	0.0	C22→24
	Total	31.9	
18:3n-6	20:3n-6	28.6	C18→20
	22:3n-6	8.0	C20→22
	24:3n-6	0.0	C22→24
	Total	36.6	
20:5n-3	22:5n-3	75.0	C20→22
	24:5n-3	4.4	C22→24
	Total	79.4	
20:4n-6	22:4n-6	53.9	C20→22
	24:4n-6	3.2	C22→24
	Total	57.1	
22:5n-3	24:5n-3	3.7	C22→24
22:4n-6	24:4n-6	3.8	C22→24

Table 3

Change in fatty acid composition (% of total fatty acids) during ontogenetic development up to 6 days after hatching (DAH).

Fatty acids (% of total FA)	**Eggs**	**0 DAH**	**1 DAH**	**2 DAH**	**3 DAH**	**4 DAH**	**5 DAH**	**6 DAH**
Total saturated	23.3 ± 0.9 ^{ab}	23.7 ± 1.2 ^{ab}	24.1 ± 1.0 ^{ab}	25.8 ± 1.5 ^{bc}	26.7 ± 2.9 ^{bc}	29.9 ± 0.8 ^{c}	35.8 ± 0.8 ^{d}	32.6 ± 1.4 ^{cd}
Total monoenes	36.1 ± 0.4 ^{a}	35.9 ± 0.6 ^{a}	35.5 ± 0.7 ^{a}	34.5 ± 1.0 ^{a}	28.2 ± 1.5 ^{b}	28.6 ± 1.7 ^{b}	28.3 ± 4.0 ^{b}	24.6 ± 1.8 ^{b}
18:2n-6	8.7 ± 0.0 ^{a}	8.8 ± 0.1 ^{a}	8.6 ± 0.1 ^{a}	8.2 ± 0.2 ^{a}	7.4 ± 0.4 ^{b}	6.2 ± 0.1 ^{c}	4.9 ± 0.2 ^{d}	5.2 ± 0.2 ^{d}
18:3n-6	0.2 ± 0.0 ^{ac}	0.1 ± 0.0 ^{ac}	0.1 ± 0.0 ^{a}	0.1 ± 0.0 ^{ac}	0.2 ± 0.0 ^{abc}	0.2 ± 0.1 ^{abc}	0.4 ± 0.1 ^{b}	0.3 ± 0.1 ^{bc}
20:2n-6	0.6 ± 0.0 ^{ab}	0.8 ± 0.0 ^{bc}	0.6 ± 0.0 ^{a}	0.6 ± 0.0 ^{a}	0.8 ± 0.1 ^{c}	0.6 ± 0.0 ^{abc}	0.5 ± 0.1 ^{a}	0.6 ± 0.1 ^{a}
20:3n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.0
20:4n-6	1.0 ± 0.0 ^{a}	1.2 ± 0.0 ^{ab}	1.5 ± 0.0 ^{bc}	1.7 ± 0.1 ^{c}	2.6 ± 0.2 ^{d}	2.7 ± 0.2 ^{d}	2.6 ± 0.2 ^{d}	3.7 ± 0.2 ^{e}
22:4n-6	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.3	0.5 ± 0.3	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
22:5n-6	0.5 ± 0.0 ^{a}	0.6 ± 0.0 ^{ab}	0.5 ± 0.0 ^{a}	0.6 ± 0.1 ^{ab}	0.8 ± 0.1 ^{ab}	0.8 ± 0.0 ^{ab}	0.9 ± 0.3 ^{ab}	1.2 ± 0.5 ^{b}
Total n-6 PUFA	11.5 ± 0.1 ^{ab}	11.9 ± 0.2 ^{ab}	11.7 ± 0.1 ^{ab}	11.8 ± 0.3 ^{ab}	12.3 ± 0.6 ^{a}	10.8 ± 0.3 ^{b}	9.7 ± 0.3 ^{c}	11.4 ± 0.7 ^{ab}
18:3n-3	2.5 ± 0.0 ^{a}	2.6 ± 0.1 ^{a}	2.2 ± 0.1 ^{ab}	2.0 ± 0.0 ^{b}	1.2 ± 0.1 ^{c}	1.0 ± 0.1 ^{cd}	0.7 ± 0.2 ^{d}	0.8 ± 0.1 ^{cd}
18:4n-3	0.4 ± 0.0 ^{a}	0.3 ± 0.0 ^{a}	0.3 ± 0.0 ^{a}	0.3 ± 0.0 ^{ab}	0.2 ± 0.0 ^{ab}	0.2 ± 0.1 ^{ab}	0.1 ± 0.2 ^{b}	0.2 ± 0.1 ^{ab}
20:3n-3	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.4	0.0 ± 0.1	0.0 ± 0.1
20:4n-3	0.3 ± 0.0 ^{a}	0.3 ± 0.0 ^{ab}	0.3 ± 0.1 ^{ab}	0.2 ± 0.0 ^{ab}	0.2 ± 0.0 ^{ab}	0.1 ± 0.1 ^{b}	0.1 ± 0.1 ^{ab}	0.1 ± 0.1 ^{ab}
20:5n-3	1.8 ± 0.1 ^{a}	1.8 ± 0.1 ^{a}	1.7 ± 0.1 ^{a}	1.7 ± 0.1 ^{a}	1.9 ± 0.1 ^{a}	3.2 ± 0.2 ^{b}	2.9 ± 0.8 ^{b}	3.0 ± 0.5 ^{b}
22:5n-3	2.6 ± 0.1 ^{a}	2.4 ± 0.1 ^{ab}	2.7 ± 0.1 ^{a}	2.4 ± 0.2 ^{ab}	2.2 ± 0.1 ^{bd}	1.5 ± 0.0 ^{c}	1.4 ± 0.1 ^{c}	1.9 ± 0.2 ^{d}
22:6n-3	20.5 ± 0.9	20.0 ± 1.4	20.3 ± 1.3	20.2 ± 1.9	25.9 ± 1.9	22.9 ± 2.5	19.5 ± 4.5	23.4 ± 1.8
Total n-3 PUFA	28.4 ± 1.2 ^{ab}	27.6 ± 1.8 ^{ab}	27.7 ± 1.6 ^{ab}	26.9 ± 2.2 ^{ab}	31.7 ± 2.1 ^{a}	29.0 ± 2.3 ^{ab}	24.8 ± 3.6 ^{b}	29.4 ± 1.6 ^{ab}
DHA/EPA	11.4 ± 0.2 ^{ab}	11.0 ± 0.4 ^{ab}	12.0 ± 0.3 ^{ab}	12.1 ± 0.3 ^{ab}	13.5 ± 0.4 ^{a}	7.4 ± 1.3 ^{b}	7.3 ± 3.9 ^{b}	8.1 ± 1.9 ^{b}
Total PUFA	40.6 ± 1.3 ^{ab}	40.5 ± 1.8 ^{ab}	40.3 ± 1.7 ^{ab}	39.7 ± 2.5 ^{ab}	45.1 ± 2.1 ^{a}	41.6 ± 2.4 ^{ab}	35.9 ± 3.7 ^{b}	42.8 ± 2.4 ^{a}

Different superscript letters in the same row indicate significant differences ($P<0.05$) between dietary regimes at the assessed age (19 or 40 DAH) (SPSS 17.0).

Table 4

Effect of diet (enriched – E – or non-enriched – NE – *Artemia* EG metanauplii) on larval and post-larval fatty acid composition (% of total fatty acids), at 19 and 40 days after hatching (DAH), respectively.

Fatty acids	***Artemia***		**19 DAH *S. senegalensis* larvae**		**40 DAH *S. senegalensis* post-larvae**			
(% of total FA)	**E**	**NE**	**E**	**NE**	**E**	**NE**	**E-NE**	**NE-E**
Total saturated	19.0	21.0	24.8 ± 0.8 ^{x}	26.6 ± 0.4 ^{y}	22.8 ± 0.5 ^{a}	26.6 ± 1.9 ^{b}	27.3 ± 1.9 ^{b}	22.4 ± 0.5 ^{a}
Total monoenes	33.9	32.7	35.2 ± 1.0 ^{x}	32.2 ± 0.9 ^{y}	31.6 ± 0.6	33.1 ± 0.8	32.5 ± 1.2	32.4 ± 0.3
18:2n-6	5.3	4.8	4.9 ± 0.2 ^{x}	5.7 ± 0.6 ^{y}	5.6 ± 0.1	6.1 ± 0.4	6.0 ± 0.3	5.7 ± 0.1
18:3n-6	0.4	0.5	0.2 ± 0.1 ^{x}	0.5 ± 0.0 ^{y}	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.2
20:2n-6	0.4	0.4	0.5 ± 0.0 ^{x}	0.4 ± 0.0 ^{y}	0.4 ± 0.0	1.1 ± 1.0	0.5 ± 0.0	0.5 ± 0.0
20:3n-6	0.1	0.2	0.2 ± 0.0 ^{x}	0.3 ± 0.0 ^{y}	0.3 ± 0.0 ^{a}	0.4 ± 0.1 ^{b}	0.4 ± 0.0 ^{b}	0.2 ± 0.0 ^{a}
20:4n-6	0.7	0.6	2.5 ± 0.1 ^{x}	3.1 ± 0.1 ^{y}	2.0 ± 0.1	1.7 ± 0.2	2.0 ± 0.3	1.8 ± 0.0
22:4n-6	0.0	0.0	0.2 ± 0.3	0.1 ± 0.0	0.1 ± 0.0 ^{ab}	0.2 ± 0.1 ^{b}	0.1 ± 0.0 ^{a}	0.1 ± 0.0 ^{a}
22:5n-6	0.1	0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0 ^{a}	0.5 ± 0.6 ^{ab}	0.2 ± 0.0 ^{b}	0.3 ± 0.0 ^{b}
Total n-6 PUFA	7.1	6.5	8.6 ± 0.4 ^{x}	10.3 ± 0.7 ^{y}	9.1 ± 0.1 ^{a}	10.5 ± 0.2 ^{b}	9.6 ± 0.7 ^{ab}	8.9 ± 0.2 ^{a}
18:3n-3	24.5	29.1	12.6 ± 1.0	13.7 ± 0.7	15.2 ± 0.5	16.5 ± 1.1	15.3 ± 0.8	16.0 ± 0.6
18:4n-3	4.2	4.9	1.6 ± 0.2	1.7 ± 0.1	2.1 ± 0.1 ^{a}	1.7 ± 0.1 ^{b}	1.6 ± 0.1 ^{b}	2.3 ± 0.1 ^{a}
20:3n-3	1.1	1.6	1.3 ± 0.1 ^{x}	1.4 ± 0.1 ^{y}	1.6 ± 0.1 ^{a}	2.4 ± 0.2 ^{b}	2.3 ± 0.2 ^{b}	1.6 ± 0.0 ^{a}
20:4n-3	0.9	0.8	0.7 ± 0.1 ^{x}	0.8 ± 0.1 ^{y}	1.0 ± 0.0 ^{a}	1.2 ± 0.1 ^{b}	1.2 ± 0.1 ^{ab}	1.0 ± 0.0 ^{a}
20:5n-3	4.1	1.7	4.8 ± 0.2 ^{x}	3.6 ± 0.0 ^{y}	3.1 ± 0.1 ^{a}	1.8 ± 0.3 ^{b}	1.9 ± 0.3 ^{b}	3.0 ± 0.0 ^{a}
22:5n-3	0.3	0.0	2.6 ± 0.2 ^{x}	2.1 ± 0.1 ^{y}	2.6 ± 0.0 ^{a}	1.3 ± 0.2 ^{b}	1.7 ± 0.3 ^{b}	2.4 ± 0.1 ^{a}
22:6n-3	3.0	0.1	6.1 ± 0.2 ^{x}	5.3 ± 0.4 ^{y}	9.4 ± 0.6 ^{a}	3.0 ± 0.6 ^{b}	4.8 ± 1.0 ^{c}	8.2 ± 0.3 ^{a}
Total n-3 PUFA	38.1	38.3	29.5 ± 1.5	28.7 ± 0.8	35.0 ± 0.4 ^{a}	27.9 ± 2.5 ^{b}	28.8 ± 2.5 ^{b}	34.5 ± 0.3 ^{a}
DHA/EPA	0.8	0.1	1.3 ± 0.1 ^{x}	1.5 ± 0.1 ^{y}	3.1 ± 0.2 ^{a}	1.7 ± 0.1 ^{b}	2.5 ± 0.2 ^{c}	2.7 ± 0.1 ^{ac}
Total PUFA	47.2	46.4	40.0 ± 1.7	41.2 ± 1.0	45.7 ± 1.0 ^{a}	40.2 ± 2.5 ^{b}	40.2 ± 3.0 ^{b}	45.2 ± 0.2 ^{ab}

Different superscript letters in the same row indicate significant differences ($P<0.05$) between dietary regimes at the assessed age (19 or 40 DAH) (SPSS 17.0).

Table 5

Growth performance and survival of *Solea senegalensis* with different dietary regimes.

	19 DAH *S. senegalensis* larvae		**40 DAH *S. senegalensis* post-larvae**			
	E	**NE**	**E**	**NE**	**E-NE**	**NE-E**
Dry weight (mg/larva)	0.34 ± 0.15^{x}	0.21 ± 0.09^{y}	2.41 ± 1.04^{a}	2.78 ± 1.26^{ab}	3.18 ± 1.44^{b}	3.29 ± 1.56^{b}
Survival (%)	23.0 ± 4.4	22.2 ± 4.1	93.0 ± 1.7	95.0 ± 2.2	91.3 ± 2.8	94.0 ± 0.6

Values are mean ± SD. Values with different superscript letters in the same row are significantly different at each age (one-way ANOVA, $P < 0.001$). Absence of letters indicates no statistical differences ($P > 0.001$).

Figure captions

Fig. 1. ClustalW2 alignment of the deduced amino acid sequences of different fish species Fads, including the sole (*S. senegalensis*) Fad characterized here (GenBank ID:JN673546), Atlantic salmon (*S. salar*) Δ5Fad (AAL82631) and Δ6Fad (AAR21624), turbot (*P.maxima*) Δ6Fad (AAS49163), rabbitfish (*S. canaliculatus*) Δ6Δ5Fad (ABR12315) and Δ4Fad (ADJ29913) and zebrafish (*D. rerio*) Δ5Δ6Fad (AAG25710). Identical residues are shaded black and similar residues (based on the Gonnet matrix, using ClustalW2 default parameters) are shaded grey. The cytochrome b5-like domain is dot-underlined, the two transmembrane regions are dash underlined, the three histidine-rich domains are solid underlined and asterisks on the top mark the hame-binding motif, HPGG.

Fig. 2. Phylogenetic tree comparing the deduced aa sequence of *Solea senegalensis* Fad with Fad proteins from fish and other organisms. The tree was constructed using the Neighbour Joining method [25] with MEGA5.05 [26]. The horizontal branch length corresponds to the aa substitution rate per site. Numbers represent the frequencies with which the tree topology presented was replicated after 1,000 bootstrap iterations.

Fig. 3. ClustalW2 alignment of the deduced amino acid sequences of different fish species Elovl5's, including the sole (*S. senegalensis*) Elovl characterized here (GenBank ID:JN793448), *P. maxima* (AAL69984), *S. canaliculatus* (ADE34561) *S. salar* (*elovl5a*; NP_001117039 and *elovl5b*; ACI62499), and *D. rerio* (NP_956747). Identical residues are shaded black and similar residues (based on the Gonnet matrix, using ClustalW2 default parameters) are shaded grey. Indicated are the conserved histidine

box motif (underlined), five (I-V) predicted membrane-spanning domains (underlined with dashed line) and asterisks mark the lysine or arginine residuals (ER retrieval signals).

Fig. 4. Functional characterization of *Solea senegalensis* putative fatty acyl desaturase in transgenic yeast (*Saccharomyces cerevisiae*) grown in the presence of n-3 FA substrates; 18:3n-3 (A); 20:4n-3 (B); and 22:5n-3 (C). Fatty acids were extracted from yeast transformed with pYES2 vector containing the ORF of the putative fatty acyl desaturase cDNA as an insert. Peaks 1-4 represent the main endogenous FAs of *S. cerevisiae*, namely 16:0 (1), 16:1n-7 (2), 18:0 (3) and 18:1n-9 (4). The remaining main additional peaks correspond to the exogenously added FAs (*) and the products of their desaturation. Vertical axis, FID response; horizontal axis, retention time.

Fig. 5. Functional characterization of *Solea senegalensis* putative fatty acyl elongase in transgenic yeast (*Saccharomyces cerevisiae*) grown in the presence of n-3 FA substrates; 18:4n-3 (A); 20:5n-3 (B); and 22:5n-3 (C). Fatty acids were extracted from yeast transformed with pYES2 vector containing the ORF of the putative fatty acyl elongase cDNA as an insert. Peaks 1-4 represent the main endogenous FAs of *S. cerevisiae*, namely 16:0 (1), 16:1n-7 (2), 18:0 (3) and 18:1n-9 (4). Peak 5 corresponds to 18:1n-7 resultant from the elongation of the yeast endogenous16:1n-7 and the remaining main additional peaks correspond to the exogenously added FAs (*) and the products of their elongation. Vertical axis, FID response; horizontal axis, retention time.

Fig. 6. Regulation of *Solea senegalensis* fatty acyl desaturase (*fad*) and elongase (*elovl*) genes during early ontogenetic development, determined by qPCR in whole eggs and

larvae. The results shown are normalized values (reference gene: *ef1a*) corresponding to a mean of n=3 with standard deviation (SD). Different letters above the columns represent significant differences (P<0.05) between ages, for each gene (SPSS 17.0).

Fig. 7. Nutritional regulation of *Solea senegalensis* fatty acyl desaturase (*fad*) and elongase (*elovl*) genes in whole larvae and post-larvae, at 19 and 40 days after hatching (DAH), respectively, determined by qPCR. Larvae were fed either enriched (E) or non-enriched (NE) *Artemia* until 19 DAH and then either kept on enriched (E) or non-enriched (NE), or switched from E to NE (E-NE) or from NE to E (NE-E) *Artemia* from 19 to 40 DAH. The results shown are normalized values (reference genes: *ubq* and *rpsa*) corresponding to a mean of n=3 with standard deviation (SD). Different letters above the columns represent significant differences (P<0.05) between the dietary regimes at the assessed age (19 or 40 DAH) (SPSS 17.0).

Figure 1

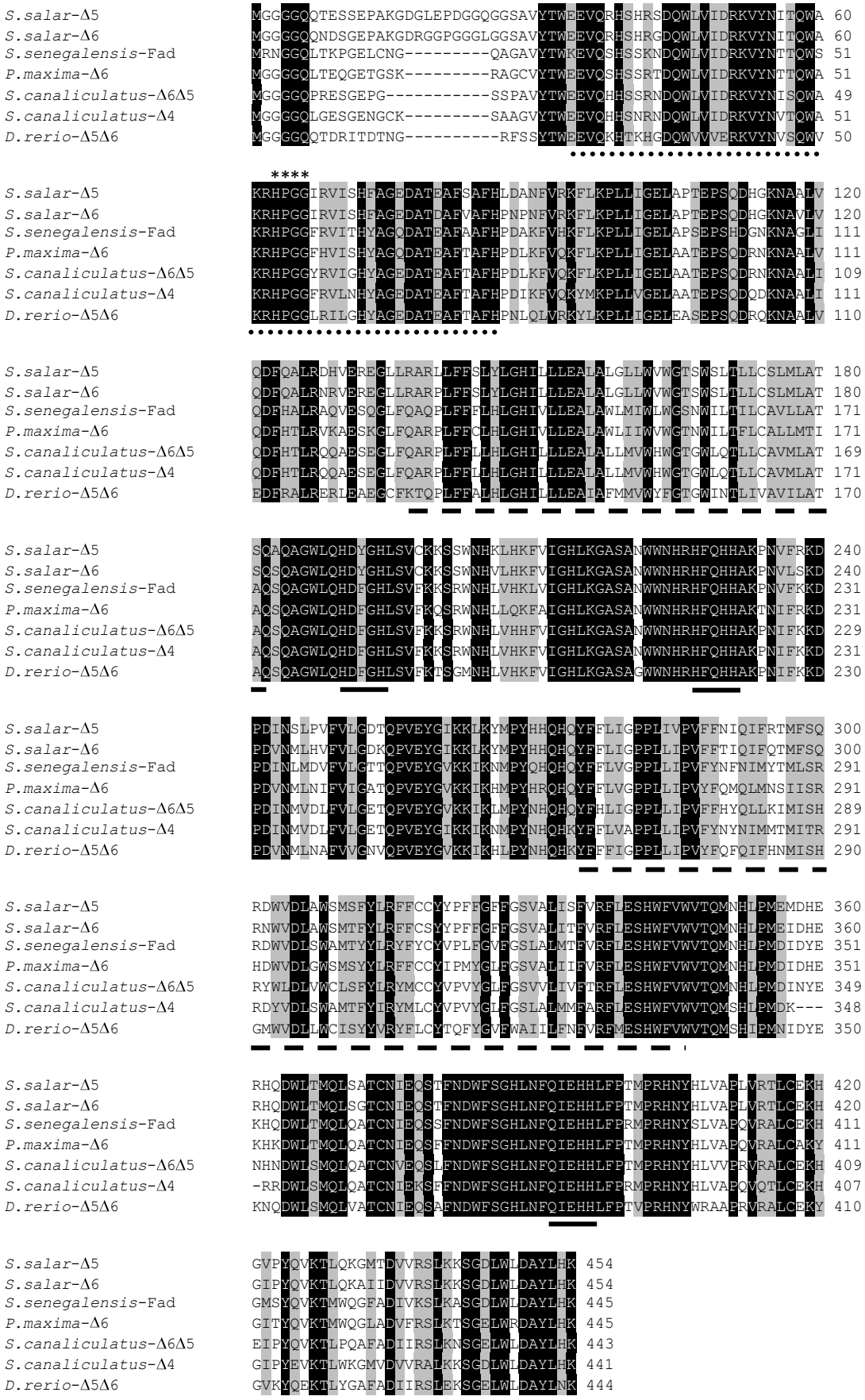

S.salar-Δ5
S.salar-Δ6
S.senegalensis-Fad
P.maxima-Δ6
S.canaliculatus-Δ6Δ5
S.canaliculatus-Δ4
D.rerio-Δ5Δ6

Figure 2

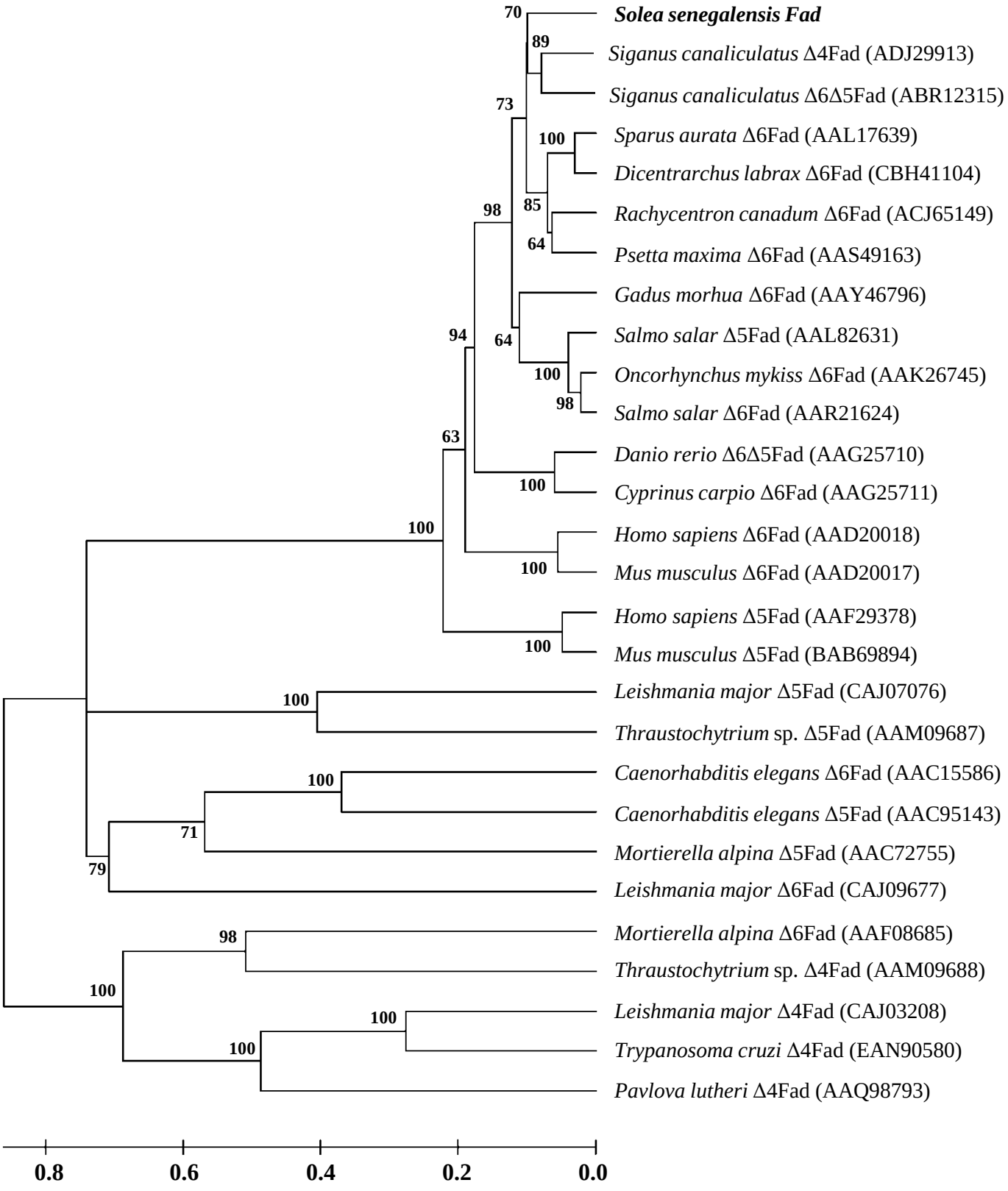

70
89
73
100
98
85
64
94
64
100
98
63
100
100
100
100
100
71
79
98
100
100
100
Solea senegalensis Fad
Siganus canaliculatus Δ4Fad (ADJ29913)
Siganus canaliculatus Δ6Δ5Fad (ABR12315)
Sparus aurata Δ6Fad (AAL17639)
Dicentrarchus labrax Δ6Fad (CBH41104)
Rachycentron canadum Δ6Fad (ACJ65149)
Psetta maxima Δ6Fad (AAS49163)
Gadus morhua Δ6Fad (AAY46796)
Salmo salar Δ5Fad (AAL82631)
Oncorhynchus mykiss Δ6Fad (AAK26745)
Salmo salar Δ6Fad (AAR21624)
Danio rerio Δ6Δ5Fad (AAG25710)
Cyprinus carpio Δ6Fad (AAG25711)
Homo sapiens Δ6Fad (AAD20018)
Mus musculus Δ6Fad (AAD20017)
Homo sapiens Δ5Fad (AAF29378)
Mus musculus Δ5Fad (BAB69894)
Leishmania major Δ5Fad (CAJ07076)
Thraustochytrium sp. Δ5Fad (AAM09687)
Caenorhabditis elegans Δ6Fad (AAC15586)
Caenorhabditis elegans Δ5Fad (AAC95143)
Mortierella alpina Δ5Fad (AAC72755)
Leishmania major Δ6Fad (CAJ09677)
Mortierella alpina Δ6Fad (AAF08685)
Thraustochytrium sp. Δ4Fad (AAM09688)
Leishmania major Δ4Fad (CAJ03208)
Trypanosoma cruzi Δ4Fad (EAN90580)
Pavlova lutheri Δ4Fad (AAQ98793)
0.8
0.6
0.4
0.2
0.0

Figure 3

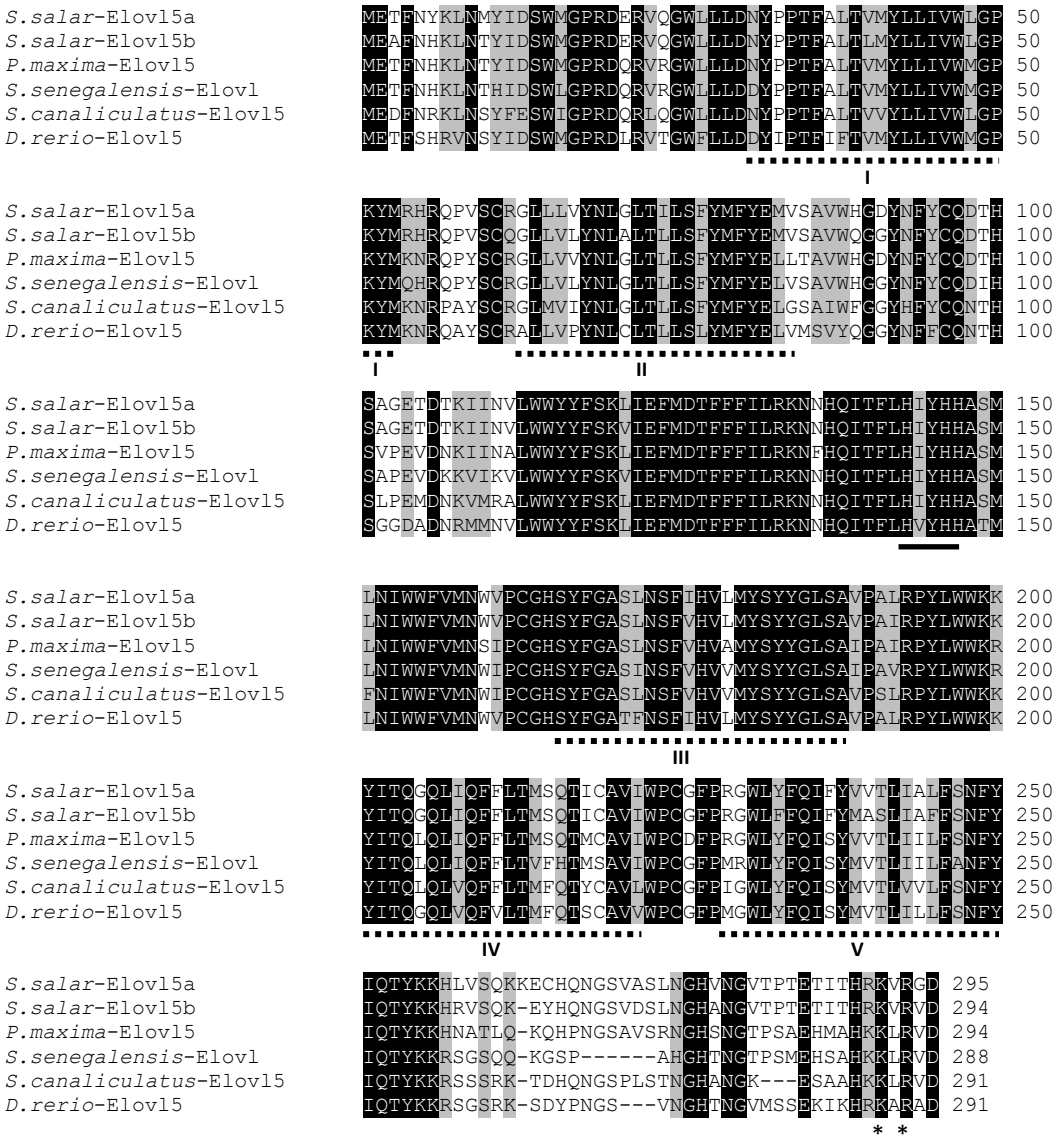

S.salar-Elovl5a
S.salar-Elovl5b
P.maxima-Elovl5
S.senegalensis-Elovl
S.canaliculatus-Elovl5
D.rerio-Elovl5
I
II
III
IV
V
* *

Figure 4

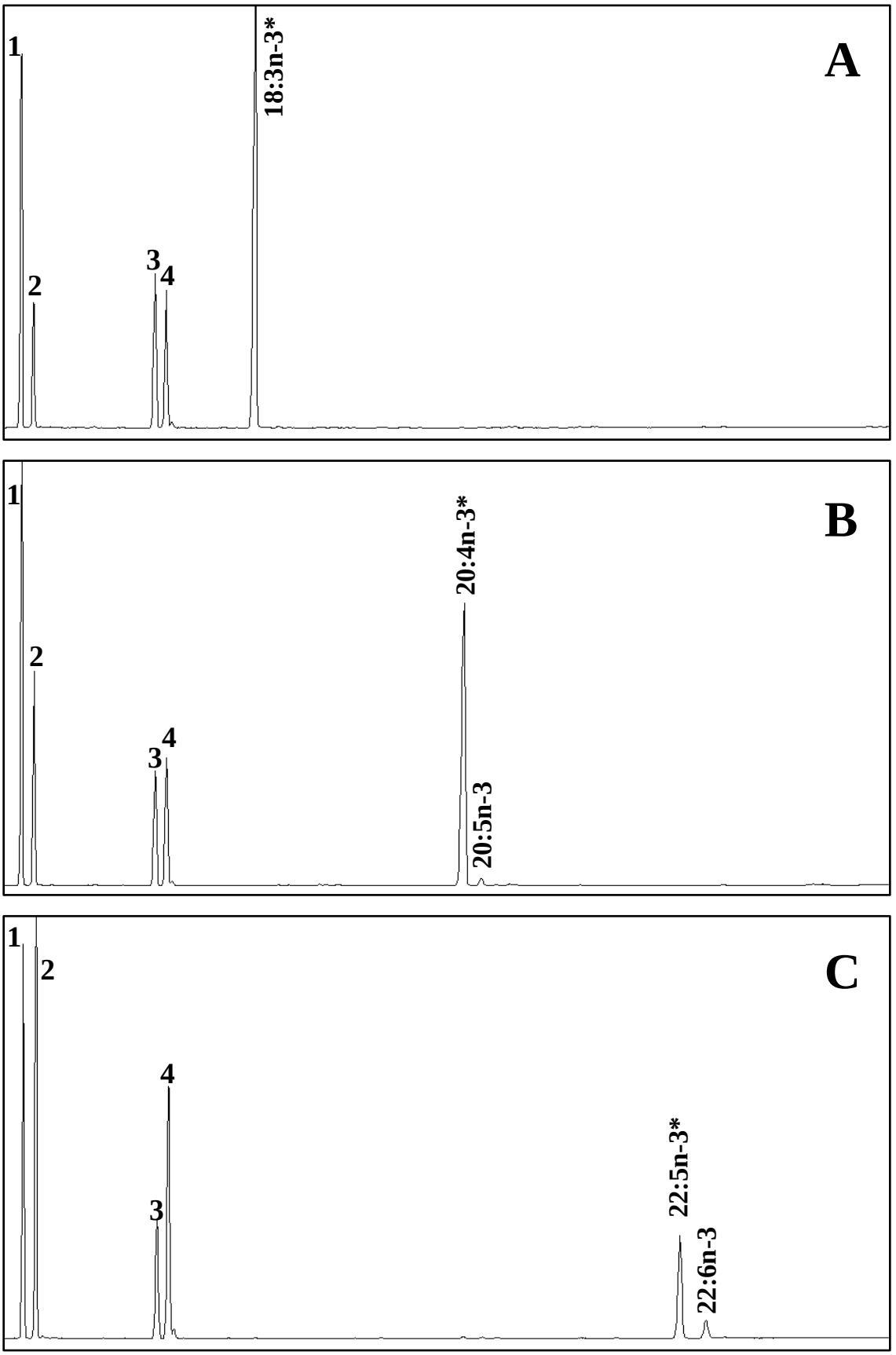


Figure 5

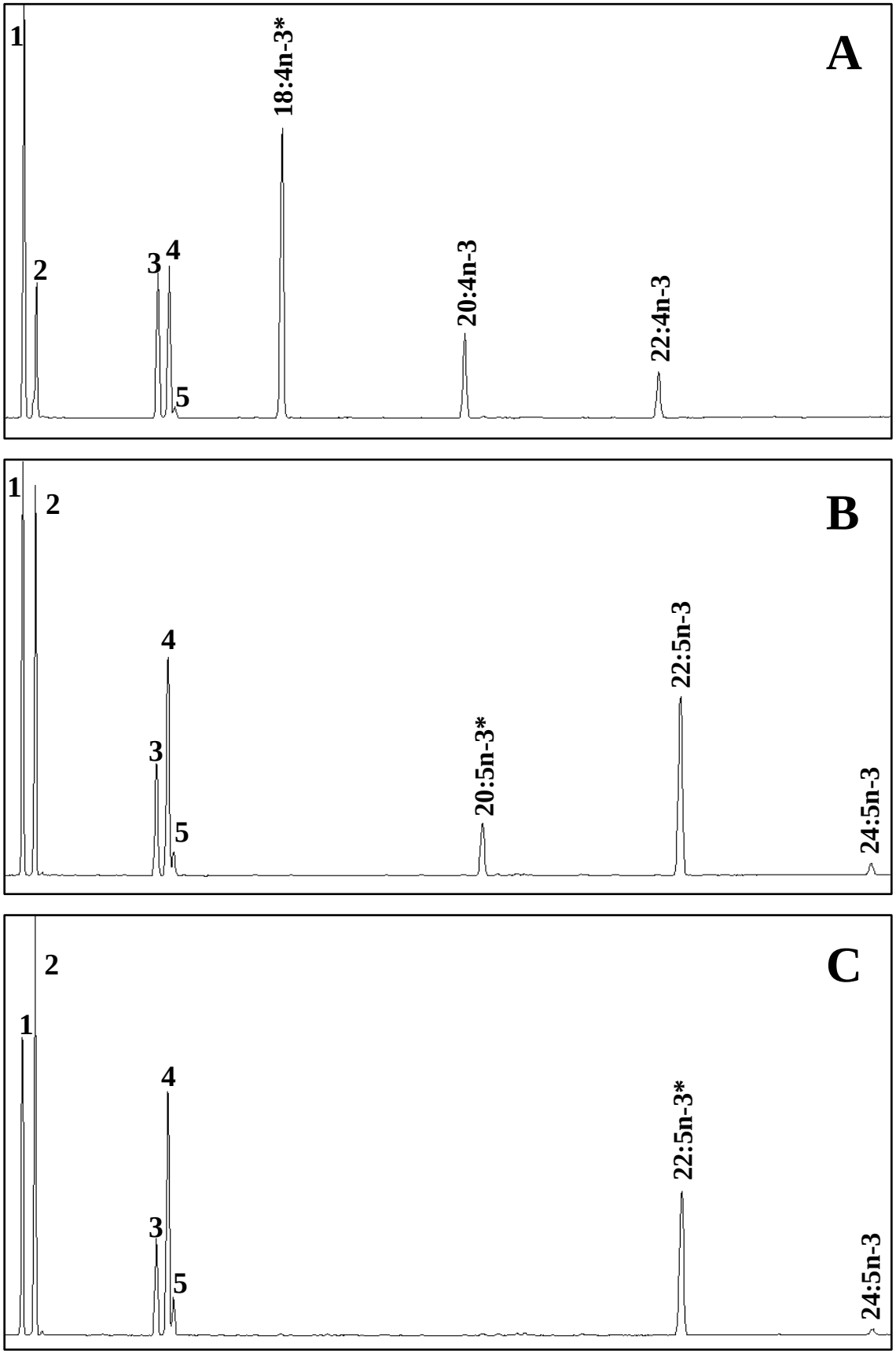

A
1
2
3
4
5
18:4n-3*
20:4n-3
22:4n-3
B
1
2
3
4
5
20:5n-3*
22:5n-3
24:5n-3
C
1
2
3
4
5
22:5n-3*
24:5n-3

Figure 6

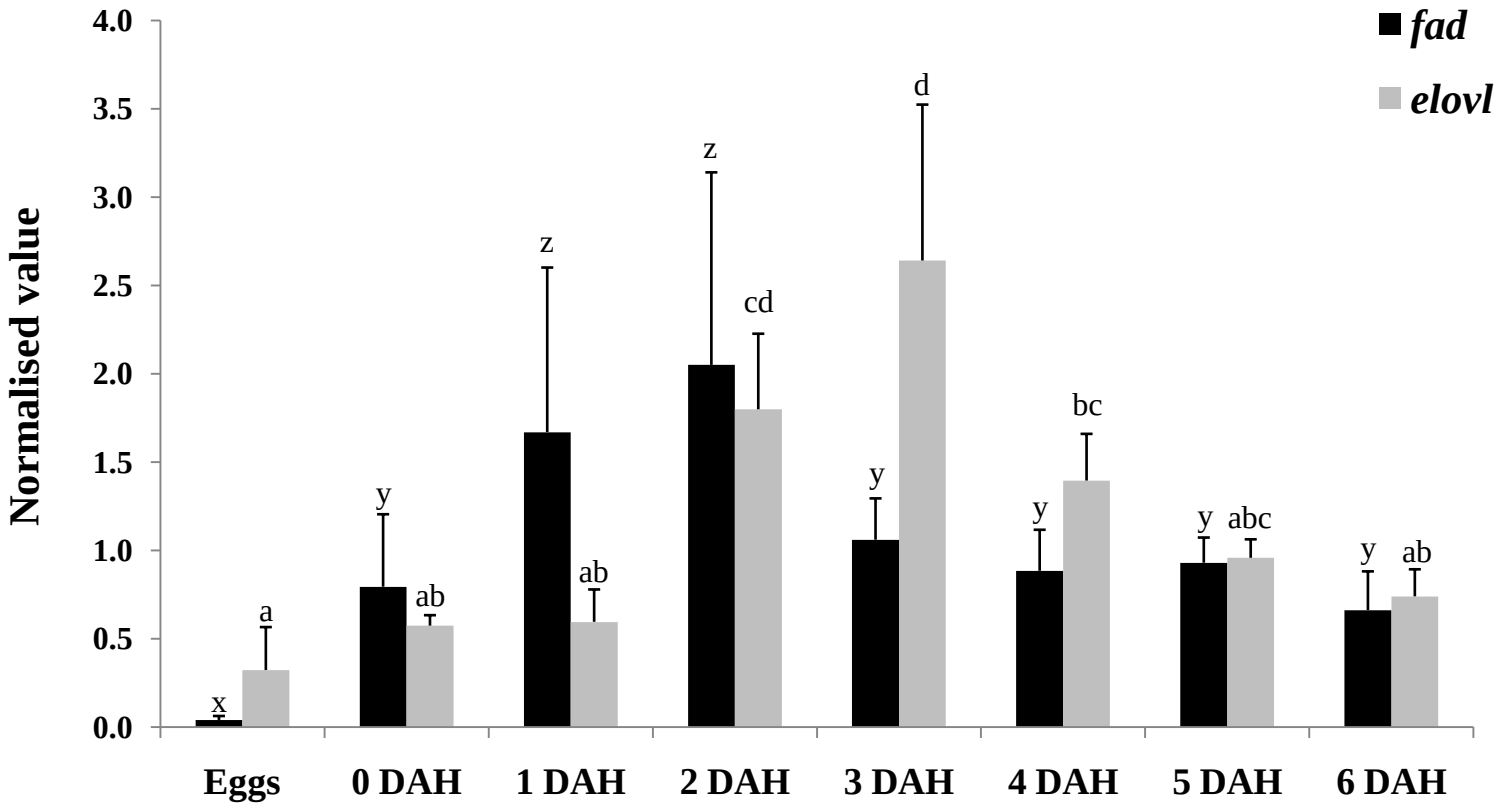
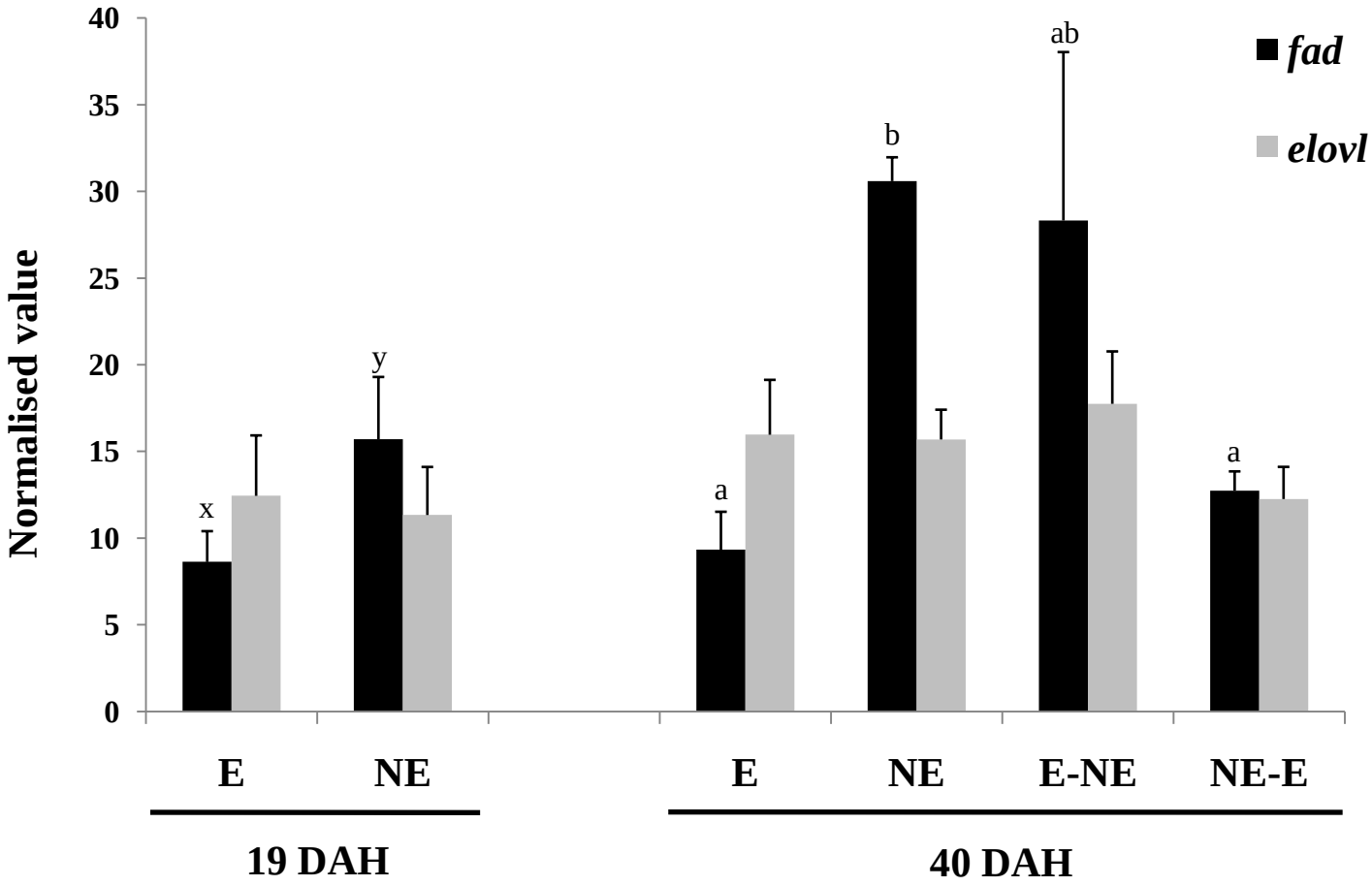

Normalised value
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
fad
elovl
x
a
y
ab
z
ab
z
cd
d
y
y
bc
y
abc
y
ab
Eggs
0 DAH
1 DAH
2 DAH
3 DAH
4 DAH
5 DAH
6 DAH

Figure 7


Normalised value
40
35
30
25
20
15
10
5
0
fad
elovl
x
y
a
b
ab
a
E
NE
E
NE
E-NE
NE-E
19 DAH
40 DAH