A complete enzymatic capacity for biosynthesis of docosahexaenoic acid (DHA, 22:6n−3) exists in the marine Harpacticoida copepod *Tigriopus californicus*.

Naoki Kabeya¹, Masanari Ogino¹, Hideki Ushio², Yutaka Haga¹, Shuichi Satoh¹, Juan C. Navarro³ and Óscar Monroig³

¹Department of Marine Biosciences, Tokyo University of Marine Science and Technology, Konan 4-5-7, Minato, Tokyo, Japan
²Department of Aquatic Bioscience, The University of Tokyo, Yayoi 1-1-1, Bunkyo, Tokyo, Japan
³Instituto de Acuicultura Torre de la Sal (IATS-CSIC), Ribera de Cabanes 12595, Castellón, Spain

NK, 0000-0002-2055-6554; YH, 0000-0002-3528-0554; JCN, 0000-0001-6976-6686; ŌM, 0000-0001-8712-0440

The long-standing paradigm establishing that global production of Omega-3 (n−3) long-chain polyunsaturated fatty acids (LC-PUFA) derived almost exclusively from marine single-cell organisms, was recently challenged by the discovery that multiple invertebrates possess methyl-end (or \( \omega_x \)) desaturases, critical enzymes enabling the biosynthesis of n−3 LC-PUFA. However, the question of whether animals with \( \omega_x \) desaturases have complete n−3 LC-PUFA biosynthetic pathways and hence can contribute to the production of these compounds in marine ecosystems remained unanswered. In the present study, we investigated the complete enzymatic complement involved in the n−3 LC-PUFA biosynthesis in *Tigriopus californicus*, an intertidal harpacticoid copepod. A total of two \( \omega_x \) desaturases, five front-end desaturases and six fatty acyl elongases were successfully isolated and functionally characterized. The *

1. Introduction

The omega-3 (α3 or n−3) long-chain (≥C20) polyunsaturated fatty acids (LC-PUFA) including eicosapentaenoic acid (EPA, 20:5n−3) and docosahexaenoic acid (DHA, 22:6n−3) have beneficial effects on human health [1,2]. Marine ecosystems have been regarded to be responsible for virtually all the global production of n−3 LC-PUFA due to the abundance of single-cell microorganisms such as photosynthetic microalgae, heterotrophic protists and bacteria, with the ability to biosynthesize n−3 LC-PUFA [3–5]. The biosynthesis of n−3 LC-PUFA in marine microbes is achieved by either anaerobic or aerobic pathways involving distinct enzymatic machineries. The polyketide synthase
(PKS) complex is involved in the anaerobic pathway existing in prokaryotes and some eukaryotic microorganisms [6]. However, most eukaryotes operate the aerobic pathway of n-3 LC-PUFA biosynthesis that entails two critical components; one is the biosynthesis of n-3 C18 polyunsaturated fatty acids (PUFA), namely α-linolenic acid (ALA, 18:3n-3), from saturated fatty acids (SFA) such as stearic acid (18:0); another is the biosynthesis of n-3 LC-PUFA including EPA and DHA from ALA (figure 1). Along with the aerobic n-3 LC-PUFA biosynthetic pathways, a varied range of enzymes catalyses multi-step desaturation (introduction of new double bonds or unsaturations) and elongation (extension of the carbon acyl chain) reactions. Initially, palmitic acid (16:0) is synthesized via the fatty acid synthase (FAS) system and then elongated to 18:0. Subsequently, a Δ9 desaturase (e.g. stearoyl-CoA desaturase) introduces the first double bond to produce the monounsaturated fatty acid (MUFA) oleic acid (18:1n-9) [7]. These enzymatic capacities enabling the biosynthesis of SFA and MUFA are virtually present in all eukaryotes [8,9]. However, enzymes enabling the de novo biosynthesis of PUFA (i.e. introducing a second double bond into oleic acid) have a more restricted distribution and, as discussed below, were believed to be largely absent from animals. Specifically, the de novo biosynthesis of PUFA typically requires the action of a particular type of desaturase enzymes called methyl-end (or αx) desaturases since they introduce a new double bond between a pre-existing one and the methyl-terminus of the carbon chain [10]. Thus, there exist αx desaturases that introduce a further double bond into LA at Δ15 position and thus produce the n-3 C18 PUFA ALA (figure 1). Both LA and ALA become substrates from which n-6 and n-3 LC-PUFA, respectively, can be biosynthesized through sequential reactions catalysed by front-end desaturases, which introduce a new double bond between the pre-existing one and the carboxyl-terminus of the fatty acid, and fatty acyl elongases, which catalyse the initial condensation step of the fatty acid elongation pathway (figure 1) [11,12]. Animals possess a varied complement of front-end desaturases and fatty acyl elongases enabling them to produce LC-PUFA from the C18 PUFA precursors LA and ALA [10,13].

Challenging the widely accepted dogma establishing that, with few exceptions (e.g. [14–17]), animals lack the ability to biosynthesize PUFA de novo, Kabeya et al. [18] demonstrated that a plethora of invertebrates including cnidarians, nematodes, lophotrochozoans and arthropods possess αx desaturases enabling them to biosynthesize PUFA de novo. Importantly, it was further established that invertebrates also have αx desaturases commonly known as ‘α3 desaturases’, which enable conversions of multiple n-6 fatty acids into the corresponding n-3 desaturated products including LC-PUFA such as EPA and DHA [18–20]. These findings have obvious ecological implications associated with the contribution that these invertebrates can have on marine ecosystems. Copepods play critical roles in the trophic ecology of marine ecosystems not only because they are the most

Figure 1. A general illustration of the PUFA and LC-PUFA biosynthetic pathways. Reactions catalysed by front-end desaturases are indicated as Δ6, Δ8, Δ5 and Δ4. Reactions catalysed by αx desaturases are indicated as ‘αx’ with their corresponding Δ regioselectivity indicated underneath. Elongase-mediated reactions are denoted as ‘Elo’.
abundant zooplanktonic crustaceans but also because they represent key prey items ensuring the transfer of essential nutrients to upper trophic level organisms. Indeed, copepods contain high levels of n-3 LC-PUFA, particularly EPA and DHA [21], and thus these crustaceans become critically important to guarantee the provision of these essential nutrients to high trophic level organisms such as fish [22]. While the abovementioned abundance of LC-PUFA in primary producers suggests that the high LC-PUFA content of copepods’ lipids can have a dietary origin, it is unclear to what extent copepods can modulate their own fatty acid profiles and contribute to their characteristic high LC-PUFA lipid profiles. Early studies using 13C-labelled fatty acids suggested some copepods showed the ability to bioconvert PUFA into LC-PUFA [23,24]. Studies involving feeding trials using LC-PUFA-deficient diets or stable isotope labelled fatty acids provided further evidence that Harpacticoida and Cyclopoida copepods possess some capacity to produce n-3 LC-PUFA endogenously [25–33]. However, these studies could not unequivocally establish that the abovementioned metabolic activities observed were indeed due to the copepod’s enzymatic complement, and hence it is difficult to completely rule out that LC-PUFA are rather synthesized by microbes that coexist within the copepod. More robust evidence has been recently collected when genes encoding ωx desaturases were found in several species from groups of copepods including Cyclopoidea, Harpacticoida and Siphonostomatoida [18]. However, with the sole exception of Lepophtheirus salmonis (Siphonostomatoida), none of copepod ωx desaturases has been functionally characterized yet, and therefore, it remains unknown whether the activities of the copepod ωx desaturases are conserved across the group or, on the contrary, have diversified during evolution as occurred for other fatty acyl desaturases [13]. In addition, it is unclear whether, beyond ωx desaturases, copepods have further enzymes such as front-end desaturases and fatty acyl elongases that, along ωx desaturases, enable complete enzymatic activities allowing the production of a variety of LC-PUFA including DHA (figure 1). Recent studies involving genomic and transcriptomic analyses identified sequences of desaturase and elongase genes with putative roles in the LC-PUFA biosynthesis in Harpacticoida and Cyclopoida copepods [31–34]. However, no functional evidence demonstrating the actual role of these enzymes in the LC-PUFA biosynthetic pathways was reported.

The marine intertidal Harpacticoida copepod Tigriopus californicus has been used as a model organism for the study in several disciplines including ecology, evolution and genetics [35–37]. In this study, we investigated the LC-PUFA biosynthetic capability of T. californicus by performing a comprehensive search for genes encoding putative ωx desaturases, front-end desaturases and fatty acyl elongases in T. californicus transcriptomic and genomic datasets. A total of 13 genes encoding two ωx desaturases, five front-end desaturases and fatty acyl elongases in T. californicus were retrieved from the NCBI database (https://i5k.nal.usda.gov/Tigriopus_californicus) and confirmed all five front-end desaturase and six fatty acyl elongase sequences were successfully found in the T. californicus genome. Apart from the T. californicus ωx1 (JW524768) and ωx2 (GBTC01004759) found by Kabeya et al. [18], no further ωx desaturases were identified in the T. californicus genome.

2.2. Isolation of putative desaturase and elongase genes from Tigriopus californicus

In addition to the two ωx desaturase sequences identified by Kabeya et al. [18], termed herein as ωx1 (NCBI acc. No. JW524768) and ωx2 (GBTC01004759), further genes encoding PUFA biosynthesizing enzymes including putative front-end desaturases and fatty acyl elongases were retrieved from the T. californicus transcriptome shotgun assembly (NCBI BioProject No. PRJNA158547 and PRJNA263967) by DELTA-BLAST. Several functionally characterized front-end desaturases and fatty acyl elongases from invertebrates including molluscs and echinoderms were used as queries [38–43]. The DELTA-BLAST search allowed the identification of a total of 21 front-end desaturase-like and 31 fatty acyl elongase-like sequences that were subsequently assembled to generate several consensus sequences. As a result, full-length open reading frames (ORF) of five putative front-end desaturases and five putative elongases were obtained, which were named as Fed1 to Fed5 and Elo1 to Elo5, respectively. After this search, a new transcriptomic dataset became available at NCBI (No. PRJNA504307). Thus, the same search against the new database was repeated and an additional elongase-like sequence was successfully obtained, which was named as Elo6. We then performed blastn search against T. californicus genome assembly (https://15k.nal.usda.gov/Tigriopus_californicus) and confirmed all five front-end desaturase and six fatty acyl elongase sequences were successfully found in the T. californicus genome. Apart from the T. californicus ωx1 (JW524768) and ωx2 (GBTC01004759) found by Kabeya et al. [18], no further ωx desaturases were identified in the T. californicus genome.

2.3. Phylogenetic analysis

Phylogenetic trees comparing the deduced amino acid (aa) sequences of front-end desaturases and fatty acyl elongases from a wide range of eukaryotic organisms were constructed using the maximum-likelihood (ML) method [44]. A comprehensive phylogenetic analysis of ωx desaturases including the two of T. californicus was carried out by Kabeya et al. [18] and hence, it was not performed in the present study. The sequence dataset for the ML phylogenetic analysis of front-end
desaturases was built through the following steps: (i) retrieval of all eukaryotic aa sequences from RefSeq-specific protein-containing ‘Deltas6-FADS-like’ domain (cd03506); (ii) selection of representative species from each taxonomic group; (iii) addition of functionally characterized genes and copepod genes; and (iv) generation of multiple sequence alignment (MSA) using MAFFT v. 7 [45] with FFT-NS-i method (− reorder −auto), and removal of all duplicated sequences and incomplete gappy sequences. A similar strategy was applied for elongases as follows: (i) retrieval of all metazoan aa sequences from RefSeq-specific protein-containing ‘ELO’ domain (pfam01151); (ii) and (iii) were the same as above and (iv) generation of cleaned MSA as described above and removal of potential non-PUFA elongase sequences. Each dataset was then aligned using MAFFT v. 7 [45] with E-INS-i method (−gappypair −maxiterate 1000) and filtered through using GUIDANCE v. 2.0 [46] with 100 bootstrap replicates, sequence and column masking cut-off threshold less than 0.5. Subsequently, the resulting MSA were filtered to delete columns containing gaps that were greater than 95% of the sequences by TrimAl [47]. The final MSA contained 254 columns and 222 sequences for front-end desaturases, and 229 columns and 184 sequences for elongases. The ML phylogenetic inference was carried out using RAxML-NG with automatic bootstrapping (−all) [48]. The protein substitution model was selected as LG + I + G4 for both gene types by ModelTest-NG (−model LG + I + G4) [49]. The resulting trees were visualized using Interactive Tree of Life (iTOL v. 5, https://itol.embl.de).

2.4. Functional characterization of the *Tigriopus californicus* desaturases and elongases

The full-length ORF of the *T. californicus* ax desaturases, front-end desaturases and fatty acyl elongases were amplified by polymerase chain reactions (PCR) using a high-fidelity DNA polymerase (PrimeSTAR Max DNA polymerase, Takara Bio Inc., Shiga, Japan). All PCR runs were carried out following the manufacturer’s default recommendations as follows: 10 s at 98°C for the initial denaturation, 35 cycles of 10 s at 98°C, 5 s at 55°C and 20 s at 72°C. The primer sequences containing restriction enzyme sites to enable further cloning into the yeast expression vector pYES2 (Thermo Fisher Scientific) are given in table 1. The resulting PCR products were purified from an agarose gel (GenElute Gel Extraction Kit, Sigma-Aldrich Japan K.K., Tokyo, Japan) and subsequently digested with the corresponding restriction enzymes (table 1). Next, the purified and digested full-length ORF were ligated into a similarly restricted pYES2 using T4 DNA ligase (Promega) and transformed into DH5α competent E. coli (Nippon Gene Co., Ltd., Tokyo, Japan). Positive transformant colonies were grown overnight in LB broth, and plasmid preparations (GenElute Plasmid Miniprep Kit, Sigma-Aldrich Japan K.K.) were sent to the DNA sequencing service (Eurofin Genomics K.K., Tokyo, Japan) to confirm their sequences. Plasmid constructs of the corresponding *T. californicus* two ax desaturases, five front-end desaturases and six fatty acyl elongases were individually transformed into INVSc1 yeast *Saccharomyces cerevisiae* (Life Technologies Japan, Tokyo, Japan) using the S.c. EasyComp yeast transformation kit (Life Technologies Japan). After growing the transformed yeast on *S. cerevisiae* minimal medium minus uracil (SCMM−ura) plates for 3 days at 30°C, one of the successful transformants from each gene was selected and used for the functional characterization assay as described below.

Each obtained transformant was individually grown in SCMM−ura broth and diluted to OD600 = 0.4 in one single Erlenmeyer flask for each potential substrate assayed. After the OD600 reached 1, the cultures were supplemented with 2% (w/v) galactose for the induction of transgene expression as well as with one of the potential PUFA substrates for each enzyme [10]. For ax desaturases, the exogenously supplied PUFA were 18:2n–6, 18:3n–6, 20:2n–6, 20:3n–6, 20:4n–6, 22:4n–6 and 22:5n–6. For front-end desaturases, PUFA substrates included 18:2n–6, 20:2n–6, 20:3n–6, 22:4n–6, 18:3n–3, 20:3n–3, 20:4n–3 and 22:5n–3, and for elongases exogenously supplied PUFA substrates were 18:2n–6, 18:3n–3, 20:4n–6, 18:3n–3, 18:4n–3, 20:5n–3 and 22:5n–3, and 22:6n–3. Since certain ax desaturases have shown the capacity to desaturate yeast endogenous FA [18–20], tendervicin yeast expressing the two *T. californicus* ax desaturases were grown in triplicate Erlenmeyer flasks in the absence of exogenously added FA substrates and their FA profiles compared with those of control yeast transformed with empty pYES2 vector (also n = 3). Additionally, to test Δ6 desaturase activity towards 24:5n–3, transgenic yeast co-expressing the zebrafish cdv2 [50] and each *T. californicus* fed gene were grown in the presence of 22:5n–3 following the method established by Oboh et al. [51]. Each FA substrate was supplemented as sodium salts at concentrations of 0.5 mM (C16), 0.75 mM (C20) and 1.0 mM (C22) to compensate for the reduced uptake efficiency with the length of the carbon chain [19]. After 48 h of incubation at 30°C and vigorous shaking, yeast cells were harvested by centrifugation (2 min, 500 × g), washed twice in double distilled water, and lyophilized prior to preparation of fatty acid methyl ester (FAME) derivatives. FAME was prepared using Fatty Acid Methyl Ester Preparation Kit (Nacalai Tesque, Kyoto, Japan) following the manufacturer’s recommendations. All fatty acids were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA), except 18:4n–3 from Larodan AB (Solna, Sweden) and 20:4n–3 from Cayman Chemicals (Ann Arbor, MI, USA).

2.5. Fatty acid analysis by gas chromatography

FAME samples were injected on a GC-2025 (Shimadzu Corporation, Kyoto, Japan) gas chromatograph equipped with a capillary column (Supelcowax 10, 30 m × 0.25 mm i.d. × 0.25 μm, Sigma-Aldrich) and a flame ionization detector (FID). The temperature conditions consisted of an initial ramp from 50°C to 180°C at a rate of 40°C min−1, 180°C to 230°C at a rate of 1°C min−1 and then 230°C for 10 min. The injection port and FID temperature were 250°C and 280°C, respectively. Helium was used as a carrier gas in constant velocity mode (30 cm sec−1). FAME was identified by the comparison of their retention times with those from commercial FAME standards. The conversion efficiency of three types of assayed enzymes towards the exogenously supplied PUFA substrates was calculated with the formula (all product area/(all product area + substrate area)) × 100 [20].

2.6. Statistical analysis

Comparisons of means of FA composition between control yeast (n = 3) and transgenic yeast expressing the *T. californicus*
Table 1. Primer sequences for the ORF amplification of each gene. Underlined nucleotides indicate the corresponding restriction sites in each primer sequence.

| target gene | NCBI accession no. | sense primer | antisense primer |
|-------------|-------------------|--------------|-----------------|
| oox1        | MT757172          | TcWx1_HindIII_F | 5'-CCCAGCTTACATGATGCACCATG-3' |
|             |                   |              | 5'-CCGAAGCTTACTGGCTCCCGATATACAG-3' |
| oox2        | MT757173          | TcWx2_HindIII_F | 5'-CCCAGCTTACATGAGTCCCAATTCCTC-3' |
|             |                   |              | 5'-CCGAAGCTTACTGGCTCCCGATATACAG-3' |
| Fed1        | MT757167          | TcFed1_BamHI_F | 5'-CCCCGAATCCAGATGCTCCGACCAAAATAAGTGC-3' |
|             |                   |              | 5'-CCGACGTCTCTTATGGTATGCACTCATTAGTGA-3' |
| Fed2        | MT757168          | TcFed2_KpnI_F | 5'-CCCCGAGCTCTACATGCTCCCAAGGGAATGATG-3' |
|             |                   |              | 5'-CCGAACGTCTGCAGACGAGTGGTCTCG-3' |
| Fed3        | MT757169          | TcFed3_Sad_F | 5'-CCCCGAGCTTGACAGAGTGGGAAAGAAGGAGGTG-3' |
|             |                   |              | 5'-CCGACGTCTCTTATGGTATGCACTCATTAGTGA-3' |
| Fed4        | MT757170          | TcFed4_HindIII_F | 5'-CCGACGTCTTTTATGACCAATGTTATG-3' |
|             |                   |              | 5'-CCGAACGTCTGCAGACGAGTGGTCTCG-3' |
| Fed5        | MT757171          | TcFed5_HindIII_F | 5'-CCGACGTCTTTTATGACCAATGTTATG-3' |
|             |                   |              | 5'-CCGAACGTCTGCAGACGAGTGGTCTCG-3' |
| Elo1        | MT757162          | TcElo1_HindIII_F | 5'-CCGACGTCTTACATGCTCCGACCAAAATAAGTGC-3' |
|             |                   |              | 5'-CCGAACGTCTGCAGACGAGTGGTCTCG-3' |
| Elo2        | MT757163          | TcElo2_HindIII_F | 5'-CCGACGTCTTACATGCTCCGACCAAAATAAGTGC-3' |
|             |                   |              | 5'-CCGAACGTCTGCAGACGAGTGGTCTCG-3' |
| Elo3        | MT757164          | TcElo3_HindIII_F | 5'-CCGACGTCTTACATGCTCCGACCAAAATAAGTGC-3' |
|             |                   |              | 5'-CCGAACGTCTGCAGACGAGTGGTCTCG-3' |
| Elo4        | MT757165          | TcElo4_HindIII_F | 5'-CCGACGTCTTACATGCTCCGACCAAAATAAGTGC-3' |
|             |                   |              | 5'-CCGAACGTCTGCAGACGAGTGGTCTCG-3' |
| Elo5        | MT757166          | TcElo5_BamHI_F | 5'-CCGACGTCTTACATGCTCCGACCAAAATAAGTGC-3' |
|             |                   |              | 5'-CCGAACGTCTGCAGACGAGTGGTCTCG-3' |
| Elo6        | MW246081          | TcElo6_HindIII_F | 5'-CCGACGTCTTACATGCTCCGACCAAAATAAGTGC-3' |
|             |                   |              | 5'-CCGAACGTCTGCAGACGAGTGGTCTCG-3' |
3. Results

3.1. Phylogeny of the *Tigriopus californicus* front-end desaturases and fatty acyl elongases

The ML phylogenetic tree of the front-end desaturases retrieved from *T. californicus* and other organisms is shown in figure 2 (a complete view is shown in electronic supplementary material, figure S1). All five *T. californicus* front-end desaturases (termed Fed1–5, figure 2) clustered together (bootstrap = 98%) with other copepod sequences from *Tigriopus japonicus* (Harpacticoida), *Callis raggeriessyi* (Siphonostomatoida) and *Paracyclopsina rubra* (Cyclopoidea). A direct sister group of the copepod clade could not be clearly identified due to insufficient resolution of the tree. However, the copepod clade clustered (bootstrap = 82%) with several taxonomically unrelated species belonging to the classes KinetoPlastea (e.g. Bodo saltans, *Trypanosoma cruzi*), Chaoflagellata (*Salpingoeca rosetta* and Haptophyta (*Rebecca salina* and *Pavlova lutheri*). This clade was further out-grouped with another containing sequences from *Sphaeroforma arctica* (Haptophyta), *Thalassiosira pseudonana* (Cyanobacteria), *Gonyaulax* sp. CCMP291 (KDG29597), and *Ciona intestinalis* (*Chordata*). Interestingly, front-end desaturases from other metazoan species including *Vertebrata*, *Echinodermata* and *Mollusca* grouped together in a clearly separated clade (bootstrap = 100%) from that containing the copepod sequences (figure 2).

oxygen desaturases (*n* = 3) were carried out using Dunnett’s test with *p* < 0.05 indicating statistical significance. The analysis was performed in R 3.4.1. (www.r-project.org).

Figure 2. Maximum-likelihood phylogenetic analysis of the *T. californicus* front-end desaturases. The tree was visualized using iTOL (https://itol.embl.de) and re-rooted at the midpoint.
In addition, Fat-3 (Δ6 desaturase, NP_001255426) and Fat-4 (Δ5 desaturase, NP_001255423) genes from the nematode Caenorhabditis elegans were also distantly located from the Copepoda clade (figure 2).

The six elongases (Elo1–6) from T. californicus were separated into three different clades in the ML phylogenetic tree (figure 3; electronic supplementary material, figure S2). The Elo1, Elo2, Elo3 and Elo5 were branched to a Pancrustacea (=Crustacea + Hexapoda)-specific elongase clade with a relatively high support value (90%). Despite its direct sister group could not be clearly established, this Pancrustacea-specific clade grouped itself with the vertebrate Elov11 and Elov17 cluster (bootstrap = 98%) (figure 3). Within the Pancrustacea clade, the relationship among each taxonomical group was not well-resolved but, along Copepoda (T. californicus, T. japonicus, P. nana, C. rogercresseyi, Caligus clemensi, Lepophtheirus salmonis and Eurytemora affinis), it included several insects (e.g. Drosophila melanogaster, Bombyx mori), a collembola (Folsomia candida), and crustaceans such as Decapoda (Scylla olivacea, Eriocheir sinensis and Penaeus vannamei), Branchiopoda (Daphnia pulex) and Amphipoda (Hyalella azteca) (figure 3). Interestingly, the T. californicus Elo1, Elo2 and Elo5 grouped with sequences from other copepods, particularly T. japonicus, while the relationship of the T. californicus Elo3 with other copepod genes could not be established (figure 3). Away from the Pancrustacea clade described above, the T. californicus Elo6 clustered within another well-supported clade (89%) containing several Arthropoda sequences including functionally characterized Elov14-like sequences from two decapods (S. olivacea and Paratana trituberculatus) [52,53] (figure 3). The T. californicus Elo4 was grouped in a completely distinct clade that out-grouped all other sequences (figure 3). This clade was comprised a wide range of metazoan sequences except bony vertebrates. The T. californicus Elo4 was the only gene from Ecdysozoa species identified in our searches.

3.2. Functional characterization of the Tigriopus californicus methyl-end desaturases

In order to fully elucidate the functions of the T. californicus ox1 desaturases, we first tested the desaturase activities towards yeast endogenous fatty acids. The fatty acid profile of the control yeast transformed with the empty pYES2 vector showed four prominent peaks corresponding to 16 : 0, 16 : 1 isomers, 18 : 0 and 18 : 1n−9 as previously reported for wild-type S. cerevisiae [54] (figure 4 and table 2). Two additional peaks corresponding to 18 : 2n−6 and 18 : 3n−3 were detected in the yeast transformed with ox1, denoting Δ12 (conversion of 18 : 1n−9 to 18 : 2n−6) and Δ15 (conversion of 18 : 2n−6 to 18 : 3n−3) desaturase activities (figure 4 and table 2). These results indicate that the T. californicus ox1 is a Δ12Δ15 desaturase. No additional peaks were observed in the yeast transformed with ox2 (table 2). Next, we tested further activities of the T. californicus ox desaturases by growing the transgenic yeast containing their ORF in the presence of exogenously added n−6 PUFA substrates, namely 18 : 2n−6, 18 : 3n−6, 20 : 2n−6, 20 : 3n−6, 20 : 4n−6, 22 : 4n−6 and 22 : 5n−6. Yeast expressing the T. californicus ox1 converted the C18 and C20 n−6 PUFA substrates (18 : 2n−6, 18 : 3n−6, 20 : 2n−6, 20 : 3n−6 and 20 : 4n−6) but not the C22 (22 : 4n−6 and 22 : 5n−6), into the corresponding n−3 products (table 3; electronic supplementary material, figure S3). Such conversions denote Δ15 and Δ17 desaturations (table 3). Overall, these results clearly show that the T. californicus ox1 holds Δ12, Δ15 and Δ17 desaturase activities. With regard to the T. californicus ox2, transgenic yeast expressing its coding region were able to desaturate all C18 and C20 n−6 PUFA to n−3 PUFA products as described above for ox1 but, in addition, was also able to convert 22 : 4n−6 into 22 : 5n−3 (table 3; electronic supplementary material, figure S3). These results show that the T. californicus ox2 has Δ15, Δ17 and Δ19 desaturase activities.

3.3. Functional characterization of the Tigriopus californicus front-end desaturases

To characterize the functions of the T. californicus front-end desaturases (Fed1 to Fed5), transgenic yeast were grown in the presence of exogenously added desaturase substrates, namely n−6 (18 : 2n−6, 20 : 2n−6, 20 : 3n−6 and 22 : 4n−6) and n−3 PUFA (18 : 3n−3, 20 : 3n−3, 20 : 4n−3 and 22 : 5n−3). Our results showed that, with the exception of the Fed1 and Fed3 that share Δ6 desaturase, each front-end desaturase characterized from T. californicus has a specific substrate preference. As mentioned above, Fed1 and Fed3 exhibited Δ6 desaturase activity since both enzymes were able to convert 18 : 2n−6 and 18 : 3n−3 into 18 : 3n−6 and 18 : 4n−3, respectively (figure 5 and table 4; electronic supplementary material, figure S3). Moreover, both Fed1 and Fed3 also showed Δ8 activity but, while Fed3 was able to desaturate 20 : 2n−6 and 20 : 3n−3 to 20 : 3n−6 and 20 : 4n−3, respectively, Fed1 only showed activity towards 20 : 3n−3 (table 4; electronic supplementary material, figure S3). The T. californicus Fed5 showed activity towards 20 : 3n−6 and 20 : 4n−3, which were converted, respectively, into the Δ5 desaturation products 20 : 4n−6 and 20 : 5n−3 (figure 5, table 4; electronic supplementary material, figure S3). The yeast expressing Fed2 were able to convert 22 : 4n−6 and 22 : 5n−3 to 22 : 5n−6 and 22 : 6n−3, respectively, indicating that the encoded enzyme is a Δ4 desaturase (figure 5, table 4; electronic supplementary material, figure S3). No detectable activity towards the exogenously added PUFA substrates was observed for the T. californicus Fed4 (table 4). None of Fed showed any detectable activity towards 24 : 5n−3 (data not shown). Furthermore, no peak corresponding to potential desaturation products of the yeast endogenous fatty acids were detected, suggesting that the T. californicus Fed studied herein do not possess desaturase activities towards yeast endogenous SFA and MUFA.

3.4. Functional characterization of the Tigriopus californicus fatty acyl elongases

Elongase activity was assessed by the analysis of fatty acid profiles of transgenic yeast expressing each of the T. californicus elongases (Elo1 to Elo6) grown in the presence of exogenously added PUFA substrates, namely n−6 (18 : 2n−6, 18 : 3n−6, 20 : 2n−6 and 22 : 4n−6) and n−3 (18 : 3n−3, 18 : 4n−3, 20 : 5n−3 and 22 : 5n−3). All elongases except Elo6 showed activity towards all C18 and C20 substrates, but not towards C22 (figure 5, table 5; electronic supplementary material, figure S3). Particularly, low conversion efficiencies were observed for Elo1 and Elo4, with conversions often below
Figure 3. Maximum-likelihood phylogenetic analysis of the *T. californicus* fatty acyl elongases. The tree was visualized by using iTOL (https://itol.embl.de) and re-rooted at the midpoint.
expressing *T. californicus* towards yeast endogenous SFA and MUFA. suggesting that none of the elongation products of the yeast endogenous FA were observed, figure S3). No obvious peaks corresponding to potential conversions towards 22:4n with low conversion efficiency (0.6%) but no activity towards 22:4n–3, respectively) (figure 5, table 5; electronic supplementary material, figure S3). Elo6 was the only elongase that showed activity towards 22:5n–3 with low conversion efficiency (0.6%) but no activity towards 22:4n–6 (table 5; electronic supplementary material, figure S3). No obvious peaks corresponding to potential elongation products of the yeast endogenous FA were observed, suggesting that none of *T. californicus* Elo has elongase capacity towards yeast endogenous SFA and MUFA.

### Table 2. Comparison of fatty acid profiles from the transgenic yeast expressing *T. californicus* ω desaturases with control yeast transformed with the empty pYES2 vector. The results are presented as area percentage of total fatty acids in each sample (mean ± s.e.m., n = 3). Asterisks (*) indicate significant differences (p < 0.05). n.d., not detected.

|       | control | ωx1 | ωx2 |
|-------|---------|-----|-----|
| 18:0  | 8.2 ± 0.1 | 9.3 ± 0.1 | 8.7 ± 0.0 |
| 18:1n–9 | 25.2 ± 0.2 | 20.1 ± 0.1* | 26.1 ± 0.1 |
| 18:2n–6 | n.d. | 0.8 ± 0.0* | n.d. |
| 18:3n–3 | n.d. | 4.6 ± 0.0* | n.d. |

1.5%. Elo2 and Elo3 had relatively high conversions for all the C_{18} and C_{20} assayed (figure 5, table 5; electronic supplementary material, figure S3). Interestingly, Elo5 had relatively low elongation conversions towards C_{18} PUFA (highest 2.2% towards 18:3n–3) and high for C_{20} (40.6% and 56.8% towards 20:4n–6 and 20:5n–3, respectively) (figure 5, table 5; electronic supplementary material, figure S3). Elo6 was the only elongase that showed activity towards 22:5n–3 with low conversion efficiency (0.6%) but no activity towards 22:4n–6 (table 5; electronic supplementary material, figure S3). No obvious peaks corresponding to potential elongation products of the yeast endogenous FA were observed, suggesting that none of *T. californicus* Elo has elongase capacity towards yeast endogenous SFA and MUFA.

#### Table 3. Substrate conversions of the transgenic yeast expressing the *T. californicus* ω desaturases. The results are presented as a percentage of the fatty acid substrate converted into the corresponding desaturated product. n.d., not detected.

| substrate | product | ωx1 (%) | ωx2 (%) | activity |
|-----------|---------|---------|---------|----------|
| 18:2n–6   | 18:3n–3 | 34.2    | 24.1    | Δ15      |
| 18:3n–3   | 18:4n–3 | 43.8    | 25.5    | Δ15      |
| 20:2n–6   | 20:3n–3 | 3.1     | 10.8    | Δ17      |
| 20:3n–3   | 20:4n–3 | 5.0     | 9.7     | Δ17      |
| 20:4n–6   | 20:5n–3 | 6.1     | 32.0    | Δ17      |
| 22:4n–6   | 22:5n–3 | n.d.    | 4.7     | Δ19      |
| 22:5n–6   | 22:6n–3 | n.d.    | n.d.    | Δ19      |

4. Discussion

Copepods, one of the most abundant groups within zooplankton, contain high levels of n–3 LC-PUFA, particularly EPA and DHA [21,55], prompting interest to elucidate to which extent biosynthesis, along diet can contribute to the abundance of these essential nutrients. While several studies provided evidence suggesting that some copepod species can indeed produce LC-PUFA endogenously, the potential contribution of microbial endosymbionts for such metabolic ability could not be completely ruled out [31–33]. In the present study, we addressed this methodological drawback by performing a comprehensive retrieval for all genes encoding desaturase and elongase enzymes with potential roles in the PUFA and LC-PUFA biosynthesis in a representative species within Harpacticoidea, *T. californicus*. We selected this species not only because of its importance as a dominant zooplanktonic component in the marine tidal area, but also because it has been recently shown to possess putative ω desaturases, enzymes limiting the ability of animals for de novo biosynthesis of PUFA, as well as being major components enabling bioconversions of n–6 substrates into n–3 LC-PUFA [18]. We herein demonstrate that *T. californicus* has multiple genes encoding ω desaturases, front-end desaturases and fatty acyl elongases whose functions enable this species to carry out all reactions required for de novo biosynthesis of PUFA and, from them, LC-PUFA up to DHA. A comprehensive phylogenetic analysis of ω desaturases performed by Kabeya et al. [18] depicted an overall distribution of these gene families in animals. The *T. californicus* ωx1 and ωx2 grouped with other copepod ωx desaturases to form a single clade, itself clustering along with sequences from lophotrochozoans including molluscs, annelids and rotifers [18]. In the present study, the phylogenetic analysis of front-end desaturase showed that the *T. californicus* Fed1–5 formed a well-supported branch including the other two
non-Harpacticoida copepods, namely *C. rogercresseyi* (Siphonostomatoida) and *P. nana* (Cyclopoida). These results suggest that the copepod front-end desaturase genes evolved from their common ancestor. Interestingly, the front-end desaturase sequences retrieved from *T. californicus* and other copepods are phylogenetically unrelated with decapod crustacean desaturases hypothesized to be Δ6 front-end desaturases [56–60]. Unlike copepod sequences, the decapod putative front-end desaturase-like sequences do not contain the conserved Delta6-FADS-like domain (cd03506) in the NCBI-specific protein database and hence did not pass the domain-specific threshold (bit-score = 113.891) and were excluded from the ML phylogenetic inference. These findings strongly suggest that the decapod desaturases are not front-end desaturases and probably explain why no functional data supporting their annotation as ‘Δ6 desaturases’ were reported [56–60]. Indeed, the absence of front-end desaturases appears to the extent to other major groups within Malacostraca crustaceans such as isopods and amphipods, according to our search strategy that did not identify any putative front-end desaturases in these groups. It is interesting to note that the copepod front-end desaturase clade is clearly separated from the well-supported branch comprising sequences from other metazoans including vertebrates, echinoderms and lophotrochozoans (see [10,13]). This result illustrates the diversity of front-end desaturase gene families that play pivotal roles in PUFA biosynthesis in metazoans. Such diversity can be partly accounted for phenomena such as horizontal gene transfer (HGT), an evolutionary mechanism that has been proposed to explain the presence of ωx desaturases [18,61] and other desaturases [62] in animals. Consistently, the copepod sequences retrieved in the present study clustered closely with front-end desaturase sequences from several protists and algae including *Leishmania major* and *P. lutheri*, both characterized as Δ4 desaturases and thus playing key roles in DHA synthesis from 22:5n–3 [63,64]. The insufficient resolution of the tree does not allow us to conclude whether the copepod front-end desaturases were acquired via HGT. However, the ever-increasing availability of genomic sequences will probably enable us to clarify their overall evolutionary history in the near future.

The ML phylogenetic analysis of elongases denoted that four out of six genes (Elo1, Elo2, Elo3 and Elo5) isolated from *T. californicus* formed a well-supported clade with other Pancrustacea sequences. This Pancrustacea-specific clade includes the Elovl7-like elongase isolated from sequences of the complete n-3 LC-PUFA biosynthetic pathway from 18:2n–6 proposed by functional analysis of the *T. californicus* ωx desaturase, front-end desaturases and fatty acyl elongases. Δy in the pathway indicates a specific carbon number from the front end of the fatty acyl chain, where the corresponding desaturase introduces a new double bond. The yeast endogenous fatty acids (16:0, 16:1 isomers, 18:0 and 18:1n–9) are indicated as 1 to 4, respectively, in all panels. Peaks corresponding to exogenously added PUFA substrates are indicated with an asterisk (*).
occuring lower trophic levels that serve as a food item for fish and other top predators. The functional assays of the two *T. californicus* ox desaturases studied here showed that harpacticoid copepods can efficiently produce ALA and, hence, play such a pivotal role in marine ecosystems. Indeed, the *T. californicus* ox desaturases contain key enzymatic activities including Δ12 (ox1) and Δ15 (ox1 and ox2) required to convert oleic acid (18:1n–9) into LA (18:2n–6) and ALA (18:3n–3), respectively (figure 1). Interestingly, the *T. californicus* ox1, along with Δ12 and Δ15 desaturase activities, further showed Δ17 activity towards C20 n–6 PUFA substrates, while ox2, in addition to Δ15, also had Δ17 and Δ19 activities towards C20 and C22 n–6 PUFA substrates, respectively. These results allow us to conclude that both ox desaturases can be categorized as ‘ω3 desaturases’ enabling desaturation of multiple n–6 substrates into the corresponding n–3 metabolic products (figure 1). Similar functions were observed in one of the ox desaturases functionally characterized from the Siphonostomatoida copepod *L. salmonis*, although this species possesses a further enzyme that exclusively exhibited Δ12 desaturase activity [18]. Beyond the phylogenetic diversification of desaturase gene/protein families pointed out above, these results further illustrate that diversification also expands to function since the enzymes

*S. olivacea*, which showed elongation capability on a range of PUFA [65]. The Pancrustacea elongase clade is closely related to that including the vertebrate Elovl1 and Elovl7 but, unexpectedly, not Elovl5, Elovl2 and Elovl4, major enzymes of PUFA elongation in vertebrates [13,66,67]. Moreover, the Pancrustacea elongase clade contained multiple insect sequences that, rather than roles in PUFA elongation, have been shown to participate in the biosynthesis of sex pheromones [68] and cuticular hydrocarbons [69]. Elo6 belonged to another well-supported Arthropoda clade, which includes functionally characterised Elovl4-like genes isolated from two decapod crustaceans, namely *S. olivacea* and *P. trituberculatus* [52,53] (figure 3). Unlike the other five elongase genes from *T. californicus*, one elongase gene (Elo4) clustered within the most diverged clade, which was an out-group of all other elongase sequences. Since Elo4 showed very low activity towards all PUFA tested in the present study, it is reasonable to speculate that Elo4 does not play a prominent role in PUFA elongation in *T. californicus*.

In marine ecosystems, high trophic level organisms such as fish have some capacity to biosynthesize n–3 LC-PUFA from the C18 n–3 PUFA ALA (18:3n–3), a phenomenon often referred to as ‘trophic up-grading’ [70]. Vital to this process is the provision of precursor ALA by organisms

### Table 4. Substrate conversions of the transgenic yeast expressing the *T. californicus* front-end desaturases (Fed1–5). The results are presented as a percentage of each fatty acid substrate converted into the corresponding desaturated product. n.d., not detected.

| substrate | product | Fed1 (%) | Fed2 | Fed3 | Fed4 | Fed5 | activity |
|-----------|---------|----------|------|------|------|------|----------|
| 18:2n–6   | 18:3n–6 | 30.9     | n.d. | 2.2  | n.d. | n.d. | Δ6       |
| 18:3n–3   | 18:4n–3 | 53.7     | n.d. | 8.4  | n.d. | n.d. | Δ6       |
| 20:2n–6   | 20:3n–6 | n.d.     | n.d. | 8.8  | n.d. | n.d. | Δ8       |
| 20:3n–3   | 20:4n–3 | 3.9      | n.d. | 13.9 | n.d. | n.d. | Δ8       |
| 20:3n–6   | 20:4n–6 | n.d.     | n.d. | n.d. | n.d. | 16.5 | Δ5       |
| 20:4n–3   | 20:5n–3 | n.d.     | n.d. | n.d. | n.d. | 28.1 | Δ5       |
| 22:4n–6   | 22:5n–6 | n.d.     | 10.1 | n.d. | n.d. | n.d. | Δ4       |
| 22:5n–3   | 22:6n–3 | n.d.     | 14.0 | n.d. | n.d. | n.d. | Δ4       |

### Table 5. Substrate conversions of the transgenic yeast expressing the *T. californicus* fatty acyl elongases (Elo1–6). The results are presented as a percentage of each fatty acid substrate converted into the corresponding elongated product. n.d., not detected.

| substrate | product | Elo1 (%) | Elo2 | Elo3 | Elo4 | Elo5 | Elo6 |
|-----------|---------|----------|------|------|------|------|------|
| 18:2n–6   | 20:2n–6 | n.d.     | 24.7 | 37.3 | 0.7  | n.d. | 3.6  |
| 18:3n–3   | 20:3n–3 | 1.1      | 59.6 | 28.1 | 3.5  | 2.2  | 2.6  |
| 18:3n–6   | 20:3n–6 | 2.0      | 64.0 | 38.1 | n.d. | n.d. | 1.8  |
| 18:4n–3   | 20:4n–3 | 0.5      | 57.1 | 43.3 | 0.6  | 0.7  | 1.6  |
| 20:4n–6   | 22:4n–6 | 0.8      | 18.2 | 54.9 | 1.3  | 40.6 | 0.7  |
| 20:5n–3   | 22:5n–3 | 1.0      | 37.6 | 45.1 | 1.5  | 56.8 | 1.5  |
| 22:4n–6   | 24:4n–6 | n.d.     | n.d. | n.d. | n.d. | n.d. | 0.6  |
| 22:5n–3   | 24:5n–3 | n.d.     | n.d. | n.d. | n.d. | n.d. | n.d. |
from relatively closely related species such as *T. californicus* and *L. salmonis* have different substrate specificities.

Along with the abovementioned ability to provide C₁₈ PUFA precursors for trophic upgrading to LC-PUFA by fish and other predators, our results demonstrated that harpacticoid copepods such as *T. californicus* can further produce LC-PUFA by themselves. They can do so by the combined action of the herein characterized front-end desaturases and elongases, which enable the biosynthesis of LC-PUFA through any of the routes described in animals to date (figure 1). For the *T. californicus* front-end desaturases, an apparent separation of regioselectivities was observed, with Δ₆ desaturase activity being contained mostly in Fed₁, Δ₈ in Fed₃, Δ₅ in Fed₅ and Δ₄ in Fed₂. Using the same set of enzymes in both the n–6 and n–3 routes, *T. californicus* can use two distinct pathways to biosynthesize both arachidonic acid (ARA) and EPA from the C₁₈ PUFA LA and ALA, respectively (figure 1). These two pathways are the so-called ‘Δ₆ pathway’, consisting of a Δ₆ desaturation, followed by an elongation and a final Δ₅ desaturation, and the ‘Δ₈ pathway’, starting with an elongation, followed by a Δ₆ desaturation and a final Δ₅ desaturation (figure 1) [71, 72]. In addition to front-end desaturases, we herein demonstrate that *T. californicus* has several elongases that can contribute to both pathways. More specifically, the *T. californicus* Elo₂ and Elo₃, with preference towards C₁₈ and C₂₀ PUFA, and Elo₅ with preference towards C₂₀ PUFA, appear to be major enzymes involved in the biosynthesis of LC-PUFA such as ARA and EPA. Although further studies will be needed to investigate physiological significance of some ‘over-lapping’ activities observed in both Fed and Elo, the apparent separation of regioselectivities/substrate specificities would imply the importance of precise modulation of LC-PUFA composition in *T. californicus*.

Copepods including harpacticoids contain lipids with particularly high levels of DHA (approximately 10 to 20%, e.g. [30, 73, 74]). Functional analyses of the *T. californicus* LC-PUFA biosynthesising genes demonstrate that some copepods have the ability to produce DHA from EPA. DHA biosynthesis in animals takes place via two different pathways, including the ‘Sprecher pathway’, reported in rats [75], fish [51, 76, 77] and the razor clam *Sinoventacula constricta* [78], and the ‘Δ₄ pathway’ that is mostly present in teleost lineages [51] (figure 1). Both pathways require the first elongation from EPA (20 : 5n–3) to 22 : 5n–3 that, in the case of *T. californicus*, can be achieved by different elongases, namely Elo₂, Elo₃ and Elo₅. However, the Sprecher pathway requires a further elongation to produce 24 : 5n–3 before this compound can be Δ₆ desaturated to 24 : 6n–3 and chain-shortened (partial β-oxidation) to DHA (figure 1). None of the *T. californicus* desaturases characterized in this study appeared to be able to desaturate 24 : 5n–3 to 24 : 6n–3, and therefore it is unlikely that this copepod can produce DHA via the Sprecher pathway. Importantly, one of the *T. californicus* front-end desaturases, the herein termed ‘Fed₂’ is a Δ₄ desaturase, an enzyme enabling the direct conversion of 22 : 5n–3 to DHA within the Δ₄ pathway. Possessing Δ₄ desaturases has been previously reported in some microalgae [4] and few vertebrates mostly teleosts [22] but, to the best of our knowledge, this key enzyme has not been hitherto identified in invertebrates. While further Investigations will help to clarify the occurrence of Δ₄ desaturases among copepods, it is reasonable to speculate that endogenous production via the Δ₄ pathway accounts for part of the DHA found in copepods’ lipids [25–33].

In conclusion, we have successfully isolated and functionally characterized two ox desaturases, five front-end desaturases and six elongases from *T. californicus*. The *T. californicus* ox desaturases are α₃ desaturases enabling (i) the biosynthesis of LA and ALA that are precursors of LC-PUFA and (ii) the conversion of multiple n–6 PUFA into the corresponding n–3 metabolic products including LC-PUFA. Upgrading from the C₁₈ PUFA LA and ALA to LC-PUFA is also possible in *T. californicus*, since the complementary action of its front-end desaturases and elongases enable multiple routes for the biosynthesis of the physiologically active compounds ARA, EPA and DHA. Collectively, the results obtained in this study demonstrate that *T. californicus* has a complete enzymatic complement enabling this species to produce n–3 LC-PUFA up to DHA endogenously. Therefore, harpacticoid copepods arise as primary producers of n–3 LC-PUFA in marine ecosystems and, given their widespread distribution and abundance at a global scale, it is likely that such contribution is not negligible.

**Data accessibility.** All isolated sequences from *T. californicus* in the present study were deposited into the NCBI GenBank with the accession numbers MT1757162 to MT1757173 and MW246081.

**Authors’ contributions.** N.K. initially conceived the study and coordinated together with J.C.N. and Ö.M. N.K., M.O., J.C.N. and Ö.M. performed experimental analyses. N.K. and M.O. prepared the initial version of the manuscript including all figures and tables, and edited with Ö.M. The manuscript was further finalized by H.U., Y.H., S.S. and J.C.N.

**Competing interests.** We declare we have no competing interests.

**Funding.** This study was partly funded through the project IMPRO-MEGA of the Ministry of Science, Innovation and Universities, Spanish Government (grant no. RTI2018-095119-B-I00, MCIU/AEI/FEDER, UE). N.K. was supported by the JSPS KAKENHI grant no. JP19K15908. The authors have no conflicts of interest related to this work.

**References.**

1. Saini RK, Keum YS. 2018 Omega-3 and omega-6 polyunsaturated fatty acids: dietary sources, metabolism, and significance—a review. Life Sci. 203, 255–267. (doi:10.1016/j.lfs.2018.04.049)
2. Zárate R, Jaber-Vazdekis N, Tejera N, Pérez JA, Rodríguez C. 2017 Significance of long chain polyunsaturated fatty acids in human health. Clin. Transl. Med. 6, 25. (doi:10.1186/s40169-017-0153-6)
3. Nichols DS. 2003 Prokaryotes and the input of polyunsaturated fatty acids to the marine food web. FEMS Microbiol. Lett. 219, 1–7. (doi:10.1016/S0168-6445(03)00058-1)
4. Khizin-Goldberg I, Iskandarov U, Cohen Z. 2011 LC-PUFA from photosynthetic microalgae: occurrence, biosynthesis, and prospects in biotechnology. Appl. Microbiol. Biotechnol. 91, 905–915. (doi:10.1007/s00253-011-3441-x)
5. Pereira SL, Leonard AE, Mukerji P. 2003 Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes. Prostaglandins Leukot. Essent. Fatty Acids 68, 97–106. (doi:10.1016/S0952-3278(02)00259-4)
6. Metz JG et al. 2001 Production of polysaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. Science 293, 290–293. (doi:10.1126/science.1059593)

7. Castro LFC, Wilson JM, Gonçalves O, Galante-Oliveira S, Rocha E, Cunha I. 2011 The evolutionary history of the steaoyl-CoA desaturase gene family in vertebrates. BMC Evol. Biol. 11, 132. (doi:10.1186/1471-2148-11-132)

8. Maier T, Leibundgut M, Ban N. 2008 The crystal structure of a mammalian fatty acid synthase. Science 321, 1315–1322. (doi:10.1126/science.1161269)

9. Bai Y, McCoy JG, Levin EJ, Sobrado P, Rajashankar KR, Fox BG, Zhou M. 2015 X-ray structure of a mammalian steaoyl-CoA desaturase. Nature 524, 252–256. (doi:10.1038/nature14549)

10. Monroig Ó, Kabeya N. 2014 Desaturases and elongases involved in polysaturated fatty acid biosynthesis in aquatic invertebrates: a comprehensive review. Fish. Sci. 84, 911–928. (doi:10.1007/s12265-012-0125-4)

11. Sperling P, Ternes P, Zank TK, Heinz E. 2003 The Δ12-desaturase from the house cricket, Acheta domesticus, Acr. Biochem. Biophys. 328, 201–206. (doi:10.1006/ajbb.2003.2020)

12. Leonard AE, Pereira S, Sprecher H, Huang Y. 2003 Elongation of long-chain fatty acids. Prog. Lipid Res. 43, 36–54. (doi:10.1016/S0163-7827(03)00040-7)

13. Castro LFC, Tocher DR, Monroig Ó. 2016 Long-chain polyunsaturated fatty acid biosynthesis in aquatic invertebrates: a comprehensive review. Phil. Trans. R. Soc. B 375, 20190654. (doi:10.1098/rstb.2019.0654)

14. Castro LFC, Tocher DR, Monroig Ó. 2019 Methyl-end desaturases with Δ12 and Δ3 regioselectivities enable the de novo PUFA biosynthesis in the cephalopod Octopus vulgaris. Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1864, 1134–1144. (doi:10.1016/j.bbalip.2019.04.012)

15. Brown MT, Müller-Norva NC, Persson J. 2009 Crustacean zooplankton fatty acid composition. In Lipids in aquatic ecosystems (eds MT Arts, MT Brett, et al.) gene expression across the transcriptome of the temperate copepod Tigriopus californicus reveals substantially reduced population differentiation at northern latitudes. Mol. Ecol. 19, 1743–1750. (doi:10.1111/j.1365-294X.2010.04922.x)

16. Schoville SD, Barreto FS, Roy GW, Wolff A, Burton RS. 2012 Investigating the molecular basis of local adaptation to thermal stress: population differences in gene expression across the transcriptome of the copepod Tigriopus californicus. BMC Evol. Biol. 12, 170. (doi:10.1186/1471-2148-12-170)

17. Willett CS. 2010 Potential fitness trade-offs for thermal tolerance in the intertidal copepod Tigriopus californicus. Evolution 64, 2521–2534. (doi:10.1111/j.1558-5646.2010.00808.x)

18. Kabeya N, Sanz-Jorquera A, Carboni S, Davie A, Obih A, Monroig Ó. 2017 Biosynthesis of polysaturated fatty acids in sea urchins: molecular and functional characterisation of three fatty acyl desaturases from Paracentrotus lividus (Lamarck 1816). PLoS ONE 12, e0169374. (doi:10.1371/journal.pone.0169374)

19. Liu H, Feng Z, Song X, Zhu W, Hu Y. 2016 Cloning, expression and functional characterization of the polysaturated fatty acid elongase (ELOV5) gene from sea cucumber (Apostichopus japonicus). Gene 593, 217–224. (doi:10.1016/j.gene.2016.08.023)

20. Liu H, Zheng H, Wang S, Wang Y, Li S, Liu W, Zhang G. 2013 Cloning and functional characterization of a polysaturated fatty acid elongase in a marine bivalve noble scallop Chlamys nobilis Reeve. Aquaculture 416, 146–151. (doi:10.1016/j.aquaculture.2013.09.015)
41. Liu H, Guo Z, Zheng H, Wang S, Wang Y, Liu W, Zhang G. 2014 Functional characterization of a Δ5-
like fatty acyl desaturase and its expression during early embryogenesis in the noble scallop Chlamys
nobilis Reeve. Mol. Biol. Rep. 41, 7437–7445. (doi:10.1007/s11033-014-3633-4)

42. Monroig Ó, Guinot D, Hontoria F, Tocher DR, Navarro JC. 2012a Biosynthesis of essential fatty acids
in Octopus vulgaris (Cuvier, 1779): molecular characterization and tissue distribution of a fatty
acyl elongase. Aquaculture 360:361, 45–53. (doi:10.1016/j.aquaculture.2012.07.016)

43. Monroig Ó, Navarro JC, Dick J, Alemany F, Tocher DR. 2012b Identification of a Δ5-like fatty acyl
desaturase from the cephalopod Octopus vulgaris (Cuvier 1797) involved in the biosynthesis of
essential fatty acids. Mar. Biotechnol. 14, 411–422. (doi:10.1007/s10126-011-9423-2)

44. Whelan S, Liò P, Goldman N. 2001 Molecular
inference. Bioinformatics 17, 262–272. (doi:10.1093/ Bioinformatics/btp348)

45. Katoh K, Standley DM. 2013 MAFFT multiple
sequence alignment software version 7: improvements in performance and usability. Mol.
Biol. Evol. 30, 772–780. (doi:10.1093/molbev/msm100)

46. Sela I, Ashkenazy H, Katoh K, Pupko T. 2015
GUIDECA2: accurate detection of unreliable
alignment regions accounting for the uncertainty of multiple parameters. Nucleic Acids Res. 43,
W7–W14. (doi:10.1093/nar/gkv318)

47. Capella-Gutierrez S, Silla-Martinez JM, Gabaldón T. 2009 trimAl: a tool for automated alignment
trimming in large-scale phylogenetic analyses. Bioinformatics 25, 1972–1973. (doi:10.1093/ bioinformatics/btp348)

48. Kozlov AM, Darriba D, Flouri T, Stamatakis A, Kozlov AM, Darriba D, Flouri T, Morel B, Stamatakis
2015 Molecular phylogenetic analysis and functional characterization of an Elolv7-like elongase
from a marine crustacean, the orange mud crab (Scylla olivacea). Comp. Biochem. Physiol. B Biochem.
Biol. 232, 60–71. (doi:10.1016/j.cbpb.2019.01.011)

49. Whelan S, Liò P, Goldman N. 2001 Molecular
inference. Bioinformatics 17, 262–272. (doi:10.1093/ Bioinformatics/btp348)

50. Kattner G, Hagen W. 2009 Lipids in marine
copepods: Latitudinal characteristics and perspective to global warming. In Lipids in aquatic ecosystems (eds MT Arts, MT Brett, M Kainz), pp. 257–280. New
York, NY: Springer.

51. Yang Z, Guo Z, Ji L, Zeng Q, Wang Y, Yang X, Cheng Y. 2013 Cloning and tissue distribution of a fatty
acyl Δ6-desaturase-like gene and effects of dietary lipid levels on its expression in the hepatopancreas
of Chinese mitten crab (Eriocheir sinensis). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 165, 99–105. (doi:10.1016/j. cbpb.2013.03.010)

52. Shi Q, Yang Z, Wang W, Yao Q, Wang Y, Cheng Y. 2015 Cloning and tissue expression of full-length cDNA in
gene encoding Δ6-desaturase fatty acyl of Portunus trituberculatus. Biol. Funct. 31, 138–145.

53. Lin Z, Hao M, Zhu D, Li S, Wen X. 2017 Molecular
cloning, mRNA expression and nutritional regulation of a Δ6 fatty acyl desaturase-like gene of mud crab,
Scylla paramamosain. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 200–209, 29–37. (doi:10.1016/ j.cbpb.2017.03.004)

54. Chen K, Li E, Li T, Xu C, Xu Z, Qin JG, Chen L. 2017
The expression of the Δ6 fatty acyl desaturase-like gene from Pacific white shrimp (Litopenaeus
vannamei) under different salinities and dietary
lipid compositions. J. Shellfish Res. 36, 501–509. (doi:10.2983/ jshr.16-0221)

55. Wu D-L, Huang Y-H, Liu Z-Q, Yu P, Gu-H, Fan B, Zhao Y-L. 2015 Molecular cloning, tissue expression
and regulation of nutrition and temperature on Δ6 fatty acyl desaturase-like gene in the red
crab clawfish (Chesurus chlorocephalus). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 225, 58–66. (doi:10.
1016/j.cbpb.2018.07.003)

56. Crisp A, Becsici T, Perry M, Tunnaccliffe A, Micklem G. 2015 Expression of multiple horizontally acquired
genes is a hallmark of both vertebrate and invertebrate genomes. Genome Biol. 16, 50. (doi:10.1186/s13059-015-0607-3)

57. Bryon A et al. 2017 Disruption of a horizontally transferred phytoene desaturase abolishes
canotoid accumulation and diapause in Tetranychus urticae. Proc. Natl. Acad. Sci. USA 114, E8671–E8880. (doi:10.1073/pnas.1706851114)

58. Liu H, Guo Z, Zheng H, Wang S, Wang Y, Liu W, Zhang G. 2014 Functional characterization of a Δ5-
like fatty acyl desaturase and its expression during early embryogenesis in the noble scallop Chlamys
nobilis Reeve. Mol. Biol. Rep. 41, 7437–7445. (doi:10.1007/s11033-014-3633-4)

59. Zhou X, Suat, Petrie JR, Frampton DMF, Mansour MP, Blackburn SJ, Nichols PD, Green AG, Singh SP. 2007 Identification and characterization of genes from the marine microalga Pavlova salmona
encoding three front-end desaturases involved in
docosahexaenoic acid biosynthesis. Phytochemistry 68, 785–796. (doi:10.1016/j.phytochemistry.2006.12. 016)

60. Mah M-Q, Kuah M-K, Ting SY, Merosa P, Janaranjani M, Dog P-T, Jaya-Ram A, Shu-Chen AC. 2019 Molecular
cloning, phylogenetic analysis and functional characterisation of an Elolv7-like elongase from a marine crustacean, the orange mud crab (Scylla olivacea). Comp. Biochem. Physiol. B Biochem.
Biol. 232, 60–71. (doi:10.1016/j.cbpb.2019.01.011)

61. Agbaga MP, Mandal MMA, Anderson RE. 2010
Retinal very long-chain PUFA: new insights from studies on ELOVL4 protein. J. Lipid Res. 51, 1624–1642. (doi:10.1194/jlr.R000525)

62. Zhang YJ, Kothapalli KS, Brenna JT. 2016 Desaturase and elongase-limiting endogenous long-
chain polysaturated fatty acid biosynthesis. Curr. Opin. Clin. Nutr. Metab. Care 19, 103–110. (doi:10.1097/MCO.0000000000000254)

63. Finck J, Berdan EL, Mayer F, Ronacher B, Geiselhardt S. 2016 Divergence of cuticular hydrocarbons in two
sympatric grasshopper species and the evolution of fatty acid syntheses and elongases across insects. Sci. Rep. 6, 36695. (doi:10.1038/srep36695)

64. Monroig O, Tocher DR, Navarro JC. 2013
Biosynthesis of polysaturated fatty acids in marine invertebrates: recent advances in molecular mechanisms. Mar. Drugs 11, 3996–4018. (doi:10.3390/ md111103998)

65. Park WJ, Kothapalli KS, Lawrence P, Tyburczy C, Brenna JT. 2009 An alternate pathway to long-chain
polysaturates: the FADS2 gene product Δ8-desaturates 20:2n-6 and 20:3n-3. J. Lipid Res. 50, 1195–1202. (doi:10.1194/jlr.M006030-JLR200)

66. Monroig O, Liu Y, Tocher, D. R. 2011 Delta-8 desaturation activity varies among fatty acyl desaturases of teleost fish: high activity in delta-6 desaturases of marine species. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 159, 206–213. (doi:10.1016/j.cbpb.2011.04.007)

67. Hiltunen M, Strandberg U, Taipale SJ, Kankaala P. 2015 Taxonomic identity and phylogenomics affect fatty acid composition of zooplankton in large lakes with differing dissolved organic carbon concentration. Limnol. Oceanogr. 60, 303–317. (doi:10.1002/lno.10028)

68. Lichi DA, Rinchard J, Kimmel DG. 2017 Changes in
zooplankton community, and seston and zooplankton fatty acid profiles at the freshwater/
saltwater interface of the Chowan River, North Carolina. PeerJ 5, e3667. (doi:10.7717/peerj.3667)

75. Sprecher H. 2000 Metabolism of highly unsaturated n-3 and n-6 fatty acids. Biochim. Biophys. Acta. 1486, 219–231. (doi:10.1016/S1388-1981(00)00077-9)

76. Buzzi M, Henderson RJ, Sargent JR. 1997 Biosynthesis of docosahexaenoic acid in trout hepatocytes proceeds via 24-carbon intermediates. Comp. Biochem. Physiol. B 116, 263–267. (doi:10.1016/S0305-0491(96)00210-6)

77. Tocher, D. R., Agaba, M., Hastings, N., Teale, A. J. 2003. Biochemical and molecular studies of the polyunsaturated fatty acid desaturation pathway in fish. In The big fish bang: Proc. of the 26th Annual Larval Fish Conf. (eds HI Browman, AB Skiftesvik), pp. 211–227. Bergen, Norway: Institute of Marine Research.

78. Ran Z et al. 2019 Biosynthesis of long-chain polyunsaturated fatty acids in the razor clam Sinonovacula constricta: characterization of four fatty acyl elongases and a novel desaturase capacity. Biochim. Biophys. Acta Mol. Cell Biol. Lipids. 1864, 1083–1090. (doi:10.1016/j.bbalip.2019.04.004)