Title

Identification of a Δ5-like fatty acyl desaturase from the cephalopod *Octopus vulgaris* (Cuvier 1797) involved in the biosynthesis of essential fatty acids

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Keywords

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Summary

Long-chain polyunsaturated fatty acids (LC-PUFA) have been identified as essential compounds for common octopus (*Octopus vulgaris*), but precise dietary requirements have not been determined due in part to the inherent difficulties of performing feeding trials on paralarvae. Our objective is to establish the essential fatty acid (EFA) requirements for paralarval stages of the common octopus through characterisation of the enzymes of endogenous LC-PUFA biosynthetic pathways. In this study we isolated a cDNA with high homology to fatty acyl desaturases (Fad). Functional characterisation in recombinant yeast showed the octopus Fad exhibited Δ5 desaturation activity towards saturated and polyunsaturated fatty acyl substrates. Thus, it efficiently converted the yeast's endogenous 16:0 and 18:0 to 16:1n-11 and 18:1n-13, respectively, and desaturated exogenously added PUFA substrates, 20:4n-3 and 20:3n-6, to 20:5n-3 (EPA) and 20:4n-6 (ARA), respectively. Although the Δ5 Fad enables common octopus to produce EPA and ARA, the low availability of its adequate substrates 20:4n-3 and 20:3n-6, either in the diet or by limited endogenous synthesis from C_{18} PUFA, might indicate that EPA and ARA are indeed EFA for this species. Interestingly, the octopus Δ5 Fad can also participate in the biosynthesis of non-methylene interrupted FA, PUFA that are generally uncommon in vertebrates but that have been found previously in marine invertebrates including molluscs, and now also confirmed to be present in specific tissues of common octopus.

Introduction

The common octopus (*Octopus vulgaris*, Cuvier 1797) is a prime candidate for diversification of marine aquaculture and extensive research efforts have been devoted over the last decade to investigate several aspects of octopus culture including husbandry (Iglesias et al., 2006), reproduction (Estefanell et al. 2010; Otero et al. 2007; Wodinsky 2008) and nutrition (Navarro and Villanueva 2000, 2003; Quintana 2006; Villanueva et al. 2009). Although considerable progress has been made and on-growing wild-captured octopus in floating cages is now possible (Iglesias et al. 2007), a major, yet unresolved, problem in octopus culture is the high mortality of paralarvae, early pelagic life stages, which massively die during metamorphosis to benthic life stages and, consequently, the octopus life cycle in captivity has not yet been closed.

Intensive investigations have been undertaken to elucidate the causes of high mortalities encountered during the paralarval stages of common octopus. Among them, nutritional studies have emphasised the importance that some dietary components including proteins and amino acids (Villanueva et al. 2004), essential and non-essential elements (Villanueva and Bustamante 2006) and vitamins (Villanueva et al. 2009) have for early life-cycle stages of common octopus. Furthermore, the lipid requirements of octopus paralarval stages were investigated by Navarro and Villanueva (2000, 2003), who concluded that increased polar lipids and cholesterol are required in the diet. Comparing the fatty acid (FA) profiles of enriched *Artemia* with those of crab zoeae, a natural prey used with relative success in paralarval cultures of common octopus (Villanueva 1994, 1995), it was suggested that octopus paralarvae have a high requirement for specific polyunsaturated fatty acids (PUFA), and that suboptimal dietary n-3 PUFA levels, stemming from the use of *Artemia*, might partly explain the low performance during early culture stages (Navarro and Villanueva 2003). These

71 results, along with the well-known importance of PUFA during early life-cycle stages of
72 organisms (Innis et al. 1999; Lauritzen et al. 2001; Monroig et al. 2009; Watts et al.
73 2003), has focussed interest in determining essential fatty acid (EFA) requirements
74 during early life stages of common octopus.

75 The specific FA that can satisfy the EFA requirements in a particular species
76 depends upon the ability for endogenous biosynthesis of PUFA through bioconversion
77 of dietary FA, which in turn is dependent on the complement of enzymes responsible
78 for such conversions (Bell and Tocher 2009). In vertebrates, the so-called elongases of
79 very long-chain fatty acids (Elovl) and fatty acyl desaturases (Fad) have been identified
80 as key enzymes involved in the conversion of the C_{18} EFA, linoleic (LOA, 18:2n-6) and
81 α-linolenic (ALA, 18:3n-3) acids, to the physiologically active long-chain PUFA (LC-
82 PUFA) arachidonic (20:4n-6, ARA), eicosapentaenoic (20:5n-3, EPA) and
83 docosahexaenoic (22:6n-3, DHA) acids. Elovl account for the condensation of malonyl-
84 CoA with activated fatty acyl chain resulting in a net 2C elongation of the preexisting
85 FA (Jakobsson et al. 2006). Fad enzymes introduce unsaturation (a double bond) in fatty
86 acyl chains at C6 (Δ6 Fad) or C5 (Δ5 Fad) from the carboxyl group. Recently, a Fad
87 isolated from the teleost *Siganus canaliculatus* has been found to have Δ4-desaturation
88 activity, so far appearing a unique case of such activity among vertebrates (Li et al.
89 2010). In vertebrates the LC-PUFA biosynthetic pathway has been extensively
90 investigated and a number of genes encoding either Elovl or Fad proteins have been
91 characterised, particularly from fish, which are the primary source of n-3 LC-PUFA in
92 the human diet. Among non-vertebrates, the eukaryotic protist *Thraustochytrium* sp.
93 (Qiu et al. 2001) and the invertebrate (nematode) *Caenorhabditis elegans* (Beaudoin et
94 al. 2000; Watts and Browse 2002) represent some of the few examples where Fad- and

95 Elovl- genes have been studied. However, as far as we are aware, neither desaturases
96 nor elongases have been previously isolated and characterised from molluscs.
97 Our overarching objective is to determine EFA requirements for paralarval
98 stages of the common octopus, so balanced diets can be formulated to improve survival
99 and development in captivity. Due to the difficulties in conducting feeding trials with
100 octopus paralarvae, alluded to above, the aim of the present study was to investigate
101 EFA requirements by characterising Fad and Elovl enzymes responsible for the LC-
102 PUFA biosynthetic pathway in this species. Here we report on the molecular cloning
103 and functional characterisation of a cDNA encoding a putative Fad from the common
104 octopus. The distribution of Fad mRNA along with fatty acid profiles were determined
105 in tissues of adult octopus in order to identify the sites of important metabolic activity.
106
107 **Materials and methods**
108 *Tissue samples*
109 Two (male and female) common octopus adult individuals (~1.5 kg) captured
110 through artisanal fisheries along the Mediterranean East Coast in Spain, were
111 transferred alive to the facilities of the Instituto de Acuicultura Torre de la Sal, cold
112 anesthetised, and sacrificed by direct brain puncture. Tissues including nerve,
113 nephridium, hepatopancreas, brain, digestive gland, gill, muscle, heart and gonad were
114 sampled and immediately frozen at -80 ºC until further analysis.
115 *Desaturase cDNA cloning*
116 Total RNA was extracted from octopus tissues using TRIzol® reagent (Gibco
117 BRL, Grand Island, NY, USA). First strand cDNA was synthesised using a Verso™
118 cDNA kit (ABgene, Rockford, IL, USA) primed with random hexamers. In order to
119 obtain the first fragment of Fad cDNA, the amino acid (aa) sequences of *Mus musculus*

FADS1 (gb|BAB69894.1|), *Danio rerio* Δ6Δ5 bifunctional Fad (gb|AAG25710.1) and desaturases from the invertebrates *Schistosoma japonicum* (emb|CAX72705.1|) and *Saccoglossus kowalevskii* (gb|XP_002736866.1|) 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/). Three EST from the Pacific oyster *Crassostrea gigas* (GenBank accession numbers CU998119.1, AM856065.1 and AM855620.1) were identified displaying high homology with Fad encoding genes. *C. gigas* EST alignment allowed the design of degenerate primers UNID5F (5'-CAYTAYGCWGGWCARGAYGC-3') and UNID5R (5'-ATYTGRAARTTVAGRTGWCC-3') that were used for polymerase chain reaction (PCR) using GoTaq® Colorless Master Mix (Promega, Southampton, UK) using brain cDNA 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 51 ºC for 30 s, extension at 72 ºC for 1 min 10 s, followed by a final extension at 72 ºC for 5 min. The PCR fragment was sequenced (CEQ-8800 Beckman Coulter Inc., Fullerton, USA) and specific primers designed for 5' and 3' rapid amplification of cDNA ends (RACE) PCR (FirstChoice® RLM-RACE kit, Ambion, Applied Biosystems, Warrington, UK) to produce full-length cDNA. Details of all primers used for RACE PCR are given in Table 1.

For 3'RACE PCR, a positive fragment was obtained by two-round PCR. The first round PCR was performed using the gene-specific sense primer OVD5F1 and the adapter-specific 3'RACE OUTER primer, 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 55 ºC for 30 s, extension at 72 ºC for 1 min 45 s, followed by a final extension at 72 ºC for 5 min

145 (GoTaq® Colorless Master Mix, Promega). First round PCR products were used as
146 template for nested PCR with primers OVD5F2 and 3'RACE INNER in a 32-cycle
147 reaction under the same thermal conditions as above. For 5'RACE PCR, a similar two-
148 round approach was followed with first round PCR performed with primers 5'RACE
149 OUTER and OVD5R1, with an initial denaturating step at 95 ºC for 1 min, followed by
150 32 cycles of denaturation at 95 ºC for 30 s, annealing at 56 ºC for 30 s, extension at 72
151 ºC for 2 min, followed by a final extension at 72 ºC for 5 min (GoTaq® Colorless
152 Master Mix, Promega). First round PCR product was then used as template for nested
153 PCR with primers 5'RACE INNER and OVD5R2, with thermal conditions as above.
154 RACE PCR products were cloned into pBluescript and sequenced as above.
155 *Tissue distribution of desaturase mRNA transcripts*
156 Expression of the octopus putative Fad was determined in adult tissues by RT-
157 PCR. Total RNA from nerve, nephridium, hepatopancreas, brain, digestive gland, gill,
158 muscle, heart, and female and male gonads was extracted as described above, and 1 µg
159 of total RNA was reverse transcribed into cDNA (M-MLV reverse transcriptase,
160 Promega). In order to determine Fad expression, the tissue cDNAs were used as
161 templates in PCR consisting of a denaturating step at 95 ºC for 1 min, followed by 35
162 cycles of denaturation at 95 ºC for 30 s, annealing at 60 ºC for 30 s, extension at 72 ºC
163 for 30 s, followed by a final extension at 72 ºC for 5 min (GoTaq® Green Master Mix,
164 Promega). Additionally, the expression of the housekeeping *β-actin* was determined to
165 check the cDNA integrity. Primers used for RT-PCR are shown in Table 1.
166 *Sequence and phylogenetic analyses*
167 An alignment of the deduced aa sequence of the newly cloned *O. vulgaris* Fad
168 cDNA with other desaturases including mammalian Δ5 (FADS1) and Δ6 (FADS2), the
169 bifunctional Δ6/Δ5 from zebrafish, and the nematode *C. elegans* Δ5 (FAT-4) was

performed using ClustalW (BioEdit). The aa sequence identity between Fad-like proteins was compared by the EMBOSS Needle Pairwise Sequence Alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). Phylogenetic analysis of the aa sequences of Fad from common octopus and other organisms including molluscs 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. Additionally, the phylogenetic tree included some stearoyl CoA desaturase (Scd) sequences, another type of membrane-bond desaturase likely to be present in molluscs.

Functional characterisation of octopus desaturase by heterologous expression in Saccharomyces cerevisiae

PCR fragments corresponding to the open reading frame (ORF) of the putative desaturase were amplified from octopus brain cDNA using the high fidelity Pfu Turbo DNA polymerase (Stratagene, Agilent Technologies, Cheshire, UK). A two-round PCR approach was used with the first round performed with specific primers OVD5U5F and OVD5U3R (Table 1). PCR conditions consisted of an initial denaturing step at 95 ºC for 2 min, followed by 32 cycles of denaturation at 95ºC for 30 s, annealing at 57 ºC for 30 s, extension at 72 ºC for 1 min 45 s, followed by a final extension at 72 ºC for 5 min. First round PCR products were used as template for the nested PCR with thermal conditions described above, and with primers containing restriction sites (underlined in Table 1) OVD5VF (*Hin*dIII) and OVD5VR (*Xho*I). The DNA fragments were then digested with the corresponding restriction endonucleases (New England BioLabs, Herts, UK) and ligated into a similarly restricted pYES2 yeast expression vector (Invitrogen, Paisley, UK). The purified plasmids (GenElute™ Plasmid Miniprep Kit, Sigma) containing the octopus desaturase ORF were then used to transform

195 *Saccharomyces cerevisiae* competent cells (S.c. EasyComp Transformation Kit,
196 Invitrogen). Transformation and selection of yeast with recombinant pYES2-OVFad
197 plasmids, and yeast culture were performed as described in detail previously (Agaba et
198 al. 2004).

199 In order to test the ability of octopus Fad to introduce double bonds into
200 saturated or monounsaturated FA, yeast transformed with pYES2 vector containing the
201 octopus desaturase as an insert (pYES2- OVFad) and no insert (control) were grown in
202 *S. cerevisiae* minimal medium$^{-uracil}$ with no exogenously added FA substrates.
203 Additionally, the ability of *O. vulgaris* Fad to desaturate PUFA substrates was tested by
204 growing pYES2-OVFad transgenic yeast in medium supplemented with one of the
205 following substrates: 18:3n-3, 18:2n-6, 20:3n-3, 20:2n-6, 20:4n-3, 20:3n-6, 22:5n-3 and
206 22:4n-6. The FA were added to the yeast cultures at final concentrations of 0.5 (C18),
207 0.75 (C20) and 1.0 (C22) mM as uptake efficiency decreases with increasing chain
208 length (Zheng et al. 2009). Yeast transformed with empty pYES2 were also grown in
209 presence of PUFA substrates as control treatments. After 2-days culture at 30 ^{o}C, yeast
210 were harvested, washed, and lipid extracted by homogenisation in chloroform/methanol
211 (2:1, v/v) containing 0.01% butylated hydroxy toluene (BHT) as antioxidant.
212 Docosapentaenoic and docosatetraenoic acids (> 98 – 99 % pure) were purchased from
213 Cayman Chemical Co. (Ann Arbor, USA) and the remaining FA substrates (> 99 %
214 pure) and chemicals used to prepare the *S. cerevisiae* minimal medium-^{uracil} were from
215 Sigma Chemical Co. Ltd. (Dorset, UK).

216 *Fatty acid analysis by GC-MS*

217 FA from the transgenic yeast were analysed by preparing methyl esters (FAME)
218 as previously described (Hastings et al. 2001). Briefly, FAME were identified and
219 quantified using a gas chromatograph (GC8000) coupled to an MD800 mass

spectrometer (ThermoFisher Scientific, Hemel Hempstead, UK). Desaturation efficiency from potential substrates including the yeast endogenous saturated FA (16:0 and 18:0) and the exogenously added PUFA substrates (18:3n-3, 18:2n-6, 20:3n-3, 20:2n-6, 20:4n-3, 20:3n-6, 22:5n-3 and 22:4n-6) were calculated by 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, picolinyl esters were prepared from FAME according to the methodology described by Destaillats and Angers (2002) and modified according to Li et al. (2010). FAME were also prepared from total lipid extracted from octopus tissues, and analysed according to Viciano et al. (2011).

Results

Octopus desaturase sequence and phylogenetics

A 1603-bp (excluding polyA tail) full-length cDNA sequence was obtained by 5' and 3' RACE PCR and deposited in the GenBank database under the accession number JN120258. It contains an ORF of 1338 bp encoding a putative protein of 445 aa, a 5' untranslated region (UTR) of 44 bp and a 3'UTR of 221 bp excluding polyA tail. *O. vulgaris* putative desaturase possesses three histidine boxes HXXXH, HXXHH and QXXHH common among Fad, the putative cytochrome b5-like domain, and the heme-binding motif, HPGG (Fig. 1).

The deduced aa sequence from the octopus desaturase cDNA predicts a protein that is 49.5-53.3 % identical to several mammalian FADS1 (Δ5) and FADS2 (Δ6) proteins including human, mouse and rat, and 48.9-51.5 % identical to teleost desaturases with various desaturation activities including Δ4, Δ5, Δ6 and bifunctional Δ6/Δ5. When compared with non-vertebrate desaturases, octopus desaturase showed

245 relatively low identity with *Thraustochytrium* sp. Δ5-like desaturases (26.0 %),
246 *Caenorhabditis elegans* Δ5- (22.8 %) and Δ6-like desaturases (26.4 %), and relatively
247 high identities with *Saccoglossus kowalevskii* (50.1 %) and *Schistosoma japonicum*
248 (49.0 %) predicted desaturases. Compared to mollusc desaturases, the octopus Fad is
249 61.9 % identical to the partial (~368 aa) desaturase sequence from the bivalve
250 *Crassostrea gigas*, and 52.2 % identical to the gastropod *Lottia gigantea* Fad-like.
251 Identities between the octopus Fad and several Scd desaturases including that of *L.*
252 *gigantea* were below 17 %.

253 *Functional characterisation*

254 The octopus putative Fad was functionally characterised by determining the FA
255 profiles of transgenic yeast *S. cerevisiae* expressing the Fad coding region and grown in
256 the presence of potential FA substrates. In order to test the ability of octopus Fad to
257 introduce double bonds into saturated or monounsaturated FA, the FA profiles of yeast
258 transformed with pYES2- OVFad or empty pYES2 (control) and grown in absence of
259 exogenously added substrate were compared (Fig. 3A and B). The results confirm that
260 octopus Fad is involved in the biosynthesis of monounsaturated FAs. Thus, FA profiles
261 of control yeast transformed with empty vector basically consisted of the main
262 endogenous FA of *S. cerevisiae*, namely 16:0, 16:1 isomers (16:1n-9 and 16:1n-7), 18:0,
263 18:1n-9 and 18:1n-7 (Fig. 3A). Importantly, pYES2-OVFad transformed yeast were
264 found to have two additional peaks identified as 16:1n-11 and 18:1n-13, thus indicating
265 a Δ5-desaturation from 16:0 and 18:0, respectively (Fig. 3B). Conversion rates for 16:0
266 and 18:0 were 20 % and 54 %, respectively (Table 2). No activity towards yeast
267 endogenous monounsaturated FA 18:1n-9 and 18:1n-7 was detected in transgenic yeast
268 containing the octopus Fad (Table 2).

In order to assess the role of octopus Fad in PUFA biosynthesis, transgenic yeast transformed with the desaturase ORF were incubated with Δ6- (18:3n-3 and 18:2n-6), Δ5- (20:4n-3 and 20:3n-6), Δ4- (22:5n-3 and 22:4n-6) and Δ8-desaturation (20:3n-3 and 20:2n-6) substrates. The FA composition of the yeast transformed with pYES2 vector containing no insert (control) was characterised by having the main endogenous yeast FA and whichever exogenously added FA substrate, this result being consistent with *S. cerevisiae* possessing no PUFA desaturation activity (Agaba et al. 2004). The transgenic yeast expressing the octopus Fad were able to convert up to 39 % of both 20:4n-3 and 20:3n-6 into 20:5n-3 ($^{\Delta5,8,11,14,17}$20:5) and 20:4n-6 ($^{\Delta5,8,11,14}$20:4), respectively (Table 2; Fig. 3C and D). The octopus Fad also exhibited the ability to efficiently convert 20:3n-3 ($^{\Delta11,14,17}$20:3) and 20:2n-6 ($^{\Delta11,14}$20:2) to their corresponding Δ5-desaturated NMI FA, namely $^{\Delta5,11,14,17}$20:4 and $^{\Delta5,11,14}$20:3, respectively (Table 2; Fig. 3E and F). These results also confirmed that the octopus Fad did not possess Δ8 desaturation activity. The ability of the octopus Fad to produce NMI FA was further confirmed by the results obtained with 18:3n-3 ($^{\Delta9,12,15}$18:3) and 18:2n-6 ($^{\Delta9,12}$18:2). Thus, small amounts of desaturated products were detected (Table 2), but these were confirmed as being NMI Δ5 desaturated products, $^{\Delta5,9,12,15}$18:4 and $^{\Delta5,9,12}$18:3, respectively, rather than Δ6 products. No desaturated products of 22:5n-3 and 22:4n-6 were detected indicating the octopus Fad possessed no Δ4-desaturation activity.

Tissue distribution of octopus desaturase mRNA transcripts

Tissue expression of common octopus desaturase was studied by RT-PCR on cDNA samples obtained from a range of tissues (Fig. 4). Transcripts of the target gene were detected in all tissues analysed, with gonads, brain, digestive gland and gill showing high expression signals. Low expression signals were detected in nerve,

293 nephridium, heart, muscle and hepatopancreas, the latter regarded as a major site for
294 lipid metabolism in molluscs (Fig. 4).
295 *Fatty acid composition of octopus tissues*
296 In order to identify sites of potentially important biosynthesis, FA profiles were
297 determined in a series of octopus tissues where expression of the Δ5 desaturase was
298 studied (Table 3). Potential Δ5-desaturated FA were detected in all tissues analysed.
299 Among monoenes, the presence of 18:1n-13 (or $^{\Delta5}$18:1) was confirmed in all tissues
300 analysed, with female and male gonad showing the highest percentages (1.9 and 1.2 %,
301 respectively). Among polyunsaturates, EPA (20:5n-3 or $^{\Delta5,8,11,14,17}$ 20:5) and ARA
302 (20:4n-6 or $^{\Delta5,8,11,14}$ 20:4) showed relatively high contents in all tissues analysed. Thus,
303 EPA was most abundant in heart (19.9 %), gill (16.8 %) and nerve (15.8 %), whereas it
304 only accounted for 0.2 % of total fatty acids in digestive gland. In contrast, ARA was
305 most abundant in brain (15.2 %) and male gonad (15.2 %), followed by gill (12.9 %)
306 and female gonad (12.9 %). The Δ5-desaturated FA, EPA and ARA, can be
307 subsequently converted by Elovl or other desaturases to FA such as 22:5n-3, 22:6n-3
308 (DHA) and 22:5n-6, also identified in octopus tissues. Particularly abundant in all
309 tissues was DHA, with eye (27.6 %) and heart (26.4 %) showing the highest
310 concentrations (Table 3). Small amounts of 20:2, 20:3 and 22:2 NMI were found in
311 nephridium, male gonad, eye and digestive gland (Table 3). Whereas the small amount
312 of the solutes meant it was not possible to unequivocally confirm the double bond
313 structure for 20:2 and 22:2 NMI, the 20:3 NMI was confirmed as $^{\Delta5,11,14}$ 20:3.
314 Dimethylacetals (DMA) of 16 and 18 carbons were also detected as previously
315 described (Rosa et al. 2004).
316
317 **Discussion**

In vertebrates the PUFA biosynthesis pathways have been extensively investigated, partly because of the critical roles that these compounds play in normal growth and development during early life-cycle stages (Innis et al. 1999; Lauritzen et al. 2001; Monroig et al. 2009). This has led to increased understanding of the biochemical and molecular mechanisms involved in the LC-PUFA pathways operating in fish, particularly farmed species, which has allowed the formulation of balanced aquafeeds tailored to the abilities of different fish species for endogenous biosynthesis. Such a strategy can be extended to new aquaculture candidates, especially those such as common octopus in which more empirical approaches through dietary trials are intrinsically difficult to undertake due to the abovementioned paralarval mortalities.

The endogenous FA biosynthetic ability of molluscs has been investigated in the past for both terrestrial (van der Horst 1973, 1974; Weinert et al. 1993; Zhu et al. 1994) and marine species (Chu and Greaves 1991; de Moreno et al. 1976; Waldock and Holland 1984; Zhukova 1986, 1991, 2007), and it is now known that it varies among species. Whereas the specific genes/enzymes responsible for individual reactions have not been characterised in any mollusc species, biochemistry and analytical approaches have allowed the identification of some critical activities (Barnathan 2009; Zhukova 2007). More specifically, three key enzymes appear to mediate the production and metabolism of essential fatty acids in molluscs, those being the elongase and two distinct desaturases: the Δ9- and Δ5-desaturases (Barnathan 2009; Kornprobst and Barnathan 2010; Zhukova 2007). The Δ9-desaturase, encoded by the so-called stearoyl CoA desaturase (Scd), is an enzymatic activity universally present in living organisms (Castro et al. 2011) including molluscs (David et al. 2005), which introduces the first double bond into saturated FAs such as 16:0 and 18:0 producing 16:1n-7 ($^{\Delta 9}$16:1) and 18:1n-9 ($^{\Delta 9}$18:1), respectively. Contrarily, the Δ5-desaturation is the catalytic activity of

343 a Fad, membrane-bond desaturases of a different gene/protein family than that of Scd
344 (Guillou et al. 2010), which act predominantly on PUFA substrates introducing a double
345 bond in the Δx carbon counting from the carboxylic group of the fatty acyl chain. For
346 that reason, Fad enzymes have been also termed 'front-end' desaturases (Napier et al.
347 1999). Below we present evidence that the newly cloned desaturase from the common
348 octopus is a Fad-like desaturase with Δ5 specificity, and represents the first molecular
349 proof of the existence of such an enzymatic activity in any mollusc species.

350 The newly cloned octopus desaturase possesses all typical features of Fad,
351 denoting that these enzymes have conserved functional domains during evolution
352 (Sperling et al. 2003). Phylogenetic analysis further supported that the octopus
353 desaturase was indeed more closely related to Fad-like than to Scd-like desaturases.
354 Previously, phylogenetic analysis of desaturases from 56 eukaryotic genomes had
355 identified four functionally distinct subfamilies with the ability to introduce double
356 bonds into saturated chains being characteristic of so-called "First Desaturases" (such as
357 Δ9 or SCD) whereas "Front-End Desaturases" (such as Fads) required pre-existing
358 double bonds for activity (Hashimoto et al., 2008). Therefore, it was interesting that
359 functional characterisation revealed that, despite being phylogenetically a Fad-like or
360 front-end desaturase, the octopus desaturase, in addition to desaturation of PUFA
361 substrates, was also able to introduce the first double bond into saturated acyl chains.

362 The common octopus Fad was clearly demonstrated to be a Δ5-desaturase, with
363 the ability to introduce new double bonds into both saturated FA and PUFA.
364 Endogenous FA in yeast including 16:0 and 18:0 were Δ5-desaturated to 16:1n-11
365 ($^{\Delta5}$16:1) and 18:1n-13 ($^{\Delta5}$18:1), respectively, by transgenic yeast expressing the octopus
366 Fad. Consistent with the catalytic ability of octopus Fad observed in vitro, the FA
367 profiles of the gastropods *Littorina littorea* and *Lunatia triseriata* indicated the

368 existence of a Δ5-desaturase accounting for the production of the monoenes 18:1n-13
369 and 20:1n-15 ($^{\Delta5}$ 20:1) (Joseph 1982). Although 20:0 was not assayed in the yeast
370 expression system, the high conversion efficiency shown on 18:0 (54 %) may suggest
371 that the octopus Fad could have the ability to desaturate 20:0 and produce 20:1n-15.
372 However, 20:1n-15 was not identified in the tissue lipids of *O. vulgaris* in the present
373 study. In vertebrates, the ability to introduce the first double bond into a saturated FA
374 appears limited to Scd-like desaturases and no Fad-like desaturase has been show to
375 possess this activity in teleosts (Hastings et al. 2001; Li et al. 2010; Monroig et al. 2010;
376 Zheng et al. 2004, 2005, 2009). An exception to this pattern is the human FADS2 (Δ6
377 Fad), which is reported to have the ability to desaturate 16:0 to sapienic acid (16:1n-10
378 or $^{\Delta6}$16:1) in specific tissues such as sebaceous glands (Ge et al. 2003). Thus, the
379 octopus Fad might have retained (conserved) the ability to desaturate saturated FA from
380 Scd, the likely ancestor of Fad-like genes (López Alonso et al. 2003).
381 In addition to the ability to act on saturated FA, the octopus Fad effectively
382 desaturated PUFA substrates in position Δ5. Thus, the results demonstrate that the
383 common octopus Δ5 Fad can participate in the biosynthesis of ARA and EPA from
384 20:3n-6 and 20:4n-3, respectively. Although this result suggests that neither ARA nor
385 EPA can be regarded strictly as EFA for octopus as they can be biosynthesised
386 endogenously, it is only possible (with this Δ5 activity) from other LC-PUFA
387 precursors. Therefore, it does not alter the fact that common octopus probably require
388 dietary sources of LC-PUFA, albeit as yet not clearly defined. Data available in the
389 literature are apparently controversial and, whereas ARA has been considered as non-
390 essential for the common octopus (Milou et al. 2006), essentiality of EPA has often
391 been suggested (Navarro and Villanueva 2000; Iglesias et al. 2007). The reason why
392 two analogous LC-PUFA (ARA and EPA) might have different essentiality status for

393 this species is unclear. However, there is evidence indicating that preformed ARA and
394 EPA are indeed required in the diet of the common octopus as their endogenous
395 biosynthesis might be limited by the availability of immediate biosynthetic precursors
396 20:3n-6 and 20:4n-3. First, the contents of 20:3n-6 and 20:4n-3 in natural and
397 experimental diets for octopus are extremely low (Navarro and Villanueva 2000; Seixas
398 et al. 2008, 2010), and consequently the endogenous production of ARA and EPA via
399 Δ5-desaturation cannot occur at physiologically significant rates. Second, the
400 endogenous production of 20:3n-6 and 20:4n-3 via biosynthesis from C_{18} PUFA (18:2n-
401 6 and 18:3n-3) might also be restricted by the absence of critical enzymatic activities.
402 As molluscs appear to possess elongases that act on PUFA substrates (Barnathan 2009;
403 Kornprobst and Barnathan 2010), we speculate that the endogenous production of
404 20:3n-6 and 20:4n-3 is limited due to the lack of desaturases with either Δ6 activity
405 operating on 18:2n-6 and 18:3n-3 prior to elongation or, alternatively, Δ8 activity acting
406 on 20:2n-6 and 20:3n-3 after elongation (Monroig et al. 2011). While the octopus Fad
407 did not show Δ6 or Δ8 activities, a second Fad could possibly be present. However, this
408 may be unlikely, as further Fad encoding genes do not appear to be present in other
409 molluscs such as the gastropod *Lottia gigantea*, whose genome seems to contain a
410 single Fad-like gene (http://genome.jgi-psf.org/Lotgi1/Lotgi1.home.html). Moreover,
411 the absence of further Fad enzymes would likely explain why DHA is regarded as an
412 EFA for the common octopus (Navarro and Villanueva 2000), as either Δ4-desaturation
413 of 22:5n-3, or Δ6-desaturation of 24:5n-3, are necessary for its biosynthesis, at least in
414 vertebrates (Li et al. 2010). While future investigations are necessary to elucidate the
415 presence or absence of other desaturation activities critical for the endogenous
416 biosynthesis of ARA, EPA and DHA, the abundance in all tissues of these LC-PUFA,
417 especially ARA that is unlikely to be purely of dietary origin, reveals their important

418 physiological functions for common octopus. Furthermore, the highest levels of ARA
419 generally corresponded to the tissues that showed the highest expression of Δ5 Fad
420 transcript supporting a potential role for this enzyme in the endogenous production of
421 ARA.

422 Thus, endogenous production of ARA may be one reason supporting the
423 retention of Δ5 desaturase activity in a carnivorous species such as octopus, where
424 preformed EPA and DHA are likely to be readily available in the natural diet. In
425 addition, however, the octopus Δ5 Fad might participate in the biosynthesis of non-
426 methylene interrupted (NMI) FA, a group of compounds with unusual unsaturation
427 features occurring in molluscs as well as other marine invertebrates (Barnathan 2009;
428 Kornprobst and Barnathan 2010). Typical mollusc NMI FA include $^{\Delta7,13}$22:2 and
429 $^{\Delta7,15}$22:2. Briefly, their synthesis has been hypothesised to derive from the initial
430 desaturation of 16:0 and 18:0 catalysed by a Scd-like desaturase to produce 16:1n-7 and
431 18:1n-9, respectively (Barnathan 2009). Subsequent elongase- and Δ5 desaturase-
432 mediated reactions account for the synthesis of C_{20} NMI including $^{\Delta5,11}$20:2 and
433 $^{\Delta5,13}$20:2, with further elongation to produce $^{\Delta7,13}$22:2 and $^{\Delta7,15}$22:2, respectively.
434 Although we cannot conclude that the octopus Fad is involved in the production of 20:2
435 NMI as potential FA substrates (20:1n-9 and 20:1n-7) were not assayed, our results
436 clearly demonstrate that the octopus Fad participates in the biosynthesis of NMI FA
437 such as $^{\Delta5,11,14,17}$20:4 and $^{\Delta5,11,14}$20:3, compounds found in bivalves (Kawashima and
438 Ohnishi 2004; Pirini et al. 2007) and gastropods (Kawashima 2005). In addition, NMI
439 FA were detected in tissues of adult octopus, including $^{\Delta5,11,14}$20:3, 20:2 and 22:2,
440 although the precise double bond positions in the latter two could not be unequivocally
441 established. The biological functions of NMI FA are not fully understood, but it has

been suggested that they play structural and protective roles in cell membranes (Barnathan 2009).

In summary, our results demonstrate that the common octopus expresses a Fad-like gene that encodes an enzyme with Δ5 desaturation activity towards saturated FA and PUFA substrates. The Fad could participate in the endogenous production of EPA and, especially, ARA from other LC-PUFA substrates. In addition the octopus Δ5 Fad participates in the biosynthesis of NMI FA, compounds previously found in a series of marine invertebrates, and now also confirmed to exist in specific tissues of common octopus.

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626

627 **Legends to Figures**

628 Fig. 1. Alignment of the deduced amino acid (aa) sequence of the newly cloned Δ5 fatty
629 acyl desaturases from *Octopus vulgaris*. The aa sequence of the octopus Fad was
630 aligned with the *Mus musculus* FADS1 (gb|NP_666206.1|), the Δ6/Δ5 bifunctional
631 desaturase from *Danio rerio* (gb| AAG25710.1|), the fatty acyl desaturase 1 from
632 *Schistosoma japonicum* (emb|CAX72705.1|), the predicted fatty acid desaturase 2-like
633 from *Saccoglossus kowalevskii* (gb|XP_002736866.1|) and the partial *Crassostrea gigas*
634 putative desaturase. Deduced aa sequences were aligned using ClustalW (Bioedit).
635 Identical residues are shaded black and similar residues are shaded grey.
636 Identity/similarity shading was based on the BLOSUM62 matrix, and the cut-off for
637 shading was 70%. The cytochrome b_5-like domain is dot-underlined and the three
638 histidine boxes (HXXXH, HXXHH and QXXHH) are highlighted with grey squares.

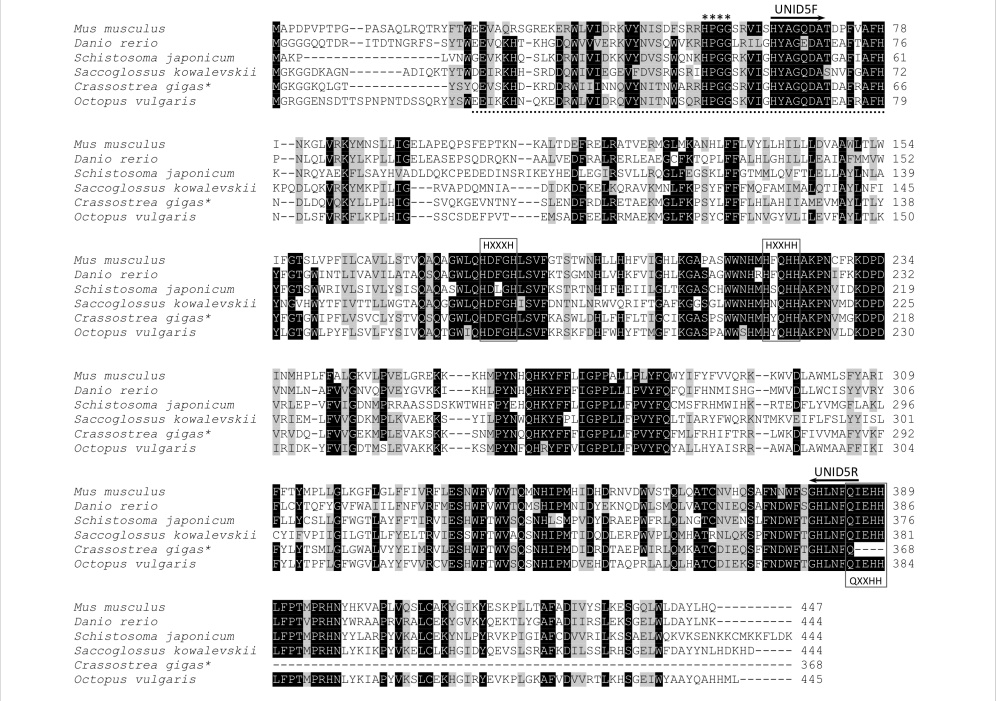
639 The asterisks on the top mark the heme-binding motif, HPGG. Conserved regions where
640 the degenerate primers UNID5F and UNID5R (see Materials and Methods section) are
641 also indicated.
642
643 Fig. 2. Phylogenetic tree comparing the deduced amino acid (aa) sequence of the newly
644 cloned *Octopus vulgaris* fatty acyl desaturase (Fad) with other Δ5- and Δ6-like Fad
645 from several organisms. Additionally, the aa sequences of several stearoyl coA
646 desaturase (Scd) were included in the analysis. The tree was constructed using the
647 Neighbour Joining method (Saitou and Nei 1987) with MEGA4. The horizontal branch
648 length is proportional to aa substitution rate per site. The numbers represent the
649 frequencies (%) with which the tree topology presented was replicated after 10000
650 iterations.
651 *Sequences derived from searches in GenBank (*C. gigas*) or *L. gigantea* Genome
652 Project website (http://genome.jgi-psf.org/Lotgi1/Lotgi1.home.html).
653
654 Fig. 3. Functional characterisation of the newly cloned *Octopus vulgaris* fatty acyl
655 desaturase (Fad) in yeast (*Saccharomyces cerevisiae*). The fatty acid (FA) profiles were
656 determined from control yeast transformed with pYES2 containing no insert (A).
657 Additionally, yeast transformed with pYES2 containing the ORF of the putative Fad
658 cDNA as an insert were grown with no substrate (B) or in the presence of one of the
659 exogenously added substrates 20:4n-3 (C), 20:3n-6 (D), 20:3n-3 (E) or 20:2n-6 (F).
660 Peaks 1-5 in all panels are the main endogenous FA of *S. cerevisiae*, namely 16:0 (1),
661 16:1 isomers (2), 18:0 (3), 18:1n-9 (4) and 18:1n-7 (5). Additionally peaks derived from
662 exogenously added substrates ("*") or desaturation products are indicated accordingly
663 in panels B-F. Vertical axis, FID response; horizontal axis, retention time.

664

665 Fig. 4. RT-PCR analyses showing the tissue distribution of octopus fatty acyl desaturase

666 (Fad) mRNA transcripts. Expression of the housekeeping gene β-actin is also shown.

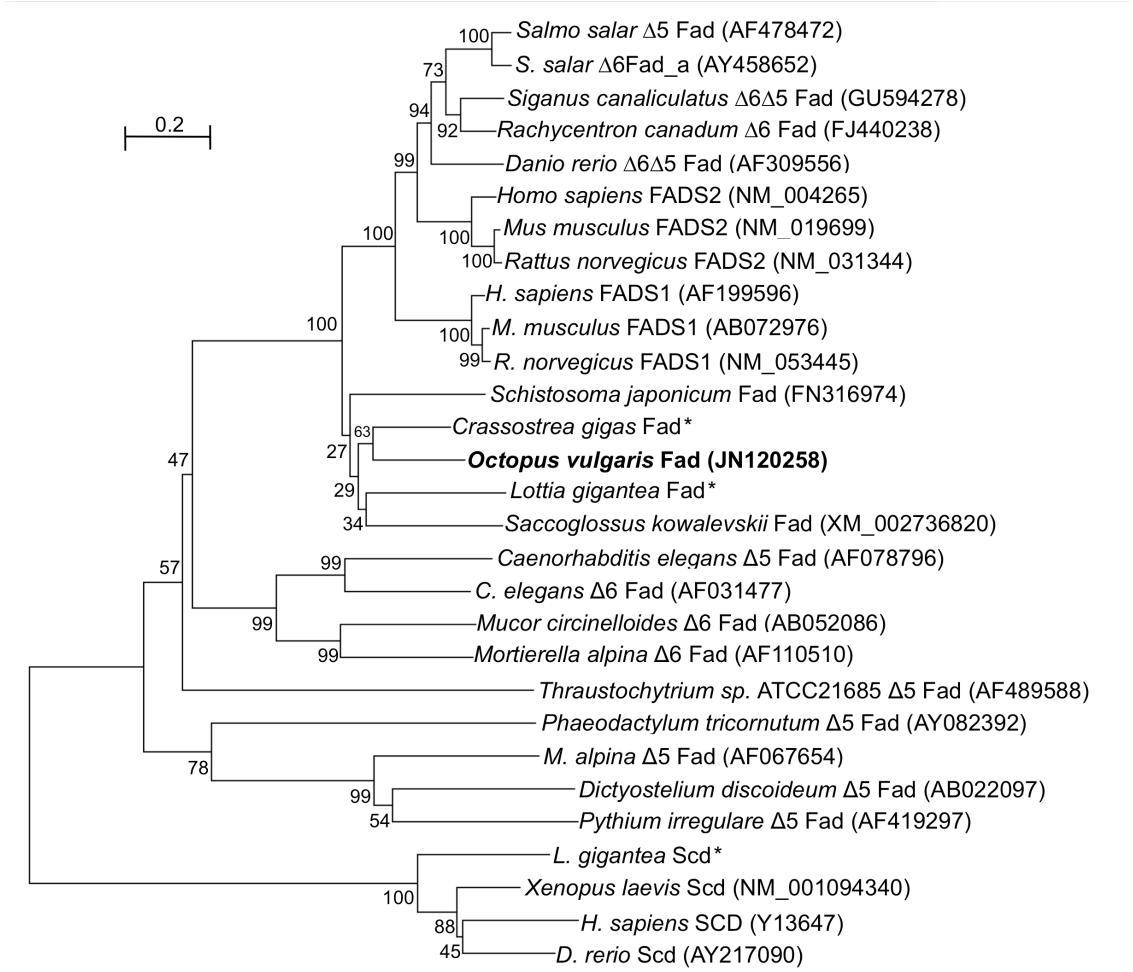
1 Fig.1


UNID5F
UNID5R
HXXXH
HXXHH
HXXHH
QXXHH
Mus musculus
Danio rerio
Schistosoma japonicum
Saccoglossus kowalevskii
Crassostrea gigas*
Octopus vulgaris

2

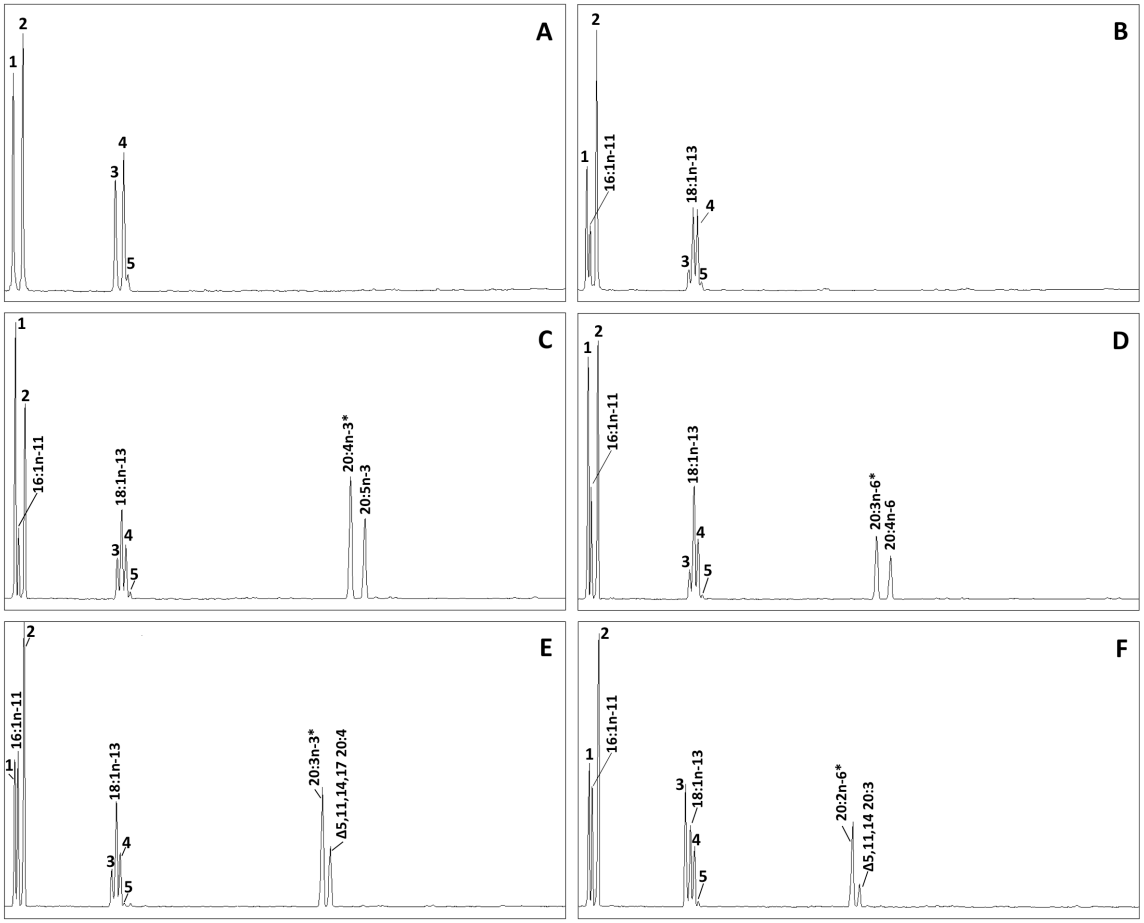
3

Fig. 2.


0.2
Salmo salar Δ5 Fad (AF478472)
S. salar Δ6Fad_a (AY458652)
Siganus canaliculatus Δ6Δ5 Fad (GU594278)
Rachycentron canadum Δ6 Fad (FJ440238)
Danio rerio Δ6Δ5 Fad (AF309556)
Homo sapiens FADS2 (NM_004265)
Mus musculus FADS2 (NM_019699)
Rattus norvegicus FADS2 (NM_031344)
H. sapiens FADS1 (AF199596)
M. musculus FADS1 (AB072976)
R. norvegicus FADS1 (NM_053445)
Schistosoma japonicum Fad (FN316974)
Crassostrea gigas Fad*
Octopus vulgaris Fad (JN120258)
Lottia gigantea Fad*
Saccoglossus kowalevskii Fad (XM_002736820)
Caenorhabditis elegans Δ5 Fad (AF078796)
C. elegans Δ6 Fad (AF031477)
Mucor circinelloides Δ6 Fad (AB052086)
Mortierella alpina Δ6 Fad (AF110510)
Thraustochytrium sp. ATCC21685 Δ5 Fad (AF489588)
Phaeodactylum tricornutum Δ5 Fad (AY082392)
M. alpina Δ5 Fad (AF067654)
Dictyostelium discoideum Δ5 Fad (AB022097)
Pythium irregulare Δ5 Fad (AF419297)
L. gigantea Scd*
Xenopus laevis Scd (NM_001094340)
H. sapiens SCD (Y13647)
D. rerio Scd (AY217090)

8 Fig. 3 .

9


A
1
2
3
4
5
B
1
2
16:1n-11
18:1n-13
3
4
5
C
1
2
16:1n-11
18:1n-13
20:4n-3*
20:5n-3
3
4
5
D
1
2
16:1n-11
18:1n-13
20:3n-6*
20:4n-6
3
4
5
E
1
2
16:1n-11
18:1n-13
20:3n-3*
Δ5,11,14,17 20:4
3
4
5
F
1
2
16:1n-11
18:1n-13
20:2n-6*
Δ5,11,14 20:3
3
4
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11

11 Fig. 4.

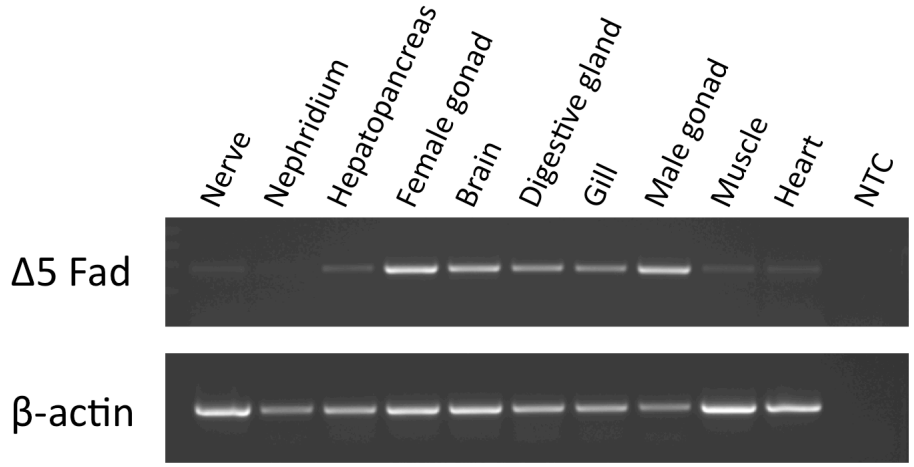

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Table 1. Sequences of the primer pairs used and accession numbers of the sequences used as references for primer design in the cloning of the octopus fatty acyl desaturase (Fad) ORF and for RT-PCR analysis of gene expression in octopus tissues.

Aim	Transcript	Primer	Primer sequence	Ac
RACE PCR	Fad	OVD5F1	5'-CCATGCGACCTGTGATATT-3'	.
		OVD5F2	5'-ATGATTGGTTTACCGGACATC-3'	
		OVD5R1	5'-ATCTCCGTCACTGGGAATTC-3'	
		OVD5R2	5'-GTGGAAAGCACGAAATGCTT-3'	
ORF cloning	Fad	OVD5U5F	5'-CCTGTTTGTTGGTGGATAAGC-3'	.
		OVD5U3R	5'-ATACACATACACACACACACGC-3'	
		OVD5VF	5'-CCCAAGCTTAAAATGGGAAGAGGCGGAGA-3'	
		OVD5VR	5'-CCGCTCGAGCTATAACATATGATGTGCTTGATA-3'	
RT-PCR	Fad	OVD5F3	5'-AGCCACATGCATTACCAACA-3'	.
		OVD5R3	5'-CAATATCACAGGTCGCATGG-3'	
	ß-actin	OVACTF	5'-CTTGACTCCGGAGATGGTGT-3'	A
		OVACTR	5'-CGCATTTCATGATGGAGTTG-3'	

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

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

FA substrates	**Product**	**Conversion rate (%)**
Saturates		
16:0	16:1n-11	20
18:0	18:1n-13	54
Polyunsaturates		
18:3n-3	$^{\Delta5,9,12,15}$ 18:4	1
18:2n-6	$^{\Delta5,9,12}$ 18:3	1
20:3n-3	$^{\Delta5,11,14,17}$ 20:4	33
20:2n-6	$^{\Delta5,11,14}$ 20:3	19
20:4n-3	20:5n-3	39
20:3n-6	20:4n-6	39
22:5n-3	22:6n-3	0
22:4n-6	22:5n-6	0

Table 3. Fatty acids and dimethyl acetal (DMA) composition (% of totals) of tissues collected from *Octopus vulgaris* adult individuals.

	Brain	Nephridium	Muscle	Male gonad	Female gonad	Skin	Nerve	Eye	Hepatopancreas	Heart	Gill	Digestive gland
14:0	0.9	0.8	1.3	0.7	3.0	1.2	1.0	0.6	3.3	0.6	0.6	2.0
15:0	0.1	nd	nd	nd	nd	0.1	nd	nd	0.2	nd	nd	0.2
16C DMA	0.5	0.2	0.3	0.1	0.2	0.4	0.5	0.2	0.5	0.2	0.6	0.5
16:0	16.1	14.0	20.1	13.4	17.9	19.0	19.2	18.0	14.9	13.8	13.3	12.4
16:1n7	0.4	0.6	1.0	0.9	1.7	0.6	0.6	0.5	4.4	0.7	0.4	3.3
16:2	0.2	0.2	0.4	0.3	0.3	0.3	0.4	0.2	0.4	0.2	0.3	0.4
17:0	1.6	2.4	1.9	1.4	1.7	1.9	1.6	1.0	1.3	1.8	1.3	1.4
16:3	0.2	nd	nd	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.2	0.2
18C DMA	4.9	1.9	2.8	1.9	2.7	4.7	4.5	2.1	3.7	3.4	6.8	5.1
18:0	11.9	12.8	11.1	7.4	9.7	14.0	10.2	6.4	10.6	12.9	10.0	12.1
18:1n-13	0.3	0.6	0.2	1.2	1.9	0.2	0.4	1.2	0.1	0.1	0.3	0.2
18:1n-9	2.0	2.1	3.4	4.3	2.8	2.1	2.7	1.2	3.1	1.5	1.4	3.7
18:1n-7	1.1	1.9	2.0	1.6	1.4	1.4	1.8	1.4	2.6	1.3	1.1	3.2
18:3n-3	0.3	nd	nd	nd	nd	nd	nd	0.1	0.5	0.1	nd	0.6
18:4n-3	0.1	nd	nd	nd	nd	nd	nd	nd	0.8	nd	nd	0.6
20:0	1.3	0.2	0.3	1.6	2.1	0.4	0.2	0.4	0.4	0.2	0.3	0.9
20:1n-9	2.3	9.4	3.2	10.5	7.1	2.6	2.5	2.1	0.9	2.4	2.8	2.3
20:1n-7	0.4	0.3	0.3	0.5	0.5	0.3	0.2	0.2	0.5	0.2	0.3	0.4
NMI 20:2	nd	1.9	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
20:2n-6	0.2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.2
NMI 20:3	nd	0.8	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
20:3n-6	0.6	0.4	0.4	0.4	0.4	0.3	0.3	0.9	0.5	0.7	0.2	0.7
20:4n-6	15.2	11.7	7.9	15.2	12.9	12.5	8.1	4.8	10.4	5.5	12.9	11.4
20:3n-3	nd	nd	nd	nd	nd	nd	0.1	14.1	0.1	0.1	nd	0.2
20:4n-3	nd	0.1	0.2	nd	nd	nd	0.2	0.1	0.4	0.1	nd	14.7
20:5n-3	11.8	9.9	14.9	7.6	8.0	11.4	15.8	12.1	14.7	19.9	16.8	0.2
22:0	0.2	nd	0.2	nd	0.2	0.3	0.2	0.1	0.4	nd	0.2	nd
22:1n-9	1.6	1.8	1.3	2.4	1.3	1.1	1.1	0.5	0.4	0.9	1.9	1.2
NMI 22:2	nd	nd	nd	1.7	nd	nd	nd	0.4	nd	nd	nd	0.6
22:4n-6	0.7	1.3	0.8	5.9	1.9	1.2	0.8	0.3	0.5	0.8	1.2	1.1
22:5n-6	0.9	1.0	1.0	0.8	1.0	1.0	1.0	0.3	0.8	1.0	1.0	0.8
22:5n-3	0.9	1.2	1.8	1.7	1.6	1.4	1.6	1.0	1.2	2.1	1.7	1.5
22:6n-3	18.7	19.9	20.7	15.0	14.0	17.4	21.4	27.6	16.4	26.4	21.3	14.0
Total	95.4	97.4	97.4	96.5	94.4	96.0	96.5	98.1	94.2	97.0	97.0	96.1

NMI: non-methylene interrupted