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Molecular characterization of biosynthesis of polyunsaturated fatty acids during different developmental stages in the copepod *Apocyclops royi*

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A R T I C L E I N F O

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A B S T R A C T

Copepod *Apocyclops royi* can biosynthesize long-chain polyunsaturated fatty acids (LC-PUFAs) when fed low-PUFA precursors. Previously, two elongases and two desaturases in the n-3 PUFA biosynthetic pathway were identified from *A. royi*. However, the complete PUFA biosynthesis pathway in this copepod species is poorly understood. Here, we report 13 genes, of which nine are novel genes, encoding PUFA biosynthesis-related enzymes belonging to the fatty acid desaturases (*Ar*D6D, *Ar*D5D, *Ar*D4D, *Ar*O3D-1, and *Ar*O3D-2) and elongases (Elovl1, 2, 3, 4, 5, 6, 7, and 8) families identified from a Thai culture of *A. royi* (*A. royi*-TH). Identification of the fatty acid contents using gas chromatography/mass spectroscopy analysis indicated that the copepodid and adult stages were high in PUFAs, with omega-3 fatty acids, while the nauplius stage had the lowest level of PUFAs. Moreover, all copepod stages of *A. royi*-TH fed *Tetraselmis suecica* contained higher levels of LC-PUFAs, including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), than the microalgae fatty acid content, which was deficient in omega-3 DHA. Changes in transcript expression levels were determined in three developmental stages of *A. royi*-TH. Interestingly, the increased gene expression of the fatty acid desaturases (*Ar*D6D, *Ar*D5D, *Ar*D4D, *Ar*O3D-1, and *Ar*O3D-2) and elongases (*Ar*Elovl3, 4, 5, 6, and 7) in the adult stages was reflected in the increased fatty acid concentration of DHA and EPA in the adult stages compared with the other developmental stages, suggesting the possible function of these genes for LC-PUFA synthesis in the different copepod developmental stages. These results indicate that the nauplius, copepodid, and adult stages are capable of synthesizing DHA from low PUFA through a LC-PUFA biosynthesis pathway.

1. Introduction

Copepods are a group of small crustaceans that play a pivotal role as consumers of primary producers and as major live prey for many aquatic species in natural food webs (Dhont et al., 2013; Williamson and Reid, 2001). The use of copepods has been reported for larval rearing of a number of marine fish species (Busch et al., 2011; McEvoy et al., 1998; Ogle et al., 2005; Olivotto et al., 2008; Payne et al., 2001; Rajkumar and Vasagam, 2006; Shields et al., 1999; Toledo et al., 2005). According to their high nutritional value and wide range of sizes and swimming motions, copepods are considered as a promising potential live feed for marine larviculture (Dhont et al., 2013; Drillet et al., 2011; Støttrup,

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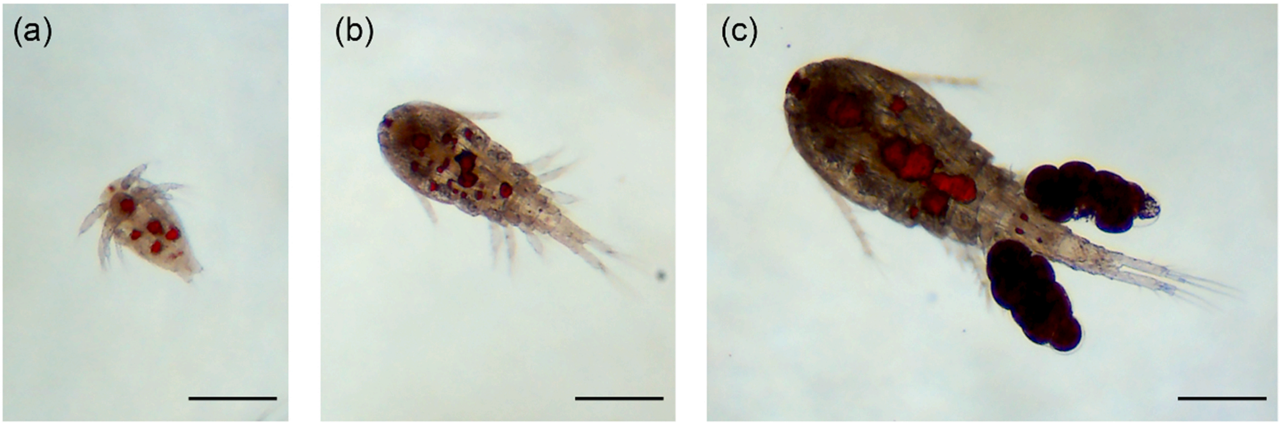

Fig. 1. Representative microscope images showing the morphology of the nauplius (a), copepodid (b), and adult (c) developmental stages of Oil Red O-stained *A. royi*-TH. Scale bars represent 200 μm.

Table 1
Nucleotide sequences of the primers used.

Primers	Sequences (5'- 3')	Purpose
Fatty acid desaturase		
D6D-F	TCGCCCTTCCCATTATCTACACGC	RT-PCR
D6D-R	TGTTCGATCTGGTAATTGAGACCTCC	RT-PCR
D5D-F	CCGTTGTCTACATCATCAGGTGGGT	RT-PCR
D5D-R	GTGTGCGTTGACTGGTCTTCTCGTG	RT-PCR
D4D-F	CGATCGGAATTCCATTCTCAGT	RT-PCR
D4D-R	GTTGAACGAAAGCTGAATCACG	RT-PCR
O3D-1-F	ACGAGATCTTCTACCCCGTGAG	RT-PCR
O3D-1-R	CCCAAGATAAGTCCAAGCCAAG	RT-PCR
O3D-2-F	AGGGTGAGGATGTTCCTTGGTA	RT-PCR
O3D-2-R	TCATTGCACTTCCTCACAAGGT	RT-PCR
D5D-TW-F	GCTGCTCTTTTCGGATTGCA	RT-PCR
D5D-TW-R	CCGAAGCTGGCCTATTTATGT	RT-PCR
Elongation of very long fatty acids protein (Elovl)		
Elovl1-F	CCGATAGCCCGAAAGCTTTACTTA	RT-PCR
Elovl1-R	ACGTTGAGGAAACCAAAGAAGGAG	RT-PCR
Elovl2-F	ACGGTCAATTCGTTAGTTCACGTC	RT-PCR
Elovl2-R	AGCATGAGGAGGCTTGTTATCAGA	RT-PCR
Elovl3-F	TTCGATTTCTTCCAGGTGGTCA	RT-PCR
Elovl3-R	CACTGGGTACGCACAGTCCTCT	RT-PCR
Elovl4-F	AGTTCCTCGAAATGTTGGACTCGT	RT-PCR
Elovl4-R	TAGTAGCCGTACATGAGGACGTGAA	RT-PCR
Elovl5-F	GGAATCCAACCTTCTTGGAAGAGAC	RT-PCR
Elovl5-R	GTTGGCGATATACAGCCTAGGTCAC	RT-PCR
Elovl6-F	GTATCACCACTTGACCGTTCTGTTG	RT-PCR
Elovl6-R	ATCTGATAGGCCCAGACGTTGACTA	RT-PCR
Elovl7-F	GATGTGTGCCGCCTACGTCTAC	RT-PCR
Elovl7-R	GCTTTGGGGTTGTCGGAGTAGT	RT-PCR
Elovl8-F	ATCGCTTACAATGCTGTTCAGGTC	RT-PCR
Elovl8-R	GACACGTTGTCGAACTTTTTCCTG	RT-PCR
Elongation factor 1-α (EF1α)		
EF1α -F	GTGTTGGACAAGCTGAAGTC	RT-PCR
EF1α -R	GGTCCAGTGATCATGTTCTTGATG	RT-PCR

2000).

Certain essential fatty acids, especially omega-3 (n-3) long-chain (≥ C20) polyunsaturated fatty acids (LC-PUFA), are essential nutrients that are considered important drivers of ecosystem health and stability (Parrish, 2013). Their significance not only impacts on animal growth, but also on other facets, including reproduction, immunity, and ion balance regulation and product quality (Glencross, 2009). Previous studies have shown that many invertebrates possess enzymes that contribute to LC-PUFA production (Kabeya et al., 2018; Monroig and Kabeya, 2018). In particular, the desaturases and elongases are key enzymes that play essential roles in the PUFA biosynthesis by aquatic invertebrates.

Three types of major enzymes that participate in the pathways of *de novo* and trophic upgrading of PUFA, namely methyl-end (or ωx) desaturases, fatty acyl (front-end) desaturases (Fads), and elongation of very long-chain fatty acid (Elovl) proteins (commonly known as elongases), have been characterized from many invertebrate species, including sponges, cnidarians, molluscs, annelids, crustaceans (including copepods), rotifers, echinoderms, and non-vertebrate chordates (amphioxus and sea squirt) (Kabeya et al., 2018; Monroig and Kabeya, 2018). In copepods, a previous study reported that methyl-end desaturase genes were exclusively found in the specific orders of Siphonostomatoida, Cyclopoida, and Harpacticoida, but not in Calanoida copepods, while the presence of front-end desaturases, as demonstrated by the ability to bioconvert eicosapentaenoic acid (EPA) to docosahexaenoic acid (DHA), has been suggested in the calanoids *Calanus finmarchicus* and *Drepanopus forcipatus* and the cyclopoid *Cyclops strenuus* (Monroig and Kabeya, 2018).

Although genomic and transcriptomic resources are available for several Harpacticoida, including *Tisbe holothuriae* (BioProject PRJEB23629), *Tigriopus californicus* (Schoville et al., 2012), *Tigriopus japonicus* (Kim et al., 2015), *Tigriopus kingsejongensis* (Kim et al., 2016; Lee et al., 2020a), and *Platychelipus littoralis* (Boyen et al., 2020), and Cyclopoida (*Paracyclopina nana*) (Lee et al., 2020b), there is no evidence demonstrating the functional role of these enzymes in the LC-PUFA biosynthetic pathways. Interestingly, a recent study in the harpacticoid copepod *T. californicus* revealed that the complete n-3 LC-PUFA biosynthetic pathways exist in a copepod, with 13 genes encoding for two ωx desaturases, five front-end desaturases, and six elongases being identified and functionally characterized (Kabeya et al., 2021). However, the molecular mechanism underlying the PUFA biosynthesis in copepods remains largely uninvestigated.

The cyclopoid *Apocyclops royi* is a tropical copepod that is suspected to possess the capability to biosynthesize n-3 LC-PUFA from short-chain PUFAs (Nielsen et al., 2019, 2020, 2021; Pan et al., 2018). Transcripts coding for four putative front-end desaturase and elongase enzymes, including Fad Δ5, Fad Δ6, Elovl4, and Elovl5, have been identified and assumed to function in the n-3 PUFA biosynthetic pathway (Nielsen et al., 2019). However, their exact function remains to be experimentally characterized. In this study, we identified 13 n-3 LC-PUFA biosynthesis-related genes encoding for methyl-end desaturase, front-end desaturase, and elongase enzymes from a Thai culture of the cyclopoid copepod *A. royi* (*A. royi*-TH). We analyzed the transcript expression levels and fatty acid compositions in the nauplius (NP), copepodid (CD), and adult (AD) developmental stages of *A. royi*-TH that had been fed on *Tetraselmis suecica*, a green microalgae that is deficient in DHA. Knowledge of the gene expression and fatty acid profile in these different development life stages of *A. royi*-TH may provide novel insights into n-3 LC-PUFA biosynthesis and metabolism in invertebrates, and may alter or enhance more efficient PUFA production to supply the demand from

Table 2
Molecular characteristics of the genes and their predicted protein products in the LC-PUFA biosynthetic pathways of the cyclopoid copepod *Apocyclops royi*.

Gene name	CDS /ORF	Closest gene (% amino acid similarity) (ACNO)	Conserved domains	Putative Biological Functions/ Reference sources
Fatty acid desaturase				
*Ar*D6D	1302 bp/ 433 aa	*Tigriopus californicus* front-end desaturase-2 (76%) (QWC69495)	Cytochrome b5-like Heme/Steroid binding domain, Five transmembrane domains, and FA_desaturase domain	Front-end desaturase: Δ6 fatty acid desaturase activity (Nielsen et al., 2019) Δ4 desaturase activity (Kabeya et al., 2021)
*Ar*D5D	1317 bp/ 438 aa	*Paracyclopina nana* delta5 desaturase (76%) (APH81338)	Cytochrome b5-like Heme/Steroid binding domain, Five transmembrane domains, and FA_desaturase domain	Front-end desaturase: Δ5 fatty acid desaturase activity (Lee et al., 2017b) Δ6 fatty acid desaturase activity (Nielsen et al., 2019)
*Ar*D4D	1026 bp/ 341 aa	*Tigriopus japonicus* delta4-desaturase (86%) (AIW65589)	Sphingolipid desaturase domain, Four transmembrane domains, and FA_desaturase domain	Front-end desaturase: Δ4 desaturase activity (Lee et al., 2020b)
*Ar*O3D-1	1149 bp/ 382 aa	*T. californicus* methyl-end desaturase-1 (72%) (QWC69499)	Five transmembrane domains and FA_desaturase domain	Methyl-end desaturase: Δ12, Δ15, and Δ17 desaturase activities (Kabeya et al., 2021)
*Ar*O3D-2	1233 bp/ 410 aa	*T. californicus* methyl-end desaturase-2 (73%) (QWC69500)	Six transmembrane domains and FA_desaturase domain	Methyl-end desaturase: Δ15, Δ17, and Δ19 desaturase activities (Kabeya et al., 2021)
Elongation of very long fatty acids protein (Elovl)				
*Ar*Elovl1	873 bp/ 290 aa	*P. nana* elongase-1 (86%) (APH81340)	Five transmembrane domains and ELO domain	Elongase activity (Lee et al., 2017b)
*Ar*Elovl2	831 bp/ 276 aa	*T. californicus* fatty acid elongase-2 (79%) (QWC69490)	Seven transmembrane domains and ELO domain	Elongase activity towards C18 and C20, but not C22 (Kabeya et al., 2021)
*Ar*Elovl3	858 bp/ 285 aa	*T. californicus* fatty acid elongase-1 (73%) (QWC69489)	Seven transmembrane domains and ELO domain	Elongase activity towards C18 and C20, but not C22 (Kabeya et al., 2021)
*Ar*Elovl4	903 bp/ 300 aa	*T. californicus* fatty acid elongase-6 (67%) (QWC69501)	Seven transmembrane domains and ELO domain	Elovl4 (Nielsen et al., 2019) Elongase activity towards C18, C20, and C22:5n–3, but not C22:4n–6 (Kabeya et al., 2021)
*Ar*Elovl5	723 bp/ 240 aa	*T. californicus* fatty acid elongase-4 (76%) (QWC69492)	Six transmembrane domains and ELO domain	Elovl5 (Nielsen et al., 2019) Elongase activity towards C18 and C20, but not C22 (Kabeya et al., 2021)
*Ar*Elovl6	879 bp/ 292 aa	*T. japonicus* elongase-2 (78%) (AIW65585)	Seven transmembrane domains and ELO domain	Elongase activity (Lee et al., 2020b)
*Ar*Elovl7	879 bp/ 292 aa	*P. nana* elongase-1 (82%) (APH81340)	Five transmembrane domains and ELO domain	Elongase activity (Lee et al., 2017b)
*Ar*Elovl8	771 bp/ 256 aa	*T. californicus* fatty acid elongase-5 (79%) (QWC69493)	Five transmembrane domains and ELO domain	Elongase activity towards C18 and C20, but not C22 (Kabeya et al., 2021)

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2. Materials and methods

2.1. Identification of PUFA biosynthesis-related genes and sequence analysis

To identify genes involved in the PUFAs biosynthesis, nucleotide sequences encoding for desaturase and elongase enzymes were retrieved from the copepod *Apocyclops royi* (*A. royi*-TH) transcriptome (Amparyup et al., unpublished data). Sequence similarity searches were performed using the BLASTx program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide and predicted amino acid sequences of the candidate genes were analyzed using the ExPASy program (https://www.expasy.org/) (The deduced amino acid sequences were shown in Fig. S1). A conserved domain search was performed using the SMART program (http://smart.embl-heidelberg.de/). The TMHMM (http://www.cbs.dtu.dk/services/TMHMM) software was used to predict transmembrane domain (TMD) helices. Multiple sequence alignments were performed using the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.2. Development stages and sample preparation of the copepod *A. royi*-TH

The *A. royi*-TH was originally isolated from Chanthaburi province, Thailand, and maintained at the Center of Excellence for Marine Biotechnology, Chulalongkorn University, Bangkok, Thailand, under controlled laboratory conditions at 28 °C in an aerated 5-L vessel containing sterile seawater at a salinity of 25 ppt (SSW), was used in this study (Fig. 1). Copepods were fed three times a week *ad libitum* with the green microalgae *Tetraselmis suecica*. To prepare samples for gene

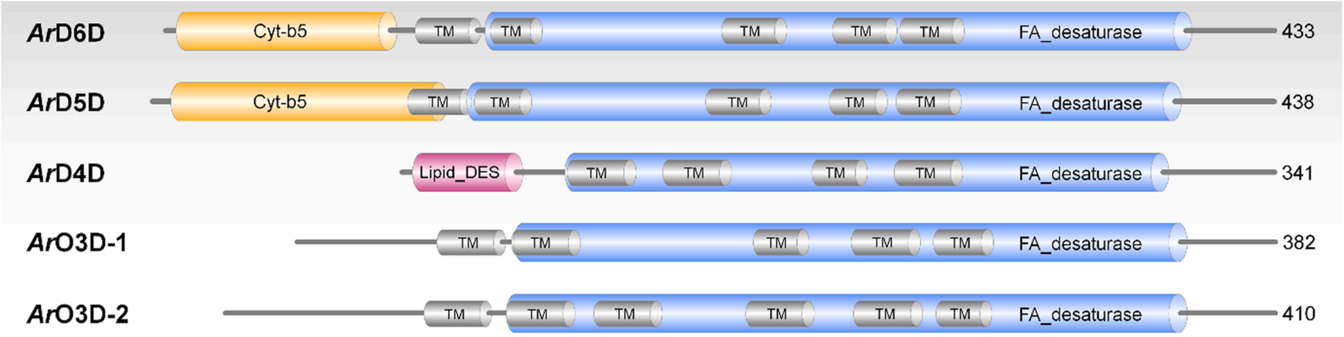

Fig. 2. Domain organization of fatty acid desaturases from *A. royi*-TH. The total number of amino acid residues is shown on the right. The cytochrome b5-like Heme/Steroid binding domain is indicated by yellow color. The transmembrane domain is indicated by gray color. The FA_desaturase domain is indicated by blue color. The sphingolipid desaturase domain is indicated by pink color. Cyt-b5, cytochrome b5-like Heme/Steroid binding domain; TM, transmembrane domain; FA_desaturase, FA_desaturase domain; Lipid_DES, sphingolipid desaturase domain.

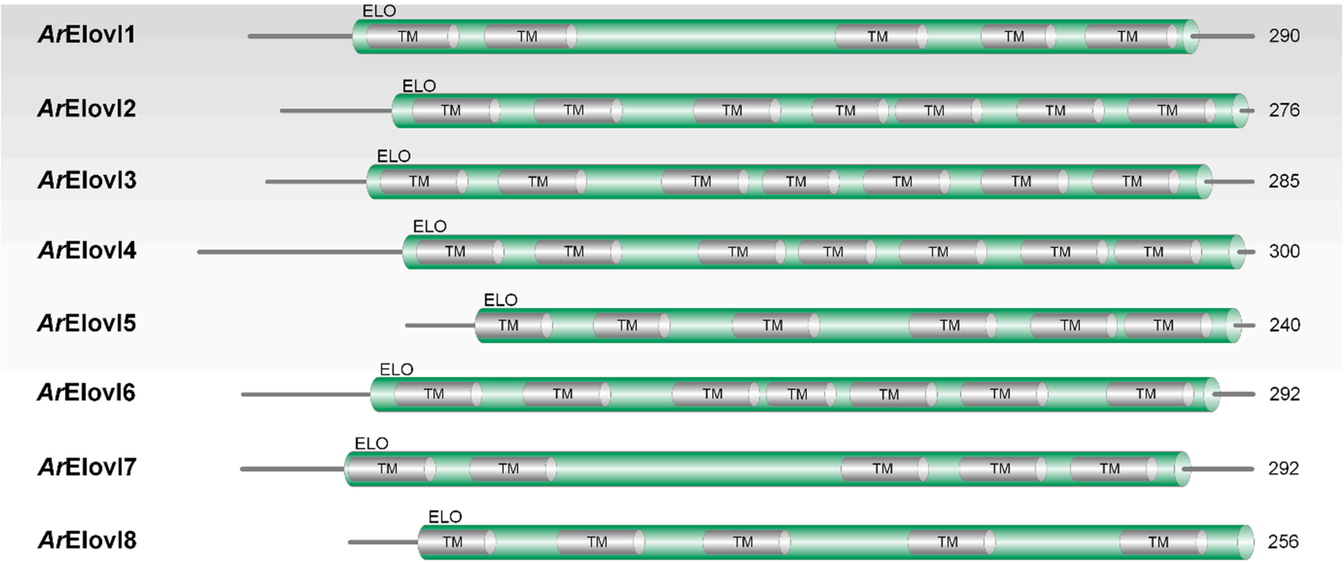

Fig. 3. Domain organization of elongation of very long fatty acids protein (Elovl) from *A. royi*-TH. The total number of amino acid residues is shown on the right. The transmembrane domain is indicated by gray color. The ELO domain is indicated by green color. TM, transmembrane domain; ELO, ELO domain.

expression analysis, three independently replicated *A. royi*-TH cultures were transferred to SSW for an additional 3 h to allow gut evacuation. Approximately 500 individuals from each of the three major development stages (NP, CD, and AD) were collected separately.

2.3. RNA isolation and cDNA synthesis of *A. royi-TH* developmental stages

Immediately after sampling, the *A. royi*-TH were homogenized in TRIzol™ Reagent (Invitrogen) with a glass tissue grinder and then the total RNA was extracted following the manufacturer's instructions. To eliminate residual genomic DNA contamination, each sample was treated with RNase-free DNase I (Promega). The RNA concentration and purity were measured with a NanoDrop (Thermo Scientific), and then 500 ng of the total RNA was used as the template to synthesize the first-strand cDNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's protocol. The cDNA samples of each developmental stage were stored at −20 °C. The amplification ability of the cDNA was evaluated by PCR amplification of the elongation factor 1-α gene (*Ar*EF1α).

2.4. Gene expression analysis

To investigate the expression patterns of the 14 desaturase and elongase genes identified from the present study (Fig. S1 and Table 1), we measured the transcript expression levels in each developmental stage of the copepod by semi-quantitative RT-PCR (sqRT-PCR). Gene-specific PCR primers (Table 1) for each gene were designed using the Primer3Plus program and the PCR reactions were performed on three replicates as previously described (Amparyup et al., 2009). Amplification products were analyzed by 1.8% (w/v) agarose gel electrophoresis after staining with ethidium bromide. The EF1α gene, a housekeeping gene, was used as a reference control in all RT-PCR experiments. All experiments were performed in triplicate (about 500 copepods in each replicate).

2.5. Fatty acid composition analysis

To analyze the fatty acid profiles of each developmental stage of the copepod using gas chromatography-mass spectrometry (GC/MS) analysis, the lipids were extracted as previously reported (Lepage and Roy, 1986), but with some modifications. Firstly, each copepod sample was ground with 2:1 (v/v) dichloromethane: methanol and incubated at room temperature for 1 h. The mixture was then filtered with filter

Table 3
The fatty acid compositions of the microalgae *Tetraselmis suecica* and the three developmental stages of the cyclopoid copepod *Apocyclops royi*-TH fed with *T. suecica*.

Fatty acid	Microalgae *T. suecica*	Copepod *A. royi*-fed *T. suecica*		
		Nauplius (NP)	Copepodid (CD)	Adult (AD)
SFA				
C8:0	–	–	0.01	0.02
C10:0	–	–	0.01	0.02
C12:0	0.03	–	0.13	0.20
C13:0	0.10	–	–	–
C14:0	0.36	4.49	0.62	0.68
C15:0	–	–	0.44	0.44
C16:0	22.06	30.82	18.54	19.43
C17:0	–	3.09	–	–
C18:0	11.12	9.05	13.48	11.69
C20:0	–	–	0.13	0.19
C23:0	–	–	0.17	0.16
C24:0	–	–	1.14	1.64
ΣSFA	33.67	47.45	43.70	34.47
MUFA				
C14:1	0.39	–	–	–
C16:1	0.62	10.82	1.18	1.78
C22:2	–	–	–	–
C22:5	–	–	0.58	0.70
C18:1n-9 cis	5.62	4.17	7.09	7.04
C20:1n-9	–	–	0.55	0.52
ΣMUFA	6.63	14.99	9.40	10.04
n-3 PUFA				
C18:3n-3	21.35	–	16.76	15.97
C20:3n-3	–	1.61	0.94	1.08
C20:5n-3 (EPA)	2.21	2.63	2.56	2.49
C22:6n-3 (DHA)	–	2.78	4.57	5.47
n-6 PUFA				
C18:2n-6 cis	11.86	6.41	12.27	12.42
C18:3n-6	0.36	–	0.57	0.54
C20:2n-6	–	–	0.39	0.48
C20:3n-6	–	–	0.21	0.26
C20:4n-6 (ARA)	0.76	–	1.40	1.44
ΣSC-PUFA	33.57	6.41	36.01	28.93
ΣLC-PUFA	2.97	7.02	10.07	11.22
Σn-3 PUFA	23.56	7.02	24.83	25.01
Σn-6 PUFA	12.98	6.41	14.84	15.14
ΣPUFA	36.54	13.43	39.67	40.15

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; SC-PUFA: short-chain polyunsaturated fatty acid; LC-PUFA: long-chain polyunsaturated fatty acid (≥C20); n-3 PUFA: omega-3 polyunsaturated fatty acid; n-6 PUFA: omega-6 polyunsaturated fatty acid; PUFA: polyunsaturated fatty acid.

paper (No. 1, Whatman, Clifton, NJ), and 0.1 M KCl was added to the filtrate. After centrifugation, the lower (organic) phase was harvested for subsequent methylation. Fatty acid methyl esters (FAME) were prepared by transesterification of the lipid extracts following the method of Lepage and Roy (1986). FAMEs were collected and fatty acids were profiled commercially by GC/MS analysis service (Halal Science Center, Chulalongkorn University, Thailand).

3. Results

3.1. Characterization of desaturase and elongation of very long fatty acids protein genes from the A. royi-TH transcriptome

To uncover the PUFA biosynthesis pathway of the copepod *A. royi*, the nucleotide sequences encoding the putative desaturases and elongation of very long fatty acids protein (Elovl) were retrieved from the *A. royi*-TH transcriptome (Amparyup et al., unpublished data). In this study, the full-length ORF sequences of five desaturase genes (*Ar*D6D, *Ar*D5D, *Ar*D4D, *Ar*O3D-1, and *Ar*O3D-2) and eight elongase genes (*Ar*Elovl1, *Ar*Elovl2, *Ar*Elovl3, *Ar*Elovl4, *Ar*Elovl5, *Ar*Elovl6, *Ar*Elovl7, and *Ar*Elovl8) of the copepod *A. royi* were characterized (Table 2).

Among the five desaturases, the BLASTx searches revealed that two of them (*Ar*D6D and *Ar*D5D) were previously known genes (Nielsen et al., 2019), while the remaining three genes, *Ar*D4D, *Ar*O3D-1, and *Ar*O3D-2, were novel putative desaturase encoded enzymes (Table 2). Surprisingly, *Ar*D6D displayed a high amino acid sequence similarity (76%) to *Tigriopus californicus* front-end desaturase-2 (QWC69495) that exhibited *in vitro* Δ4 desaturase activity (Kabeya et al., 2021), while *Ar*D5D showed 76% similarity to *Paracyclopina nana* delta5 desaturase (APH81338). Moreover, the primary sequence of both *Ar*D6D and *Ar*D5D exhibited the typical features of front-end desaturases involved in PUFA synthesis. The *Ar*D6D and *Ar*D5D proteins have a characteristic fused cytochrome b5-like Heme/Steroid binding domain at their N-terminus, five TMDs, and a FA_desaturase domain (Fig. 2). Analysis of the obtained novel desaturase sequences showed that *Ar*D4D displays the highest sequence similarity (86%) to *Tigriopus japonicus* delta4-desaturase (AIW65589). Interestingly, the *Ar*D4D is composed of an N-terminal sphingolipid desaturase domain, four TMDs, and a C-terminal FA_desaturase domain (Fig. 2). For *Ar*O3D-1, the sequence analysis demonstrated that it exhibited 72% similarity to *T. californicus* methyl-end desaturase-1 (QWC69499), while *Ar*O3D-2 exhibited 73% similarity to *T. californicus* methyl-end desaturase-2 (QWC69500). The characteristics of both *Ar*O3D-1 and *Ar*O3D-2 contain a conserved domain of the methyl-end desaturase that is comprised of the respective five and six TMDs and a FA_desaturase domain (Fig. 2).

With respect to the elongase genes, all sequences were BLASTx searched against the NCBI database. The results showed that two genes (*Ar*Elovl4 and *Ar*Elovl5) have been reported previously (Nielsen et al., 2019), while the remaining six genes, including *Ar*Elovl1, *Ar*Elovl2, *Ar*Elovl3, *Ar*Elovl6, *Ar*Elovl7, and *Ar*Elovl8, are novel genes (Table 2). The BLASTx searches also revealed that *Ar*Elovl6 showed the highest sequence similarity (78%) with the elongases-2 of *T. japonicus* (AIW65585). The cDNA sequences encoding for *Ar*Elovl1 and *Ar*Elovl7 were found to display 86% and 82% sequence similarity with *P. nana* elongases (APH81340), respectively, while *Ar*Elovl2, *Ar*Elovl3, and *Ar*Elovl8 had the highest sequence similarities with *T. californicus* fatty acid elongase-2 (QWC69490; 79%), *T. californicus* fatty acid elongase-1 (QWC69489; 73%), and *T. californicus* fatty acid elongase-5 (QWC69493; 79%), respectively.

Further in silico analysis was conducted to predict whether these *A. royi* elongases were transmembrane proteins. The prediction showed that the *Ar*Elovl2, *Ar*Elovl3, *Ar*Elovl4, and *Ar*Elovl6 encoded putative proteins with seven TMDs and an ELO domain, whereas *Ar*Elovl1, *Ar*Elovl7, and *Ar*Elovl8 have five TMDs and an ELO domain. Additionally, *Ar*Elovl5 contains six TMDs and an ELO domain (Fig. 3).

3.2. Fatty acid analysis in the different developmental stages of A. royi-TH

It is well known that fatty acids have many important functions in the normal development and energy storage in copepods (Pond, 2012). Thus, the fatty acid compositions in three developmental stages (NP, CD, and AD) of *A. royi*-TH fed with the microalga *T. suecica* were examined, and the results are summarized in Table 3. In general, the fatty acids of copepods were categorized into saturated fatty acids (SFAs), comprised of 47.45%, 43.70%, and 34.47% of the total fatty acids in the NP, CD, and AD stages, respectively; monounsaturated fatty acids (MUFAs), comprised of 14.99%, 9.40%, and 10.04%, respectively; and PUFAs, comprised of 13.43%, 39.67%, and 40.15%, respectively. For MUFA, the NP stage also contained a higher level of palmitoleic acid (C16:1), at 10.82%, compared to others.

Of the overall detected PUFAs, the CD (39.67%) and AD (40.15%) stages were high in PUFAs, with omega-3 fatty acids (24.83% and 25.01%, respectively), while the NP stage (13.43%) had the lowest levels of PUFAs. Moreover, most *A. royi*-TH fed with *T. suecica* contained

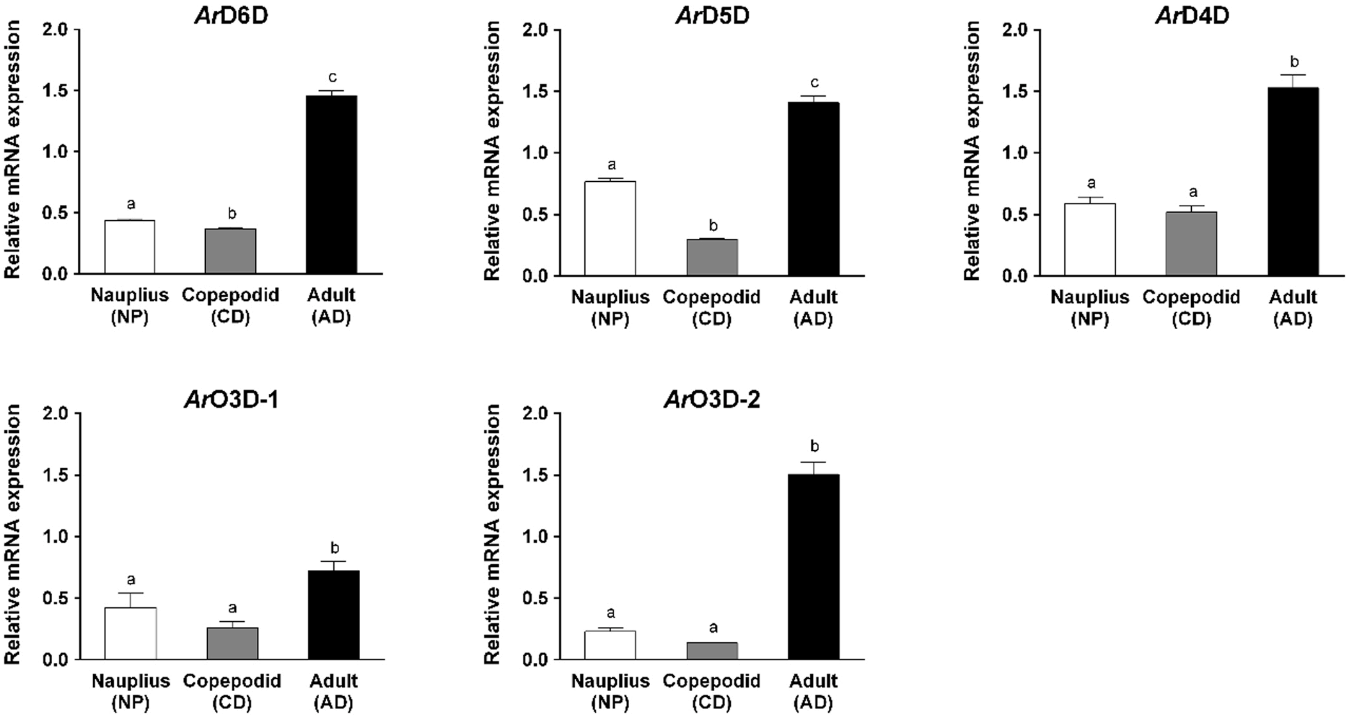

Fig. 4. Developmental expression profile of the indicated fatty acid desaturase mRNA in *A. royi*-TH. The expression profile from the nauplius (NP), copepodid (CD), and adult (AD) developmental stages were analyzed by semi-quantitative RT-PCR analysis. The elongation factor 1-α gene (*Ar*EF1α) served as an internal reference gene. Data represents the mean ± standard deviation (error bars) of triplicate samples. Means with different lowercase letter (above each bar) are significantly different ($P < 0.05$; one-way ANOVA with Duncan's multiple range tests).

a higher level of LC-PUFAs compared to the fatty acid content of *T. suecica*. The LC-PUFAs of each copepod stage (NP, CD, and AD stages) were 7.02%, 10.07%, and 11.22%; with a fairly high DHA (C22:6n-3) content at 2.78%, 4.57%, and 5.47%, and EPA (C20:5n-3) at 2.63%, 2.56%, and 2.49%, respectively.

3.3. Gene expression profiles in different developmental stages of A. royi-TH

Little is known about the relationship between the gene expression levels of fatty acid desaturase and elongase genes and the fatty acid composition profiles during the developmental stages in copepods. In the present study, in order to identify potential genes involved in the PUFA biosynthesis during copepod development, we analyzed their relative expression patterns in NP, CD, and AD developmental stages using sqRT-PCR (Figs. 4 and 5). The results indicated that five desaturase genes (*Ar*D6D, *Ar*D5D, *Ar*D4D, *Ar*O3D-1, and *Ar*O3D-2) and four elongase genes (*Ar*Elovl4, *Ar*Elovl5, *Ar*Elovl6, and *Ar*Elovl7) were significantly up-regulated in AD compared to NP and CD stages. For *Ar*Elovl3, the transcript level was significantly up-regulated in both the AD and CD stages compared to that for the NP. However, the transcript levels of *Ar*Elovl1, *Ar*Elovl2, and *Ar*Elovl8 genes were not significantly different between the three different developmental stages of *A. royi*-TH. Interestingly, the previously reported expression of *Ar*D5D-TW (Nielsen et al., 2019) was not detected in *A. royi*-fed *T. suecica* in this study, even when using RT-PCR.

4. Discussion

Based on the nutritional value, copepods are potentially the most important live feed for marine hatcheries due to their rich content of PUFAs that have important impacts on animal health (Støttrup, 2000). To date, several studies have shown that copepods have metabolic pathways involved in the biosynthesis of fatty acid synthesis that can produce the omega-6 and omega-3 series of LC-PUFAs (Kabeya et al., 2021; Nielsen et al., 2019, 2020, 2021; Pan et al., 2018). Understanding the molecular pathway of PUFA biosynthesis is critical for establishing strategies that allow further physiological manipulation of PUFA levels in copepods. According to Nielsen et al. (2019), *A. royi* is able to synthesize n-3 LC-PUFA using the Elovl4, Elovl5, Fad Δ5, and Fad Δ6 enzymes. However, evidence supporting the molecular mechanisms of the PUFA biosynthesis pathway in the cyclopoid copepod *A. royi* are not completely understood.

In this study, we report the characterization of the genes involved in PUFA biosynthesis by *A. royi*-TH (the *A. royi* species/cultivar used in this study). Based on our analysis, 13 PUFA synthesis-related genes, including five desaturases (*Ar*D6D, *Ar*D5D, *Ar*D4D, *Ar*O3D-1, and *Ar*O3D-2) and eight elongases (*Ar*Elovl1, *Ar*Elovl2, *Ar*Elovl3, *Ar*Elovl4, *Ar*Elovl5, *Ar*Elovl6, *Ar*Elovl7, and *Ar*Elovl8), were identified. This suggests that there are multienzyme complexes of PUFA biosynthesis in *A. royi*. These data are in agreement with those observed previously (Kabeya et al., 2021), where a complete set of multienzyme complexes composed of seven desaturases (Fed1, Fed2, Fed3, Fed4, Fed5, ωx1, and ωx2) and six elongases (Elo1, Elo2, Elo3, Elo4, Elo5, and Elo6) for LC-PUFA biosynthesis were reported in the marine harpacticoida copepod *T. californicus*.

In this study, 13 full-length ORF of three front-end desaturases (*Ar*D6D, *Ar*D5D, and *Ar*D4D), two methyl-end desaturases (*Ar*O3D-1 and *Ar*O3D-2), and eight elongases (*Ar*Elovl1, *Ar*Elovl2, *Ar*Elovl3, *Ar*Elovl4, *Ar*Elovl5, *Ar*Elovl6, *Ar*Elovl7, and *Ar*Elovl8) were successfully identified in *A. royi*-TH. The molecular characteristics of these genes are shown in Table 2; the results suggested that these enzymes belong to the fatty acid desaturases (*Ar*D6D, *Ar*D5D, *Ar*D4D, *Ar*O3D-1, and *Ar*O3D-2) and elongases (*Ar*Elovl1, 2, 3, 4, 5, 6, 7, and 8) families. Interestingly, different expression patterns of five desaturases (*Ar*D6D, *Ar*D5D, *Ar*D4D, *Ar*O3D-1, and *Ar*O3D-2) and five elongases (*Ar*Elovl3, *Ar*Elovl4, *Ar*Elovl5, *Ar*Elovl6 and *Ar*Elovl7) were observed in different developmental stages of *A. royi*-TH. These transcript expression levels were down-

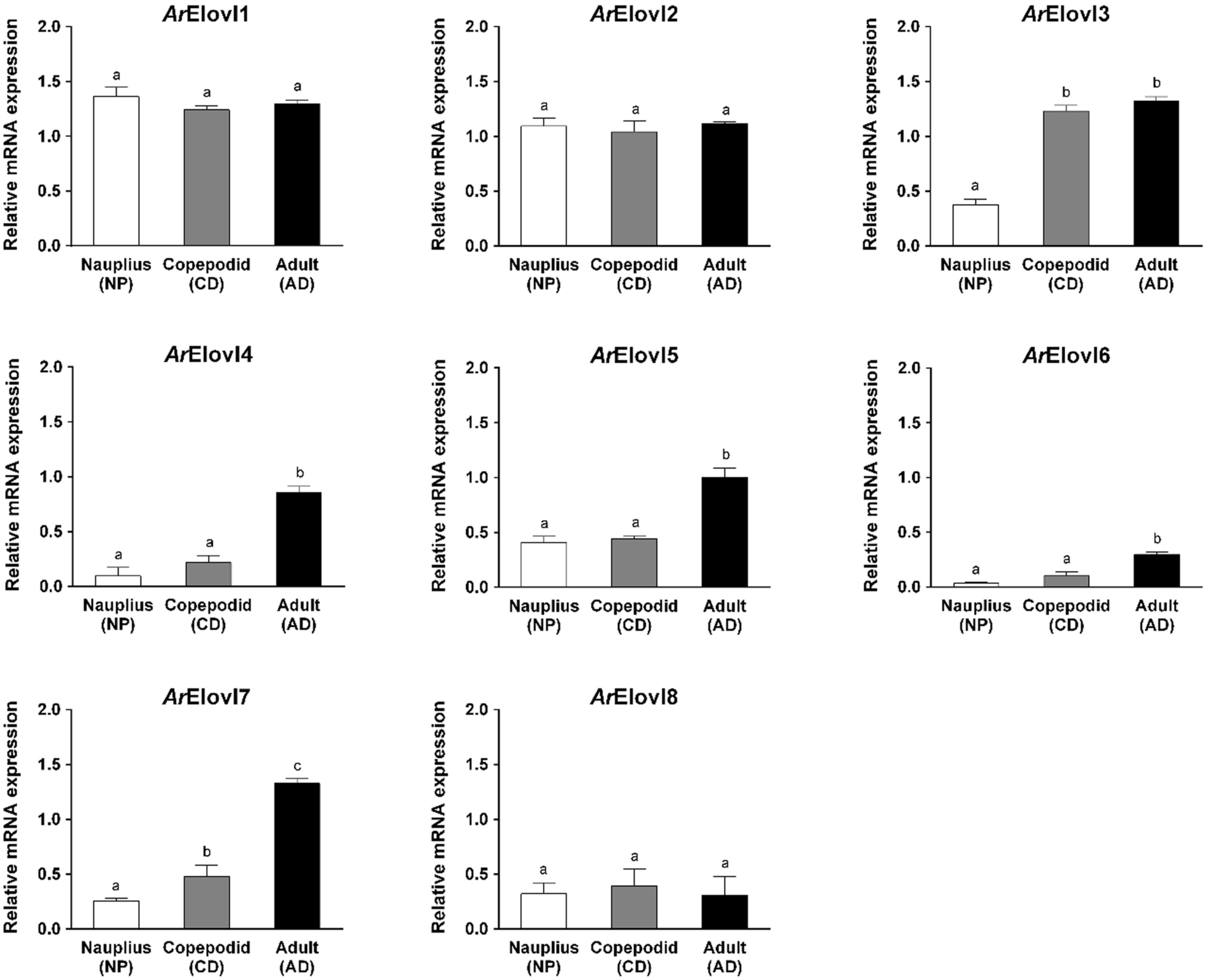

Fig. 5. Developmental expression profile of the indicated elongation of very long fatty acids protein (Elovl) mRNA in *A. royi*-TH. The expression profile from the nauplius (NP), copepodid (CD), and adult (AD) developmental stages were analyzed by semi-quantitative RT-PCR analysis. The elongation factor 1-α gene (*Ar*EF1α) served as an internal reference gene. Data represents the mean ± standard deviation (error bars) of triplicate samples. Means with different lowercase letter (above each bar) are significantly different ($P < 0.05$; one-way ANOVA with Duncan's multiple range tests).

regulated in the NP and CD developmental stages, but were up-regulated in the AD stage, except for *Ar*Elovl3 which was up-regulated in both the AD and CD stages, suggested that these genes are the principal candidate desaturase and elongase genes for PUFA synthesis and their expression is likely to be crucial to guarantee the very high level of DHA in AD stage of *A. royi*-TH fed with *T. suecica* (lacks a DHA content).

In contrast, three elongases (*Ar*Elovl1, *Ar*Elovl2, and *Ar*Elovl8) showed no significant differential expression among all three developmental stages of *A. royi*-TH. However, the highly conserved nature and expression of these elongases in all three developmental stages suggests their role in the lipid biosynthetic pathway. In this research, we propose a model for PUFA biosynthesis in *A. royi* (Fig. 6), based on the analysis of their amino acid sequences for homology and gene expression profiles.

In a previous report, based on gene expression in response to diets containing a high DHA content (*Isochrysis galbana*) and a lack of DHA and EPA content (*Dunaliella tertiolecta*) (Nielsen et al., 2019), it seemed that two different desaturases (Δ6 desaturase, a homolog of the *Ar*D6D, and Δ6 desaturase isoform 1, a homolog of the *Ar*D5D) were the main genes. Thus, the typical front-end desaturase-like domain cytochrome b5 contributes to the n-3 PUFA biosynthesis.

For Fad Δ5 of *A. royi*, transcriptome annotation from *A. royi*-TH (this study) using the BLASTx program revealed that the *A. royi*-TH homolog of *A. royi*-TW Fad Δ5 was not present. In order to identify Fad Δ5 genes in *A. royi*-TH, specific primers for this gene were designed. Interestingly, Fad Δ5 transcripts were not detected in all developmental stages of *A. royi*-TH fed with *T. suecica* (data not shown), suggesting that this gene is not directly involved in the PUFA synthesis by *A. royi*-TH, which is not congruent with that previously reported in *A. royi*-TW (the *A. royi* species/cultivar reported previously (Nielsen et al., 2019)).

The fatty acid profiles in three developmental stages of *A. royi* obtained in this study is novel and also likely to be highly important for copepod production. The fatty acid composition in *A. royi*-TH fed with *T. suecica* had a higher level of SFAs than MUFAs and PUFAs in all three developmental stages, which is in accord with previous studies on the copepod *Apocyclops dengizicus* fed with *Tetraselmis tetrathele*, which contained a higher level of SFAs (41.2%) than MUFAs (13.02%) and PUFAs (37.56%) (Farhadian et al., 2009).

Palmitic acid (C16:0) was the predominant fatty acid in all three developmental stages of *A. royi*-TH fed *T. suecica* (NP, 30.82%; CD, 18.54%; and AD, 19.43%), similar to that reported previously for *A. royi* fed with *D. tertiolecta* and *T. suecica* at 22.7% and 21.23% palmitic acid, respectively (Nielsen et al., 2019). The second most abundant fatty acid

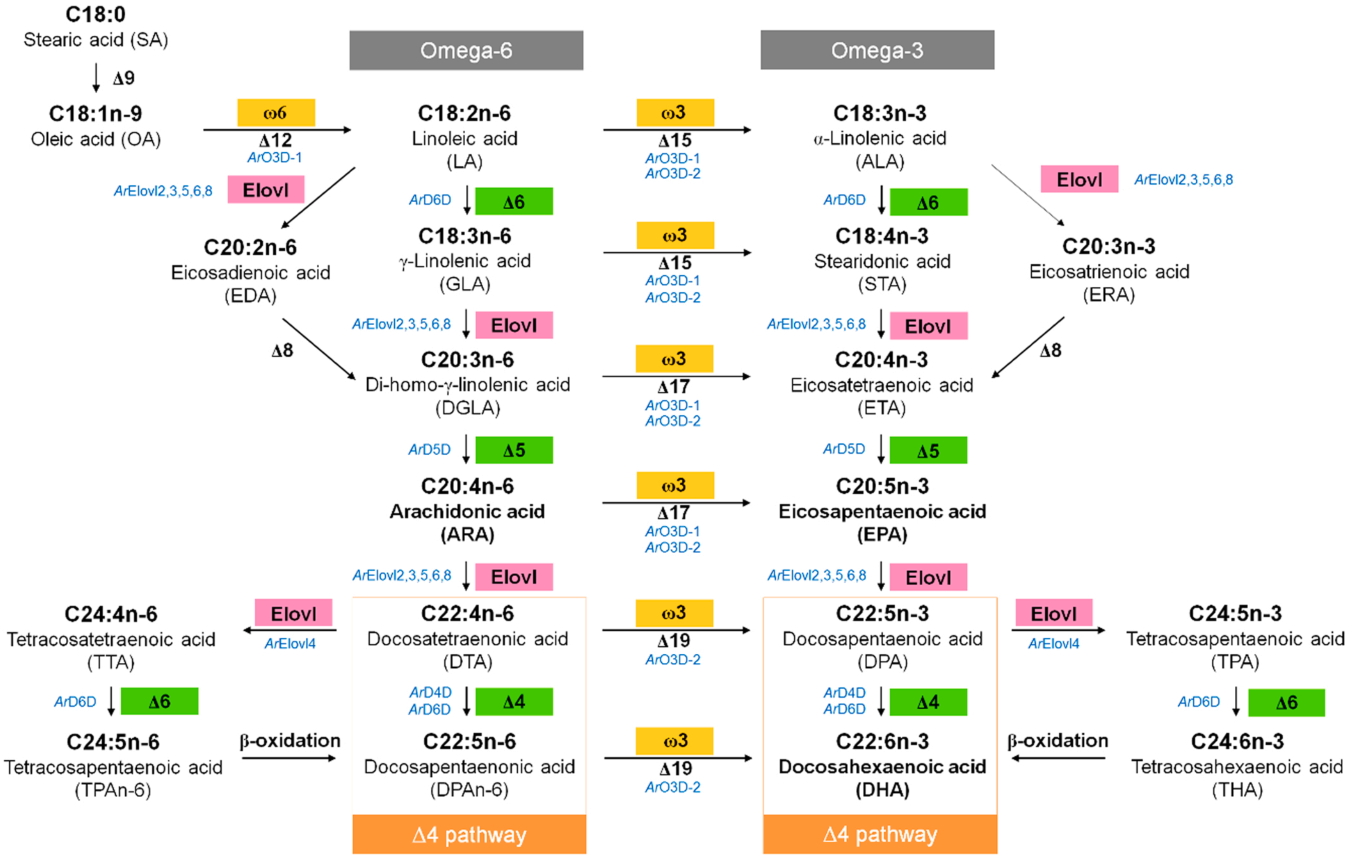

Fig. 6. Proposed biosynthesis pathway of long-chain polyunsaturated fatty acids (LC-PUFAs) in the copepod *A. royi*. Desaturation reactions catalyzed by front-end desaturases (*Ar*D6D, *Ar*D5D, and *Ar*D4D) and methyl-end desaturases (*Ar*O3D-1 and *Ar*O3D-2) are indicated by green and yellow colors, respectively. Elongation reactions mediated by elongation of very long-chain fatty acid proteins (*Ar*Elovl2, *Ar*Elovl3, *Ar*Elovl5, *Ar*Elovl6 and *Ar*Elovl8) are indicated by pink color.

was linolenic acid (18:3n-3), which is an essential fatty acid belonging to the omega-3 fatty acids, and was highest in the CD (16.76%) and AD (15.97%) stages, but was not present in the NP stage. Compared to the PUFA values previously reported for *A. royi*-TH fed with *T. suecica* (53.37%) (Nielsen et al., 2019), the PUFA content was lower in this study (40.15%). However, a higher SC-PUFA content was found in two stages of *A. royi*-TH in the current study (CD, 36.01%; AD, 28.93%) compared to the previous study (14.79%). Although copepods can synthesize PUFAs, such as EPA and DHA, using the desaturase and elongase activities in the PUFA biosynthetic pathway, the dietary composition (fatty acid profile of algae) and culture conditions (salinity, temperature or cell density) can significantly impact on the fatty acid composition of the total lipids in copeopods (Lee et al., 2017a; Rasdi et al., 2016).

The transcript expression level of *Ar*D4D desaturase was highlighted since it exhibited an expression pattern similar to *Ar*D6D, showing that these two genes were up-regulated in the AD stage of *A. royi*-TH fed with *T. suecica* (lacks a DHA content). This increased expression corresponded to a significant rise in the LC-PUFA composition. According to gene expression in response to nutritionally rich diets, sphingolipid Δ4 desaturases (Δ4 FAD) and Δ6 FAD in the prawn *Macrobrachium nipponense* were up-regulated by linolenic acid (ALA, 18:3n-3)-rich diets (Luo et al., 2018). Similarly, the expression of Δ6 desaturase-like genes in several crustacean species, including crab *Eriocheir sinensis* (Yang et al., 2013), *Scylla paramamosain* (Lin et al., 2017), crab *Portunus trituberculatus* (Wang et al., 2014), lobster *Sagmariasus verreauxi* (Shu-Chien et al., 2017), and shrimp *Litopenaeus vannamei* (Chen et al., 2017), were also found to be nutritionally responsive genes, suggested that the role of these genes in LC-PUFA biosynthesis.

In summary, in the present study we successful identified and characterized a number of possible conserved desaturase and elongase genes that are likely to be involved in PUFA biosynthesis in the copepod *A. royi*. Differentially expressed genes were found between the NP/CD stages and the AD stages of *A. royi* (*Ar*D6D, *Ar*D5D, *Ar*D4D, *Ar*O3D-1, *Ar*O3D-2, *Ar*Elovl3, 4, 5, 6, and 7), although three elongases (*Ar*Elovl1, *Ar*Elovl2, and *Ar*Elovl8) were not differentially expressed. At the same time, the high level of desaturase and elongase expression in the AD stage of copepod development, in accordance with the process of LC-PUFA accumulation, indicates that PUFA from *T. suecica* (lack DHA) is actively converted into LC-PUFA (high DHA), probably due to the high desaturase and elongase activity. However, further in-depth analysis on the *in vivo/in vitro* functional role of these enzymes in the PUFA biosynthesis pathway in *A. royi* is required to better understand the relationship between the metabolic enzyme level and fatty acid composition in copepod development, which is important in the design of dietary microalgae to improve culture methods for copepods as live feed for aquaculture hatcheries.

CRediT authorship contribution statement

Piti Amparyup: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Supakarn Sungkaew**: Conceptualization, Formal analysis, Investigation. **Walaiporn Charoensapsri**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Writing – original draft, Writing – review & editing. **Paveena Tapaneeyaworawong**: Investigation. **Parichat Chumtong**: Investigation. **Patchari Yocawibun**: Investigation. **Prarthana Pantong**: Investigation. **Ratree Wongpanya**: Formal analysis.

Chanprapa Imjongjirak: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Sorawit Powtongsook**: Resources, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2022.101064.

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