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Title

Investigating the essential fatty acids in the common cuttlefish *Sepia officinalis* (Mollusca, Cephalopoda): Molecular cloning and functional characterisation of fatty acyl desaturase and elongase

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Abstract

Some polyunsaturated fatty acids (PUFA) play critical roles in physiology and are essential for normal growth and development of aquatic animals including cephalopods like the common cuttlefish *Sepia officinalis*. This is particularly true for early life stages when neuronal tissues are rapidly developing and accumulating PUFA. The specific fatty acids (FAs) that can satisfy the essential requirements of any animal species depend upon the enzymatic capability to convert dietary FA into those physiologically important FAs required for normal function. In order to identify the dietary essential FA for the common cuttlefish, we have performed the molecular cloning and functional characterisation of two key enzymes involved in PUFA biosynthesis in this species, namely a fatty acyl desaturase (Fad) and an elongation of very long-chain fatty acid (Elovl) protein. The gene product of the cuttlefish Fad exhibited Δ5-desaturase activity, enabling this species to potentially biosynthesise the physiologically essential FAs eicosapentaenoic (20:5n-3, EPA) and arachidonic (20:4n-3, ARA) acids from 20:4n-3 and 20:3n-6, respectively. However, the cuttlefish Fad did not show Δ6 or Δ8 activity and therefore the biosynthesis of EPA and ARA from C18 PUFA precursors could be limited, suggesting that both may be essential FAs for cuttlefish. Moreover, the cuttlefish Fad also lacked Δ4-desaturase activity suggesting that DHA biosynthesis was not possible and thus DHA is also an essential FA for this species. The cuttlefish Δ5 Fad was able to produce non-methylene-interrupted (NMI) FA, a group of PUFAs typically found in marine invertebrates. The cuttlefish Elovl was able to elongate C18 and C20 PUFA substrates, but showed no activity towards C22 PUFA. Overall the results obtained in the present study allowed the prediction of the biosynthetic pathways of PUFA, including NMI FA, in the common cuttlefish. It was concluded that EPA, ARA

and DHA were likely to be essential dietary FA for this species as endogenous production from precursor FA appears to be limited.

Keywords

Elongase; essential fatty acids; desaturase; polyunsaturated fatty acids; *Sepia officinalis*.

Introduction

The common cuttlefish or European common cuttlefish, *Sepia officinalis* Linnaeus 1758, is a cephalopod found in the Eastern Atlantic and the Mediterranean (Boletzky, 1983). It attains high market values in countries including Spain and Italy. Production is based on fisheries, although its potential for aquaculture has long been recognised (Barnabé, 1996; Boucaud-Camou, 1989). Cuttlefish has several characteristics that make it one of the most promising cephalopod species for aquaculture: (1) large eggs, which are easily transported and maintained, (2) hatchlings do not have a paralarval phase and feed on relatively large live prey, (3) survival of hatchlings is usually high, compared to other species (Domingues et al., 2001). Moreover, *S*. *officinalis* is resistant to crowding and disease (Forsythe et al., 1994), as well as handling, and can be easily shipped, thus it is used as an animal model in biological and biomedical research (Sykes et al., 2014). Since it exhibits fast growth and a relatively short life cycle, cuttlefish are readily available within relatively short periods. For instance, it is possible to produce 50 g individuals with great commercial interest within 45-60 days. Despite the above, Sykes et al. (2014) highlighted some major bottlenecks hindering the transition from experimental to industrial scale culture, including lack of control of reproduction in captivity, as well as nutritional issues related to the dependence on live prey in early developmental stages, and also lack of an adequate pelleted diet for grow-out stages. Thus, it is critical to understand the essential nutrient requirements of cephalopod species so that adequate provision of critical compounds is satisfied through the diet.

Some polyunsaturated fatty acids (PUFA), including the eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), have been previously suggested as critical dietary components for early life-cycle stages of cephalopods, like cuttlefish hatchlings (Navarro and Villanueva, 2000, 2003; Almansa et al., 2006; Sykes et al.,

2009). This is consistent with the formation of neural tissues accumulating these compounds in early development of animals (Monroig et al., 2009; Tocher, 2010). The identification of EPA and DHA as essential fatty acids (EFA) for cuttlefish hatchlings was based on the high contents of these fatty acids (FA) in hatchlings (Navarro and Villanueva, 2000). Recently, the EFA requirements of *Octopus vulgaris* have been recently characterised through the characterisation of genes encoding enzymes involved in PUFA biosynthetic pathways including fatty acyl desaturases (Fad) and elongation of very long-chain fatty acid (Elovl) proteins (Monroig et al., 2012a, 2012b). This approach proved an invaluable tool to identify the specific FA fulfilling the EFA requirements for any animal species (Bell and Tocher, 2009), but was particularly useful in cephalopod early life-cycle stages where availability and viability of individuals to run feeding trials cannot always be guaranteed, and where there is total reliance on live prey-based diets where levels of PUFA cannot be fully controlled (Guinot et al., 2013a, 2013b).

A cDNA encoding a cephalopod Fad enzyme was first isolated from *O. vulgaris*. The common octopus Fad showed Δ5 desaturation activity towards saturated and polyunsaturated fatty acyl substrates (Monroig et al., 2012a). Likewise, a full-length cDNA sequence encoding an Elovl-like protein identified from *O. vulgaris* had high homology to vertebrate Elovl2 and Elovl5 enzymes (Monroig et al., 2012b). Consistent with vertebrate Elovl5 and Elovl2, two elongases with demonstrated roles in PUFA biosynthesis in vertebrates (Monroig et al. 2011a), the octopus Elovl was also very efficient in the elongation of C18 and C20 PUFA substrates. While further studies on other molluscs, the gastropod *Haliotis discus hannai* (Li et al., 2013) and the bivalve *Chlamys nobilis* (Liu et al., 2013, 2014a), have confirmed similar substrate specificities to those described for the *O. vulgaris* genes, it remained unclear if the functions of

homologous enzymes from species belonging to other cephalopod orders like *S. officinalis* (order Sepiida) have different functionalities that could confer a distinctive ability to biosynthesise PUFA.

The overarching aim of the present study was to identify the dietary essential FA for the cuttlefish through the isolation and functional characterisation of Fad and Elovl cDNA sequences involved in PUFA biosynthesis. Here we report on the molecular and functional characterisation of a Fad and an Elovl with high homologies to the octopus orthologs previously investigated. Moreover, the results obtained from the common cuttlefish, a cephalopod of the order Sepiida, are compared with those obtained from the order Octopoda representative *O. vulgaris*, as well as with those reported from other mollusc classes including gastropods and bivalves.

Materials and methods

Sample collection and RNA preparation

Samples (~100 mg) from hepatopancreas and gonad, proven to be major metabolic sites for PUFA biosynthesis (Monroig et al., 2012a, 2012b) were collected from an adult male cuttlefish (~110 g) captured through artisanal fisheries along the Mediterranean East Coast in Spain. The cuttlefish was anesthetised by placing it in ice water (~2 ºC) and thereafter sacrificed by disrupting the brain with a scalpel blade. Samples were immediately frozen in dry ice and stored at -80 ºC until further analysis. Total RNA from each tissue sample (one single replicate per tissue) (~100 mg) was extracted by homogenisation in Tri Reagent (Sigma-Aldrich, Alcobendas, Spain) following manufacturer's instructions. cDNA was synthesised from 2 μg total RNA using a GoScript Reverse Transcription System (Promega, Madison, WI, USA) using random primers.

Molecular cloning of the desaturase cDNA

In order to obtain the nucleotide sequence of the cuttlefish Fad cDNA, the amino acid (aa) sequence of the *O. vulgaris* Fad (gb|AEK20864.1|) was used for *in silico* searches of cuttlefish expressed sequence tags (ESTs) using NCBI tblastn tool (http://www.ncbi.nlm.nih.gov/). An EST (emb|FO198762.1|) from *S. officinalis*, consisting of a 669 bp fragment within the open reading frame (ORF) of the cuttlefish putative Fad, was identified as having high homology to the octopus desaturase. Gene-specific primers were designed for 5' and 3' rapid amplification of cDNA ends (RACE) PCR (FirstChoice® RLM-RACE kit, Ambion, Applied Biosystems, Warrington, UK) to produce full-length cDNA. For 5'RACE PCR, a positive fragment was obtained by two-round PCR. First round PCR was run on cDNA prepared with a mixture of hepatopancreas and gonad RNA, and using the adapter-specific 5'RACE OUTER primer and the gene-specific antisense primer SODR1 (Table 1). PCR consisted of an initial denaturing step at 95 ºC for 2 min, followed by 32 cycles of denaturation at 95 ºC for 30 s, annealing at 55 ºC for 30 s, extension at 72 ºC for 1 min 15 s, followed by a final extension at 72 ºC for 7 min (GoTaq® Colorless Master Mix, Promega). First round PCR products were used as template for nested PCR with primers 5'RACE INNER and SODR2 and in a 35-cycle reaction under the same thermal conditions as above. Similarly, the 3' end of the Fad cDNA was obtained through a two-round approach with first round using the primers SODF1 and 3'RACE OUTER for a PCR consisting of a first denaturation step at 95 ºC for 1 min, followed by 32 cycles of denaturation at 95 ºC for 30 s, annealing at 58 ºC for 30 s, extension at 72 ºC for 1 min 15 s, followed by a final extension at 72 ºC for 7 min. Primers SODF2 and 3'RACE INNER were used for the nested PCR with first round PCR product as template and

with the same conditions as the first round PCR. RACE PCR products were cloned into pGEM®T-Easy vector (Promega) and sequenced in the DNA Sequencing Service of the IBMCP-UPV (Valencia, Spain). Details of all primers used for the desaturase RACE PCRs are given in Table 1.

Molecular cloning of the elongase cDNA

The common octopus Elovl aa deduced sequence (gb|AFM93779.1|) was used for tblastn searches of cuttlefish ESTs. Four ESTs (gb|FO193872.1|, gb|FO202346.1|, gb|FO180217.1| and gb|FO200591.1|) with high homology to the *O. vulgaris* Elovl were identified and aligned (BioEdit v7.0.9, Tom Hall, Department of Microbiology, North Carolina State University, USA) to produce a contig of 781 bp comprising 219 bp of the 5' untranslated region (UTR) and approximately 563 bp of the ORF of the putative *S. officinalis* Elovl. Similarly to the Fad cloning strategy, a 3'RACE PCR enabled us to obtain the 3' end of the Elovl cDNA. The first-round PCR, with sense gene-specific primer SOEF1 and the adapter-specific 3' RACE OUTER primer (FirstChoice® RLM-RACE Ambion kit), consisted of an initial denaturing step at 95 ºC for 2 min, followed by 35 cycles of denaturation at 95 ºC for 30 s, annealing at 58 ºC for 30 s, extension at 72 ºC for 45 s, followed by a final extension at 72 ºC for 7 min. A 3' RACE positive product was obtained by nested PCR with primers SOEF2 and 3' RACE INNER and using the first PCR product as template and identical thermal conditions over 35 cycles. Details of primers used for the elongase 3'RACE PCR are given in Table 1.

Sequence and phylogenetic analyses

The deduced aa sequence identities of the newly cloned *S. officinalis* Fad and Elovl cDNAs were compared to homologous sequences from vertebrates (mammals and fish)

and non-vertebrate organisms, using the EMBOSS Needle Pairwise Sequence Alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle). Moreover, deduced aa sequences of the cloned *S. officinalis* cDNAs were aligned with homologous sequences using Bioedit. Thus, the common cuttlefish Fad was aligned with the *Mus musculus* FADS1 (gb|NP_666206.1|), *Danio rerio* dual Δ6Δ5 Fads2 (gb|NP_571720.2|) and two Fad functionally characterised as Δ5 desaturases from molluscs including *O. vulgaris* (gb|AEK20864.1|) and *C. nobilis* (gb|AIC34709.1|) (Liu et al., 2014a; Monroig et al., 2012a). Additionally, the common cuttlefish Elovl was aligned with *D. rerio* Elovl2 (gb|NP_001035452.1|) and Elovl5 (gb|NP_956747.1|), and the mollusc Elovl cDNAs from *O. vulgaris* (gb|AFM93779.1|) and *C. nobilis* (gb|AGW22128.1|) that have been functionally characterised to date (Liu et al., 2013; Monroig et al., 2012b). For phylogenetic analyses, the deduced aa sequences of the cuttlefish Fad and Elovl cDNAs and those from vertebrate and non-vertebrate organisms were compared by constructing a tree using the neighbor-joining method (Saitou and Nei, 1987), with confidence in the resulting tree branch topology measured by bootstrapping through 10,000 iterations. For the cuttlefish Fad, both Fad and stearoyl-CoA desaturases (Scd) from a variety of organisms were considered for the phylogenetic analysis. For the cuttlefish elongase, both invertebrate (molluscs) Elovl-like sequences and vertebrate Elovl from all subfamilies described to date (Elovl1-7) were included in the phylogenetic analysis (Jakobsson et al., 2006).

Functional characterisation of the cuttlefish Fad and Elovl by heterologous expression in Saccharomyces cerevisiae

PCR fragments corresponding to the ORF of the putative Fad and Elovl were amplified from a mixture of cDNA synthesised from hepatopancreas and gonad RNA

extracts, and using the high fidelity *Pfu* DNA Polymerase (Promega). PCR conditions consisted of an initial denaturing step at 95 ºC for 2 min, followed by 35 cycles of denaturation at 95 ºC for 30 s, annealing at 52 ºC for 30 s, extension at 72 ºC for 3 min, followed by a final extension at 72 ºC for 5 min. The primers containing restriction sites (underlined in Table 1) SODVF (*Hin*dIII) and SODVR (*Xho*I) for Fad, and SOEVF (*EcoR*I) and SOEVR (*Xho*I) for Elovl, were used for PCRs. DNA fragments obtained were purified, digested with the corresponding restriction enzymes (Promega), and ligated into a similarly restricted pYES2 yeast expression vector (Invitrogen, Paisley, UK). The purified plasmids (GenElute™ Plasmid Miniprep Kit, Sigma) containing the cuttlefish desaturase (pYES2-SOFad) and elongase (pYES2-SOElovl) ORF were then used to transform *S. cerevisiae* competent cells (S.c. EasyComp Transformation Kit, Invitrogen). Transformation and selection of yeast with recombinant pYES2-SOFad or pYES2-SOElovl plasmids, and yeast culture were performed as described in detail previously (Monroig et al., 2012a, 2012b).

In order to test the ability of the common cuttlefish Fad to act on saturated or monounsaturated FA, yeast transformed with pYES2 vector containing the cuttlefish desaturase as an insert (pYES2-SOFad) and no insert (control) were grown in *S. cerevisiae* minimal medium-uracil (SCMM$^{-uracil}$) with no exogenously added FA substrates. In order to investigate the function of the cuttlefish Fad to desaturate PUFA substrates, yeast transformed with pYES2-SOFad were grown in the presence of exogenously added substrates including C18 (18:3n-3 and 18:2n-6), C20 (20:3n-3, 20:2n-6, 20:4n-3 and 20:3n-6) and C22 (22:5n-3 and 22:4n-6) FAs. Exogenously added substrates were supplied to the yeast cultures at final concentrations of 0.5 (C18), 0.75 (C20) and 1.0 (C22) mM as uptake efficiency decreases with increasing chain length (Zheng et al., 2009). Similarly, the ability of the cuttlefish Elovl cDNA ORF to elongate

either saturated or monounsaturated FA, yeast transformed with either pYES2-SOElovl or empty pYES2 (control) were grown without exogenously added substrates. The ability of the cuttlefish Elovl to elongate PUFA substrates was tested by growing pYES2-SOElovl transgenic yeast in SCMM$^{-uracil}$ supplemented with FA substrates including C18 (18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6), C20 (20:5n-3 and 20:4n-6) and C22 (22:5n-3 and 22:4n-6).

Fatty acid analysis by GC-MS

FAs from the transgenic yeast were analysed by preparing methyl esters (FAME) as previously described (Monroig et al., 2013). Briefly, FAME were identified and quantified using an Agilent 6850 Gas Chromatograph coupled to a 5975 series Mass Selective Detector (MSD, Agilent Technologies, Santa Clara, CA, USA). The desaturation of potential substrates including the yeast endogenous saturated (16:0 and 18:0) and monounsaturated (16:1n-7, 18:1n-9, 18:1n-7) FA, as well as the exogenously added PUFA substrates (18:3n-3, 18:2n-6, 20:3n-3, 20:2n-6, 20:4n-3, 20:3n-6, 22:5n-3 and 22:4n-6) was calculated from the proportion of substrate (FA) converted to desaturated FA product as [product areas/(product areas+substrate area)] ×100. Similarly, the ability of the cuttlefish Elovl cDNA for elongation of yeast endogenous FAs and the exogenously added PUFA substrates (18:3n−3, 18:2n−6, 18:4n−3, 18:3n−6, 20:5n−3, 20:4n−6, 22:5n−3 and 22:4n−6) was calculated as described above for the desaturase. When further confirmation of double bond positions was required, GC-MS analysis of FA picolinyl esters, prepared from FAME according to Destaillats and Angers (2002) and modified by Li et al. (2010) was performed.

Materials

All FA substrates (all > 98-99 % pure) used for the functional characterisation assays, except for stearidonic acid (18:4n-3) and eicosatetraenoic acid (20:4n-3), were obtained from Nu-Chek Prep, Inc (Elysian, MN, USA). Stearidonic acid (> 99 % pure) and BHT, fatty acid-free BSA, galactose, 3-(hydroxymethyl) pyridine, nitrogen base, raffinose, tergitol NP-40 and uracil dropout medium were obtained from Sigma-Aldrich, Alcobendas, Spain. Eicosatetraenoic acid was purchased from Cayman Chemical Co., Ann Arbor, USA. Thin-layer chromatography (TLC) (20 x 20 cm x 0.25 mm) plates pre-coated with silica gel 60 were purchased from Merck, Darmstadt, Germany. All solvents were HPLC grade and were from VWR, Llinars del Vallés, Spain.

Results

S. officinalis Fad and Elovl sequences and phylogenetics

The cuttlefish Fad cDNA encoded a putative protein of 445 aa with three histidine boxes (HXXXH, HXXHH and QXXHH), a putative cytochrome b5-like domain and a heme-binding motif (HPGG), all typical features of "front-end" desaturases (Fig. 1). The deduced aa sequence of the cuttlefish Fad was 80.7 % identical to the *O. vulgaris* Fad (Monroig et al., 2012a), 60.2 % identical to bivalve *C. nobilis* Fad (Liu et al., 2014a) and 53.6-65.8 % identical to homologues form the gastropods *Lottia gigantea* and *H. discus hannai* (Li et al., 2012). Lower identities (< 52.0 %) were observed when the cuttlefish Fad was compared with vertebrate (mammals and fish) front-end desaturases (Fads). This similarity pattern was reflected in the phylogenetic tree (Fig. 2). The common cuttlefish Fad grouped together with other Fad-like sequences from molluscs, which form a cluster basal to the three fatty acyl desaturases described in vertebrate linages, namely Fads1, Fads2 and Fads3. Moreover, other desaturases including the Δ5 desaturases from *Thraustochytrium* sp. (gb|AAM09687.1|) and *C.*

elegans (gb|AAC95143.1) clustered more distantly to the mollusc Fad group. The cuttlefish Fad sequence was deposited in the GenBank database with accession number KP260645.

The cuttlefish Elovl cDNA consisted of a protein of 295 aa, whose deduced aa sequence contained the diagnostic histidine box (HXXHH) conserved in all members of the Elovl protein family, as well as two lysine (K) residues at the carboxyl terminus (KKXX), regarded as putative endoplasmic reticulum retrieval signals (Jakobsson et al., 2006) (Fig. 3). The deduced aa sequence of the cuttlefish Elovl showed 77.7 % identity to the common octopus Elovl previously characterised (Monroig et al., 2012b), with lower identity scores to homologues from the bivalve *C. nobilis* (noble scallop) (49.5 %) (Liu et al., 2013) and the gastropod mollusc *L. gigantea* (57.2 %). It is important to note that lower identity scores were obtained when the *S. officinalis* Elovl sequence was compared to another elongase sequence existing in molluscs with high homology to vertebrate Elovl4. In agreement, the phylogenetic tree, constructed on the basis of aa sequence comparisons, grouped the cuttlefish Elovl very closely to other Elovl-like sequences from molluscs, particularly those from the cephalopods *Euprymna scolopes* and *O. vulgaris* (Fig. 4). Interestingly, this group of mollusc Elovl including the newly cloned cuttlefish elongase clustered basal to Elovl2 and Elovl5 proteins from vertebrates. Some molluscs such as the gastropods *L. gigantea* and *Aplysia californica* possess putative Elovl proteins with high homology to vertebrate Elovl4, an elongase with demonstrated roles in the biosynthesis of very long chain (>C24) fatty acids (Agbaga et al., 2008; Monroig et al., 2010, 2011b). The cuttlefish Elovl sequence was deposited in the GenBank database with accession number KP260646.

Functional characterisation of the S. officinalis Fad and Elovl in yeast

Functional characterisation by heterologous expression in yeast revealed that the cuttlefish Fad and Elovl cDNAs have similar functions to those of the octopus homologues (Monroig et al., 2012a, 2012b). The activity of common cuttlefish putative Fad was determined by expressing its ORF in yeast *S. cerevisiae* that were grown in the presence of potential desaturase FA substrates. The ability of the cuttlefish Fad to introduce double bonds into saturated or monounsaturated FA was investigated by comparing the FA profiles of yeast transformed with pYES2-SOFad or empty pYES2 (control). Typically, the FA profile of yeast transformed with empty vector (empty pYES2) consisted of the main endogenous FA of *S. cerevisiae*, namely 16:0, 16:1 isomers (16:1n-9 and 16:1n-7), 18:0, 18:1n-9 and 18:1n-7 (data not shown) and the exogenously added PUFA (data not shown). This is consistent with the well-established inability of the endogenous desaturases and elongases of *S. cerevisiae* to metabolise PUFA (Hastings et al., 2001; Agaba et al., 2004). Importantly, the FA profiles of yeast transformed with pYES2-SOFad had two additional peaks identified by GC-MS as 16:1n-11 and 18:1n-13, corresponding to the Δ5-desaturation products of the yeast endogenous FA 16:0 and 18:0, respectively. Both Δ5-desaturated monoenes were detected regardless of exogenously added PUFA substrate (all panels in Fig. 5). Conversions of 16:0 and 18:0 to 16:1n-11 and 18:1n-13, respectively, by transgenic yeast were determined as 25 % and 69 %, respectively (Table 2). Clearly, these results confirmed that the common cuttlefish Fad could be involved in the biosynthesis of monounsaturated FAs. No activity towards yeast endogenous monounsaturated FA 18:1n-9, 18:1n-7 and 16:1n-7 was detected in transgenic yeast expressing the *S. officinalis* Fad.

The role of the common cuttlefish Fad in PUFA biosynthesis was determined by growing the transgenic yeast transformed with pYES2-SOFad with a range of substrates

including 18:3n-3, 18:2n-6, 20:3n-3, 20:2n-6, 20:4n-3, 20:3n-6, 22:5n-3 and 22:4n-6. No activity towards 18:3n-3, 18:2n-6, 22:5n-3 and 22:4n-6 was detected. However, the transgenic yeast expressing the ORF of the cuttlefish Fad converted 20:4n-3 and 20:3n-6 to 20:5n-3 ($^{\Delta5,8,11,14,17}$20:5) and 20:4n-6 ($^{\Delta5,8,11,14}$20:4), respectively (Fig. 5A and B), both being Δ5-desaturation products. Furthermore, the cuttlefish Fad also exhibited Δ5-desaturation ability towards 20:3n-3 ($^{\Delta11,14,17}$20:3) and 20:2n-6 ($^{\Delta11,14}$20:2), which were converted into the non-methylene interrupted (NMI) FA $^{\Delta5,11,14,17}$20:4 and $^{\Delta5,11,14}$20:3, respectively (Table 2; Fig. 5C and D).

The common cuttlefish Elovl-like cDNA was also functionally characterised in yeast. Thus, yeast transformed with pYES2-SOElovl were able to elongate some of the PUFA substrates assayed. Thus, the exogenously added C18 (18:3n-3, 18:2n-6, 18:4n-3 and 18:3n-6) and C20 (20:5n-3 and 20:4n-6) substrates were elongated to C20 (20:3n-3, 20:2n-6, 20:4n-3 and 20:3n-6) and C22 (22:5n-3 and 22:4n-6) products, respectively (Table 3; Fig. 6). Conversions to elongated products exhibited by the common cuttlefish Elovl were above 30 %, except for the elongation on 20:5n-3, of which 8 % was converted to 22:5n-3. Interestingly, the common cuttlefish Elovl did not show elongation activity towards any of the C22 PUFA substrates assayed, namely 22:5n-3 and 22:4n-6 (Table 3). The cuttlefish Elovl showed some activity towards monounsaturated FAs, as yeast endogenous 16:1n-7 (peak 2 in Fig. 6) appeared to be elongated to 18:1n-7 (shoulder on peak 4 in Fig. 6).

Discussion

The study of the quantitative EFA requirements in early life stages of aquatic species like cephalopods is challenging due to a lack of understanding of their physiology and metabolism. In addition to the inherent difficulty in obtaining individuals for

performing feeding trials and the lack of adequate diets where varying levels of PUFAs can be established, the most fundamental question that must be answered is which specific FAs satisfy the essential requirements in that particular species. In cephalopods, as for any animal, the specific EFAs that must be supplied in the diet to satisfy normal growth and development depend upon the enzymatic capabilities of desaturation and elongation existing in that species (Bell and Tocher, 2009). Following a similar approach as that taken for investigating EFA requirements in *O. vulgaris*, here we report on the molecular cloning and functional characterisation of two enzymes that participate in the biosynthesis of PUFA in the common cuttlefish *S. officinalis*, namely a Fad and an Elovl. Altogether, the investigations on both *O. vulgaris* and *S. officinalis* have allowed the prediction of the putative PUFA biosynthetic pathways in cephalopods (Fig. 7).

The phylogenetic analysis comparing the amino acid (aa) sequence of the newly cloned *S. officinalis* Fad revealed its high homology with other Fad-like enzymes from a range of molluscs including the cephalopod *O. vulgaris*, the gastropods *L. gigantea*, *H. discus hannai*, *A. californica*) and the bivalves *C. nobilis* and *Crassostrea gigas*. In agreement with our observations from the molecular cloning of the *O. vulgaris* Fad (Monroig et al., 2012a), there was no evidence to support common cuttlefish possessing any other Fad apart from that cloned in the present study. However, it cannot be completely eliminated the possibility that another Fad exists in the cuttlefish genome as, contrary to previous hypotheses based on *in silico* searches among *L. gigantea* and *A. californica* genomes (Monroig et al., 2012a), some molluscs possess more Fad genes in their genomes. Thus, it has been recently published that the gastropod *H. discus hannai* has two distinct Fad-encoding genes (Li et al., 2013). Interestingly, the gene products of the two *H. discus hannai* Fad cDNAs were functionally characterised as Δ5 desaturases

(Li et al., 2013) but it remains unclear what advantage this could bring to overall PUFA biosynthetic capacity. More clearly though, our present data show that, regardless of the number of desaturases, all Fad-like enzymes identified from molluscs formed a group that was basal to vertebrate Fads-like desaturases including Fads1 and Fads2, enzymes with well-demonstrated roles in long-chain (≥ C20) PUFA biosynthesis (Guillou et al., 2010). These results support a putative role for the newly cloned *S*. *officinalis* Fad in the endogenous production of PUFA and thus would be potentially critical in determining the EFA in this species.

Similar to mollusc Fad phylogeny, the Elovl isolated from cuttlefish, as well as others from molluscs, clustered as a group at the base of the vertebrate Elovl2 and Elovl5 family groups. This indicated that the cuttlefish Elovl was closely related to the Elovl families Elovl5 and Elovl2 with well-demonstrated roles in the biosynthesis of long-chain PUFA in vertebrates (Jakobsson et al., 2006). However, the phylogenetic analysis delineating the cuttlefish and other mollusc Elovl as basal sequences to the distinct vertebrate families Elovl5 and Elovl2 led us to term this protein as "Elovl5/2" (Monroig et al., 2013). Supporting the phylogenetic data, the aa sequence analysis of the newly cloned Fad and Elovl showed they possess all typical features among these protein families. Thus, for the cuttlefish Fad, conserved functional domains including three histidine boxes HXXXH, HXXHH and QXXHH, the putative cytochrome b5-like domain, and the heme-binding motif, HPGG, could be predicted in common with front-end desaturases (Sperling et al., 2003). For the Elovl, five trans-membrane regions, an endoplasmic reticulum retrieval signal at the C terminus (KKXX) and a diagnostic histidine box (HXXHH) were identified (Jakobsson et al., 2006; Leonard et al., 2004). The phylogenetic and sequence analyses, as well as the functional characterisation

assays conducted in yeast, strongly support that the Fad and Elovl cDNAs isolated from *S. officinalis* encoded enzymes with a role in the biosynthesis of PUFA including EFA.

The gene product of the cuttlefish Fad cDNA showed Δ5-desaturase specificity when expressed in yeast as previously reported for the *O. vulgaris* Fad (Monroig et al., 2012a). The cuttlefish Δ5 Fad was able to desaturate 20:4n-3 and 20:3n-6 to EPA (20:5n-3) and ARA (20:4n-3), respectively. Thus, strictly speaking, neither EPA nor ARA can be regarded as dietary EFA for the cuttlefish and other cephalopods like the common octopus, as they can be produced from adequate FA precursors by endogenous enzymes. However, as discussed for the octopus Δ5 Fad, it is highly unlikely that dietary supply of 20:4n-3 and 20:3n-6 can satisfy the physiological demands for EPA and ARA. Additionally, biosynthesis of 20:4n-3 and 20:3n-6 themselves appears to be not possible, as no desaturases with the Δ6 or Δ8 activities required to produce 20:4n-3 and 20:3n-6 from precursors appear to exist in cephalopods (Monroig et al., 2012a). Interestingly, Liu et al. (2014b) have recently reported that *C. nobilis* possesses a desaturase with Δ8 activity that was able to biosynthesise 20:4n-3 and 20:3n-6 from 20:3n-3 and 20:2n-6, respectively, opening thus the possibility that other molluscs might also have similar desaturases. Moreover, a recent study suggested that the Δ6 [1-^{14}C]-labelled FA substrates 18:3n-3 and 18:2n-6 could not be desaturated by *O. vulgaris* hatchlings (Reis et al., 2014). While current data suggest that Δ6 desaturase appear not to exist in cephalopods and putative Δ8 desaturase have not been yet confirmed, we postulate that EPA and ARA are indeed EFA for cephalopods including *S. officinalis*.

Beyond its role in the biosynthesis of PUFA including EPA and ARA, the cuttlefish Fad was demonstrated to have other roles in FA biosynthesis. Firstly, the cuttlefish Δ5 Fad has the ability to efficiently introduce a Δ5 desaturation into saturated fatty acids including 16:0 and 18:0 producing 16:1n-11 ($^{\Delta5}$16:1) and 18:1n-13 ($^{\Delta5}$18:1),

respectively. Such desaturation capability seems to be widespread among molluscs as the Δ5 Fad characterised from *C*. *nobilis* (Liu et al., 2014b) and the FA profiles from *Lunatia triseriata* and *Littorina littorea* containing both 18:1n-13 and 20:1n-5 ($^{\Delta5}$20:1) suggested the presence of Δ5 desaturases (Joseph, 1982). Second, the cuttlefish Δ5 Fad has a role in the biosynthesis of NMI FA, a group of PUFA with particular desaturation patterns that have been reported to occur in marine invertebrates but whose functions are yet to be identified (Barnathan, 2009; Kornprobst and Barnathan, 2010). Consistent with the octopus Fad, the cuttlefish Δ5 Fad showed the ability to desaturate the FA substrates 20:3n-3 ($^{\Delta11,14,17}$20:2) and 20:2n-6 ($^{\Delta11,14}$20:2), respectively, into $^{\Delta5,11,14,17}$20:4 and $^{\Delta5,11,14}$20:3. The presence of NMI FA in tissues collected from the common octopus has been demonstrated (Monroig et al., 2012a, 2012b), but, as far as we are concerned, there are no data on NMI FA from *S*. *officinalis* available in the literature. Compared to other invertebrates including molluscs (Barnathan, 2009), the abundance of NMI FA in the tissues of *O*. *vulgaris* was relatively low and thus it may be that NMI FA in cephalopod tissues derives from dietary origin rather than from endogenous biosynthesis. Indeed, both *S*. *officinalis* and *O*. *vulgaris*, particularly their juvenile stages, feed on other invertebrates that themselves accumulate large amounts of NMI FA that are consequently accumulated in their tissue lipids. In the case of *S*. *officinalis*, fish are a preferred dietary source for adult stages (Castro and Guerra, 1990) and thus it is expected that the levels of NMI FA are even lower than those detected in *O*. *vulgaris* (Monroig et al., 2012a, 2012b).

Molluscs have been regarded as having active FA elongation pathways and studies in *O*. *vulgaris* have shown that this is also the case in cephalopods (Monroig et al., 2012b; Reis et al., 2014). Indeed, the present data clearly showed that the cuttlefish Elovl had the ability to elongate C18 and C20 PUFA substrates, but showed no activity towards

C22 PUFA. These results were consistent with the substrate specificities of the common octopus Elovl5/2 characterised previously (Monroig et al., 2012b) and, more recently, the scallop *C. nobilis* (Liu et al., 2013). The efficiency exhibited by the cuttlefish Elovl to elongate C18 PUFA suggests that all the required elongase activities required for biosynthesis of EPA and ARA from C18 PUFA exist in this species. On the contrary, the Elovl reported here lacked any ability to elongate C22 PUFA to C24. Thus, although EPA can to some extent be elongated to 22:5n-3, in the absence of any other elongase, the Elovl5/2 can only contribute to the biosynthesis of DHA if there is subsequent Δ4 desaturation of 22:5n-3. As described above, such a desaturase capability is yet to be found in molluscs. Alternatively, as accepted for most vertebrates (Sprecher, 2000), biosynthesis of DHA could proceed from 22:5n-3 through a further elongation step to 24:5n-3 by the action of another elongase distinct to the here cloned Elovl5/2. Preliminary data obtained from the common octopus suggested that cephalopods posses an Elovl4 with the ability to elongate 22:5n-3 to 24:5n-3 (Monroig et al., 2014). However, lack of a desaturase with Δ6 desaturase activity would constrain the biosynthesis of DHA and, in conclusion, DHA also appears to be an EFA for the cuttlefish.

In summary, the present study demonstrated that the common cuttlefish expresses Fad- and Elovl-like genes that participate in PUFA biosynthesis pathways. Functional characterisation of the cuttlefish Fad and Elovl cDNAs enabled to hypothesise the PUFA biosynthetic pathways in this species (Fig. 7). Thus, it is suggested that EPA and ARA are essential dietary FA for *S. officinalis* as their biosynthesis from 20:4n-3 and 20:3n-6, respectively, is likely to be limited. First, the availability of 20:4n-3 and 20:3n-6 in natural diets for cuttlefish are extremely low and, second, the endogenous production of 20:4n-3 and 20:3n-6 via biosynthesis from C18 PUFA (18:2n-6 and

18:3n-3) appears to be restricted by the apparent absence of key desaturase (Δ6 or Δ8) activities. Similarly, lack of Δ4- or Δ6-desaturase activities strongly suggested that DHA cannot be endogenously biosynthesised by cuttlefish and therefore it must be supplied in the diet to ensure normal growth.

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Figure legends

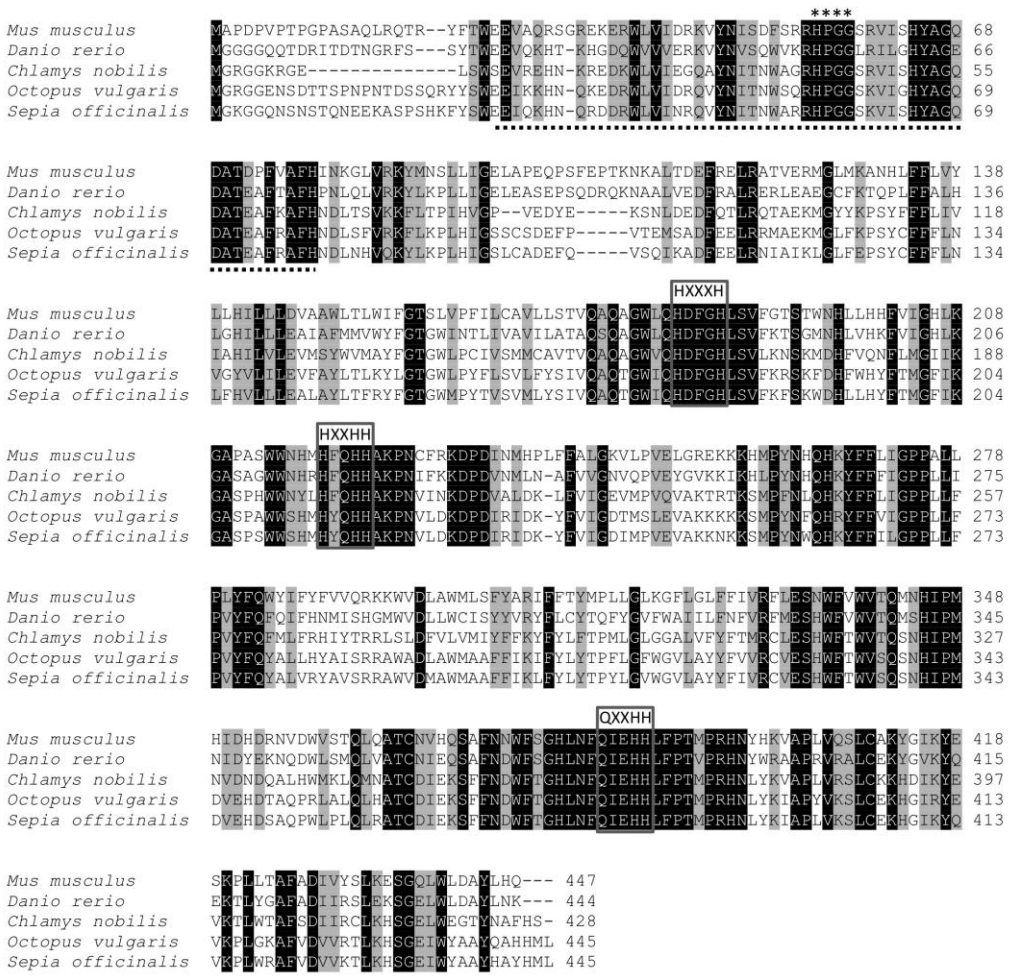

Fig 1. Alignment of the deduced amino acid (aa) sequence of the newly cloned Δ5 fatty acyl desaturase (Fad) from the common cuttlefish *Sepia officinalis*. The aa sequence of the cuttlefish Fad was aligned with the *Mus musculus* FADS1 (gb|NP_666206.1|), the dual Δ6Δ5 Fads2 *Danio rerio* (gb|NP_571720.2|), and two mollusc Δ5 desaturases functionally characterised from *Octopus vulgaris* (gb|AEK20864.1|) and *Chlamys nobilis* (gb|AIC34709.1|). Deduced aa sequences were aligned using ClustalW (Bioedit). Identical residues are shaded black and similar residues are shaded grey. Identity/similarity shading was based on the BLOSUM62 matrix. The cytochrome b_5-like domain is dot-underlined and the three histidine boxes (HXXXH, HXXHH and QXXHH) are highlighted with grey squares. The asterisks on the top indicate the heme-binding motif, HPGG.

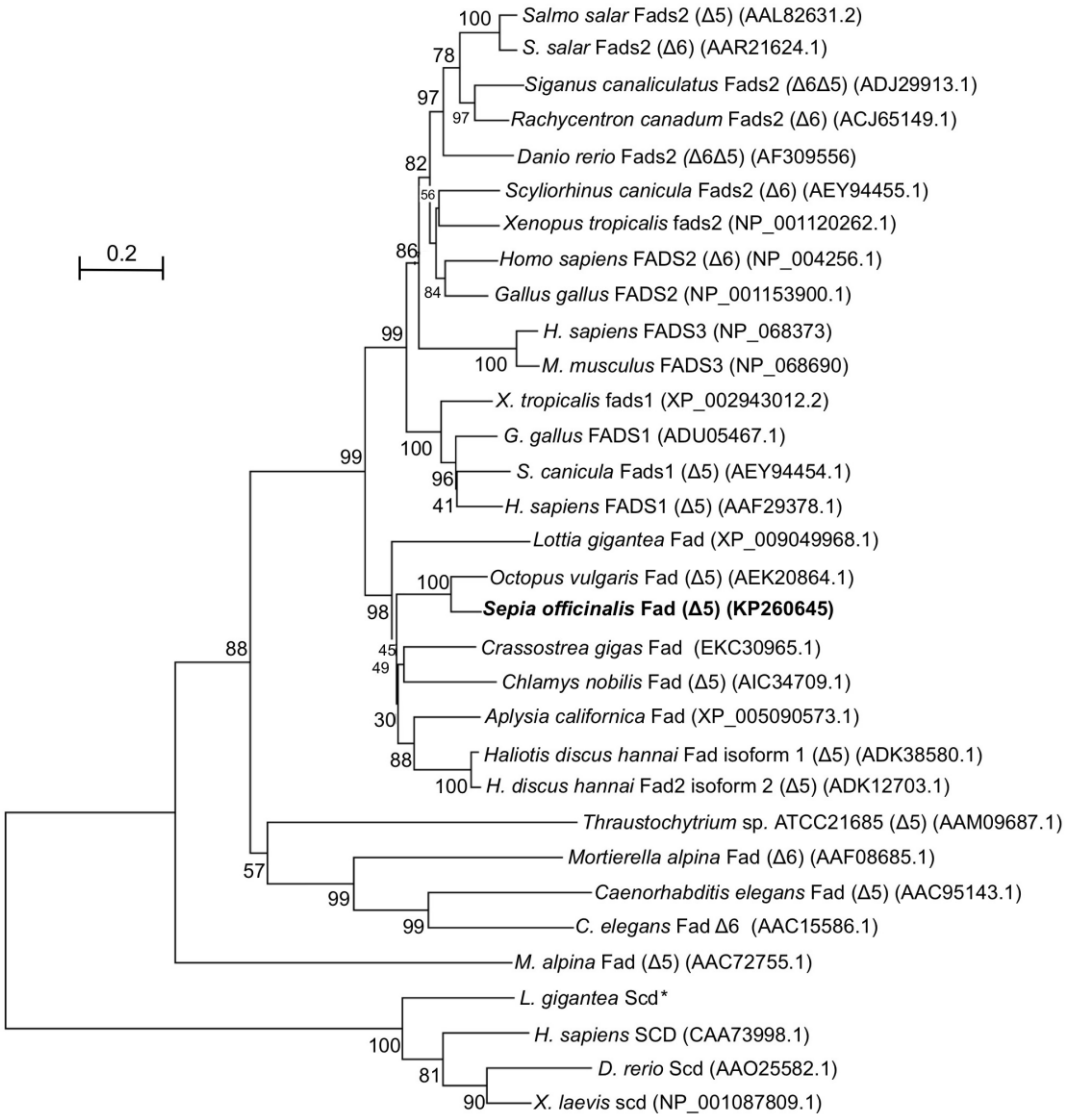

Fig 2. Phylogenetic tree comparing the deduced amino acid (aa) sequence of the newly cloned *Sepia officinalis* fatty acyl desaturase (Fad) with other Δ5- and Δ6-like Fad from several organisms. Additionally, the aa sequences of several stearoyl-CoA desaturase (Scd) were included in the analysis. The tree was constructed using the neighbour-joining method (Saitou and Nei, 1987) with MEGA4. The horizontal branch length is proportional to aa substitution rate per site. The numbers represent the frequencies (%) with which the tree topology presented was replicated after 10,000 iterations.

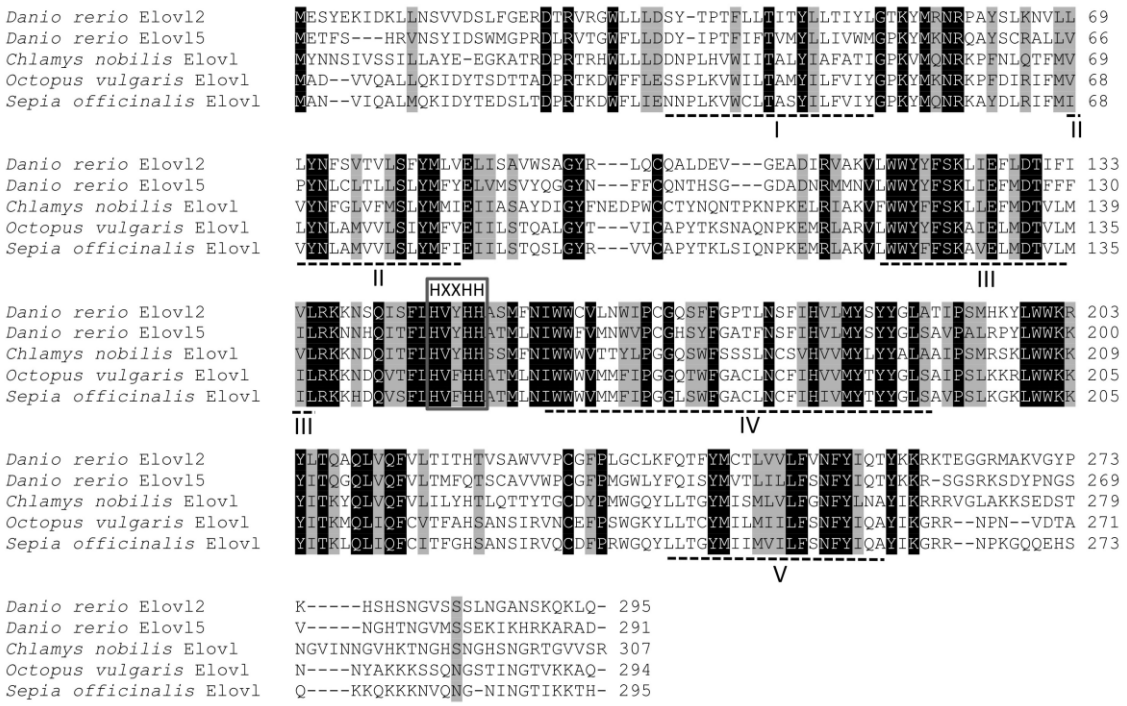

Fig 3. Alignment of the deduced amino acid (aa) sequence of the newly cloned elongation of very long-chain fatty acid (Elovl) protein from the common cuttlefish *Sepia officinalis*. The aa sequence of the cuttlefish Elovl was aligned with the *Danio rerio* Elovl2 (gb|NP_001035452.1|) and Elovl5 (gb|NP_956747.1|), and two mollusc Elovl-like sequences functionally characterised from *Octopus vulgaris* (gb|AFM93779.1|) and *Chlamys nobilis* (gb|AGW22128.1|). Deduced aa sequences were aligned using ClustalW (Bioedit). Identical residues are shaded black and similar residues are shaded grey. Identity/similarity shading was based on the BLOSUM62 matrix. The histidine box (HXXHH) conserved among Elovl family members is highlighted with a grey square. Five (I-V) transmembrane-regions predicted by InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) are dot-underlined.

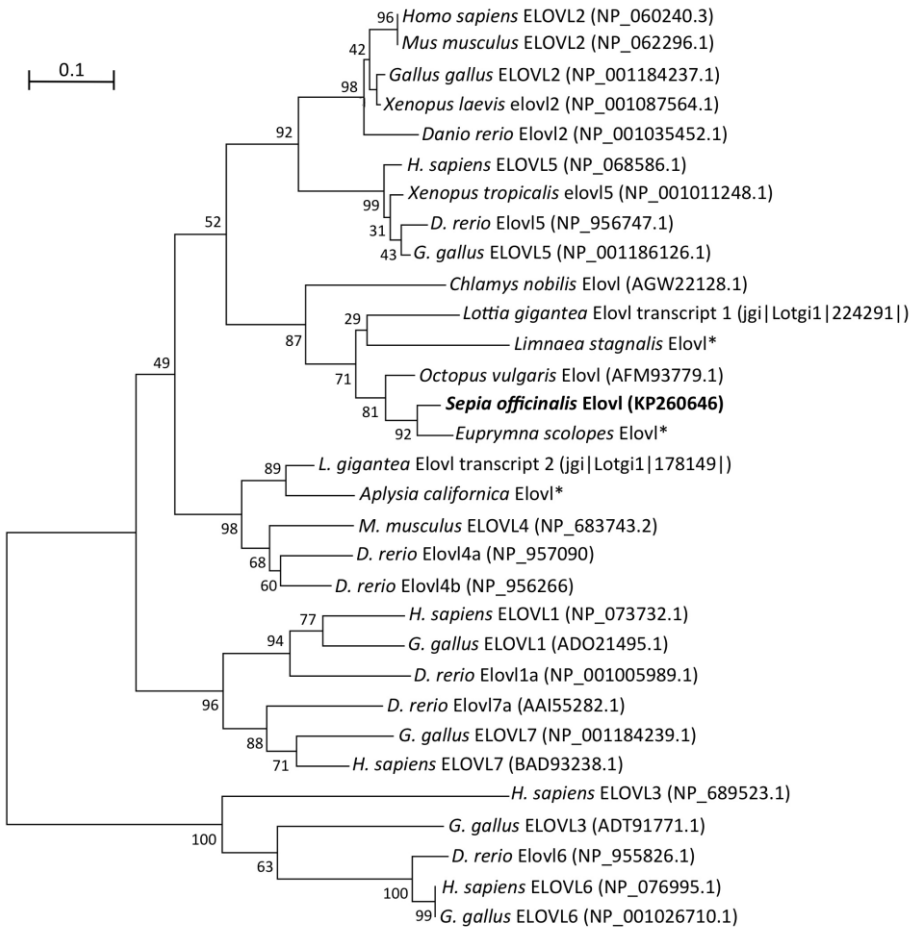

Fig 4. Phylogenetic tree comparing the deduced amino acid (aa) sequence of the *Sepia officinalis* elongation of very long-chain fatty acids (Elovl) protein with a series of sequences including representatives of the seven (1-7) Elovl subtypes and other Elovl-like sequences from invertebrate organisms. All accession numbers are from GenBank database, except for *Lottia gigantea* elongases where JGI protein ID are given (http://genome.jgi-psf.org/Lotgi1/Lotgi1.home.html). Asterisks indicate the aa sequences deduced from searches and subsequent assembly of expressed sequence tags (EST) using NCBI tblastn tool (http://www.ncbi.nlm.nih.gov/). The tree was constructed using the neighbour-joining method (Saitou and Nei 1987) with MEGA4. The horizontal branch length is proportional to aa substitution rate per site. The numbers represent the frequencies (%) with which the tree topology presented was replicated after 10,000 iterations.

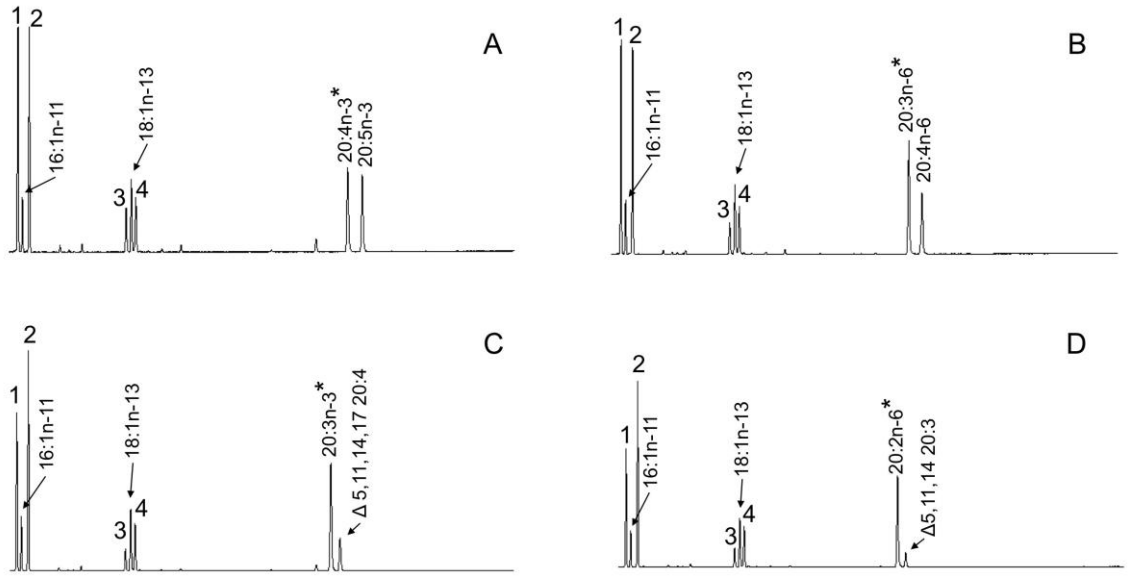

Fig 5. Functional characterisation of the newly cloned *S*. *officinalis* Fad in yeast. The FA profiles were determined from yeast expressing the coding region of the common cuttlefish Fad cDNA as an insert and grown in the presence of one of the exogenously added substrates 20:4n-3 (A), 20:3n-6 (B), 20:3n-3 (C) or 20:2n-6 (D). Peaks 1-4 in all panels are the main endogenous FA of *S*. *cerevisiae*, namely 16:0 (1), 16:1 isomers (2), 18:0 (3), 18:1n-9 (4). Additionally, peaks derived from exogenously added substrates (*) or desaturation products are indicated accordingly. Peaks corresponding to the Δ5-mononenes 16:1n-11 and 18:1n-13 are also indicated in all panels. Vertical axis, FID response; horizontal axis, retention time.

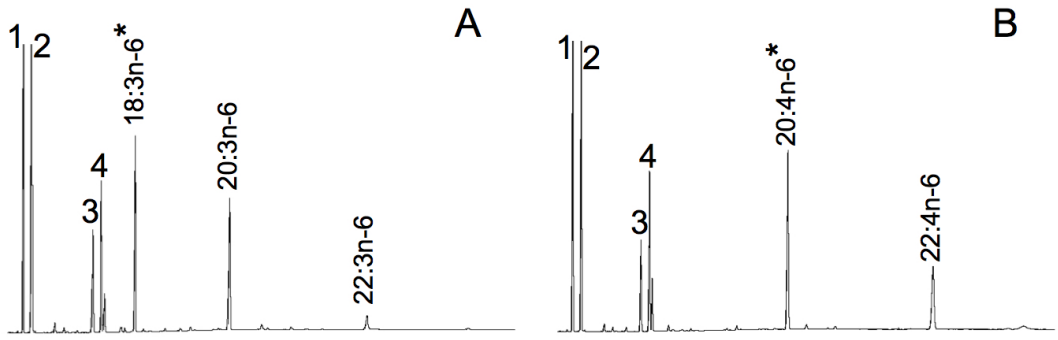

Fig 6. Functional characterisation of the *S*. *officinalis* Elovl in yeast *S*. *cerevisiae*. Yeast expressing the coding region of the cuttlefish Elovl were able to elongate the exogenously added PUFA 18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6 (A), 20:5n-3, 20:4n-6 (B), but showed no activity towards 22:5n-3 and 22:4n-6. Peaks 1-4 in all panels are the main endogenous FA of *S*. *cerevisiae*, namely 16:0 (1), 16:1 isomers (2), 18:0 (3), 18:1n-9 (4). Additionally, peaks derived from exogenously added substrates (*) or elongation products are indicated accordingly. Small peaks immediately after 18:1n-9 (peak no. 4) corresponds to 18:1n-7 and thus denotes that the cuttlefish Elovl had the ability to elongate the yeast endogenous 16:1n-7. Vertical axis, FID response; horizontal axis, retention time.

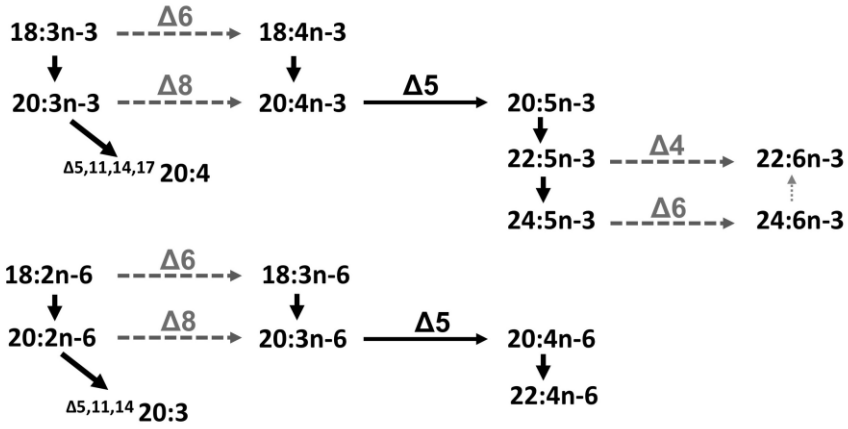

Fig 7. Putative PUFA biosynthetic pathways in the common cuttlefish (*S*. *officinalis*) from C18 PUFA, α-linolenic acid (18:3n-3) and linoleic acid (18:2n-6). Solid arrows indicate demonstrated activities, whereas dashed arrows show vertebrate-based activities not determined in the common cuttlefish. Horizontal arrows are desaturation reactions and vertical downwards arrows denote elongation reactions.

Tables

Table 1. Sequences of the primer pairs used and accession numbers of the sequences used as references for primer design in the cloning of the cuttlefish fatty acyl desaturase (Fad) and elongation of very long-chain fatty acid (Elovl) protein cDNA sequences. Restriction sites for *Hin*dIII and *Xho*I are underlined in SODVF and SODVR sequences, respectively, whereas restriction sites for *Eco*RI and *Xho*I are underlined in SOEVF and SOEVR sequences, respectively.

Aim	Transcript	Primer	Primer sequence	Accession No[1].
5' RACE PCR	*Fad*	SODR3	5'-TGCTGCCAGTTATATGGCAT-3'	KP260645
		SODR2	5'-TCAGGGTCTTTATCCAGCACA-3'	
3' RACE PCR	*Fad*	SODF1	5'-AGTCTGGGGTGTATTGGCATA-3'	KP260645
		SODF2	5'-ACTTGTTCCCAACAATGCCA-3'	
	Elovl	SOEF1	5'-CCAAAGCTGTTGAACTAATGGAC-3'	KP260646
		SOEF2	5'-ATTTGGTGGTGGGTCATGAT-3'	
ORF cloning	*Fad*	SODVF	5'- CCCAAGCTTAATATGGGTAAAGGAGGACA-3'	KP260645
		SODVR	5'- CCGCTCGAGTTATAACATATGATAAGCATG-3'	
	Elovl	SOEVF	5'-CCCGAATTCAAAATGGCAAATGTTATAC-3'	KP260646
		SOEVR	5'-CCGCTCGAGCAAAAGTTAATGTTAATGGGT-3'	

[1]GenBank accession numbers.

Table 2. Substrate conversions of yeast Saccharomyces cerevisiae transformed with pYES2-SOFad containing the open reading frame (ORF) of the cuttlefish desaturase. Transgenic yeast were grown in presence of yeast endogenous saturated (16:0 and 18:0) and monounsaturated (16:1n-7, 18:1n-9 and 18:1n-7) fatty acid (FA) substrates, and the exogenously added polyunsaturated FA substrates 18:3n-3, 18:2n-6, 20:3n-3, 20:2n-6, 20:4n-3, 20:3n-6, 22:5n-3 and 22:4n-6. Only FA substrates that were converted to desaturated products are shown. Results are expressed as a percentage of total FA substrate converted to desaturated product. FA are designated using the 'n-' nomenclature, except for non-methylene interrupted FA where the 'Δ' nomenclature was used.

FA substrate	**Product**	**Conversion (%)**
Saturates		
16:0	16:1n-11	25
18:0	18:1n-13	69
Polyunsaturates		
20:3n-3	$^{\Delta 5,11,14,17}$ 20:4	24
20:2n-6	$^{\Delta 5,11,14}$ 20:3	14
20:4n-3	20:5n-3	48
20:3n-6	20:4n-6	39

Table 3. Functional characterisation of the cuttlefish elongation of very long-chain fatty acid (Elovl) protein in *Saccharomyces cerevisiae*. Results are expressed as a percentage of total fatty acid (FA) substrate converted to elongated products.

FA Substrate	**Product**	**% Conversion**	**Activity**
18:3n-3	20:3n-3	32	C18→20
18:2n-6	20:2n-6	50	C18→20
18:4n-3	20:4n-3	51	C18→20
18:3n-6	20:3n-6	51	C18→20
20:5n-3	22:5n-3	8	C20→22
20:4n-6	22:4n-6	37	C20→22
22:5n-3	24:5n-3	0	C22→24
22:4n-6	24:4n-6	0	C22→24