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Title

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

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Keywords

Elongases; essential fatty acids; non-methylene interrupted fatty acids; *Octopus vulgaris*; polyunsaturated fatty acids.

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 establish the essential fatty acid (FA) requirements for octopus paralarvae through determination of the enzymes of endogenous PUFA biosynthetic pathways. We here 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 mediate the elongation of FA including PUFA. Our results suggested 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. 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 analogous 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.

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 for 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; Quintana, 2006; Villanueva and Bustamante, 2006; 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. While limited success in the production of juvenile octopuses has been achieved (Villanueva, 1995; Iglesias et al., 2002, 2004; Lenzi et al., 2009), 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) have been previously suggested to be critical dietary components for octopus paralarvae (Navarro and Villanueva, 2003). We have recently initiated a series of studies to establish the essential fatty acid (FA) requirements for octopus paralarvae, so that balanced diets matching their endogenous biosynthetic capability can be formulated and thus promote paralarval survival and development. Due to the obvious difficulties in conducting feeding trials with octopus paralarvae, our approach aims to characterise the gene products encoding enzymes involved in the PUFA biosynthetic pathway, which themselves dictate the ability of

70 species to endogenously produce long-chain PUFA (LC-PUFA) (Bell and Tocher,
71 2009).

72 Previously, we reported the molecular cloning and functional characterisation of a
73 fatty acyl desaturase (Fad) from *O. vulgaris* (Monroig et al., 2012a). The substrate
74 specificity of the octopus Fad revealed that this enzyme was a Δ5-like Fad and thus we
75 provided for first time molecular evidence of such an enzymatic activity in molluscs
76 (Monroig et al., 2012a). Interestingly, the Δ5 Fad enables the common octopus to
77 endogenously convert 20:4n-3 and 20:3n-6 into eicosapentaenoic acid (EPA, 20:5n-3)
78 and arachidonic acid (ARA, 20:4n-6), respectively, the latter regarded as critical LC-
79 PUFA in a variety of physiological processes ensuring normal cellular function (Funk,
80 2001). Rather than a role in the biosynthesis of EPA, we hypothesised that Δ5 Fad
81 activity may actually be retained in the octopus for the endogenous biosynthesis of
82 ARA, as high contents of ARA encountered in adult octopus tissues were unlikely to be
83 exclusively from dietary origin. In addition to the potential participation of the octopus
84 Δ5 Fad in ARA biosynthesis, the common octopus Δ5 Fad might also have a role in the
85 biosynthesis of non-methylene interrupted (NMI) FA, compounds with unusual
86 unsaturation features that have been found in a variety of marine invertebrates
87 (Barnathan, 2009; Kornprobst and Barnathan, 2010).

88 The biosynthesis of PUFA including NMI FA in marine molluscs has been
89 investigated previously (De Moreno et al., 1976; Waldock and Holland, 1984; Zhukova,
90 1986, 1991, 2007). Although the biosynthetic ability of molluscs was species-specific
91 (Waldock and Holland, 1984), it has been shown that some molluscs have active PUFA
92 biosynthetic pathways and, in addition to the above mentioned Δ5 desaturase, active FA
93 elongation systems also appear to be present. Using radioactive FA, it was reported that
94 the clam *Mesoderma mactroides* could elongate both 18:3n-3 and 18:2n-6 (De Moreno

et al., 1976). Later, Waldock and Holland (1984) demonstrated that the Pacific oyster *Crassostrea gigas* had the ability to produce 20:5n-3 and 22:6n-3. Investigations in other bivalves like *Scapharca broughtoni*, *Callista brevisiphonata* and *Mytilus edulis* (Zhukova, 1986, 1991) demonstrated that the biosynthesis of the NMI dienes Δ7,13 22:2 and Δ7,15 22:2 was achieved by elongation from Δ5,11 20:2 and Δ5,13 20:2, respectively. 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 dienes Δ7,13 22:2 and Δ7,15 22:2, respectively, by chain elongation and subsequent Δ5 desaturation (Kawashima, 2005).

The elongases of very long-chain fatty acids (Elovl), a protein family with seven distinct members (Elovl 1-7) in vertebrates, account for the condensation of 2C into activated preexisting fatty acyl chains (Jakobsson et al., 2006). Investigation of FA biosynthetic pathways has allowed the molecular and functional characterisation of a number of genes encoding Elovl enzymes from vertebrates (see reviews by Jakobsson et al., 2006; Guillou et al., 2010; Monroig et al., 2011a). In contrast, studies of elongase-encoding genes from non-vertebrate organisms are scarce, with only 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), and no elongases from molluscs have been reported.

In order to expand our knowledge of EFA requirements of common octopus, the present study reports the molecular cloning, functional characterisation and tissue distribution of transcripts (mRNA) of a cDNA encoding a putative elongase involved in PUFA biosynthesis. In order to understand a potential role of the newly cloned elongase

120 in the NMI FA biosynthesis in the common octopus, we also analysed the double bond
121 features of NMI FA found in specific tissues of octopus adult specimens.

122 **Materials and methods**

123 *Tissue samples*

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

130 *Elongase cDNA cloning*

131 Total RNA was extracted from octopus tissues using TRIzol® (Gibco BRL, Grand
132 Island, NY, USA) reagent following manufacturer's instructions. Subsequently, first
133 strand cDNA was synthesised using a Verso™ cDNA kit (ABgene, Rockford, IL, USA)
134 primed with random hexamers. In order to amplify the first fragment of the elongase
135 cDNA, the amino acid (aa) sequences of Elovl5 proteins from *Homo sapiens*
136 (NP_068586.1), *Rattus norvegicus* (NP_599209.1), *Bos taurus* (NP_001040062.1),
137 *Danio rerio* (NP_956747.1) and *Pagrus major* (ADQ27303.1) were aligned using
138 BioEdit v5.0.6 (Tom Hall, Department of Microbiology, North Carolina State
139 University, USA). Conserved regions were used for *in silico* searches of mollusc
140 expressed sequence tags (EST) using NCBI tblastn tool (http://www.ncbi.nlm.nih.gov/).
141 Several EST displaying high homology with Elovl encoding genes were identified from
142 the molluscs *Mytilus galloprovinciallis* (gb|FL495089.1| and gb|FL499406.1|),
143 *Euprymna scolopes* (gb|DW256301.1|), and *Lymnaea stagnalis* (gb|FC701557.1|,
144 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® Colorless Master Mix (Promega, Southampton, UK), and using a mixture of cDNA from gonads, brain, nerve and caecum as template. The PCR 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.

For 5'RACE PCR, a positive fragment was obtained by two-round PCR. The first round PCR was performed using the adapter-specific 5'RACE OUTER primer and the gene-specific forward 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 two-round 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 product was then used as template for nested PCR with primers 5'RACE INNER and OVEloF2, 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 those 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 Neighbour Joining method (Saitou and Nei 1987), with confidence in the resulting tree branch topology measured by bootstrapping through 10000 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, extension at 72 ºC for 2 min 15 s, followed by a final extension at 72 ºC for 5 min. The primers containing restriction sites (underlined in Table 1) OVEloVF (*Hin*dIII) and OVEloVR (*Sac*I) 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).

In order to test the ability of octopus Elovl cDNA ORF to elongate either saturated or monounsaturated FA, 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$^{-uracil}$ with no exogenously added FA substrates. Additionally, the ability of *O. vulgaris* Fad to desaturate 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 uptake efficiency decreases with increasing chain length (Zheng et al., 2009). Yeast transformed with empty pYES2 were also grown in presence of PUFA substrates as control treatments. After 2-day culture at 30 ^{o}C, yeast were harvested, washed, and lipid extracted by homogenisation in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxy toluene (BHT) as antioxidant. All fatty acid substrates, except stearidonic acid (18:4n-3), were purchased from Nu-Chek Prep,

220 Inc (Elysian, MN, USA). Stearidonic acid and chemicals used to prepare the *S.*
221 *cerevisiae* minimal medium-^{uracil} were from Sigma Chemical Co. Ltd. (Dorset, UK),
222 except for the bacteriological agar obtained from Oxoid Ltd. (Hants, UK).

223 *Tissue distribution of elongase transcripts*

224 Expression of the octopus elongase was examined in adult tissues by RT-PCR. Total
225 RNA from a series of tissues including nerve, nephridium, hepatopancreas, brain,
226 caecum, gill, muscle, heart, and female and male gonads was extracted as described
227 above, and 1 μg of total RNA was reverse transcribed into cDNA (M-MLV reverse
228 transcriptase, Promega). In order to determine the mRNA distribution of the octopus
229 elongase, the tissue cDNAs were used as templates in PCR consisting of a denaturating
230 step at 95 ºC for 1 min, followed by 35 cycles of denaturation at 95 ºC for 30 s,
231 annealing at 58 ºC for 30 s, extension at 72 ºC for 30 s, followed by a final extension at
232 72 ºC for 5 min (GoTaq® Green Master Mix, Promega). Additionally, the expression of
233 the housekeeping β-actin was determined to check the cDNA integrity. Primers used for
234 RT-PCR are shown in Table 1.

235 *Fatty acid analysis by GC-MS*

236 FA from the transgenic yeast were analysed by preparing methyl esters (FAME) as
237 previously described (Hastings et al. 2001). Briefly, FAME were identified and
238 quantified using an Agilent 6850 Gas Chromatograph system coupled to a 5975 series
239 MSD (Agilent Technologies, Santa Clara, CA, USA). The elongation efficiency from
240 potential substrates including the yeast endogenous FA and the exogenously added
241 PUFA substrates (18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3 and
242 22:4n-6) were calculated by the proportion of substrate FA converted to elongated FA
243 product as [product area/(product area + substrate area)] x 100. When further
244 confirmation of double bond positions was required, 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 nephrydium, male gonad, eye and caecum were applied to 20x20 silica gel plates (Merck, Darmstadt, Germany) and eluted 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 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* consisted of 885 bp encoding a putative protein of 294 aa. Its sequence was deposited in the GenBank database with accession number JX020803. The deduced aa sequence from the octopus elongase showed identity scores ranging from 39.3 and 43.2 % with several Elovl proteins (Elovl2, Elovl4 and Elovl5) from vertebrates including *H. sapiens*, *X. 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 *A. 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 streches were predicted in 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 types (Elovl 1-7) from a variety of vertebrates (Fig. 2). Our results showed that the octopus Elovl protein clustered with other Elovl-like from molluscs including the cephalopod *E. scolopes* and the gastropods *L. stagnalis* and *L. gigantea* ('transcript 1'), altogether forming a group close to Elovl2 and Elovl5 proteins from vertebrates. More distantly, 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 encoding cDNA was functionally characterised by expressing the ORF in yeast *S. cerevisiae*. The FA composition of wild yeast consists basically 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 (Monroig et al., 2010a). Total lipid of yeast transformed with the empty pYES2 vector (control) contained these FA together with

295 whichever exogenous FA (if any) was added as substrate (data not shown), indicating
296 that no elongase activity towards any of the exogenously added PUFA substrates
297 assayed. This result is in agreement with the well-know inability of *S. cerevisiae*
298 elongases to operate towards PUFA substrates (Hastings et al., 2001; Agaba et al.,
299 2004).

300 In order to test the ability of the octopus Elovl to elongate saturated and
301 monounsaturated FA, yeast transformed with pYES2-OVElovl were grown in absence
302 of exogenously added substrates. Our results showed that none of the yeast endogenous
303 FA, whether saturated or monounsaturated, were elongated. Conversely, yeast
304 transformed with pYES2-OVElovl showed activity towards PUFA substrates producing
305 the corresponding 2-carbon elongation product. As shown in Fig. 3, the exogenously
306 added C18 (18:3n-3, 18:2n-6, 18:4n-3 and 18:3n-6) and C20 (20:5n-3 and 20:4n-6)
307 substrates were elongated to C20 (20:3n-3, 20:2n-6, 20:4n-3 and 20:3n-6) and C22
308 (22:5n-3 and 22:4n-6) products, respectively. Conversion rates derived from the yeast
309 assays suggested that the octopus Elovl generally elongated n-6 PUFA substrates more
310 efficiently than n-3 substrates for each pair of homologous substrates considered. Thus,
311 the substrates 18:2n-6, 18:3n-6, 20:4n-6 were consistently elongated at higher rates than
312 the corresponding n-3 PUFA substrates 18:3n-3, 18:4n-3 and 20:5n-3, respectively.
313 Interestingly, no activity towards C22 (22:5n-3 and 22:4n-6) PUFA substrates was
314 detected.

315 *Tissue distribution of octopus elongase transcripts*

316 Tissue expression of the common octopus Elovl was studied by RT-PCR on cDNA
317 samples obtained from a range of tissues (Fig. 4). Except for nephridium, transcripts of
318 the octopus Elovl gene were detected in all tissues analysed. Although RT-PCR

319 analyses should not be regarded as strictly quantitative data, our results indicate that
320 both the male and female gonads showed higher expression signals.
321 *Fatty acid composition from polar lipids of adult octopus tissues*
322 FA from PL were analysed in several tissues of adult octopus individuals (Table 3).
323 DHA appeared the most abundant FA for each tissue considered, with up to 27.0 % of
324 total FA in eye PL. Other PUFA relatively abundant in the tissues studied were ARA
325 (with up to 16.4 % in male gonad PL) and EPA (up to 13.7 % in caecum PL).
326 Interestingly, 20:3n-3 content in eye was 13.5 % of total FA in the PL fraction.
327 GC-MS analysis of picolinyl esters enabled us to identify four different NMI FA in
328 the octopus tissues, namely Δ5,11 20:2, Δ7,13 20:2, Δ5,11,14 20:3 and Δ7,13 22:2
329 (Table 3). Although we specifically analysed the PL fractions, where NMI FA are
330 believed to accumulate (Klingensmith, 1982; Pirini et al., 2007), the amounts of all the
331 NMI FA identified were generally low, and only relatively higher contents were
332 detected for Δ5,11 20:2 in nephrydium (1.8 %) and its corresponding elongation product
333 Δ7,13 22:2 in male gonad (2.2 %).
334 **Discussion**
335 The FA biosynthesis pathways have been investigated in both terrestrial (van der
336 Horst 1973, 1974; Weinert et al. 1993; Zhu et al. 1994) and aquatic mollusc species
337 (Chu and Greaves 1991; de Moreno et al. 1976; Waldock and Holland 1984; Zhukova
338 1986, 1991, 2007; Delaporte et al., 2005). It was shown that some molluscs have active
339 FA elongation systems (Waldock and Holland, 1984; Zhukova, 1986; Delaporte et al.,
340 2005). In the present study we provide compelling evidence of the existence of an Elovl
341 cDNA that encodes an enzyme potentially involved in the biosynthesis of PUFA in the
342 cephalopod *O. vulgaris*.

343 The deduced aa sequence of the Elovl-like cDNA from *O. vulgaris* contains all the
344 features of the vertebrate Elovl protein family members, including five membrane-
345 spanning regions, an ER retrieval signal at the C terminus containing lysine residues
346 (KKXX) and a diagnostic histidine box (HXXHH) (Leonard et al., 2004; Jakobsson et
347 al., 2006). Moreover, the histidine (H) box and its N-terminal side (QVTFLHVFHH)
348 show a typical aa pattern of the PUFA elongase subfamily of eukaryotic elongases, with
349 a glutamine (Q) at position -5 and a leucine (L) at position -1 from the first H
350 (Hashimoto et al., 2008). Further evidence supporting a potential role of this octopus
351 Elovl cDNA in the PUFA biosynthetic pathways was provided by phylogenetic
352 analysis. Thus, the octopus Elovl aa sequence, as well as those of other mollusc
353 elongases, obtained by *in silico* searches, including the cephalopod *Euprymna scolopes*
354 and the gastropods *Lymnaea stagnalis* and *Lottia gigantea* (transcript 1), showed great
355 similarity to the sequences of Elovl2 and Elovl5 proteins, critical enzymes participating
356 in the biosynthesis of LC-PUFA in vertebrates (Leonard et al., 2004; Jakobsson et al.,
357 2006). More distantly, the other elongase identified in the *L. gigantea* genome
358 (transcript 2) and also other Elovl-like proteins from *A. californica* and *M.*
359 *galloprovincialis* grouped together with vertebrate Elovl4 elongases, another type of
360 elongase involved in the biosynthesis of very long-chain FA (C>24) including both
361 saturates and polyenes (Agbaga et al., 2008; Monroig et al., 2010b, 2011b, 2012b).
362 While these results suggest that another elongase with similarity to Elovl4 might also be
363 present in the common octopus, the functional characterisation of the present Elovl
364 cDNA confirmed, not only its participation in the PUFA elongation pathway, but also
365 that it has substrate specificities more similar to Elovl5 than Elovl2.
366 Clearly, transgenic yeast expressing the octopus Elovl efficiently converted C18 and
367 C20 PUFA substrates to their corresponding 2-carbon elongated products, but no

368 activity towards C22 PUFA was detected. Generally, this pattern of substrate specificity
369 of the octopus elongase is consistent with that of vertebrate Elovl5 proteins (Jakobsson
370 et al., 2006). For instance, the human ELOVL5 (also termed HELO1) and the rat
371 ELOVL5 (also termed rELO1) were shown to efficiently elongate C18 and C20 PUFA,
372 whereas C22 PUFA did not appear to be substrates for these enzymes (Leonard et al.,
373 2000; Inagaki et al., 2002). Similarly, fish Elovl5 demonstrated high activity for the
374 elongation of C18 and C20 PUFA substrates, whereas C22 substrates were only
375 elongated to a lesser extend (Agaba et al., 2004; Morais et al., 2009; Mohd-Yusof et al.,
376 2010; Monroig et al., 2012b). Importantly, elongation of C22 PUFA including 22:5n-3
377 and 22:4n-6 in vertebrates is basically mediated by Elovl2, whose substrate chain-length
378 specificity also includes C20, but not C18, PUFA substrates, the latter being only
379 marginally or not elongated (Tvrdik et al., 2000; Leonard et al., 2002; Monroig et al.,
380 2009; Morais et al., 2009). Overall it can be concluded that the *O. vulgaris* elongase
381 cloned here is phenotypically an Elovl5-like elongase, but its sequence similarity to
382 vertebrate Elovl2 suggests an interesting evolutionary scenario that is worth exploring
383 in future investigations.

384 The functional characterisation of the octopus Elovl revealed, however, that the gene
385 product might have conserved/acquired a different PUFA family specificity compared
386 to vertebrate Elovl5 proteins during evolution. Unlike mammalian (Leonard et al., 2000;
387 Inagaki et al., 2002) and fish Elovl5 (Agaba et al., 2005; Mohd-Yusof et al., 2010;
388 Morais et al., 2011; Monroig et al., 2012b), which are generally more efficient in
389 elongating n-3 rather than n-6 FA substrates, the octopus Elovl exhibited higher
390 elongation rates towards n-6 compared to n-3 substrates for each homologous pair
391 considered. Thus 18:2n-6, 18:3n-6, 20:4n-6 were all elongated at higher rates than the
392 corresponding n-3 FA, namely 18:3n-3, 18:4n-3 and 20:5n-3, respectively. These results

393 emphasise that n-6 FA in general, and especially ARA (20:4n-6), might play
394 particularily important physiological roles in the common octopus. Consitent with this,
395 several studies have reported unexpectedly high levels of ARA in tissues of common
396 that were unlikely to derive purely from dietary origin and, thus, an active biosynthesis
397 of ARA in the common octopus was postulated (Milou et al., 2006; García-Garrido et
398 al., 2010; Monroig et al., 2012a). In the present study, the efficiency shown by the
399 octopus Elovl to elongate certain PUFA substrates indicates that this enzyme could
400 contribute to the endogenous biosynthesis of ARA in this species.

401 In vertebrates, ARA is biosynthesised from the dietary essential C18 PUFA 18:2n-6
402 through two alternative pathways, the 'classical' Δ6-pathway (Δ6 desaturation →
403 elongation → Δ5 desaturation), or alternatively through the so-called 'Δ8-pathway'
404 (elongation → Δ8 desaturation → Δ5 desaturation) (Monroig et al., 2011c). In addition
405 to the ability of the formerly characterised Fad cDNA to mediate the Δ5-desaturation
406 steps of these pathways (Monroig et al., 2012a), we here demonstrate that the newly
407 cloned octopus Elovl can efficiently catalyse the elongation reactions required for ARA
408 biosynthesis from the dietary essential 18:2n-6, namely 18:3n-6 → 20:3n-6 for the Δ6-
409 pathway and 18:2n-6 → 20:3n-6 for the Δ8-pathway. Although genes responsible for
410 elongation and Δ5 desaturation steps of these pathways have now been identified in
411 octopus, no Fad cDNA with Δ6 or Δ8-desaturase activity has yet been identified and,
412 consequently, it remains unclear whether the common octopus can biosynthesise ARA
413 from the dietary essential 18:2n-6. This appears to be the case for some abalone species
414 (Dunstan et al., 1996; Durazo-Beltrán et al., 2003) but other species like *C. gigas*
415 (Waldock and Holland, 1984) and *Mytilus edulis* (Zhukova, 1991) appear unable to
416 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 non-methylene-interrupted (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 20:2. 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 summary, 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 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 particularily pivotal physiological roles in the common octopus. Moreover, the Elovl might be involved in the biosynthesis of NMI FA, although the quantitative significance of this biosynthetic pathways in *O. vulgaris* requires further investigation.

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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	Ac
RACE PCR	Elovl	OVEloF1	5'-GACTTGGTTCGGTGCTTGTT-3'	
		OVEloF2	5'-ATGGCCTGTCTGCTATACCAT-3'	
		OVEloR1	5'-ATGGTATAGCAGACAGGCCAT-3'	
		OVEloR2	5'-ATGATGGAAGACATGCAGGAA-3'	
ORF cloning	Elovl	OVEloVF	5'-CCCAAGCTTAAAATGGCGGACGTTGTG-3'	
		OVEloVR	5'-CCGGAGCTCCTATTGAGCTTTCTTCACC-3'	
RT-PCR	Elovl	OVEloF1	5'-GACTTGGTTCGGTGCTTGTT-3'	
		OVEloR3	5'-GTCTGCCTTTGATGTAAGCCTG-3'	
	ß-actin	OVACTF	5'-CTTGACTCCGGAGATGGTGT-3'	
		OVACTR	5'-CGCATTTCATGATGGAGTTG-3'	

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

Table 2. Functional characterisation of the octopus 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

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

	Nephrydium	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

699 nd, no detected.
700

Legends to Figures

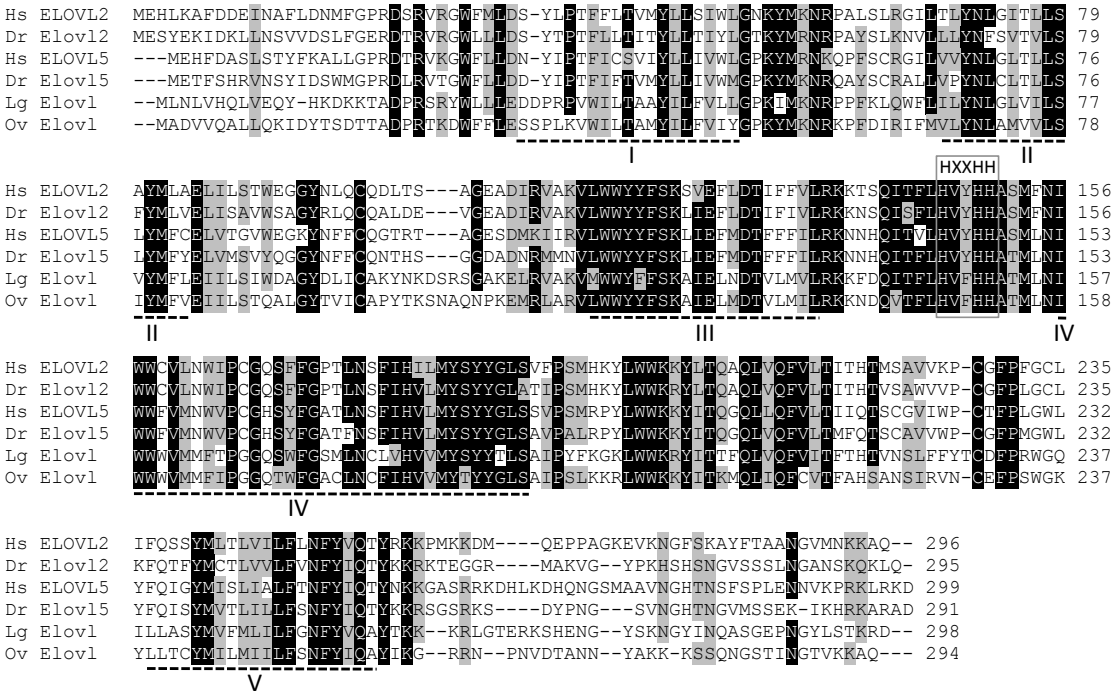


Fig. 1. Alignment of the deduced amino acid (aa) sequence of the elongase from *Octopus vulgaris* (Ov). The aa sequence of the octopus Elovl-like protein was aligned with the *Homo sapiens* (Hs) ELOVL2 (gb|NP_060240|) and ELOVL5 (gb|NP_068586|), the *Danio rerio* (Ds) Elovl2 (gb|NP_001035452|) and Elovl5 (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.

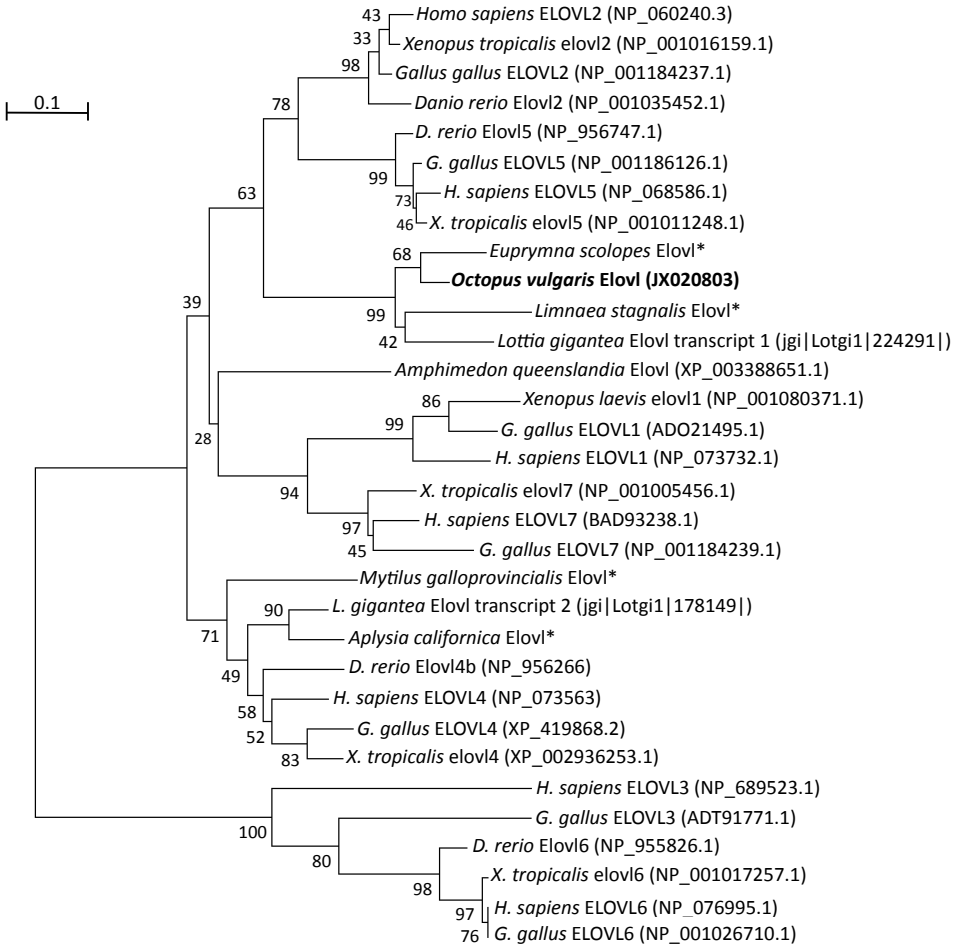
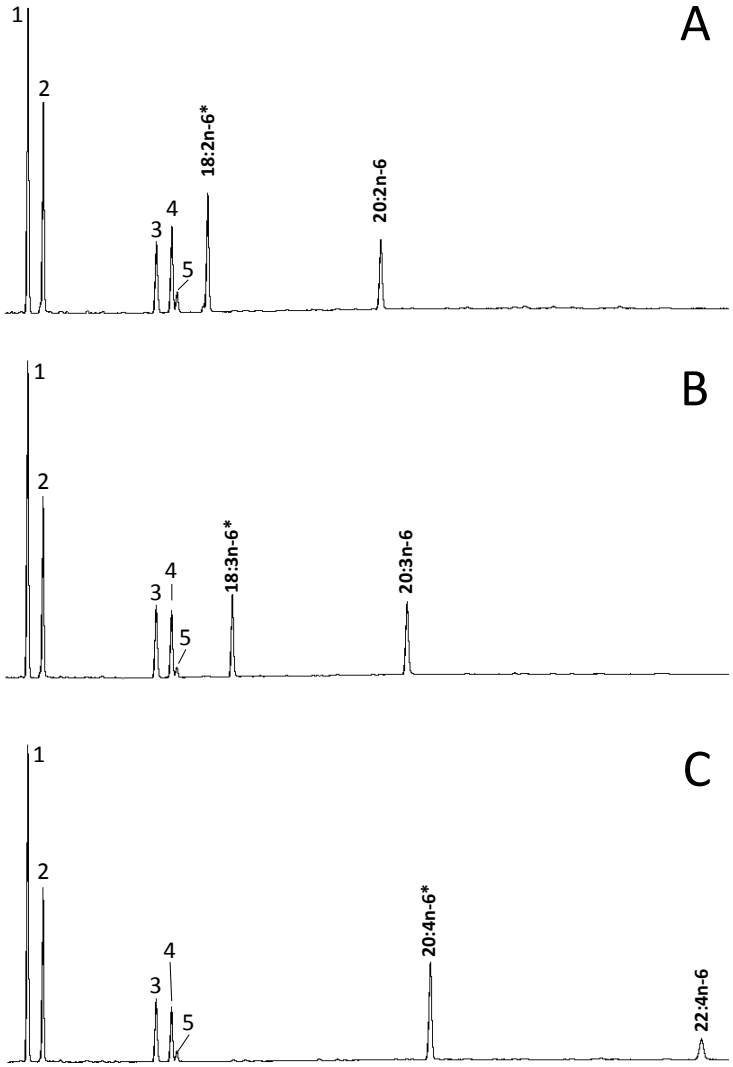


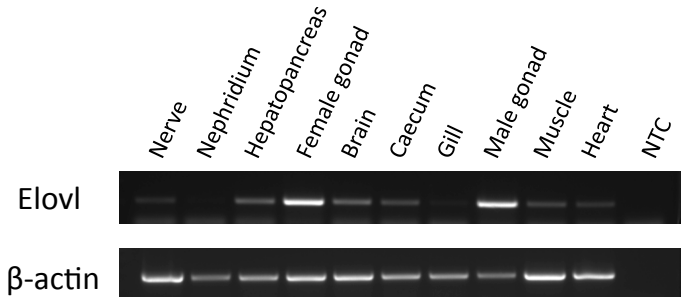
Fig. 2. Phylogenetic tree comparing the deduced amino acid (aa) sequence of the *Octopus vulgaris* elongase of very long-chain fatty acids (Elovl)-like with a series of protein sequences including representatives of the seven (1-7) Elovl subtypes and other Elovl-like sequences from invertebrate organisms. 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 10000 iterations.

726



727

728 Fig. 3. Functional characterisation of the *Octopus vulgaris* elongase of very long-chain
729 fatty acids (Elovl) in yeast (*Saccharomyces cerevisiae*). The fatty acid (FA) profiles of
730 yeast transformed with pYES2 containing the ORF of the putative Elovl cDNA as an
731 insert, were determined after the yeast was grown in the presence of one of the
732 exogenously added substrates 18:2n-6 (A), 18:3n-6 (B) and 20:3n-6 (C). Peaks 1-5 in all
733 panels are the main endogenous FA of *S. cerevisiae*, namely 16:0 (1), 16:1 isomers (2),
734 18:0 (3), 18:1n-9 (4) and 18:1n-7 (5). Additionally peaks derived from exogenously
735 added substrates ("*") or elongation products are indicated accordingly in panels A-C.
736 Vertical axis, FID response; horizontal axis, retention time.



737

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