Methyl-end desaturases with $\Delta 12$ and $\omega 3$ regioselectivities enable the *de novo* PUFA biosynthesis in the cephalopod *Octopus vulgaris*

Diego Garrido¹, Naoki Kabeya², Francisco Hontoria³, Juan C. Navarro³, Diana B. Reis⁴, M. Virginia Martín¹, Covadonga Rodríguez⁴, Eduardo Almansa¹, Óscar Monroig^{3*}

¹Centro Oceanográfico de Canarias, Instituto Español de Oceanografía, Santa Cruz de Tenerife, Tenerife, Spain

²Department of Aquatic Bioscience, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo, Japan

³Instituto de Acuicultura Torre de la Sal (IATS-CSIC), Ribera de Cabanes, Castellón, Spain

⁴Departamento de Biología Animal, Edafología y Geología, Universidad de La Laguna, San Cristóbal de La Laguna, Tenerife, Spain

*Corresponding author: Óscar Monroig

oscar.monroig@csic.es

Abstract

The interest in understanding the capacity of aquatic invertebrates to biosynthesise omega-3 (ω 3) long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA) has increased in recent years. Using the common octopus *Octopus vulgaris* as a model species, we previously characterised a Δ 5 desaturase and two elongases (i.e. Elovl2/5 and Elovl4) involved in the biosynthesis of LC-PUFA in molluscs. The aim of this study was to characterise both molecularly and functionally, two methyl-end (or ω x) desaturases that have been long regarded to be absent in most animals. *O. vulgaris* possess two ω x desaturase genes encoding enzymes with Δ 12 and ω 3 regioselectivities enabling the *de novo* biosynthesis of the C_{18} PUFA 18:2 ω 6 (LA, linoleic acid) and 18:3 ω 3 (ALA, α -linolenic acid), generally regarded as dietary essential for animals. The *O. vulgaris* Δ 12 desaturase (“ ω x2”) mediates the conversion of 18:1 ω 9 (oleic acid) into LA, and subsequently, the ω 3 desaturase (“ ω x1”) catalyses the Δ 15 desaturation from LA to ALA. Additionally, the *O. vulgaris* ω 3 desaturase has Δ 17 capacity towards a variety of C_{20} ω 6 PUFA that are converted to their ω 3 PUFA products. Particularly relevant was the affinity of the ω 3 desaturase towards 20:4 ω 6 (ARA, arachidonic acid) to produce 20:5 ω 3 (EPA, eicosapentaenoic acid), as supported by yeast heterologous expression, and enzymatic activity exhibited *in vivo* when paralarvae were incubated in the presence of [1- 14 C]20:4 ω 6. These results confirmed that several routes enabling EPA biosynthesis are operative in *O. vulgaris* whereas ARA and docosahexaenoic acid (DHA, 22:6 ω 3) should be considered essential fatty acids since endogenous production appears to be limited.

Keywords

Biosynthesis; essential fatty acids; methyl-end desaturases; *Octopus vulgaris*

Highlights

1. *O. vulgaris* possesses two methyl-end desaturases enabling *de novo* PUFA biosynthesis.
2. The *O. vulgaris* ω x1 encodes an ω 3 desaturase with Δ 15, Δ 17 and Δ 19 activities.
3. The *O. vulgaris* ω x2 encodes a desaturase with Δ 12 desaturase activity.
4. Biosynthesis of EPA, but not ARA or DHA, appears to be operative in *O. vulgaris*.

Introduction

The omega-3 (ω 3) long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic acid (EPA, 20:5 ω 3) and docosahexaenoic acid (DHA, 22:6 ω 3) have beneficial roles in human health [1–3] and this has prompted interest in understanding the mechanisms involved in their biosynthesis [4]. Some eukaryotes can biosynthesise LC-PUFA via the anaerobic pathway involving the polyketide synthase (PKS), but more often LC-PUFA biosynthesis occurs through an aerobic pathway in which desaturases are one of the key enzymes involved [5,6]. In addition to Δ 9 desaturases like stearoyl-CoA desaturases (Scd) that catalyse the biosynthesis of 18:1 ω 9 (oleic acid) from 18:0 (stearic acid) (Fig. 1), further double bonds required to produce polyunsaturated fatty acids (PUFA) (i.e. fatty acids with at least two double bonds) are inserted by ω x (or methyl-end) desaturases, enzymes that introduce a new double bond between a pre-existing one and the methyl terminus of the fatty acid (FA) (Fig. 1). Hence, an ω x desaturase with Δ 12 desaturase activity catalyses the conversion of 18:1 ω 9 to 18:2 ω 6 (LA, linoleic acid), whereas an ω x desaturase with Δ 15 activity is required to convert the latter to 18:3 ω 3 (ALA, α -linolenic acid) [7] (Fig. 1). LA and ALA are regarded as dietary essential FA in vertebrates because they lack desaturases with Δ 12 and Δ 15 activities. However, the C_{18} PUFA can be converted to C_{20-22} LC-PUFA such as EPA and DHA by sequential reactions mediated by front-end desaturases and fatty acyl elongases (Fig. 1). Despite the term “front-end desaturases” refers to protein families with potentially different evolutionary origin [8], a common characteristic of these enzymes is that they introduce the incipient double bond between an existing one and the carboxylic group. Importantly, while front-end desaturases are widely distributed in Metazoa [5,9], genes encoding ω x desaturases have been considered to have a more restricted distribution and, in addition to their absence in vertebrates alluded to above, they have been regarded to be absent in most animals, with only few exceptions reported in the nematode *Caenorhabditis elegans* [10–12] and insects [13–15].

Consequently, virtually all the primary production of $\omega 3$ LC-PUFA in the marine ecosystem has been historically associated to algae, heterotrophic protists and bacteria, organisms possessing ωx desaturases [16,17].

Figure 1. Biosynthesis of long-chain polyunsaturated fatty acids in animals. The ωx desaturases with $\Delta 12$ and $\Delta 15$ desaturase activity catalyse reactions for the *de novo* production of 18:2 $\omega 6$ (linoleic acid) and 18:3 $\omega 3$ (α -linolenic acid) from 18:1 $\omega 9$. Consequently, 18:2 $\omega 6$ and 18:3 $\omega 3$ can be further desaturated and elongated by front-end desaturases and elongases, respectively. The ωx desaturase with multiple regioselectivity towards a variety of $\omega 6$ substrates can be called “ $\omega 3$ desaturase”. Front-end desaturases with $\Delta 4$, $\Delta 5$, $\Delta 6$ and $\Delta 8$ can be found in animals [5,8]. In *O. vulgaris*, stearoyl-CoA desaturase ($\Delta 9$), $\Delta 5$ front-end desaturase ($\Delta 5$), elongase of very long chain fatty acids (Elovl2/5 and Elovl4) had been functionally characterised prior the present study [29,30,32]. β -ox, β -oxidation.

Recently, it has been demonstrated that ω x desaturases are more widespread than initially believed, being found across different taxa such as cnidarians, rotifers, nematodes, molluscs, arthropods and annelids [18], the latter also including the giant tubeworm *Riftia pachyptila* in which an ω x desaturase had been also characterised [19]. The newly uncovered animal ω x desaturases have distinct evolutionary origins [18], but a fairly conserved pattern that consists of animal species having two ω x desaturase genes whose combined desaturase capabilities enable the *de novo* biosynthesis of ω 3 PUFA (Fig. 1). In some species like *Acropora millepora* (cnidarian), *Patella vulgata* (mollusc), *Lepeophtheirus salmonis* (crustacean arthropod) and *Platynereis dumerilii* (polychaete annelid) one of the ω x desaturases encodes an ω x desaturase with Δ 12 activity enabling the biosynthesis of LA. A second gene encodes an ω 3 desaturase that converted LA into ALA (*de novo* ω 3 PUFA production), but, additionally, this desaturase is also efficient in converting a range of ω 6 PUFA substrates into their corresponding C₁₈₋₂₂ ω 3 PUFA products since it shows Δ 15, Δ 17 and Δ 19 desaturase activities (Fig. 1) [18]. A different pattern was observed in the rotifer *Adineta vaga*, whose two ω x desaturase genes encodes desaturases that contains simultaneously Δ 12 and Δ 15 activities, as well as Δ 17 activity [18]. The presence of ω 3 PUFA biosynthesising enzymes in multiple invertebrates has important ecological implications since ecosystems, where animals with ω x desaturases are abundant, can significantly contribute to the ω 3 production at a global scale [18]. At a physiological level, the presence of ω x desaturases enables certain species to produce physiologically important FA that, otherwise, are required to be supplied in the diet to prevent deficiency symptoms.

The common octopus (*Octopus vulgaris*) is a promising candidate to diversify the marine aquaculture [20]. However, production of *O. vulgaris* in captivity has not been fully developed because of the high mortalities occurring in early developmental stages (paralarvae), in part because of nutrition-related factors that are not yet solved [21]. Empirically, LC-PUFA had been identified as physiologically essential nutrients required for normal growth and

development of cephalopods although the underlying mechanisms had not been elucidated [22–27]. Since dietary essential FA can be identified from the enzymatic capacity for PUFA biosynthesis existing in a particular species [28], several studies aiming to characterise desaturases and elongases involved in the LC-PUFA biosynthetic pathways in *O. vulgaris* reported the existence of a front-end $\Delta 5$ desaturase ($\Delta 5$ Fad) [29] and two elongases, namely Elov12/5 [30,31] and Elov14 [32]. On one hand, the *O. vulgaris* $\Delta 5$ Fad desaturates 20:3 ω 6 and 20:4 ω 3 to arachidonic acid (ARA, 20:4 ω 6) and EPA, respectively. Among elongases, the *O. vulgaris* Elov12/5 can elongate C₁₈₋₂₀ PUFA substrates [30,31], whereas Elov14 shows preferential elongase activity towards C₂₂ PUFA [32]. Importantly, the interrogation of *Octopus bimaculoides* genome [33] suggests that, in addition to the above mentioned enzymes, *O. vulgaris* might also possess ω x desaturases with putative roles in the PUFA biosynthetic pathways in cephalopods [18]. Therefore, the present study aimed at the molecular cloning and functional characterisation of two ω x desaturases from *O. vulgaris*, as well as to assess their enzymatic activity in *O. vulgaris* paralarvae.

Materials and methods

Tissue samples, RNA extraction and cDNA synthesis

This study was carried out in compliance with the Spanish Real Decreto 53/2013, which is based on the European Union Directive 2010/63/EU for protection of animals used for scientific purposes. Four adult males of common octopus (2.2 ± 0.2 kg) were captured in Tenerife, Spain, from local fisheries using artisanal traps and maintained at the facilities of Centro Oceanográfico de Canarias (IEO, Tenerife, Spain) until tissue sampling. Octopuses were maintained and sacrificed according to the methodology used in previous studies [26] and approved by the ethical committee (registration number: CEIBA2014-0108). Tissues including nerve, nephridium, hepatopancreas, caecum, gill, heart, gonad, and eye were collected and immediately kept into RNAlater, first 24 h at 4 °C and subsequently at -80 °C until further analysis. Total RNA was extracted from octopus tissues using TRI Reagent (Sigma-Aldrich, Dorset, UK) following the manufacturer's instructions. First strand cDNA was synthesised from 2 µg of total RNA using High Capacity cDNA Reverse Transcription Kits (AB Applied Biosystems, California, USA) and oligo(dT) primers.

*Molecular cloning of *O. vulgaris* ω desaturase cDNA sequences*

A tblastn search was carried out against *O. vulgaris* transcriptome shotgun assembly (TSA, NCBI BioProject number: PRJNA79361) using the amino acid (aa) sequence of the *P. dumerilii* $\Delta 12$ desaturase (ATV93525) as a query [18]. The ω desaturase-like sequence (JR459845) obtained was then used for designing primers to perform Rapid Amplification of cDNA Ends (RACE) polymerase chain reaction (PCR) (Table 1). The RACE cDNA synthesis was conducted using FirstChoice® RLM-RACE kit (Thermo Fisher Scientific, Warrington, UK) following the manufacturer's instructions. The RACE PCR were carried out with GoTaq® Green Master Mix (Promega, Southampton, UK). The thermocycling conditions and primer

set used for RACE PCR are given in Table 1. The amplified DNA fragments were purified on an agarose gel (Illustra™ GFX™ PCR DNA and Gel Band Purification kit, GE Healthcare Life Sciences, Buckinghamshire, UK) and sequenced (GATC Biotech, Konstanz, Germany). The sequences obtained from RACE PCR were assembled with the abovementioned tblastn hit (JR459845) to generate a putative full-length cDNA using BioEdit v7.2.5 [34]. This desaturase will be thereafter referred to as “ ω x1”.

After the first *O. vulgaris* ω x desaturase-like sequence was isolated (see above), the genomic assembly of *Octopus bimaculoides* was released in NCBI (GCA_001194135.1) [33]. A further tblastn search was conducted against *O. bimaculoides* genome using as a query the deduced aa sequence of the *O. vulgaris* ω x1 desaturase. A first hit (KOF99091) with high identity (96 %) to the *O. vulgaris* ω x desaturase was found in *O. bimaculoides*, suggesting these sequences were orthologous. In addition, a second putative ω x desaturase was found in *O. bimaculoides* (KOF62429) and its relatively low aa identity (51 %) with the cloned *O. vulgaris* putative ω x desaturase suggested this was a distinct ω x desaturase that could also exist in *O. vulgaris*. Several primer sets were designed in random locations along the *O. bimaculoides* KOF62429 sequence as a reference in order to amplify the first fragment of the second *O. vulgaris* ω x desaturase (Table 1). The first fragment of the *O. vulgaris* second ω x desaturase was successfully amplified using GoTaq (Promega) with nerve and hepatopancreas cDNA as templates and using one of the primer sets as indicated in Table 1. The PCR conditions and primer sequences are described in Table 1. The full length of the second *O. vulgaris* ω x desaturase was obtained as described above. RACE PCR primers and conditions are described in Table 1. This desaturase will be thereafter referred to as “ ω x2”.

Table 1. Sequences of the primer pairs and corresponding PCR conditions for the cloning and qPCR of *Octopus vulgaris* ω x desaturases. Restrictions sites for the ORF cloning primers are underlined (Fw: *Bam*HI, Rv: *Xho*I).

Target	Step	Primers sequence	Temperature in °C (duration in sec)			Cycles	Enzyme
			Denaturation	Annealing	Extension		
ω x1 desaturase (similar to <i>O. bimaculoides</i> KOF99091)	5'RACE first	Rv: 5'-GTCGGAATTATGCGTTCGTT-3'	95 (30)	53 (30)	72 (90)	35	GoTaq
	5'RACE nested	Rv: 5'-AGGCATCGAAGGAAAGAGGT-3'	95 (30)	55 (30)	72 (90)	35	GoTaq
	3'RACE first	Fw: 5'-TGGTATAAGAGCGAAGAGTGGA-3'	95 (30)	55 (30)	72 (60)	35	GoTaq
	3'RACE nested	Fw: 5'-CCTCACCCATTCCATCGACA-3'	95 (30)	57 (30)	72 (60)	35	GoTaq
	ORF cloning first	Fw: 5'-ATTACACCAGGCAACACCAC-3' Rv: 5'-TTTGCAGTGTCTGTGGATT-3'	95 (30)	55 (30)	72 (150)	35	Pfu
	ORF cloning nested	Fw: 5'-CCCGGATCCAAAAATGGTGACAGCGATTCCA-3' Rv: 5'-CCGCTCGAGCTATTTATACCAATGTATTAAGG-3'	95 (30)	55 (30)	72 (150)	35	Pfu
	qPCR	Fw: 5'-GTAATTACGGTTGGGCGCAC-3' Rv: 5'-GACAAGCTGAGGGAAGCCT-3'	95 (15)	55 (30)	72 (30)	40	HiGreen
	1st fragment	Fw: 5'-GTCGGCAGGAATTGTACATAT-3' Rv: 5'-TTTTTCGGAATGGCTAGTGG-3'	95 (30)	55 (30)	72 (70)	35	GoTaq
ω x2 desaturase (similar to <i>O. bimaculoides</i> KOF62429)	5'RACE first	Rv: 5'-ACAGGGTTCCTTGTATCGT-3'	95 (30)	60 (30)	72 (60)	35	GoTaq
	5'RACE nested	Rv: 5'- ACCATGTCCACAGTCGTGTC-3'	95 (30)	60 (30)	72 (60)	35	GoTaq
	3'RACE first	Fw: 5'-CCCCACTATCATCTCCAGAAAGCC-3'	95 (30)	60 (30)	72 (30)	35	GoTaq
	3'RACE nested	Fw: 5'- TTCCACATTTGGTGCACTATT-3'	95 (30)	55 (30)	72 (30)	35	GoTaq
	ORF cloning first	Fw: 5'-CATCGGGCTATTTACATGGTAGTT-3' Rv: 5'-TGACACTATTAACAACAATCATTGTGA-3'	95 (30)	55 (30)	72 (150)	35	Pfu
	ORF cloning nested	Fw: 5'-CCCGGATCCACCATGGTTTGGGAAGAAGA-3' Rv: 5'-CCGCTCGAGTCATTGTAAACATGTATCTGGGTA-3'	95 (30)	53 (30)	72 (150)	35	Pfu
	qPCR	Fw: 5'-GTCGGCAGGAATTGTACATAT-3' Rv: 5'-ACAGGGTTCCTTGTATCGT-3'	95 (15)	60 (30)	72 (30)	40	HiGreen
	18S Ribosomal RNA	Fw: 5'-GCGTTCGCTTCGATGAC-3' Rv: 5'-GCCCTTCCGTCAAATCC-3'	95 (15)	58 (30)	72 (30)	40	HiGreen

Sequence and phylogenetic analysis

Multiple animal ω x desaturase sequences for the maximum likelihood (ML) phylogenetic analysis were collected from NCBI. In addition to the known 58 sequences isolated from Lophotrochozoa/Arthropoda identified by [18], six further sequences were retrieved from the TSA database of five Cephalopoda species (*Pterygioteuthis hoylei*, *Idiosepius notoides*, *Dosidicus gigas*, *Octopoteuthis deletron* and *Vampyroteuthis infernalis*). The multiple sequence alignment (MSA) of deduced aa sequences was carried out using MAFFT v7.402 [35] under the parameter E-INS-i (--reorder --maxiterate 1000 --retree 1 --genafpair). Subsequently, the obtained MSA was filtered through to remove all the columns containing gaps using trimAl [36]. The most appropriate model of protein evolution was selected to LG+G+I by Smart Model Selection (SMS) [37] with Akaike's information criterion. The ML

phylogenetic inference was carried out using PhyML [38] with 1000 bootstrap replicates. The obtained tree was visualised using CLC Main Workbench 8 (CLC bio, Aarhus, Denmark).

Functional characterisation of O. vulgaris ωx desaturases by heterologous expression in Saccharomyces cerevisiae

The open reading frame (ORF) of the putative ωx desaturases were amplified from *O. vulgaris* nerve and hepatopancreas cDNA mixture, using the high-fidelity *Pfu* DNA polymerase (Promega). The PCR conditions and primers are shown in Table 1. The PCR fragments were subsequently digested with *Bam*HI and *Xho*I following the manufacturer's instructions (New England Biolabs, Herts, UK) and purified as described above. The restricted and purified PCR products were then ligated into similarly restricted pYES2 vector (Invitrogen, Paisley, UK) for heterologous expression in yeast *S. cerevisiae*. Transformation and culture of yeast were conducted as previously described [39,40].

To test the desaturation capacity of the newly cloned *O. vulgaris* ωx desaturases, yeast transformed with pYES2 containing the ORF of the *O. vulgaris* ωx desaturases (pYES2-OVωx1 and pYES2-OVωx2) were grown in the absence and presence of exogenously added PUFA substrates including 18:2ω6, 18:3ω6, 20:4ω6, 20:2ω6, 20:3ω6, 22:4ω6 and 22:5ω6. Each PUFA was supplied to the yeast culture at a final concentration of 0.5 (C₁₈), 0.75 (C₂₀) and 1.0 (C₂₂) mM, as uptake efficiency decreases with increasing chain length [41]. A control treatment consisting of yeast transformed with empty pYES2 was also run under the same conditions as above. After 48 h culture at 30 °C, yeasts were harvested, washed twice in distilled water and homogenised in chloroform/methanol (2:1, v/v) containing 0.01 % (w/v) butylated hydroxyl toluene (BHT, Sigma-Aldrich) as antioxidant before total lipids (TL) were extracted according to [42]. All PUFA substrates were from Nu-Chek Prep, Inc. (Elysian, MN).

Chemicals used to prepare yeast media were from Sigma-Aldrich, except for the bacteriological agar obtained from Oxoid Ltd. (Hants, UK).

Fatty acid analysis of yeast samples by gas chromatography

Fatty acid methyl esters (FAME) were prepared from the entire TL fraction obtained from 5 mL yeast subcultures [39,40]. FAME were subsequently identified and quantified using a Fisons GC-8160 (Thermo Fisher Scientific) gas chromatograph equipped with a 60 m x 0.32 mm i.d. x 0.25 μ m ZB-wax column (Phenomenex, Cheshire, UK) and flame ionisation detector. Functions of the *O. vulgaris* ω x desaturases were established by comparing the FA profiles of ω x desaturase transformed yeast with those of the controls. Desaturation efficiencies from potential substrates were calculated by the proportion of substrate FA converted to desaturated FA product as [product area / (product area + substrate area)]*100.

In vivo incubations of O. vulgaris paralarvae with [1-¹⁴C]20:4 ω 6

A total of 360 *O. vulgaris* 0 days old paralarvae were obtained from eggs of one female broodstock maintained at the facilities of Centro Oceanográfico de Canarias (IEO, Santa Cruz de Tenerife) using the same methodology described previously [31]. Paralarvae were incubated in flat-bottom 6-well tissue culture plates (SARSTEDT AG & CO., Nümbrecht, Germany) at a density of 90 paralarvae per well (n=4) in 10 mL of seawater (36 ‰). Incubations were performed for 6h with 0.2 μ Ci (0.3 μ M) of [1-¹⁴C]20:4 ω 6 (American Radiolabeled Chemicals Inc., St. Louis, Missouri, USA) as described by [31]. A survival rate of 100 % was obtained over all incubations.

Paralarvae were sampled and processed as described by [31] prior extraction of TL. An aliquot of 1.7 mg of TL from *O. vulgaris* paralarvae collected from the *in vivo* assays was used to prepare FAME. FAME were separated by thin-layer chromatography (TLC) using pre-

coated TLC plates SIL G-25 (20 cm × 20 cm; Macherey-Nagel GmbH & Co. KG) pre-impregnated with a solution of 2 g silver nitrate in 20 mL acetonitrile followed by activation at 110 °C for 30 min. The plates were developed in toluene/acetonitrile (95:5, v/v), which resolves the FAME into discrete bands based on both degree of unsaturation and chain length [43]. Developed TLC plates were placed in closed Expouser Cassette-K (BioRad, Madrid, Spain) in contact with a radioactive-sensitive phosphorus screen (Image Screen-K, BioRad) for one week. The screens were scanned with an image acquisition system (Molecular Imager FX, BioRad) and bands resultant from the elongase/desaturase enzymatic activity identified and quantified (as percentage of total) by image analysis software (Quantity One, BioRad).

Tissue expression of ω x desaturases

Expression of genes encoding the *O. vulgaris* ω x desaturases was determined in adult octopus tissues by quantitative real-time PCR (qPCR) and using 18S as reference gene. Total RNA from nerve, nephridium, hepatopancreas, caecum, gill, heart, gonad, and eye were extracted as described above, with 1 μ g being used in each cDNA synthesis reaction (High Capacity cDNA Reverse Transcription Kits, AB Applied Biosystems). In order to determine the efficiency of the primer pairs, serial dilutions of pooled cDNA were carried out. All qPCR were performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate at total volumes of 20 μ L containing 10 μ L of Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific, Hemel Hempstead, UK), 1 μ L of each primer (10 μ M), 3 μ L of molecular biology grade water, and 5 μ L cDNA (1/10 and 1/40 dilutions for target and reference genes, respectively). Moreover, no template controls (NTC) using molecular biology grade water instead of cDNA, were also run systematically in each plate. The qPCR thermocycling conditions and primers used are described in Table 1.

Finally, a melting curve with 1 °C increments during 6 s from 60 to 95 °C was systematically run after each qPCR run in order to check the presence of a single product in each reaction.

Absolute copy numbers of the ω x desaturase genes and 18S reference gene in each sample were calculated from linear standard curve constructed as described [40]. Briefly, solutions of known copy numbers were obtained by estimating the molecular weights of previously linearised constructs, namely pYES2-OV ω x1 and pYES2-OV ω x2 for candidate genes, or pGEM-T-18S for reference gene, according to relation 660 g bp per length (bp) of the plasmid constructs, divided all of them by Avogadro's number. Expression levels for both ω x desaturase genes were normalised by expression of the reference gene (18S).

Statistical analysis

Results are presented as means $\pm$ standard error (qPCR) or standard deviation (in vivo experiments). Data of functional characterisation of the *O. vulgaris* ω x desaturases were checked for normal distribution with the one-sample Kolmogorov-Smirnov test. T-test was used to assess differences in fatty acid profiles of yeast from functional assays of ω x desaturases [44,45]. $P < 0.05$ was considered significantly different.

Results

*Molecular characterisation and phylogenetics of the *O. vulgaris* ω x desaturases*

The sequences of the two newly cloned ω x desaturases from *O. vulgaris* were deposited in GenBank under the accession numbers MH492794 (ω x1) and MH492795 (ω x2). The *O. vulgaris* ω x1 consists of an ORF of 1257 bp that encodes a peptide of 419 amino acids (aa). Moreover, the *O. vulgaris* ω x2 has an ORF of 1104 bp equivalent to 368 aa. The aa identity between the *O. vulgaris* ω x1 and ω x2 was 44 %. Both desaturases contained multiple transmembrane domains and three histidine boxes, whose aa composition were HDCGH for

H-box 1, HRNHH (ω x1) and HKNHH (ω x2) for H-box 2, and HQVHH (ω x1) and HQIHH (ω x2) for H-box 3 (Fig. 2).

The ML phylogenetic tree using aa sequence comparisons of the *O. vulgaris* ω x desaturases and other metazoan ω x desaturases is shown in Fig. 3. The *O. vulgaris* ω x desaturases are placed in a well-supported Lophotrochozoa/Arthropoda clade (bootstrap = 92%) (Clade 3) outgrouped by Clade 2 (Cnidaria) and Clade 1 (Nematoda) as denoted by [18] (Fig. 3). Within Clade 3, Cephalopoda sequences form two separate clades themselves (both bootstrap = 100%) (“Clades 3.I and 3.II” in Fig. 3), with each *O. vulgaris* ω x desaturase cloned herein clustering within each of these cephalopod specific clades. Additionally, it is worth noting that Octopodiformes (octopuses) and Decapodiformes (cuttlefishes & squids) sequences were clearly separated in each Cephalopoda clades (Fig. 3).

Figure 2. Multiple sequence alignment of the *Octopus vulgaris* ωx desaturase sequences with those from *Patella vulgata*, *Acropora millepora* and *Caenorhabditis elegans*. The height of each amino acid in sequence logo indicates a proportion of its frequency in the alignment. Three histidine boxes (H-box1-3) are indicated.

Figure 3. Maximum likelihood phylogenetic analysis of the *Octopus vulgaris* ω x desaturases. Multiple ω x desaturases from animals were used for the analysis. Clades 1-3 are consistent with nomenclature used by [18]. Two Cephalopoda sequence groups, namely Clade 3.I and Clade 3.II, are also indicated. The newly cloned *O. vulgaris* ω x1 ($\omega 3$) and ω x2 ($\Delta 12$) are highlighted in bold.

Functional characterisation of O. vulgaris ω x desaturases in yeast and in vivo enzymatic activity

The *O. vulgaris* ω x desaturases were functionally characterised by heterologous expression in yeast *S. cerevisiae*. The FA profiles of transgenic yeast expressing each ω x desaturase and grown in the absence of exogenously added substrates, were compared with those of yeast carrying the empty pYES2. Our results show that, while the FA profile of the transgenic yeast expressing the ω x1 desaturase did not differ from that of the control yeast (data not shown), the FA profile of yeast expressing the ω x2 desaturase showed a statistically significant increase of 18:2 ω 6 suggesting that ω x2 desaturase had Δ 12 desaturase activity (Fig. 4A, B; Table 2). Further desaturase activities were tested by growing the transgenic yeast expressing the *O. vulgaris* ω x desaturase in the presence of a variety of ω 6 PUFA substrates. None of the ω 6 PUFA substrates exogenously supplemented was desaturated by the *O. vulgaris* ω x2 desaturase (data not shown) and consequently this desaturase can be categorised as a Δ 12 desaturase. Importantly, yeast expressing the *O. vulgaris* ω x1 desaturase showed the activity towards all ω 6 PUFA substrates assayed since all were converted into the corresponding ω 3 product (Table 3). More specifically, conversion of 18:2 ω 6 and 18:3 ω 6 into 18:3 ω 3 and 18:4 ω 3, respectively, denoted a Δ 15 desaturase activity; conversion of 20:2 ω 6, 20:3 ω 6 and 20:4 ω 6 to 20:3 ω 3, 20:4 ω 3 and 20:5 ω 3, respectively, denoted Δ 17 desaturase activity; and conversion of 22:4 ω 6 to 22:5 ω 3 denoted Δ 19 desaturase activity (Table 3). No Δ 19 desaturase activity towards 22:5 ω 6 was detected (Table 3). These results confirm that the *O. vulgaris* ω x1 can be categorised as an ω 3 desaturase.

Table 2. Functional characterisation of the *Octopus vulgaris* ω 2 desaturase in *Saccharomyces cerevisiae*. Results are expressed as an area percentage of total fatty acids (FA) found in yeast transformed with either empty pYES2 vector (Control) or *O. vulgaris* ω 2 desaturase ORF (mean $\pm$ SEM, n=3). (*) Indicate significant differences ($P<0.05$).

FA	Control	ω 2
16:0	34.6 $\pm$ 0.7	31.2 $\pm$ 1.0*
16:1 ω 7	23.2 $\pm$ 0.8	27.0 $\pm$ 0.8*
18:0	21.2 $\pm$ 0.7	17.8 $\pm$ 0.7*
18:1 ω 9	21.1 $\pm$ 0.5	18.1 $\pm$ 0.8*
18:2 ω 6	n.d.	6.0 $\pm$ 0.1*

n.d., not detected

Table 3. Substrate conversions of ω 1 desaturase from *Octopus vulgaris* by heterologous expression in the yeast *Saccharomyces cerevisiae*. Results are expressed as a percentage of total fatty acid (FA) substrate converted to desaturated product.

FA substrate	FA product	% conversion	Activity
18:2 ω 6	18:3 ω 3	1.9	Δ 15
18:3 ω 6	18:4 ω 3	4.2	Δ 15
20:2 ω 6	20:3 ω 3	5.2	Δ 17
20:3 ω 6	20:4 ω 3	5.5	Δ 17
20:4 ω 6	20:5 ω 3	13.4	Δ 17
22:4 ω 6	22:5 ω 3	1.8	Δ 19
22:5 ω 6	22:6 ω 3	n.d.	Δ 19

n.d., not detected

The yeast assay denoted a particularly high conversion of ARA towards EPA (Fig. 4C; Table 3) and thus an enzymatic activity assay by incubating *O. vulgaris* paralarvae with [1- 14 C]20:4 ω 6 was conducted to test whether the results of the *in vitro* yeast assay were confirmed *in vivo*. The majority of [1- 14 C]20:4 ω 6 incorporated into *O. vulgaris* hatchlings TL was recovered as unmodified FA substrate (Table 4). Nonetheless, it was possible to identify two

additional bands corresponding to an elongation product (22:4 ω 6) and to a Δ 17 desaturation product (20:5 ω 3) (Fig. S1).

Figure 4. Functional characterisation of the *Octopus vulgaris* ω x desaturases by heterologous expression in yeast. (A) Gas chromatogram of fatty acid methyl esters (FAME) derived from the yeast expressing the *O. vulgaris* ω x2. (B) Gas chromatogram of FAME from yeast transformed empty pYES2 (control). Peaks 16:0, 16:1 ω 7, 18:0 and 18:1 ω 9 are major fatty acids (FA) in *S. cerevisiae*. Peak corresponding to the Δ 12 desaturation product 18:2 ω 6 from 18:1 ω 9 was higher in yeast expressing the *O. vulgaris* ω x2 (A) compared to control yeast (B). (C) Gas chromatogram of FAME derived from the yeast expressing *O. vulgaris* ω x1 desaturase grown in the presence of 20:4 ω 6. The peak of 20:5 ω 3 corresponds to the Δ 17 desaturation product of 20:4 ω 6.

Supplementary Figure S1. Autoradiogram of fatty acids (FA) from *Octopus vulgaris* paralarvae incubated with $[1-^{14}\text{C}]20:4\omega6$ (indicated with an asterisk) ($n=4$; Figure shows samples 1 to 3 in lanes 1-3). A standard consisting of a mixture of radiolabeled FA was also run (lane on the right). Radioactivity was recovered with the exogenously supplied substrate $20:4\omega6$ (ARA), its elongation product ($22:4\omega6$) and the $\Delta 17$ desaturase product $20:5\omega3$ (EPA).

Table 4. Recovery of radioactivity from $[1-^{14}\text{C}]20:4\omega6$ substrate in fatty acid metabolites in *Octopus vulgaris* paralarvae. Results represent means $\pm$ SD; $n=4$. Data of recovery are given in percentage.

Substrate	Products	% Recovery
$[1-^{14}\text{C}]20:4\omega6$	$20:4\omega6$	97.4 ± 0.2
	$22:4\omega6$	1.1 ± 0.1
	$20:5\omega3$	1.5 ± 0.1

Tissue distribution of ω x desaturases in adult O. vulgaris

Tissue expression of the ω x desaturases in adult *O. vulgaris* were assessed by qPCR on cDNA samples collected from eight tissues (nerve, nephridium, hepatopancreas, caecum, gill, heart, gonad, and eye) (Fig. 5). High expression levels were detected in eye and nerves (Fig. 5).

Figure 5. Tissue expression of ω x desaturases in adult *Octopus vulgaris*. Expression levels for each gene were normalised by expression of the reference gene (18S). Data are shown as mean $\pm$ standard error (n=4).

Discussion

The present study builds on previous investigations aimed to identify the essential FA for *O. vulgaris*, which resulted in the cloning and functional characterisation of a $\Delta 5$ Fad and the elongases Elov12/5 and Elov14 [29,30,32]. Some of these enzymatic activities had been confirmed through *in vivo* assays using *O. vulgaris* paralarvae [31]. While Elov12/5 and Elov14 guarantee all elongation capacity required for LC-PUFA biosynthesis from C₁₈ PUFA precursors [32], presence of one sole front-end desaturase ($\Delta 5$ Fad) in its genome [33], appears to limit the overall pathways in *O. vulgaris* since key $\Delta 4$, $\Delta 6$ and/or $\Delta 8$ desaturase activities appear to be missing (Fig. 1). It was concluded that both ARA and EPA should be regarded as dietary essential FA for *O. vulgaris* since, despite the $\Delta 5$ Fad their biosynthesis from 20:3 ω 6 and 20:4 ω 3, respectively, these precursors occur in insufficient levels in the *O. vulgaris* natural diet to meet the physiological demands. Similarly, the above-mentioned absence of front-end desaturases with $\Delta 6$ or $\Delta 4$ activities confirmed that DHA was also an essential FA for *O. vulgaris* [31,32]. The recent discovery of ω x desaturases being widespread among animals including molluscs [18], and the availability of a reference genome from *O. bimaculoides* [33], have enabled us to identify two ω x desaturase enzymes and thus revise our current understanding of the LC-PUFA biosynthesis in cephalopods.

The *O. vulgaris* ω x1 and ω x2 desaturases possess three histidine boxes (H-box 1-3) that are conserved in all membrane-bound FA desaturases [46]. The composition and structure of the histidine boxes vary depending on the type of desaturases [46] and, consistently with other ω x desaturases, both *O. vulgaris* ω x1 and ω x2 enzymes contained the three histidine boxes as HXXXH, HXXHH and HXXHH. This pattern can also be found in sphingolipid desaturases [46] but there are some distinctive traits that enable discrimination between these two types of desaturases. More specifically, the aa occurrence frequency within the first histidine box consists of the third and fourth aa residues being mainly cysteine (C) and glycine (G),

respectively, in ω x desaturases, whereas sphingolipid desaturases have mostly isoleucine (I) and serine (S) residues in the same positions [46]. In agreement, both *O. vulgaris* desaturases investigated herein contain HDCGH as the H-box 1. Interestingly, valine (V) is almost exclusively the second aa in H-box 3 (HVXHH) for ω x desaturases according to [46]. However, that is not the case for any of the two *O. vulgaris* ω x desaturases, which contain a glutamine (Q) instead (HQXHH). Such Q is a diagnostic residue characteristic of H-box 3 from ω x desaturases within Clade 3 (see Fig. S2 from [18]), a cluster that included sequences from Arthropoda and Lophotrochozoa, namely Rotifera, Annelida and Mollusca. Collectively, the H-box aa composition and occurrence frequency of the *O. vulgaris* ω x desaturases is characterised by sharing the pattern of H-box 1 commonly to all ω x desaturase types identified to date, as well as having the diagnostic H-box 3 pattern of ω x desaturases from molluscs and other invertebrate phyla within Clade 3 [18].

As mentioned above, the *O. vulgaris* ω x desaturases investigated herein clustered within Clade 3 containing sequences retrieved from Mollusca, Rotifera, Annelida and Arthropoda in agreement with previous findings [18]. Within Mollusca, the *O. vulgaris* ω x desaturases grouped together with other Cephalopoda sequences, which form themselves two distinct clades (Clades 3.I and 3.II). The phylogenetic relationship between those two Cephalopoda clades and with other groups contained within Clade 3 could not be clearly established due to low bootstrap values. Thus, it remained unclear the specific evolutionary events responsible for the presence of two distinct genes encoding ω x desaturases in cephalopods. Previously, ω x desaturases from the nematode *C. elegans* (Fat-1 and Fat-2) and the whitefly *Bemisia tabaci* were hypothesised to derive from horizontal gene transfer (HGT) [18,47], and this phenomenon might be also responsible for the presence of ω x desaturases in cephalopods. This hypothesis is supported by the “intronless” organisation of the *O. bimaculoides* ω x desaturases (see Fig. 2 in [18]), a feature suggesting a gene origin via RNA retrotransposition-mediated HGT [48].

Occurrence of two ω x desaturases appears to be a common feature among animals including other classes of molluscs like the Gastropoda *P. vulgata*, as well as other phyla such as Cnidaria (e.g. *A. millepora*), Nematoda (e.g. *C. elegans*), Rotifera (e.g. *A. vaga*), Annelida (e.g. *P. dumerilii*) and Arthropoda (e.g. *L. salmonis*) [18]. Whereas the currently available data do not allow us to clarify the evolutionary events resulting in the presence of two ω x desaturase genes in animal genomes, it is more clear that this represents an advantageous trait when the two co-existing desaturases enable both $\Delta 12$ and $\Delta 15$ desaturations required for *de novo* biosynthesis of LA and ALA. Our functional data clearly show that such physiological innovation exist in *O. vulgaris* through the enzymatic capacities of the two ω x desaturases characterised in the present study.

We here demonstrate that *O. vulgaris* possesses two functional ω x desaturases with $\Delta 12$ and $\omega 3$ regioselectivities enabling them to *de novo* synthesise PUFA such as 18:2 ω 6 (LA) and 18:3 ω 3 (ALA). Thus, the *O. vulgaris* ω x2 mediates the $\Delta 12$ desaturation required to convert 18:1 ω 9 into 18:2 ω 6, whereas the ω x1 catalyses the subsequent reaction ($\Delta 15$ desaturation) leading to the production of 18:3 ω 3 (Fig. 6). These results confirm that neither LA nor ALA can be strictly categorised as essential FA for *O. vulgaris* since they can be produced endogenously. Subsequently, both 18:2 ω 6 and 18:3 ω 3 can be further elongated to 20:2 ω 6 and 20:3 ω 3, respectively, by the *O. vulgaris* Elovl2/5. Both 20:2 ω 6 and 20:3 ω 3 are precursors of the non-methylene interrupted FA $\Delta 5,11,14$ 20:3 and $\Delta 5,11,14,17$ 20:4, respectively [29]. Moreover, 20:2 ω 6 can be also desaturated to 20:3 ω 3 by the action of the *O. vulgaris* ω x1 (Fig. 6). Thus, the *O. vulgaris* ω x1 exhibits activity towards a wide range of substrates specificities and, along 18:2 ω 6 and 20:2 ω 6, it also had activity towards 18:3 ω 6, 20:3 ω 6, 20:4 ω 6 and 22:4 ω 6, which were converted into their corresponding $\omega 3$ products and thus demonstrating an $\omega 3$ regioselectivity. These conversions denote $\Delta 15$, $\Delta 17$ and $\Delta 19$ desaturation capacities of the *O. vulgaris* ω x1 desaturase (Fig. 1) as previously shown in the gastropod *P. vulgata* and other

non-mollusc species such as *A. millepora* (Cnidaria), *P. dumerilii* (Annelida) and *L. salmonis* (Arthropoda) [18]. In contrast, other aquatic invertebrates possess ωx desaturases with different capacities like the annelid *R. pachyptila* (Δ15 desaturase) [19], and the rotifer *A. vaga*, with two genes encoding Δ12/Δ15/Δ17 ωx desaturases [18]. Overall, the functional analyses performed on animal ωx desaturases indicate that these enzymes are highly diverse.

Figure 6. Biosynthesis of long-chain ($\geq C_{20}$) polyunsaturated fatty acids in *Octopus vulgaris* hypothesised from yeast functional assays of the herein characterised $\omega x1$ and $\omega x2$ desaturases, and other desaturase and elongase enzymes performed in previous studies [29,30,32]. Biosynthesis of the non-methylene interrupted fatty acids $\Delta 5,11,14$ 20:3 and $\Delta 5,11,14,17$ 20:4 from 20:2 $\omega 6$ and 20:3 $\omega 3$, respectively, is catalysed by the $\Delta 5$ front-end desaturase [29]. Elongation reactions involved in the biosynthesis of very long-chain ($>C_{24}$) polyunsaturated fatty acids by the *O. vulgaris* Elov14 [32] have been omitted for simplicity. $\Delta 4$ and $\Delta 6$

desaturations required for 22:6 ω 3 (DHA) biosynthesis are indicated with grey dotted arrows to denote that these activities have not been demonstrated in *O. vulgaris*.

Δ 9, Δ 9 desaturation by stearyl-CoA desaturase; Δ 5, desaturation by the Δ 5 front-end desaturase; Elovl, elongations by Elovl2/5 and/or Elovl4; β -ox, β -oxidation.

The herein uncovered desaturation abilities contained within the two *O. vulgaris* ω x desaturases have allowed us to revise the dietary essential FA for this species. Considering the activities of the elongases Elovl2/5 [30] and Elovl4 [32], the desaturase Δ 5 Fad [29] and the herein characterised ω x1 and ω x2 desaturases, *O. vulgaris* can biosynthesise EPA from 18:3 ω 6 (γ -linolenic acid) via two possible pathways, namely Δ 15 desaturation – elongation – Δ 5 desaturation, and elongation – Δ 17 desaturation – Δ 5 desaturation (Fig. 6). The combined action of Δ 5 and ω 3 desaturases in the production of EPA has been also observed in the oomycete *Pythium aphanidermatum* [49]. Neither of these two routes would overcome the apparent lack of Δ 6 desaturase capacity of *O. vulgaris* [29–31] required to produce 18:3 ω 6, and supply of the latter through diet might not meet the demands judging from its low occurrence in natural preys of *O. vulgaris* [25,50]. Alternatively, EPA can be directly biosynthesised from 20:4 ω 6 (ARA) as demonstrated by our *in vivo* enzymatic assays, with the *O. vulgaris* ω x1 (ω 3) desaturase arising as the most likely candidate accounting for such reaction according from high affinity shown in the yeast experiments. Unlike 18:3 ω 6, ARA is relatively abundant in natural preys [50] and thus it can serve as a precursor for the biosynthesis of EPA when high physiological demands are not met through the diet. The widespread expression pattern of the two *O. vulgaris* ω x desaturases can ensure that such endogenous supply of EPA is guaranteed throughout the entire body.

Investigations of the molecular mechanisms of LC-PUFA biosynthetic pathways in aquatic invertebrates can provide interesting tools in the form of enzymes with novel functionalities,

amenable for production of ω 3-rich products that contribute to alleviate the pressure on wild fisheries which nowadays supply most of the fish oil consumed globally [49,51]. In this regard, ω x desaturases from animals, especially with ω 3 regioselectivity such as the *O. vulgaris* ω x1, can be particularly interesting due to their Δ 19 desaturase capacity, an ability apparently absent from most microorganism ω 3 desaturases [52,53]. Moreover, Δ 19 desaturase capacity is also required for the conversion of 22:5 ω 6 to 22:6 ω 3 (DHA), an activity that was not detected when the *O. vulgaris* ω x1 was functionally characterised. In the absence of evidence confirming the Δ 19 desaturase to produce DHA from 22:5 ω 6, DHA should be still regarded as an essential FA for *O. vulgaris* as previously postulated [32], since alternative routes involving front-end desaturases, i.e. the “ Δ 4 pathway” or the “Sprecher pathway” (Fig. 1), appear not to be present in cephalopods.

In conclusion, we demonstrated that the cephalopod *O. vulgaris* possesses two distinct ω x desaturase enzymes with Δ 12 and ω 3 regioselectivities. The *O. vulgaris* Δ 12 desaturase (ω x2) catalyses the conversion of 18:1 ω 9 into 18:2 ω 6, the latter acting as precursor for the ω 3 desaturase (ω x1) to produce 18:3 ω 3 via Δ 15 desaturation. Both 18:2 ω 6 and 18:3 ω 3 are precursors of the LC-PUFA through further enzymatic capacities previously demonstrated in this species. In addition to Δ 15 desaturation, the *O. vulgaris* ω 3 desaturase also showed Δ 17 capacity, which was particularly relevant in the conversion from 20:4 ω 6 to 20:5 ω 3 as supported by heterologous expression and *in vivo* enzymatic activity analyses. The herein uncovered enzymatic capabilities confirmed that several routes enabling EPA biosynthesis are operative in *O. vulgaris*. With regards to ARA and DHA, current evidence strongly suggests that these are dietary essential FA since endogenous production does not seem to account for the high physiological demands associated to these compounds in animals.

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