Title

Biosynthesis of essential fatty acids in *Octopus vulgaris* (Cuvier, 1797): Molecular cloning, functional characterisation and tissue distribution of a fatty acyl elongase

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Summary

Polyunsaturated fatty acids (PUFA) have been identified as key nutrients for the common octopus (*Octopus vulgaris*), particularly for its early life-cycle stages (paralarvae). Our overarching aim is to identify the dietary essential fatty acid (FA) for octopus paralarvae through characterisation of the enzymes of endogenous PUFA biosynthetic pathways. Here we report on the molecular cloning and functional characterisation of a cDNA encoding a putative elongase of very long-chain fatty acids (Elovl), a critical enzyme that catalyses the elongation of FA including PUFA. Our results suggest that the octopus Elovl is phylogenetically related to Elovl5 and Elovl2, two elongases with demonstrated roles in PUFA biosynthesis in vertebrates. Further evidence supporting a role of the octopus Elovl in PUFA biosynthesis was provided through functional characterisation of its activity in yeast. It was confirmed that expression of the octopus Elovl conferred on yeast the ability to elongate some C18 and C20 PUFA, while C22 PUFA substrates remained unmodified. Therefore, the substrate specificities exhibited by the octopus elongase were consistent with those of vertebrate Elovl5. Interestingly, the octopus Elovl elongated n-6 PUFA substrates more efficiently than their homologous n-3 substrates, suggesting that n-6 PUFA may have particular biological significance in *O. vulgaris*. Finally, we investigated the potential role of the newly cloned Elovl in the biosynthesis of non-methylene-interrupted FA, compounds typically found in marine invertebrates and confirmed to be also present in the common octopus.

Keywords

Essential fatty acids; fatty acyl elongase; non-methylene interrupted fatty acids; *Octopus vulgaris*; polyunsaturated fatty acids

Abbreviations

ARA, arachidonic acid

BHT, butylated hydroxy toluene

DHA, docosahexaenoic acid

Elovl, elongase of very long-chain fatty acids

EPA, eicosapentaenoic acid

EST, expressed sequence tag

FA, fatty acid

Fad, fatty acyl desaturase

FAME, fatty acid methyl ester

GC-MS, gas chromatography-mass spectrometry

NMI FA, non-methylene interrupted fatty acid

ORF, open reading frame

OD, optical density

PCR, polymerase chain reaction

PUFA, polyunsaturated fatty acid

Introduction

Cephalopods have emerged as prime candidates for diversifying aquaculture. Among the species studied, the common octopus (*Octopus vulgaris*, Cuvier, 1797) has received special attention and relevant aspects of its culture such as husbandry (Iglesias et al., 2006; Estefanell et al., 2012), behaviour (Di Cristo et al., 2005; Valverde and García, 2005), reproduction (Otero et al., 2007; Wodinsky, 2008; Estefanell et al., 2010), pathologies (Castellanos-Martínez and Gestal, 2011) and nutrition (Villanueva, 1994, Navarro and Villanueva, 2000, 2003; Villanueva et al., 2004, 2009; Villanueva and Bustamante, 2006; Quintana, 2009; Seixas et al., 2010; Estefanell et al., 2011; Fuentes et al., 2011; Viciano et al., 2011) have been studied. Despite considerable effort, the production of the common octopus in captivity is limited to on-growing wild-captured specimens in floating cages (Iglesias et al., 2007), as the octopus life cycle has not yet been closed at commercial scale. While limited success in the production of juvenile octopuses has been achieved (Villanueva, 1995; Iglesias et al., 2002, 2004), the massive mortalities occurring during early life-cycle stages (paralarvae) have become an, as yet, unresolved zootechnical issue that requires further investigation.

Polyunsaturated fatty acids (PUFA), in particular docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), have been previously suggested as critical dietary components for octopus paralarvae (Navarro and Villanueva, 2003). We have recently initiated a series of studies to identify the dietary essential fatty acids (FA) for octopus paralarvae. Due to the obvious difficulties in conducting feeding trials with octopus paralarvae, our approach is to characterise the enzymes involved in PUFA biosynthesis as they dictate the ability of a certain species to endogenously produce PUFA (Bell and Tocher, 2009). These studies will help us to design balanced diets for octopus paralarvae that do not compromise their endogenous capabilities for PUFA biosynthesis.

Previously, we reported the molecular cloning and functional characterisation of a fatty acyl desaturase (Fad) from *O. vulgaris* (Monroig et al., 2012a). The substrate specificity of the octopus Fad revealed that this enzyme was a Δ5-like Fad and thus we provided for the first time molecular evidence of such an enzymatic activity in molluscs (Monroig et al., 2012a). Interestingly, the Δ5 Fad potentially enables the common octopus to endogenously convert 20:4n-3 and 20:3n-6 to 20:5n-3 (EPA) and arachidonic acid (ARA, 20:4n-6), respectively. The latter are regarded as critical PUFA in a variety of physiological processes ensuring normal cellular function (Funk, 2001). Rather than a role in the biosynthesis of EPA, we hypothesised that the Δ5 Fad activity may actually contribute to the endogenous biosynthesis of ARA in the octopus, as high concentrations of ARA encountered in adult octopus tissues were unlikely to be exclusively of dietary origin. In addition to the potential participation of the octopus Δ5 Fad in ARA biosynthesis, the common octopus Δ5 Fad might also have a role in the biosynthesis of non-methylene interrupted fatty acids (NMI FA), compounds with unusual unsaturation features that have been found in a variety of marine invertebrates (Barnathan, 2009; Kornprobst and Barnathan, 2010).

The biosynthesis of PUFA including NMI FA in marine molluscs has been investigated previously (De Moreno et al., 1976; Waldock and Holland, 1984; Zhukova, 1986, 1991, 2007). The PUFA biosynthetic capability of molluscs seems to vary among species according to enzymatic activities present in each species. However, it has been shown that some molluscs have active PUFA biosynthetic pathways and, in addition to the above mentioned Δ5 desaturase, active FA elongation systems also appear to be present. Using radioactive FA, De Moreno et al. (1976) were able to show that the clam

Mesoderma mactroides could elongate both 18:3n-3 and 18:2n-6. Later, Waldock and Holland (1984) demonstrated that the Pacific oyster *Crassostrea gigas* had the ability to desaturate and elongate ^{14}C-labeled PUFA precursors provided through the diet (microalgae) to PUFA including 20:5n-3 and 22:6n-3. Investigations with ^{14}C substrates demonstrated that other molluscs such as *Scapharca broughtoni*, *Callista brevisiphonata* and *Mytilus edulis* can biosynthesise the NMI Δ7,13 22:2 and Δ7,15 22:2 by elongation from Δ5,11 20:2 and Δ5,13 20:2, respectively (Zhukova, 1986, 1991). In addition to biochemical assays with radiotracers, indirect evidence of FA elongase activity in molluscs was provided analytically (Joseph, 1982). For instance, the unusual NMI FA Δ5,9,15 24:3 and Δ5,9,17 24:3 found in the limpets *Cellana grata* and *Collisella dorsuosa* were suggested to derive from the typical NMI FA Δ7,13 22:2 and Δ7,15 22:2, respectively, by chain elongation and subsequent Δ5 desaturation (Kawashima, 2005).

In vertebrates, elongase of very long-chain fatty acids (Elovl) enzymes catalyse the addition of 2 carbons to a preexisting fatty acyl chains (Jakobsson et al., 2006). There are seven distinct member of the ELOVL protein family in vertebrates (designated Elovl 1-7) and many of them have been functionally characterised (see reviews by Jakobsson et al., 2006; Guillou et al., 2010; Monroig et al., 2011a). In contrast, studies of Elovl genes and proteins from non-vertebrate organisms are scarce, with only a few examples such as elongases from the nematode *Caenorhabditis elegans* (Beaudoin et al., 2000) and the marine protist *Thraustochytrium* sp. (Heinz et al., 2001; Jiang et al., 2008). To date, no elongases from molluscs have been reported.

As a further step towards understanding the EFA requirements of common octopus, the present study reports the molecular cloning, functional characterisation and tissue distribution of transcripts encoding a putative elongase involved in PUFA biosynthesis.

In order to investigate a potential role of the newly cloned elongase in NMI FA biosynthesis in the common octopus, we also analysed the double bond features of NMI FA found in specific tissues of octopus adult specimens.

Materials and methods

Tissue samples

Tissue samples from common octopus were obtained from the dissection of two (male and female) adult individuals (~1.5 kg) captured by artisanal fisheries along the Mediterranean East coast of Spain. The octopuses were anesthetised by inmersion in seawater at 4 ºC and sacrificed by direct brain puncture and tissues including nerve, nephridium, hepatopancreas, brain, caecum, gill, muscle, heart and gonad were sampled and immediately frozen at -80 ºC until further analysis.

Fatty acyl elongase cDNA cloning

Total RNA was extracted from octopus tissues using TRIzol® (Gibco BRL, Grand Island, NY, USA) reagent following manufacturer's instructions. Subsequently, first strand cDNA was synthesised from 1 μg total RNA using a Verso™ cDNA kit (ABgene, Rockford, IL, USA) primed with random hexamers. In order to amplify the first fragment of the elongase cDNA, the amino acid (aa) sequences of Elovl5 proteins from *Homo sapiens* (NP_068586.1), *Rattus norvegicus* (NP_599209.1), *Bos taurus* (NP_001040062.1), *Danio rerio* (NP_956747.1) and *Pagrus major* (ADQ27303.1) were aligned using BioEdit v5.0.6 (Tom Hall, Department of Microbiology, North Carolina State University, USA). Conserved regions were used for *in silico* searches of mollusc expressed sequence tags (EST) using NCBI tblastn tool (http://www.ncbi.nlm.nih.gov/). Several ESTs displaying high similarity with Elovl encoding genes were identified from the molluscs *Mytilus galloprovinciallis* (gb|FL495089.1| and gb|FL499406.1|), *Euprymna scolopes* (gb|DW256301.1|), and *Lymnaea stagnalis* (gb|FC701557.1|, gb|FC773093.1|, gb|FC770692.1| and gb|FC696214.1|). Additionally, a search of the owl limpet *Lottia gigantea* genome was performed using the zebrafish Elovl5 (NP_956747.1) sequence with the tblastn tool at http://genome.jgi-psf.org/Lotgi1/Lotgi1.home.html. After processing, the mollusc Elovl-like sequences were aligned (Bioedit) for the design of the primers UNIEloF (5'-TTGTGGTGGTATTACTTCTC-3') and UNIEloR (5'-GTAATATACTTTTTCCACCA-3') that were used for polymerase chain reaction (PCR) using GoTaq® Green Master Mix (Promega, Southampton, UK), and using a mixture of cDNA from gonads, brain, nerve and caecum as template. The PCR cycling 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 50 ºC for 30 s, extension at 72 ºC for 1 min, followed by a final extension at 72 ºC for 5 min. The PCR fragment was sequenced at the DNA Sequencing Service of the IBMCP-UPV (Valencia, Spain) and 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 a full-length cDNA. Details of all primers used for RACE PCR are given in Table 1.

(TABLE 1)

For 5'RACE PCR, a positive fragment was obtained by nested PCR approach. The first round PCR was performed using the adapter-specific 5'RACE OUTER primer and the gene-specific reverse primer OVEloR1, with an initial denaturing step at 95 ºC for 2 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 75 s, followed by a final extension at 72 ºC for 5 min (GoTaq® Colorless Master Mix, Promega). First round PCR products were used as template for nested PCR with primers 5'RACE INNER and OVEloR2 in a 32-cycle reaction under the same thermal conditions as above. For 3'RACE PCR, a similar nested approach was followed with first round PCR performed with primers OVEloF1 and 3'RACE OUTER, with an initial denaturating step at 95 ºC for 1 min, followed by 32 cycles of denaturation at 95 ºC for 30 s, annealing at 56 ºC for 30 s, extension at 72 ºC for 2 min, followed by a final extension at 72 ºC for 5 min (GoTaq® Colorless Master Mix, Promega). First round PCR products were then used as template for nested PCR with primers OVEloF2 and 3'RACE INNER, with thermal conditions as above. RACE PCR products were cloned into pGEM-T Easy Vector (Promega) and sequenced as above.

Sequence and phylogenetic analyses

Using ClustalW (Bioedit), the deduced aa sequence of the newly cloned *O. vulgaris* elongase cDNA was aligned with that of a predicted elongase found in the gastropod owl limpet (termed '*L. gigantea* Elovl transcript 1', jgi|Lotgi1|224291|), as well as those of protein homologues including the human ELOVL5 (gb|NP_068586|) and ELOVL2 (gb|NP_060240|), and the zebrafish Elovl5 (gb|NP_956747|) and Elovl2 (gb|NP_001035452|). The aa sequence identity between Elovl-like proteins was compared using the EMBOSS Needle Pairwise Sequence Alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). Phylogenetic analysis of the aa sequences deduced from the Elovl-like cDNA from common octopus and those from other organisms including several marine invertebrates was performed 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.

Functional characterisation of the octopus elongase by heterologous expression in Saccharomyces cerevisiae

PCR fragments corresponding to the open reading frame (ORF) of the putative elongase were amplified from a mixture of cDNA synthesised from gonads, brain, nerve and caecum 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 95ºC for 30 s, annealing at 57 ºC for 30 s, and extension at 72 ºC for 2 min 15 s, followed by a final extension at 72 ºC for 5 min. The primers containing restriction enzyme sites (underlined in Table 1) were OVEloVF (*Hin*dIII) and OVEloVR (*Sac*I) and they were used for PCR, and the DNA fragments produced were subsequently 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 octopus elongase ORF were then used to transform *Saccharomyces cerevisiae* competent cells (S.c. EasyComp Transformation Kit, Invitrogen). Transformation and selection of yeast with recombinant pYES2-OVElo plasmids, and yeast culture were performed as described in detail previously (Agaba et al., 2004; Monroig et al., 2012b).

One single colony of yeast transformed with pYES2 vector containing the octopus elongase as an insert (pYES2-OVElo) and no insert (control) were grown in *S. cerevisiae* minimal medium-uracil broth and diluted to OD600 of 0.4 in one single Erlenmeyer flask for each potential substrate assayed. In order to test the ability of the octopus Elovl cDNA ORF to elongate either saturated or monounsaturated FA, pYES2-OVElo and empty pYES2 yeast were grown with no exogenously added substrates. Additionally, the ability of *O. vulgaris* Elovl to elongate PUFA substrates was tested by growing pYES2-OVElo transgenic yeast in medium supplemented with one of the following 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. The FA were added to the yeast cultures at final concentrations of 0.5 (C18), 0.75 (C20) and 1.0 (C22) mM as FA uptake efficiency has been shown to decrease with increasing chain length (Zheng et al., 2009). Yeast transformed with empty pYES2 vector were also grown in the presence of the PUFA substrates as control treatments. After 2-days culture at 30 ^{o}C, the yeast were harvested, washed, and cellular lipid was extracted by homogenisation in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxy toluene (BHT) as antioxidant. All FA substrates, except stearidonic acid (18:4n-3), were purchased from Nu-Chek Prep, Inc (Elysian, MN, USA). Stearidonic acid and chemicals used to prepare the *S. cerevisiae* minimal medium-^{uracil} were from Sigma Chemical Co. Ltd. (Dorset, UK), except for the bacteriological agar obtained from Oxoid Ltd. (Hants, UK). In order to confirm the results, the assay was repeated with a different yeast colony transformed with pYES2-OVElo, but using only n-6 PUFA as potential substrates.

Tissue distribution of elongase transcripts

Expression of the octopus elongase was examined in adult tissues by reverse transcriptase PCR (RT-PCR). Total RNA from a series of tissues including nerve, nephridium, hepatopancreas, brain, caecum, gill, muscle, heart, and female and male gonads was extracted as described above, and 1 µg of total RNA was reverse transcribed into cDNA (M-MLV reverse transcriptase, Promega). In order to determine the mRNA distribution of the octopus elongase, the tissue cDNAs were used as templates in PCR consisting of a denaturating step at 95 ºC for 1 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 30 s, followed by a final extension at 72 ºC for 5 min (GoTaq® Green Master Mix, Promega). Additionally, the expression of the housekeeping gene β-actin was also determined. Primers used for RT-PCR are shown in Table 1.

Fatty acid analysis

FA from the transgenic yeast were analysed by preparing methyl esters (FAME) as previously described (Hastings et al. 2001). 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 elongation efficiencies for potential substrates including the yeast endogenous FA 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) were calculated from the proportion of substrate FA converted to elongated FA product as [product area/(product area + substrate area)] x 100. When further confirmation of double bond positions was required, FA picolinyl esters were prepared from FAME according to the methodology described by Destaillats and Angers (2002) and modified according to Li et al. (2010).

In order to investigate the potential participation of the octopus elongase in the biosynthesis of NMI FA, the FA compositions of specific tissues in which we had previously detected NMI FA (Monroig et al., 2012a) were determined through preparation of both methyl and picolinyl ester derivatives from polar lipid (PL) fractions prepared as follows. Lipid extracts (2 mg) from nephridium, male gonad, eye and caecum were applied to 20x20 silica gel plates (Merck, Darmstadt, Germany) and the plates were developed with a solvent mixture of n-hexane / diethyl ether / glacial acetic acid (85:15:1.5, v/v/v). PL fractions, identified by comparison with known standards, were scraped from the plates, and FAME prepared (Monroig et al., 2012a) and analysed as described above. FAME samples were subsequently derivatised to FA picolinyl esters prepared for identification of the double bond patterns in NMI FA.

Results

Octopus elongase sequence and phylogenetics

The ORF of the newly cloned Elovl from *O. vulgaris* consists of 885 bp encoding a putative protein of 294 aa. The sequences of the ORF and the untranslated regions (UTRs) were deposited in the GenBank database with the accession number JX020803. The deduced aa sequence from the octopus elongase showed identity scores ranging from 39.3 to 43.2 % with several Elovl proteins (Elovl2, Elovl4 and Elovl5) from vertebrates including *H. sapiens*, *Xenopus tropicalis* and *D. rerio*. When compared with the two full-length elongases found in the genome of the gastropod *L. gigantea*, the octopus Elovl was 58.1 % identical to the so-called '*L. gigantea* Elovl transcript 1' and 39.5 % identical to the 'Elovl transcript 2'. When the octopus Elovl aa sequence was compared with incomplete elongase sequences from *E. scolopes*, *L. stagnalis*, *M. galloprovincialis* and *Aplysia californica* the identity scores were relatively low, ranging from 31.9 to 43.9 %.

Similar to vertebrate Elovl-like proteins, the deduced aa sequence of the octopus elongase contained the diagnostic histidine box (HXXHH) conserved in all members of the Elovl protein family (Fig. 1). It also possessed two lysine (K) residues at the carboxyl terminus (KKXX), regarded as putative ER retrieval signals. Additionally, five putative transmembrane-spanning regions containing hydrophobic aa stretches were predicted corresponding to residues 32-50, 65-83, 117-137, 158-192 and 239-259 by InterProScan (version 4.2) (Fig. 1).

A phylogenetic tree was constructed on the basis of aa sequence comparisons of the octopus Elovl and other predicted elongases from molluscs, as well as several Elovl family members (Elovl 1-7) from a variety of vertebrates (Fig. 2). Our results show that the octopus Elovl protein clustered with other Elovl-like proteins from molluscs including the cephalopod *E. scolopes* and the gastropods *L. stagnalis* and *L. gigantea* ('transcript 1'). Together these formed a group close to Elovl2 and Elovl5 from vertebrates. More distantly related, three main clusters could be distinguished including Elovl3/Elovl6, Elovl1/Elovl7 and Elovl4 representatives. Interestingly, the Elovl4 cluster included the well-studied proteins from vertebrates, but also other mollusc Elovl-like proteins from *L. gigantea* ('transcript 2'), *A. californica* and *M. galloprovincialis*.

Functional characterisation in yeast

The octopus Elovl-like cDNA was functionally characterised by expressing the ORF in yeast (*S. cerevisiae*). The FA composition of wild type yeast consists basically of 16:0, 16:1 isomers (16:1n-9 and 16:1n-7), 18:0, 18:1n-9 and 18:1n-7 (Monroig et al., 2010a). Similarly, in the present study, the FA profile of *S. cerevisiae* transformed with the empty pYES2 vector (control) consisted of these FA together with whichever exogenous FA (if any) was added as substrate (data not shown). This confirms the well-known inability of *S. cerevisiae* elongases to operate on PUFA substrates (Hastings et al., 2001; Agaba et al., 2004).

In order to test the ability of the octopus Elovl to elongate saturated and monounsaturated FA, yeast transformed with pYES2-OVElovl were grown in absence of exogenously added substrates. The results showed that none of the yeast endogenous FA, whether saturated or monounsaturated, was elongated. Conversely, yeast transformed with pYES2-OVElovl showed activity towards PUFA substrates producing the corresponding 2C elongation product. 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 2; Fig. 3). Conversion rates derived from the yeast assays suggested that the octopus Elovl generally elongated n-6 PUFA substrates more efficiently than n-3 substrates for each pair of homologous substrates considered (Table 2). Thus, the substrates 18:2n-6, 18:3n-6, 20:4n-6 were consistently elongated at higher rates than the corresponding n-3 PUFA substrates 18:3n-3, 18:4n-3 and 20:5n-3, respectively. Interestingly, no activity towards C22 (22:5n-3 and 22:4n-6) PUFA substrates was detected.

Tissue distribution of octopus elongase transcripts

Tissue expression of the common octopus Elovl was studied by RT-PCR on cDNA samples obtained from a range of tissues (Fig. 4). Except for nephridium, transcripts of the octopus Elovl gene were detected in all tissues analysed. Although RT-PCR analyses should not be regarded as strictly quantitative data, our results indicate that both the male and female gonads showed higher expression signals.

Fatty acid composition from polar lipids of adult octopus tissues

FA from PL were analysed in several tissues of adult octopus individuals (Table 3). DHA appeared to be the most abundant FA for each tissue considered, with up to 27.0 % of total FA in eye PL. Other PUFA relatively abundant in the tissues studied were ARA (with up to 16.4 % in male gonad PL) and EPA (up to 13.7 % in caecum PL). Interestingly, 20:3n-3 content in eye was 13.5 % of total FA in the PL fraction.

GC-MS analysis of picolinyl esters enabled us to identify four different NMI FA in the octopus tissues, namely Δ5,11 20:2, Δ7,13 20:2, Δ5,11,14 20:3 and Δ7,13 22:2 (Table 3). Although we specifically analysed the PL fractions, where NMI FA are believed to accumulate (Klingensmith, 1982; Pirini et al., 2007), the amounts of all the NMI FA identified were generally low, and only relatively higher contents were detected for Δ5,11 20:2 in nephridium (1.8 %) and its potential elongation product Δ7,13 22:2 in male gonad (2.2 %).

Discussion

The FA biosynthesis pathways have been investigated in both terrestrial (van der Horst 1973, 1974; Weinert et al. 1993; Zhu et al. 1994) and aquatic mollusc species (Chu and Greaves 1991; de Moreno et al. 1976; Waldock and Holland 1984; Zhukova 1986, 1991, 2007; Delaporte et al., 2005). It was shown that some molluscs have active FA elongation systems (Waldock and Holland, 1984; Zhukova, 1986; Delaporte et al., 2005). In the present study we provide compelling evidence of the existence of an Elovl cDNA that encodes an enzyme potentially involved in the biosynthesis of PUFA in the cephalopod *O. vulgaris*.

The deduced aa sequence of the Elovl-like cDNA from *O. vulgaris* contains all the features of the vertebrate Elovl protein family members, including five membrane-spanning regions, an ER retrieval signal at the C terminus containing lysine (K) residues (KKXX) and a diagnostic histidine box (HXXHH) (Leonard et al., 2004; Jakobsson et al., 2006). Moreover, the histidine (H) box and its N-terminal side (QVTFLHVFHH) show a typical aa pattern of the PUFA elongase subfamily of eukaryotic elongases, with a glutamine (Q) at position -5 and a leucine (L) at position -1 from the first H (Hashimoto et al., 2008). Further evidence supporting a potential role of the octopus Elovl cDNA in the PUFA biosynthetic pathways was provided by phylogenetic analysis. Thus, the octopus Elovl aa sequence, as well as those of other mollusc elongases, obtained by *in silico* searches, including the cephalopod *E. scolopes* and the gastropods *L. stagnalis* and *L. gigantea* (transcript 1), showed great similarity to the sequences of Elovl2 and Elovl5 proteins, critical enzymes participating in the biosynthesis of LC-PUFA in vertebrates (Leonard et al., 2004; Jakobsson et al., 2006). More distantly, the other elongase identified in the *L. gigantea* genome (transcript 2) and also other Elovl-like proteins from *A. californica* and *M. galloprovincialis* grouped together with vertebrate Elovl4 elongases, another type of elongase involved in the biosynthesis of very long-chain FA (C>24) including both saturates and polyenes (Agbaga et al., 2008; Monroig et al., 2010b, 2011b, 2012b). While these results suggest that another elongase with similarity to Elovl4 might also be present in the common octopus, the functional characterisation of the present Elovl cDNA confirmed, not only its participation in the PUFA elongation pathway, but also that it has substrate specificities more similar to Elovl5 than Elovl2.

Clearly, transgenic yeast expressing the octopus Elovl efficiently converted C18 and C20 PUFA substrates to their corresponding 2C elongated products, but no activity towards C22 PUFA was detected. Generally, this pattern of substrate specificity of the octopus elongase is consistent with that of vertebrate Elovl5 proteins (Jakobsson et al., 2006). For instance, the human ELOVL5 (also termed HELO1) and the rat ELOVL5 (also termed rELO1) were shown to efficiently elongate C18 and C20 PUFA, whereas C22 PUFA did not appear to be substrates for these enzymes (Leonard et al., 2000; Inagaki et al., 2002). Similarly, fish Elovl5 demonstrated high activity for the elongation of C18 and C20 PUFA substrates, whereas C22 substrates were only elongated to a lesser extend (Agaba et al., 2004; Morais et al., 2009; Mohd-Yusof et al., 2010; Monroig et al., 2012b). Importantly, elongation of C22 PUFA including 22:5n-3 and 22:4n-6 in vertebrates is basically mediated by Elovl2, whose substrate chain-length specificity also includes C20, but not C18, PUFA substrates, the latter being only marginally or not elongated (Tvrdik et al., 2000; Leonard et al., 2002; Monroig et al., 2009; Morais et al., 2009). Overall it can be concluded that the *O. vulgaris* elongase cloned here is phenotypically an Elovl5-like elongase, but its sequence similarity to vertebrate Elovl2 suggests an interesting evolutionary scenario that is worth exploring in future investigations.

The functional characterisation of the octopus Elovl revealed, however, that the gene product might have conserved/acquired a different PUFA family specificity compared to vertebrate Elovl5 proteins during evolution. Unlike mammalian (Leonard et al., 2000; Inagaki et al., 2002) and fish Elovl5 enzymes (Agaba et al., 2005; Mohd-Yusof et al., 2010; Morais et al., 2011; Monroig et al., 2012b), which are generally more efficient in elongating n-3 rather than n-6 FA substrates, the octopus Elovl appeared to exhibit higher elongation rates towards n-6 compared to n-3 substrates for each homologous pair considered. Thus, 18:2n-6, 18:3n-6, 20:4n-6 were all elongated at higher rates than the corresponding n-3 FA, namely 18:3n-3, 18:4n-3 and 20:5n-3, respectively. These results emphasise that n-6 FA in general, and especially ARA (20:4n-6), might play particularly important physiological roles in the common octopus. Consistent with this, several studies have reported unexpectedly high levels of ARA in tissues of common octopus that were unlikely to derive purely from dietary origin and, thus, an active biosynthesis of ARA in this species was postulated (Milou et al., 2006; García-Garrido et al., 2010; Monroig et al., 2012a). In the present study, the efficiency shown by the octopus Elovl to elongate certain PUFA substrates indicates that this enzyme could contribute to the endogenous biosynthesis of ARA in this species.

In vertebrates, ARA is biosynthesised from the dietary essential C18 PUFA 18:2n-6 through two alternative pathways, the 'classical' Δ6-pathway (Δ6 desaturation → elongation → Δ5 desaturation), or alternatively through the so-called 'Δ8-pathway' (elongation → Δ8 desaturation → Δ5 desaturation) (Monroig et al., 2011c). In addition to the ability of the formerly characterised Fad cDNA to mediate the Δ5-desaturation steps of these pathways (Monroig et al., 2012a), we here demonstrate that the newly cloned octopus Elovl can efficiently catalyse the elongation reactions required for ARA biosynthesis from the dietary essential 18:2n-6, namely 18:3n-6 → 20:3n-6 for the Δ6-

pathway and 18:2n-6 → 20:3n-6 for the Δ8-pathway. Although genes responsible for elongation and Δ5 desaturation steps of these pathways have now been identified in octopus, no Fad cDNA with Δ6 or Δ8-desaturase activity has yet been identified and, consequently, it remains unclear whether the common octopus can biosynthesise ARA from the dietary essential 18:2n-6. This appears to be the case for some abalone species (Dunstan et al., 1996; Durazo-Beltrán et al., 2003), but other species like *C. gigas* (Waldock and Holland, 1984) and *Mytilus edulis* (Zhukova, 1991) appear unable to biosynthesise ARA from 18:2n-6.

In addition to the biosynthesis of conventional PUFA, the octopus Elovl can also have a role in the production of NMI FA. Thus, the biosynthesis of Δ7,13 22:2 encountered in male gonad, eye and caecum may be accounted for by the elongation of Δ5,11 20:2, as described for other marine invertebrates (Kornprobst and Barnathan, 2010). Although we cannot directly conclude that the octopus Elovl has the ability to elongate Δ5,11 20:2 as this substrate was not available, some of our results suggest a role for the elongase in the production of Δ7,13 22:2 from Δ5,11 20:2. First, the increased expression signal of Elovl in the male gonad is consistent with this tissue containing the highest amount of Δ7,13 22:2. Second, it is reasonable to assume that, similar to the elongation rates exhibited towards other C20 PUFA like 20:4n-3 and 20:3n-6, the octopus Elovl might also efficiently operate towards another C20 PUFA like Δ5,11 20:2. Whereas these circumstantial data suggest that the octopus Elovl may contribute to the endogenous biosynthesis of NMI FA in this cephalopod, the extent to which this biosynthetic pathway is operative in the common octopus is difficult to predict. On one hand, the ability of the octopus Δ5 Fad to convert 20:3n-3 (Δ11,14,17 20:3) and 20:2n-6 (Δ11,14 20:2) to the NMI FA Δ5,11,14,17 20:4 and Δ5,11,14 20:3, respectively (Monroig et al., 2012a), supports the hypothesis of a notable production of

NMI FA by *O. vulgaris* itself. On the other, the endogenous biosynthesis of NMI FA in the common octopus appears to be limited as, despite the likely intake of preformed NMI FA through the diet, they still present relative low levels compared to those found in some bivalves (Klingensmith, 1982) or nudibranchs (Zhukova, 2007).

In conclusion, the present study demonstrates that the common octopus possesses an Elovl-like cDNA with high homology to vertebrate Elovl5 and Elovl2 enzymes. The functions of the octopus Elovl, while generally consistent with those of vertebrate Elovl5, have some novel particularities. Thus, the octopus Elovl showed higher elongation efficiency towards n-6 than n-3 PUFA suggesting that these compounds, and especially ARA, might play particularly pivotal roles in the common octopus. Moreover, the Elovl might be involved in the biosynthesis of NMI FA, although the quantitative significance of these biosynthetic pathways in *O. vulgaris* requires further investigation.

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References

Agaba, M., Tocher, D.R., Dickson, C., Dick, J.R., Teale, A.J., 2004. Zebrafish cDNA encoding multifunctional fatty acid elongase involved in production of eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids. Mar. Biotechnol.

6, 251-261.

Agaba, M.K., Tocher, D.R., Dickson, C.A., Zheng, X., Dick, J.R., Teale, A.J., 2005. Cloning and functional characterisation of polyunsaturated fatty acid elongases from marine and freshwater teleost fish. Comp. Biochem. Physiol. 142B, 342–352.

Agbaga, M.P., Brush, R.S., Mandal, M.N.A., Henry, K., Elliott, M.H., Anderson, R.E., 2008. Role of Stargardt-3 macular dystrophy protein (ELOVL4) in the biosynthesis of very long chain fatty acids. Proc. Natl. Acad. Sci. USA. 105, 12843-12848.

Barnathan, G., 2009. Non-methylene-interrupted fatty acids from marine invertebrates: Ocurrence, characterization and biological properties. Biochimie 91, 671-678.

Beaudoin, F., Michaelson, L.V., Lewis, M.J., Shewry, P.R., Sayanova, O., Napier, J.A., 2000. Production of C20 polyunsaturated fatty acids (PUFAs) by pathway engineering: identification of a PUFA elongase component from *Caenorhabditis elegans*. Biochem. Soc. Trans. 28, 661-663.

Bell, M.V., Tocher, D.R, 2009. Biosynthesis of polyunsaturated fatty acids in aquatic ecosystems: general pathways and new directions, in: Arts, M.T., Brett, M., Kainz, M. (Eds.), Lipids in Aquatic Ecosystems. Springer-Verlag, New York.

Castellanos-Martinez, S., Gestal, C., 2011. Inmune response of *Octopus vulgaris* against the infection by the gastrointestinal parasite *Aggregata octopiana*. J. Shellfish 30, 997-998.

Chu, F.E., Greaves, J., 1991. Metabolism of palmitic, linoleic, and linolenic acid in adult oyster, *Crassostrea virginica*. Mar. Biol. 110, 229–236.

Delaporte, M., Soudant, P., Moal, J., Kraffe, E., Marty, Y., Samain, J.F., 2005. Incorporation and modification of dietary fatty acids in gill polar lipids by two bivalves species *Crassostrea gigas* and *Ruditapes philippinarum*. Comp. Biochem. Physiol. 140A, 460–470.

De Moreno, J.E.A., Moreno, V.J., Brenner, R.R., 1976. Lipid metabolism of the yellow clam, *Mesodesma mactroides*: 2-polyunsaturated fatty acid metabolism. Lipids 11, 561-566.

Destaillats, F., Angers, P., 2002. One-step methodology for the synthesis of FA picolinyl esters from intact lipids. J. Am. Oil Chem. Soc. 79, 253–256.

Di Cristo, C., Van Minnen, J., Di Cosmo, A., 2005. The presence of APGWamide in *Octopus vulgaris*: a possible role in the reproductive behavior. Peptides 26, 53-62.

Dunstan, G.A., Baillie, H.J., Barrett, S.M., Volkman, J.K., 1996. Effect of diet on the lipid composition of wild and cultured abalone. Aquacult. Abalone Cult. 140, 115–127.

Durazo-Beltrán, E., D'Abramo, L.R., Toro-Vazquez, J.F., Vasquez-Peláez, C., Viana, M.T., 2003. Effect of triacylglycerols in formulated diets on growth and fatty acid composition in tissue of green abalone (*Haliotis fulgens*). Aquaculture 224, 257–270.

Estefanell, J., Socorro, J., Roo, F.J., Fernández-Palacios, H., Izquierdo, M., 2010. Gonad maturation in *Octopus vulgaris* during ongrowing, under different conditions of sex ratio. ICES J. Mar. Sci. 67, 1487–1493.

Estefanell, J., Socorro, J., Tuya, F., Izquierdo, M., Roo, J., 2011. Growth, protein retention and biochemical composition in *Octopus vulgaris* fed on different diets based on crustaceans and aquaculture by-products. Aquaculture 322, 91-98.

Estefanell, J., Roo, J., Fernandez-Palacios, H., Izquierdo, M., Socorro, J., Guirao, R., 2012. Comparison between individual and group rearing systems in *Octopus vulgaris* (Cuvier, 1797). J. World Aquacult. Soc. 43, 63-72.

Fuentes, L., Sanchez, F.J., Lago, M.J., Iglesias, J., Pazos, G., Linares, F., 2011. Growth and survival of *Octopus vulgaris* (Cuvier 1797) paralarvae fed on three Artemia-based diets complemented with frozen fish flakes, crushed zooplankton and marine microalgae. Sci. Mar. 75, 771-777.

Funk, C.D., 2001. Prostaglandins and leukotrienes: Advances in eicosanoid biology. Science 294, 1871–1875.

García-Garrido, S., Hachero-Cruzado, I., Garrido, D. Rosas, C., Domingues, P., 2010. Lipid composition of the mantle and digestive gland of *Octopus vulgaris* juveniles (Cuvier, 1797) exposed to prolonged starvation. Aquacult. Int. 18,1223–1241.

Guillou, H., Zadravec, D., Martin, P.G.P., Jacobsson, A., 2010. The key roles of elongases and desaturases in mammalian fatty acid metabolism: Insights from transgenic mice. Prog. Lipid Res. 49, 186-199.

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

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

Heinz, E., Zank, T., Zaehringer, U., Lerchl, J., Renz, A., 2001. Patent: WO 0159128-A.

Iglesias, J., Otero, J.J., Moxica, C., Fuentes, L., Sánchez, F.J., 2002. Paralarvae culture of octopus (*Octopus vulgaris* Cuvier) using *Artemia* and crab zoeas and first data on juvenile growth up to eight months of age. European Aquaculture Society. Special Publication 32, 268–269.

Iglesias, J., Otero, J.J., Moxica, C., Fuentes, L., Sánchez, F.J., 2004. The completed life cycle of the octopus (*Octopus vulgaris*, Cuvier) under culture conditions: paralarval rearing using *Artemia* and zoeae, and first data on juvenile growth up to 8 months of age. Aquac. Int. 12, 481–487.

Iglesias, J., Fuentes, L., Sánchez, J., Otero, J.J., Moxica, C., Lago, M.J., 2006. First feeding of *Octopus vulgaris* Cuvier, 1797 paralarvae using *Artemia*: Effect of prey size, prey density and feeding frequency. Aquaculture 261, 817–822.

Iglesias. J., Sánchez, F.J., Bersano, J.G.F., Carrasco, J.F., Dhont, J., Fuentes, L., Linares, F., Muñoz, J.L., Okumura, S., Roo, J., van der Meeren, T., Vidal, E.A.G., Villanueva, R., 2007. Rearing of *Octopus vulgaris* paralarvae: Present status, bottlenecks and trends. Aquaculture 266, 1–15.

Inagaki, K., Aki, T., Fukuda, Y., Kawamoto, S., Shigeta, S., Ono, K., Suzuki, O., 2002. Identification and expression of a rat fatty acid elongase involved in the biosynthesis of C18 fatty acids. Biosci, Biotechnol, Biochem, 66, 613–21.

Jakobsson, A., Westerberg, R., Jacobsson, A., 2006. Fatty acid elongases in mammals: Their regulation and roles in metabolism. Prog. Lipid Res. 45, 237-249.

Jiang, X., Qin, L., Tian, B., Shu, Z., Huang, J., 2008. Cloning and expression of two elongase genes involved in the biosynthesis of docosahexaenoic acid in *Thraustochytrium* sp. FJN-10. Acta microbiologica Sinica 48, 176-83.

Joseph, J.D., 1982. Lipid composition of marine and estuarine invertebrates. Part II: Mollusca. Prog. Lipid Res. 21, 109-153.

Kawashima, H., 2005. Unusual minor nonmethylene-interrupted di-, tri-, and tetraenoic fatty acids in limpet gonads. Lipids 40, 627-630.

Klingensmith, J., 1982. Distribution of methylene and nonmethylene-interrupted dienoic fatty acids in polar lipids and triacylglycerols of selected tissues of the hardshell clam (*Mercenaria mercenaria*). Lipids 17, 976–981.

Kornprobst, J.M., Barnathan, G., 2010. Demospongic acids revisited. Mar. Drugs 8, 2569-2577.

Leonard, A.E., Kelder, B., Bobik, E.G., Kroeger, P.E., Chuang, L.-T., Thurmond, J.M., Parker-Barnes, J.M., Kopchick, J.J., Huang, Y.-S., Murkerji, P., 2000. cDNA cloning and characterisation of human Δ5 desaturase involved in the synthesis of arachidonic acid. Biochem. J. 347, 719–724.

Leonard, A.E., Kelder, B., Bobik, E.G., Chuang, L., Lewis, C.J., Kopchick, J.J., Mukerji, P., Huang, Y., 2002. Identification and expression of mammalian long-chain PUFA elongation enzymes. Lipids 37, 733–740.

Leonard, A.E., Pereira, S.L., Sprecher, H., Huang, Y.-S., 2004. Elongation of long-chain fatty acids. Prog. Lipid Res. 43, 36–54.

Li, Y., Monroig, Ó., Zhang, L., Wang, S., Zheng, X., Dick, J.R., You, C., Tocher, D.R., 2010. Vertebrate fatty acyl desaturase with Δ4 activity. Proc. Natl. Acad. Sci. USA 107, 16840-16845.

Milou, H., Fintikaki, M., Tzitzinakis, M., Kountouris, T., Virriopoulos, G. 2006. Fatty acid composition of the common octopus, *Octopus vulgaris*, in relation to rearing temperature and body weight. Aquaculture 256, 311-322.

Mohd-Yusof, N.Y., Monroig, Ó., Mohd-Adnan, A., Wan, K.-L., Tocher, D.R., 2010. Investigation of highly unsaturated fatty acid metabolism in the Asian sea bass, *Lates calcarifer*. Fish Physiol. Biochem. 3, 827–843.

Monroig, Ó., Rotllant, J., Sánchez, E., Cerdá-Reverter, J.M., Tocher, D.R., 2009. Expression of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis genes during zebrafish *Danio rerio* early embryogenesis. Biochim. Biophys. Acta 1791, 1093–1101.

Monroig, Ó., Zheng, X., Morais, S., Leaver, M.J., Taggart, J.B., Tocher, D.R., 2010a. Multiple genes for functional Δ6 fatty acyl desaturases (Fad) in Atlantic salmon (*Salmo salar* L.): Gene and cDNA characterization, functional expression, tissue distribution and nutritional regulation. Biochim. Biophys. Acta 1801, 1072–1081.

Monroig, Ó., Rotllant, J., Cerdá-Reverter, J.M., Dick, J.R., Figueras, A., Tocher, D.R., 2010b. Expression and role of Elovl4 elongases in biosynthesis of very long-chain fatty acids during zebrafish *Danio rerio* early embryonic development. Biochim. Biophys. Acta 1801, 1145-1154.

Monroig, Ó., Navarro, J.C., Tocher, D.R., 2011a. Long-chain polyunsaturated fatty acids in fish: recent advances on desaturases and elongases involved in their biosynthesis, in: Cruz-Suarez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Hernández-Hernández, L.H. (Eds), Proceedings of the XI International Symposium on Aquaculture Nutrition, 23-25 November 2011, San Nicolás de los Garza, N.L. Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, México. ISBN 978-607-433-775-4. Universidad Autónoma de Nuevo León, Monterrey, México. pp. 257-282.

Monroig, Ó., Webb, K., Ibarra-Castro, L., Holt, G.J., Tocher, D.R. 2011b. Biosynthesis of long-chain polyunsaturated fatty acids in marine fish: Characterization of an Elovl4-like elongase from cobia *Rachycentron canadum* and activation of the pathway during early life stages. Aquaculture 312, 145–153.

Monroig, Ó., Li, Y., Tocher, D.R., 2011c. Delta-8 desaturation activity varies among fatty acyl desaturases of teleost fish: high activity in delta-6 desaturases of marine species. Comp. Biochem. Physiol. 159B, 206-213.

Monroig, Ó., Navarro, J.C., Dick, J.R., Alemany, F., Tocher, D.R., 2012a. Identification of a Δ5-like fatty acyl desaturase from the cephalopod *Octopus vulgaris* (Cuvier 1797) involved in the biosynthesis of essential fatty acids. Mar. Biotechnol. 14, 411–422.

Monroig, Ó., Wang S., Zhang L., You, C., Tocher D.R., Li Y., 2012b. Elongation of long-chain fatty acids in rabbitfish *Siganus canaliculatus*: Cloning, functional characterisation and tissue distribution of Elovl5- and Elovl4-like elongases. Aquaculture 350-353, 63–70.

Morais, S., Monroig, Ó., Zheng, X., Leaver, M.J., Tocher, D.R., 2009. Highly unsaturated fatty acid synthesis in Atlantic salmon: characterization of Elovl5- and Elovl2-like elongases. Mar. Biotechnol. 11, 627–639.

Morais, S., Mourente, G., Ortega, A., Tocher, J.A., Tocher, D.R., 2011. Expression of fatty acyl desaturase and elongase genes, and evolution of DHA:EPA ratio during development of Atlantic bluefin tuna (*Thunnus thynnus* L.). Aquaculture 313, 129-139.

Navarro, J.C., Villanueva, R., 2000. Lipid and fatty acid composition of early stages of cephalopods: an approach to their lipid requirements. Aquaculture 183:161–177.

Navarro, J.C., Villanueva, R., 2003. The fatty acid composition of *Octopus vulgaris* paralarvae reared with live and inert food: deviation from their natural fatty acid profile. Aquaculture 219, 613–631.

Otero, J., González, Á.F., Sieiro, M.P., Guerra, Á., 2007. Reproductive cycle and energy allocation of *Octopus vulgaris* in Galician waters, NE Atlantic. Fish. Res. 85, 122-129.

Pirini, M., Manuzzi, M.P., Pagliarani, A., Trombetti, F., Borgatti, A.R., Ventrella, V., 2007. Changes in fatty acid composition of *Mytilus galloprovincialis* (Lmk) fed on microalgal and wheat germ diets. Comp. Biochem. Physiol. 147B, 616–626.

Quintana, D., 2009. Valoración de los requerimientos nutricionales de reproductores de pulpo común (*Octopus vulgaris*). PhD Thesis. Universidad de La Laguna.

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

Seixas, P., Otero, A., Valente, L.M.P., Dias, J., Rey-Méndez, M., 2010. Growth and fatty acid composition of *Octopus vulgaris* paralarvae fed with enriched *Artemia* or co-fed with an inert diet. Aquacult. Int. 18, 1121-1135.

Tvrdik, P., Westerberg, R., Silve, S., Asadi, A., Jakobsson, A., Cannon. B., Loison, G., Jacobsson, A., 2000. Role of a new mammalian gene family in the biosynthesis of very long chain fatty acids and sphingolipids. J. Cell Biol. 149, 707-18.

Valverde, J.C., Garcia, B.G., 2005. Suitable dissolved oxygen levels for common octopus (*Octopus vulgaris,* Cuvier, 1797) at different weights and temperatures: analysis of respiratory behaviour. Aquaculture 244, 303-314.

Van der Horst, D.J., 1973. Biosynthesis of saturated and unsaturated fatty acids in the pulmonate land snail *Cepaea nemoralis* (L.). Comp. Biochem. Physiol. 46B, 551–560.

Van der Horst, D.J., 1974. In vivo biosynthesis of fatty acids in the pulmonate land snail *Cepaea nemoralis* (L.) under anoxic conditions. Comp. Biochem. Physiol. 47B, 181-187.

Viciano, E., Iglesias, J., Lago, M.J., Sánchez, F.J., Otero, J.J., Navarro, J.C., 2011. Fatty acid composition of polar and neutral lipid fractions of *Octopus vulgaris* Cuvier, 1797 paralarvae reared with enriched on-grown *Artemia*. Aquacult. Res. 42, 704-709.

Villanueva, R., 1994. Decapod crab zoeae as food for rearing cephalopod paralarvae. Aquaculture 128, 143-152.

Villanueva, R., 1995. Experimental rearing and growth of planktonic *Octopus vulgaris* from hatching to settlement. Can. J. Fish. Aquat. Sci. 52, 2639–2650.

Villanueva, R., Riba, J., Ruíz-Capillas, C., González, A.V., Baeta, M., 2004. Amino acid composition of early stages of cephalopods and effects of amino acid dietary treatments on *Octopus vulgaris* paralarvae. Aquaculture 242, 455–478.

Villanueva, R., Bustamante, P., 2006. Composition in essential and non-essential elements of early stages of cephalopods and dietary effects on the elemental profiles of *Octopus vulgaris* paralarvae. Aquaculture 261, 225-240.

Villanueva, R., Escudero, J.M., Deulofeu, R., Bozzano, A., Casoliva. C., 2009. Vitamin A and E content in early stages of cephalopods and their dietary effects in *Octopus vulgaris paralarvae*. Aquaculture 286, 277-282.

Waldock, M.J., Holland, D.L, 1984. Fatty acid metabolism in young oysters, *Crassostrea gigas*: Polyunsaturated fatty acids. Lipids 19, 332-336.

Weinert, J., Blomquist, G.J., Borgeson, C.E., 1993. De novo biosynthesis of linoleic acid in two non-insect invertebrates: the land slug and the garden snail. Experientia 49, 919-921.

Wodinsky, J., 2008. Reversal and transfer of spermatophores by *Octopus vulgaris* and *O. hummelincki*. Mar. Biol. 155, 91-103.

Zheng, X., Ding, Z., Xu, Y., Monroig, O., Morais, S., Tocher, D.R., 2009. Physiological roles of fatty acyl desaturase and elongase in marine fish: Characterisation of cDNAs of fatty acyl Δ6 desaturase and Elovl5 elongase of cobia (*Rachycentron canadum*). Aquaculture 290, 122-131.

Zhu, N., Dai, X., Lin, D.S., Connor, W.E., 1994. The lipids of slugs and snails: Evolution, diet and biosynthesis. Lipids 29, ,869-875.

Zhukova, N.V., 1986. Biosynthesis of non-methylene-interrupted dienoic fatty acids from ^{14}C-acetate in molluscs. Biochim. Biophys. Acta 878, 131-133.

Zhukova, N.V., 1991. The pathway of the biosynthesis of non-methylene-inter- rupted dienoic fatty acids in Molluscs. Comp. Biochem. Physiol. 100B, 801–804.

Zhukova, N.V, 2007. Lipid classes and fatty acid composition of the tropical nudibranch mollusks *Chromodoris* sp. and *Phyllidia coelestis*. Lipids 42, 1169–1175.

Legends to Figures

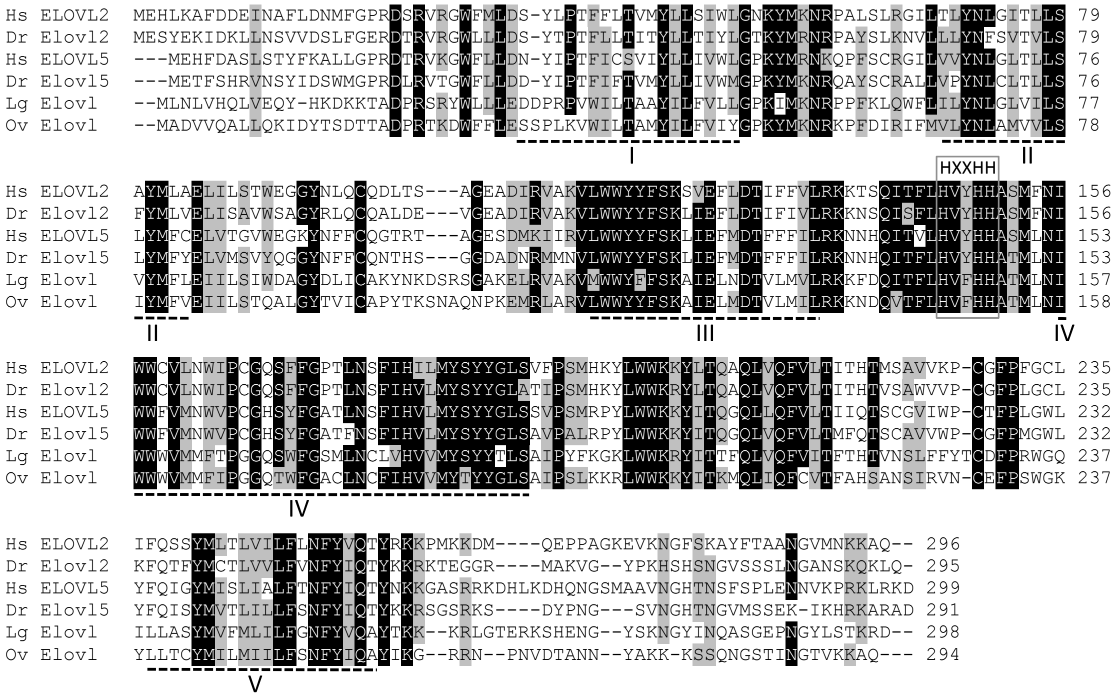

Fig. 1. Alignment of the deduced amino acid (aa) sequence of the fatty acyl elongase from *Octopus vulgaris* (Ov). The aa sequence of the octopus Elovl-like protein was aligned with those of the *Homo sapiens* (Hs) ELOVL2 (gb|NP_060240|) and ELOVL5 (gb|NP_068586|), the *Danio rerio* (Ds) Elovl2 (gb|NP_001035452|) and Elovl5 (gb|NP_956747|) and the so-called Elovl-like transcript 1 (jgi|Lotgi1|224291|) from *Lottia gigantea* (Lg) using ClustalW (Bioedit). Identical residues are shaded black and similar residues are shaded grey. Identity/similarity shading was based on the BLOSUM62 matrix, and the cut-off for shading was 70%. 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.

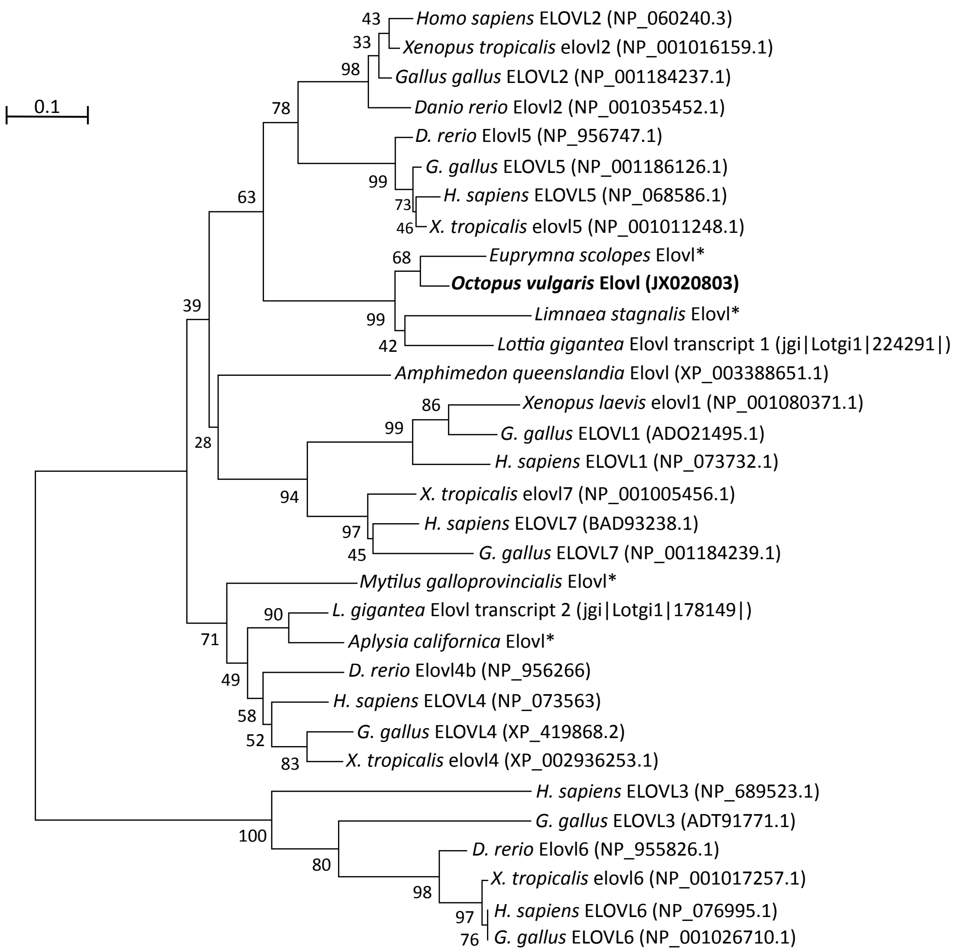

Fig. 2. Phylogenetic tree comparing the deduced amino acid (aa) sequence of the *Octopus vulgaris* elongase of very long-chain fatty acids (Elovl)-like protein with a series of protein sequences including representatives of the seven (1-7) Elovl subtypes and other Elovl-like sequences from invertebrate 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/) as described in Materials and Methods. 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.

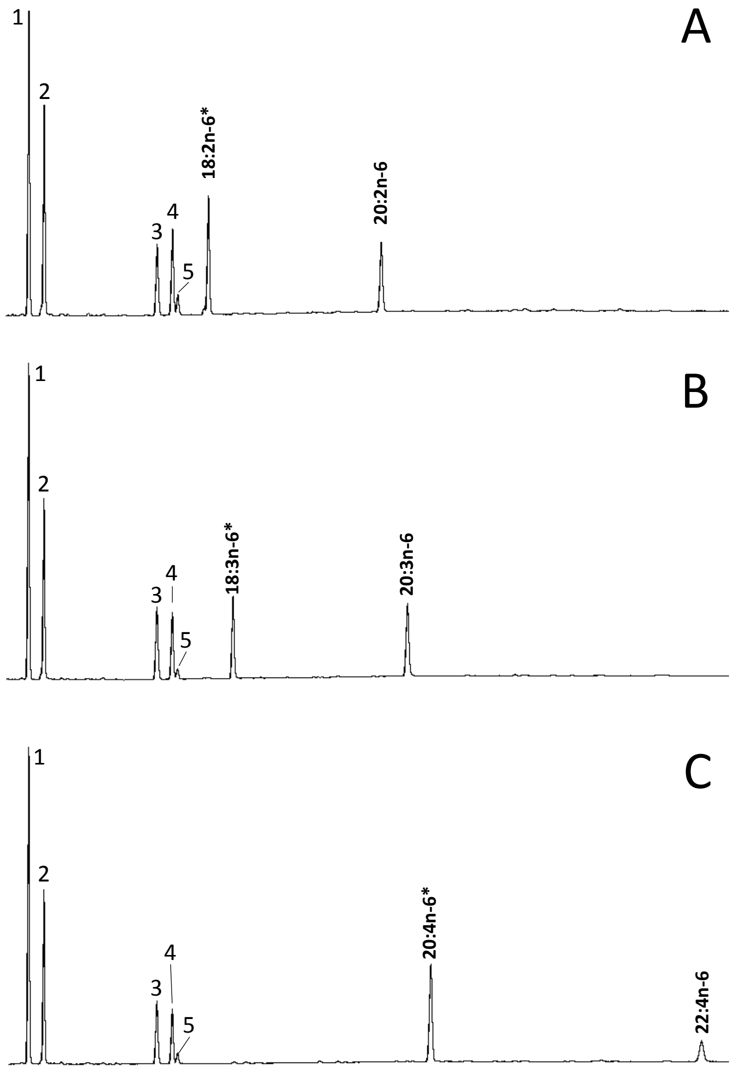

Fig. 3. Functional characterisation of the *Octopus vulgaris* elongase of very long-chain fatty acids (Elovl) in yeast (*Saccharomyces cerevisiae*). The fatty acid (FA) profiles of yeast transformed with pYES2 containing the ORF of the putative Elovl cDNA as an insert, were determined after the yeast was grown in the presence of one of the exogenously added substrates 18:2n-6 (A), 18:3n-6 (B) and 20:3n-6 (C). Peaks 1-5 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) and 18:1n-7 (5). Additionally peaks derived from exogenously added substrates ("*") or elongation products are indicated accordingly in panels A-C. Vertical axis, FID response; horizontal axis, retention time.

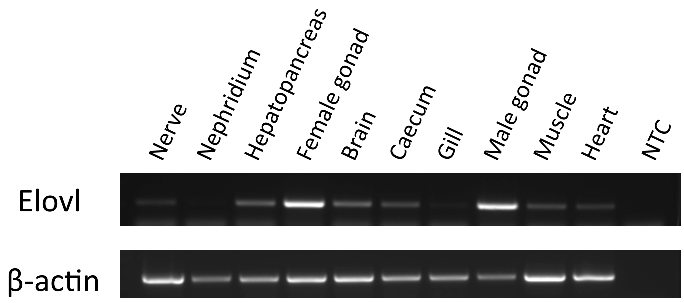

Fig. 4. RT-PCR analyses showing the tissue distribution of octopus elongase of very long-chain fatty acids (Elovl) transcripts. Expression of the housekeeping gene β-actin is also shown. NTC, no template control.

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 octopus elongase of very long-chain fatty acids (Elovl) ORF and for RT-PCR analysis of gene expression in octopus tissues.

Aim	Transcript	Primer	Primer sequence	Accession No[a].
3' RACE PCR	Elovl	OVEloF1	5'-GACTTGGTTCGGTGCTTGTT-3'	JX020803
		OVEloF2	5'-ATGGCCTGTCTGCTATACCAT-3'	
5' RACE PCR		OVEloR1	5'-ATGGTATAGCAGACAGGCCAT-3'	
		OVEloR2	5'-ATGATGGAAGACATGCAGGAA-3'	
ORF cloning	Elovl	OVEloVF	5'-CCCAAGCTTAAAATGGCGGACGTTGTG-3'	JX020803
		OVEloVR	5'-CCGGAGCTCCTATTGAGCTTTCTTCACC-3'	
RT-PCR	Elovl	OVEloF1	5'-GACTTGGTTCGGTGCTTGTT-3'	JX020803
		OVEloR3	5'-GTCTGCCTTTGATGTAAGCCTG-3'	
	ß-actin	OVACTF	5'-CTTGACTCCGGAGATGGTGT-3'	AB053937
		OVACTR	5'-CGCATTTCATGATGGAGTTG-3'	

[a] GenBank (http://www.ncbi.nlm.nih.gov/)

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Table 2. Functional characterisation of the octopus fatty acyl elongase 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	13.4	C18→20
18:2n-6	20:2n-6	40.8	C18→20
18:4n-3	20:4n-3	36.9	C18→20
18:3n-6	20:3n-6	52.3	C18→20
20:5n-3	22:5n-3	2.4	C20→22
20:4n-6	22:4n-6	15.9	C20→22
22:5n-3	24:5n-3	0.0	C22→24
22:4n-6	24:4n-6	0.0	C22→24

Table 3. Fatty acid (FA) composition (% of totals) from the polar lipids of tissues collected from *Octopus vulgaris* adult individuals. FAs are designated using the 'n-' nomenclature, except for non-methylene-interrupted FA where the 'Δ' nomenclature was used.

	Nephridium	Male gonad	Eye	Caecum
14:0	0.7	0.5	0.7	1.3
15:0	0.3	0.3	0.3	0.3
16:0	14.2	14.7	18.9	14.8
16:1n-9	nd	0.5	0.2	0.1
16:1n-7	0.5	0.4	0.4	1.9
16:0 iso	0.2	0.1	0.2	0.2
16:0 anteiso	0.2	nd	0.1	nd
17:0	2.7	1.4	1.2	1.9
17:1	nd	0.1	0.2	nd
17:0 iso	0.3	nd	0.2	0.2
18:0	13.6	8.4	7.4	14.9
18:1n-13	0.6	0.9	1.4	0.3
18:1n-9	2.0	2.1	1.1	3.1
18:1n-7	2.0	1.3	1.4	2.6
18:1n-5	0.3	0.1	nd	nd
18:2n-6	0.1	nd	0.8	0.3
18:3n-3	nd	nd	0.1	0.2
18:4n-3	nd	nd	nd	0.1
20:0	0.1	0.1	0.1	0.2
20:1n-11	0.5	0.5	0.5	1.0
20:1n-9	9.2	10.5	2.4	2.8
20:1n-7	0.2	0.2	0.1	0.2
Δ5,11 20:2	1.8	nd	nd	nd
Δ7,13 20:2	nd	0.2	0.1	0.1
20:2n-6	0.3	0.1	0.8	0.4
Δ5,11,14 20:3	0.8	nd	nd	nd
20:3n-6	0.1	nd	0.2	0.1
20:4n-6	11.9	16.4	5.1	13.6
20:3n-3	0.1	nd	13.5	0.1
20:5n-3	10.0	7.3	11.4	13.7
22:0	0.2	0.1	0.2	0.3
22:1n-11	2.0	2.3	0.4	1.8
22:1n-9	0.1	0.1	nd	0.3
Δ7,13 22:2	nd	2.2	0.4	0.8
21:5n-3	0.1	nd	0.1	nd
22:2n-6	nd	nd	nd	0.3
22:4n-6	1.1	7.7	0.3	1.1
22:5n-6	1.0	0.8	0.2	0.8
22:5n-3	1.3	2.0	1.0	1.5
22:6n-3	20.3	17.4	27.0	15.5
24:1n-9	0.1	0.1	0.2	0.4

nd, not detected.