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Biochemical adaptation by the tropical copepods *Apocyclops royi* and *Pseudodiaptomus annandalei* to a PUFA-poor brackish water habitat

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ABSTRACT: The cyclopoid *Apocyclops royi* (Lindberg 1940) and the calanoid *Pseudodiaptomus annandalei* (Sewell 1919) are 2 tropical copepods suspected of having the capability to biosynthesize the physiologically important n-3 polyunsaturated fatty acids (n-3 PUFAs) eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA). We demonstrated this suspected ability using $^{13}C_{18}$ α-linolenic acid (C18:3n-3, ALA) fed to the copepods through liposomes and a subsequent fatty acid (FA) analysis by GC-MS at 3 different time points (0, 24, and 48 h). Two different diets were applied post liposome exposure: baker's yeast *Saccharomyces cerevisiae* and the microalga *Dunaliella tertiolecta.* For both copepods, further elongated and desaturated ^{13}C n-3 PUFAs were found at all time points. At T_{48h}, *A. royi* and *P. annandalei* contained ^{13}C-labelled DHA contents (±SD) of 1.3 ± 0.2 and 0.7 ± 0.3 µg ^{13}C-FA $mgC_{copepod}^{-1}$ when fed baker's yeast, respectively, and 1.2 ± 0.1 and 1.6 ± 0.5 µg ^{13}C-FA $mgC_{copepod}^{-1}$ when fed *D. tertiolecta*, respectively, with significant differences observed only between *P. annandalei* diet treatments. The ^{13}C-labelled EPA content of *A. royi* and *P. annandalei* at T_{48h} was 0.6 ± 0.4 and 0.7 ± 0.4 µg ^{13}C-FA $mgC_{copepod}^{-1}$ when fed baker's yeast and 0.8 ± 0.2 and 0.3 ± 0.1 µg ^{13}C-FA $mgC_{copepod}^{-1}$ when fed *D. tertiolecta*, with significant differences only between copepods fed *D. tertiolecta*. *A. royi* and *P. annandalei* exhibited an ability to produce n-3 PUFAs from the precursor ALA in comparatively large quantities. This ability enables these 2 species to inhabit habitats characterized by PUFA-poor particulate material.

KEY WORDS: Copepod · Docosahexaenoic acid · DHA · Biosynthesis · Isotope labelling

1. INTRODUCTION

In aquatic food webs, certain phytoplankton are the biggest contributors to the production of polyunsaturated fatty acids (PUFAs, ≥2 double bonds) (Taipale et al. 2013, Jónasdóttir 2019), including the physiologically important fatty acids (FAs) eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA). EPA and DHA are crucial for zooplankton as they are important for survival, growth, and reproduction (Kattner et al. 2007). However, EPA- and DHA-synthesizing primary producers may not always be present in sufficient quantities, which may cause a need for consumers to biosynthesize their own n-3 PUFAs.

In recent years, several studies have challenged the general perception that only primary producers can produce long-chain PUFAs (LC-PUFAs, ≥C20 and ≥2 double bonds) in significant amounts (Bell & Tocher 2009, Monroig et al. 2013). Not only has a limited capability of trophic upgrade (further elongation and desaturation of a dietary FA) of n-6 and n-3 PUFAs

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been found in mammals (Burdge & Calder 2005) and fish (Monroig et al. 2013), but strong indicia of such capability have also been found in several invertebrates (Monroig et al. 2013). Furthermore, a recent study of genes encoding for n-3 desaturases has shown that many species of invertebrates are, to a certain extent, capable of biosynthesizing linoleic acid (C18:2n-6, LNA) and α-linolenic acid (C18:3n-3, ALA), the precursors of n-6 and n-3 LC-PUFAs, respectively (Kabeya et al. 2018). Interestingly, this has until recently been thought mostly impossible for animals (Bell & Tocher 2009, Kabeya et al. 2018). These findings suggest limited knowledge at present of n-3 PUFA biosynthesis and how environmental pressures presumably can affect it.

Copepods exhibit high contents of n-3 PUFAs, with their presence being typically considered a result of the FA profile found in their diet (Dalsgaard et al. 2003). Therefore, the common phrase 'you are what you eat' is often applied when discussing copepod FA content. However, several species of copepods are thought to actually possess the capability to perform trophic upgrade on n-3 PUFAs. The calanoids *Calanus finmarchicus* (Bell et al. 2007), *Pseudodiaptomus annandalei* (Rayner et al. 2017), and *Paracalanus parvus* (Moreno et al. 1979); the harpacticoids *Tisbe holothuriae* (Norsker & Støttrup 1994), *Tisbe* sp. (Nanton & Castell 1999, Arndt & Sommer 2014), *Tachidius discipes* (Arndt & Sommer 2014), *Amonardia* sp. (Nanton & Castell 1999), *Microarthridion littorale* (De Troch et al. 2012), and *Platychelipus littoralis* (Werbrouck et al. 2017); and the cyclopoids *Eucyclops serrulatus* (Desvilettes et al. 1997), *Paracyclopina nana* (K. Lee et al. 2006), and *Apocyclops royi* (Pan et al. 2018, Nielsen et al. 2019) are all to various degrees suggested to possess this particular trait. Bell et al. (2007) concluded that the trace amounts of D_5-labelled DHA found in *C. finmarchicus* were ecologically insignificant. *C. finmarchicus* is found mostly in temperate and polar marine ecosystems (Melle et al. 2014). In these high latitudes, the primary producers are often rich in n-3 PUFAs, especially EPA and DHA (Dalsgaard et al. 2003, Colombo et al. 2017). Furthermore, high-latitude copepods develop lipid storage as an overwintering strategy, which is made possible by the PUFA-rich diet they consume during spring and summer and their low metabolic rate during winter dormancy (R. Lee et al. 2006). In contrast, subtropical and tropical copepods do not develop such large lipid stores, as food is somewhat constant throughout the year, and the high temperatures cause high rates of metabolism. While food particles are available throughout the year in subtropical and tropical systems, they are less likely to possess the same high-quality n-3 PUFAs as in the higher latitudes (Dalsgaard et al. 2003, Colombo et al. 2017). It is therefore likely that regionality and differing survival strategies could influence the n-3 PUFA biosynthetic capabilities of copepods.

The tropical copepods *A. royi* and *P. annandalei* are suspected of producing n-3 LC-PUFAs at ecologically significant amounts (Rayner et al. 2017, Pan et al. 2018, Nielsen et al. 2019). These 2 species of copepods are the dominant mesozooplankton in southern Taiwanese artificial aquaculture ponds (Su et al. 2005, Blanda et al. 2015), which are characterized by environmentally harsh conditions such as extreme hypoxia, low PUFA content, and overall poor water quality (Blanda et al. 2015). It has been hypothesized that the dominance of these 2 copepod species in the ponds is partly due to effective trophic upgrade capabilities of n-3 PUFAs (Nielsen et al. 2019). This was supported by the demonstration that *A. royi* showed no significant difference in absolute content of DHA when fed *Dunaliella tertiolecta* (63.4 ± 11.2 ng ind.$^{-1}$), a microalga containing no n-3 PUFAs except ALA and stearidonic acid (C18:4n-3, SDA), compared to when fed the n-3 LC-PUFA-rich *Rhodomonas salina* (97.8 ± 26.2 ng ind.$^{-1}$) (Nielsen et al. 2019). Rayner et al. (2017) reported a DHA content of 5.4% of total FAs in *P. annandalei* when fed for several months on *Tetraselmis chuii,* a microalga lacking DHA, and Blanda et al. (2017) found a DHA content in *P. annandalei* ranging from 12.3 ± 0.7 to 46.6 ± 5.3% of total FAs while the DHA content of the seston ranged from 2.0 ± 1.1 to 7.5 ± 2.3% of total FAs throughout the year. Because of the high DHA content in *A. royi* and *P. annandalei* despite the low contents or complete absence of DHA in their diets, and because of the dominance of the 2 copepods in the aquaculture ponds (Su et al. 2005, Blanda et al. 2015), we believe that these 2 species of copepods are able to produce adequate amounts of DHA possibly to cover their own physiological functions, a trait only demonstrated in few other marine animals.

While indications of n-3 LC-PUFA biosynthesis have been found in both copepods through FA profile comparisons, and transcriptomic annotation in the case of *A. royi* (Nielsen et al. 2019), the final evidence is still lacking. This is because neither a functional analysis of enzymes nor an isotope-tracing experiment has yet been performed. An isotope-tracing experiment with an isotope-labelled ALA going through the n-3 PUFA biosynthetic pathway could potentially illustrate the direct amounts of DHA pro-

duced by the copepods themselves. So far, isotope-tracing experiments have only been conducted on the calanoid copepods *P. parvus* (Moreno et al. 1979), *C. finmarchicus*, and *Calanus acutus* (Bell et al. 2007), but not on any cyclopoid copepods. The results of these studies demonstrated production of DHA by *P. parvus* and *C. finmarchicus*, albeit in negligible amounts. However, this fits well with the general assumption that calanoids, compared to harpacticoids and cyclopoids, are not very efficient in their trophic upgrade of n-3 PUFAs (Norsker & Støttrup 1994).

We hypothesized that both *A. royi* and *P. annandalei* are capable of biosynthesizing DHA from ALA. Hence, in the present study, we attempted to demonstrate this capability through an isotope-labelling experiment using $^{13}C_{18}$-ALA fed as liposomes to *A. royi* and *P. annandalei*, a setup inspired by Bell et al. (2007). Additionally, we aimed to quantify this biosynthesis, compare it to the capabilities of the few other reported copepods, and explain this crucial trait as a biochemical adaptation to a PUFA-poor habitat.

2. MATERIALS AND METHODS

2.1. Stock cultures

The marine microalga *Dunaliella tertiolecta* (K-0591) was kept at Roskilde University, Denmark, as a pure strain. It was cultivated in 0.2 µm UV-filtrated salinity 35 seawater at 17°C, with aeration, and continuous 50–65 µmol photons m^{-2} s^{-1}. The f/2 growth medium (Guillard 1975, without cobalt sensu Thoisen et al. 2018) was administered daily. We chose *D. tertiolecta* because of its lack of n-3 LC-PUFAs, possibly inducing a higher production of LC-PUFAs in the copepods (Nielsen et al. 2019).

Apocyclops royi and *Pseudodiaptomus annandalei* were obtained from Tungkang Biotechnology Research Center, Taiwan, and Cam Ranh Centre for Tropical Marine Research and Aquaculture, Nha Trang University, Vietnam, respectively. Upon arrival at Roskilde University, the copepods were cultivated in 50 l tanks in a walk-in climate-controlled room under dark conditions. The cultures were kept in an aerated mixture of 0.2 µm UV-filtrated seawater and demineralized water at 25°C, salinity of 20. The copepods were fed *D. tertiolecta* for >3 generations (>5 wk) every other day ad libitum as evidenced by residual algae found in the cultivation tanks immediately before feeding.

2.2. Liposome preparation and labelling

To document the biosynthesis of LC-PUFAs, liposomes were used as carriers in order to deliver the isotope-labelled FAs into the copepods through ingestion and thereby ensure tissue marking. The liposomes mimicking food particles, e.g. microalgae, were prepared freshly before the experiments according to the method described by Bell et al. (2007) with a few adjustments. VWR International supplied all chemicals except for the marked FAs. Approximately 2.02 µmol of $^{13}C_{18}$-ALA methyl ester (633 µg) (Cambridge Isotope Laboratories) or ALA methyl ester (596 µg), 93 µg C18:0 phosphatidyl choline, and 13 µg Nile red were mixed into 2 ml glass vials. To this mixture, an aliquot of 2.7 µg antioxidant solution containing 6% (w/v) butylated hydroxy anisole, 6% (w/v) propyl gallate, and 4% (w/v) citric acid in propylene glycol were added. The resulting solution was dried under a gentle nitrogen stream, and 40 µl diethyl ether and 400 µl of 0.2 µm filtrated UV-treated seawater (salinity 20) were added. The contents were stirred vigorously, and liposome droplets were formed. The liposome size distribution was measured on a Beckman Coulter Multisizer 4e, resulting in a size range of 2–35 µm equivalent spherical diameter (ESD) with a mean of approximately 7 µm ESD. This particle size range is likely retainable by the copepods (Berggreen et al. 1988). Preliminary experiments were done to test if the copepods would feed on the liposomes.

2.3. Experimental setup

The experimental setup by Bell et al. (2007) yielded a generally low uptake of the added isotope-labelled FA (<1 µg $replicate^{-1}$, i.e. <0.2% of FA added) by *Calanus finmarchicus*. To optimize the intake of liposomes, preliminary observations were made on the colouration of the copepods as an estimation of intake with differing liposome densities and differing starvation periods. Based on these observations, the copepods in the current study were fed a density of >200 000 liposomes ml^{-1}, which is an estimated factor of 7 higher than the density used by Bell et al. (2007). Furthermore, the copepods in the current study were starved for 24 h prior to the liposome exposure to induce grazing activity, which was not done in the study by Bell et al. (2007).

Adult copepods and advanced copepodites were gently separated from the stock cultures using a

250 µm mesh. They were then placed in clean culture water to starve for 24 h. Then, 24 beakers with 50 ml of clean culture water were prepared for each species of copepod. Two liposome treatments were arranged; labelled and non-labelled, with 4 replicates per time point T_{0h}, T_{24h}, and $T_{48h,}$ where the samples were terminated for FA analysis. Individuals of *A. royi* (n = 50) and *P. annandalei* (n = 30) were placed into each replicate. A single dose (~440 µl) of liposome suspension was added to each beaker. The water was aerated and the beakers were covered with parafilm to reduce evaporation. After 20 h of exposure to liposomes, T_{0h} was terminated, and 3.5 mg of suspended baker's yeast *Saccharomyces cerevisiae* were added to the remaining beakers, leaving them with ad libitum feed without naturally occurring ALA (Aloklah et al. 2014) for the remaining 24 and 48 h. After 24 and 48 h, T_{24h} and T_{48h} were terminated. Upon termination, the copepods were filtered through a 100 µm mesh filter and rinsed with clean culture water and filtered onto 25 mm Whatman GF/C filters. The filters were placed in 7.5 ml Pyrex vials and stored at −80°C for later analysis.

The experiment was later duplicated with *D. tertiolecta* as feed for both *A. royi* and *P. annandalei* after liposome exposure instead of baker's yeast, as unexpectedly high mortality was observed in *P. annandalei* on a diet of baker's yeast. Results from both copepods fed both diet treatments are presented.

The contents of ^{13}C-labelled FAs (^{13}C-FAs) in the copepods were estimated, and the relative conversion calculated as the percentages of specific ^{13}C-FAs out of the total amount of ^{13}C-FAs. Furthermore, to compare the capabilities of the 2 different-sized copepods, C-specific content was calculated as µg ^{13}C-FA mg$C_{copepod}^{-1}$. The C content of *A. royi* and *P. annandalei* was calculated with the C-length regression $C = 1.24 \times 10^{-6} \times L^{2.259}$ (T. A. Rayner & B. W. Hansen unpubl.) and $C = 2.19 \times 10^{-9} \times L^{3.136}$ (Rayner et al. 2015), respectively, where C is µg C, and L is prosome length (combined length of the cephalosome and metasome) in µm. *A. royi* yielded an estimated carbon content of 1.6 µg C ind.$^{-1}$, with the prosome length estimated as the average of adults (600 µm) and advanced copepodites (400 µm) (Chang & Lei 1993), and *P. annandalei* yielded an estimated carbon content of 2.6 µg C ind.$^{-1}$, with the prosome length estimated as the average of adults (900 µm) and advanced copepodites (600 µm) (Grønning et al. 2019).

2.4. FA methyl ester analysis

The samples were freeze dried for 24 h in a Christ-Alpha 1-2 equipped with a vacuum pump. We added 3 ml of a 2:1 (v:v) chloroform:methanol solution to the vials in accordance with Folch et al. (1957). An internal standard (C23:0) was added to the vials, which were then stored at −20°C for 24 h. Approximately 1.7 ml of the solutions were transferred to 2 ml GC-vials and dried under a gentle nitrogen stream on a heating block at 60°C. We then added 1000 µl of a 66:85:15 (v:v:v) solution of toluene:methanol:acetyl chloride to these vials. The vials were capped and left on a heating block at 95°C for 2 h for transesterification. After de-capping, 500 µl of 5% $NaHCO_3$ (w/v) prepared with a few minutes of nitrogen bubble-through were added to remove excess acid from the organic phase. The solutions were mixed and left to phase-separate before transferring the organic phases to new GC vials. The contents of the original vials were washed twice with 500 µl of heptane, and the new organic phases were transferred to the new GC vials. The vials were then dried under a gentle nitrogen stream on a heating block at 60°C before 500 µl of chloroform were added. The vials were capped and kept at −20°C until analysis.

The samples, now derivatized into FA methyl esters (FAMEs), were hereafter analysed on an Agilent GC 6890 N with an Agilent J&W DB-23 column (60 m × 250 µm × 0.25 µm) with He as a carrier gas. Initial temperature was 50°C and increased at a rate of 25°C min^{-1} until reaching 200°C, and kept constant for 10 min. Hereafter, the temperature increased at a rate of 5°C min^{-1} until 250°C, and kept constant for 3 min. The GC was connected to an Agilent Mass Selective 5975 detector with positive electron ionization at 70 eV. Standard calibration curves were created using FAMEs in varying concentrations while keeping the internal standard C23:0 constant. The samples were analysed in MSD Chemstation E.02.02.1431 (Agilent Technologies). Samples were run using the following specific ion monitoring (SIM) protocol; m/z = 55, 74, 79, and 81 (Drillet et al. 2006), and non-labelled n-3 PUFA content was measured through standard curves of the m/z = 79 ion abundance. This ion was chosen because it is the base peak for n-3 PUFAs, and is indicative of polyunsaturation, and thus the position of the unsaturated ion is so far down the carbon-chain that the ^{13}C- FAs elongated with non-labelled carbon will not have the same ion signature. In addition, instead of monitoring the M^+ peaks which often have weak signals in n-3 PUFAs, the specific ions m/z = 108 and

116 were also monitored. These were chosen in accordance with the predictions of splitting patterns by Mjøs (2004), who predicted an n-3 methyl end (C_8H_{12}) at m/z = 108, and therefore a fully ^{13}C-labelled n-3 methyl end at m/z = 116. Because the methyl end is unlikely to be altered through the experiment, other isotopologues were not considered. ^{13}C-FA content was determined through the relative peak intensities of the isotopologues (peak intensity of m/z = 116: peak intensity of m/z = 108). The parallel treatment of copepods fed non-labelled liposomes acted as controls, and the mean relative peak intensities of the controls were subtracted from the relative peak intensities of the isotope-labelled samples, resulting in the isotopologue ratio ($^{116:108}R$). For every sample, the mass of every isotope-labelled n-3 PUFA ($m_{13C\text{-}FA}$) was calculated as in the following equation:

$$m_{13C-FA} = {}^{116:108}R \cdot m_{12C-FA} \quad (1)$$

where $m_{12C\text{-}FA}$ is the measured mass of the corresponding non-labelled FA.

2.5. Statistics

All mean values in the text are presented ± SD. Significant differences in ^{13}C-FA content were tested with time, copepod species, and diet as independent variables in a 3-way ANOVA. Normality was tested with Shapiro-Wilk tests, and equal variances were tested with Brown-Forsythe tests. To meet ANOVA assumptions, the data were ln(x + 1) transformed. However, the assumption of normality was still not met for SDA, but as ANOVA is robust to the violations of normality assumptions, the test was carried out regardless of the violation. As post hoc tests, Tukey tests were completed. The significance level for all tests was set at 0.05. All FA content tests were done in GraphPad Prism 8.

3. RESULTS

Nile-red-stained liposomes were observed in the gut of both *Apocyclops royi* and *Pseudodiaptomus annandalei* (Fig. 1). Furthermore, stained faecal pellets were observed for both species, and egg-sacks were clearly stained red. Therefore, the liposomes were considered ingested and metabolized. Mortality was not observed for *A. royi* during the experiments. However, *P. annandalei* had a high mortality rate (~33–50 %) after the addition of baker's yeast, but a much lower mortality (~10 %) when fed *Dunaliella tertiolecta*. Unfortunately, mortality was not measured, but an estimation was applied as 33 % mortality at T_{24h} and 50 % at T_{48h} for *P. annandalei* fed baker's yeast. Furthermore, a conservative estimation was applied to mortality at T_{24h} and T_{48h} of *P. annandalei* fed *D. tertiolecta* (both 15 %).

^{13}C-ALA was metabolized and the n-3 LC-PUFA derivatives were found in all treatments (Table 1). The lowest total amount of ^{13}C-FA was found at T_{48h} in *A. royi* fed *D. tertiolecta* after initial liposome exposure (90 ± 32 ng ^{13}C-FA ind.$^{-1}$), and the highest total amount was found at T_{0h} in *P. annandalei* fed *D. tertiolecta* (1520 ± 367 ng ^{13}C-FA ind.$^{-1}$). Due to the body size differences between *A. royi* and *P. annandalei,* the total amounts of ^{13}C-FAs were normalized between species as total µg ^{13}C-FA $mgC_{copepod}^{-1}$. The carbon-specific ^{13}C-FA contents were similar at T_{0h} in both *A. royi* and *P. annandalei* fed baker's yeast (166.9 ± 103.2 and 161.1 ± 73.0 µg ^{13}C-FA $mgC_{copepod}^{-1}$, respectively), but differed when fed *D. tertiolecta* (263.7 ± 91.7 and 589.0 ± 142.2 µg ^{13}C-FA $mgC_{copepod}^{-1}$,

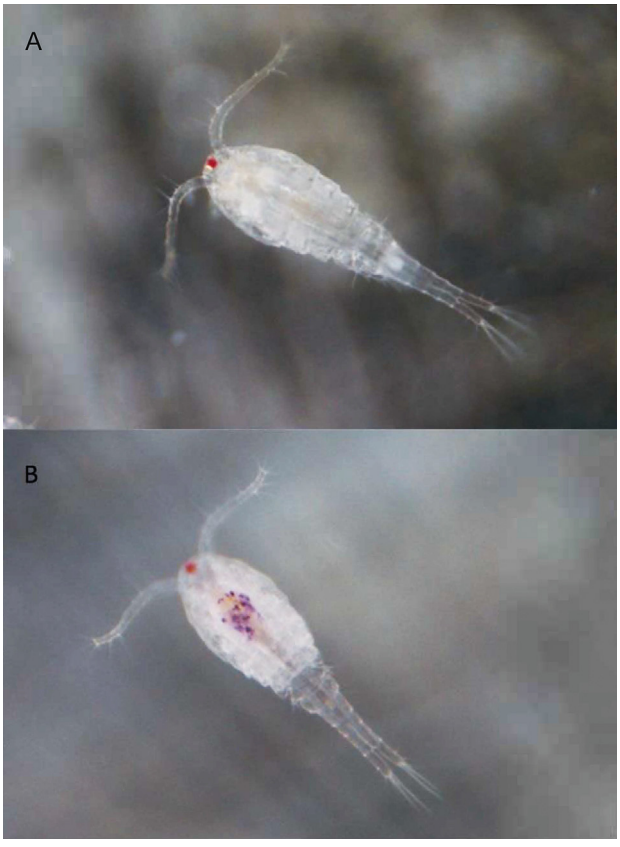

Fig. 1. *Apocyclops royi* (A) starved for 24 h and (B) fed Nile-red-stained liposomes

Table 1. Relative distribution of isotope-labelled fatty acids (FAs) in copepods at 0, 24, and 48 h after initial incubation with liposomes, presented as mean ± SD (n = 4). The masses of the isotope-labelled FAs are not presented with the additional weight of ^{13}C. Total weight (ng) of isotope-labelled FAs is calculated as the sum of α-linolenic acid (C18:3n-3, ALA), stearidonic acid (C18:4n-3, SDA), eicosatrienoic acid (C20:3n-3, ETE), eicosapentaenoic acid (C20:5n-3, EPA), and docosahexaenoic acid (C22:6n-3, DHA). The relative distribution is presented as percent labelled FA of total labelled FAs

Diet	Species	n	Time (h)	Total ng ^{13}C-FA ind.$^{-1}$	Total µg ^{13}C-FA mgC$_{copepod}^{-1}$	^{13}C-labelled FAs (% of total) ALA	SDA	ETE	EPA	DHA
Baker's yeast	*Apocyclops royi*	50	0	274 ± 169	166.9 ± 103.2	99.51 ± 0.26	nd	0.44 ± 0.24	0.01 ± 0.01	0.04 ± 0.03
		50	24	161 ± 23	98.4 ± 14.2	96.77 ± 1.27	0.64 ± 0.14	2.09 ± 0.84	0.25 ± 0.23	0.25 ± 0.2
		50	48	173 ± 57	105.7 ± 34.9	93.41 ± 1.39	1.49 ± 0.46	3.23 ± 1.09	0.58 ± 0.15	1.29 ± 0.45
	Pseudodiaptomus annandalei	30	0	414 ± 188	161.1 ± 73.0	99.13 ± 0.22	0.03 ± 0.01	0.73 ± 0.25	0.04 ± 0.02	0.06 ± 0.03
		~20	24	1033 ± 312	400.4 ± 120.8	97.39 ± 0.85	0.41 ± 0.67	2.11 ± 0.86	0.04 ± 0.05	0.05 ± 0.09
		~15	48	1048 ± 752	406.4 ± 291.3	95.09 ± 2.09	0.13 ± 0.11	4.31 ± 1.93	0.22 ± 0.11	0.25 ± 0.16
Dunaliella tertiolecta	*A. royi*	50	0	432 ± 150	263.7 ± 91.7	98.39 ± 1.09	0.17 ± 0.05	1.23 ± 1.05	0.03 ± 0.04	0.18 ± 0.04
		50	24	255 ± 27	155.3 ± 16.5	94.63 ± 1.49	0.91 ± 0.03	3.70 ± 1.49	0.45 ± 0.12	0.32 ± 0.08
		50	48	90 ± 32	55.1 ± 19.8	87.12 ± 1.91	2.91 ± 0.92	5.98 ± 2.09	1.64 ± 0.48	2.35 ± 0.85
	P. annandalei	30	0	1520 ± 367	589.0 ± 142.2	99.03 ± 0.27	0.05 ± 0.01	0.82 ± 0.24	0.02 ± 0.01	0.07 ± 0.04
		~25	24	369 ± 198	168.1 ± 90.4	96.36 ± 1.43	0.26 ± 0.19	2.45 ± 0.75	0.12 ± 0.10	0.81 ± 0.57
		~25	48	117 ± 14	53.5 ± 6.5	90.60 ± 2.42	0.38 ± 0.11	5.38 ± 1.00	0.55 ± 0.20	3.09 ± 1.32

respectively), despite T_{0h} being experimentally the same. For all treatments except *P. annandalei* fed baker's yeast, total contents of ^{13}C-FAs decreased over time.

The predominant ^{13}C-FA in all treatments was ALA, with a relative content of >85% of total ^{13}C-FAs measured. The second-most prominent ^{13}C-FA was ETE, with the highest relative content found at T_{48h} in *A. royi* fed *D. tertiolecta* (6.0 ± 2.1% of total ^{13}C-FAs). EPA and DHA were found at all time points in all treatments, increasing in relative content with time. The highest relative content of DHA was found at T_{48h} in *P. annandalei* fed *D. tertiolecta* (3.1 ± 1.3% of total ^{13}C-FAs), while the highest relative content of EPA was found at T_{48h} in *A. royi* fed *D. tertiolecta* (1.6 ± 0.5% of total ^{13}C-FAs) (Table 1).

The C-specific contents of the different ^{13}C-FAs varied between diet treatments and time points for *A. royi* and *P. annandalei* (Fig. 2, Table 2). The SDA content was affected by the interaction of time × species and species × diet ($p < 0.05$). The SDA content only increased significantly over time for *A. royi* ($p < 0.05$), and the SDA content generally increased more for *P. annandalei* when fed baker's yeast compared to *D. tertiolecta,* and vice versa for *A. royi.* The ETE content was also affected by the interaction of time × species and species × diet but was in addition affected by the interaction of all 3 factors, time × species × diet ($p < 0.05$). The ETE content increased in both species, except for *P. annandalei* fed *D. tertiolecta*, and *P. annandalei* fed baker's yeast increased more than *A. royi* fed baker's yeast. The ETE content in *A. royi* generally increased more when fed *D. tertiolecta* compared to when fed baker's yeast, albeit not significantly ($p > 0.05$). The EPA content was affected by the interaction of time × species, time × diet, and species × diet. The EPA content was generally higher in *A. royi* fed *D. tertiolecta* compared to when fed baker's yeast, but the same was not true for *P. annandalei.* Furthermore, the EPA content did not increase significantly over time for *P. annandalei* when fed *D. tertiolecta* ($p > 0.05$). The DHA content was affected by the interaction of species × diet and time × species × diet ($p < 0.05$). The DHA content was generally higher when the copepods were fed *D. tertiolecta* compared to when they were fed baker's yeast, and *P. annandalei* had generally higher DHA content compared to *A. royi* when fed *D. tertiolecta,* albeit not significantly ($p > 0.05$). However, the increase in DHA content over time was higher for *A. royi* fed baker's yeast compared to when fed *D. tertiolecta.* The same tendency was not observed for *P. annandalei.*

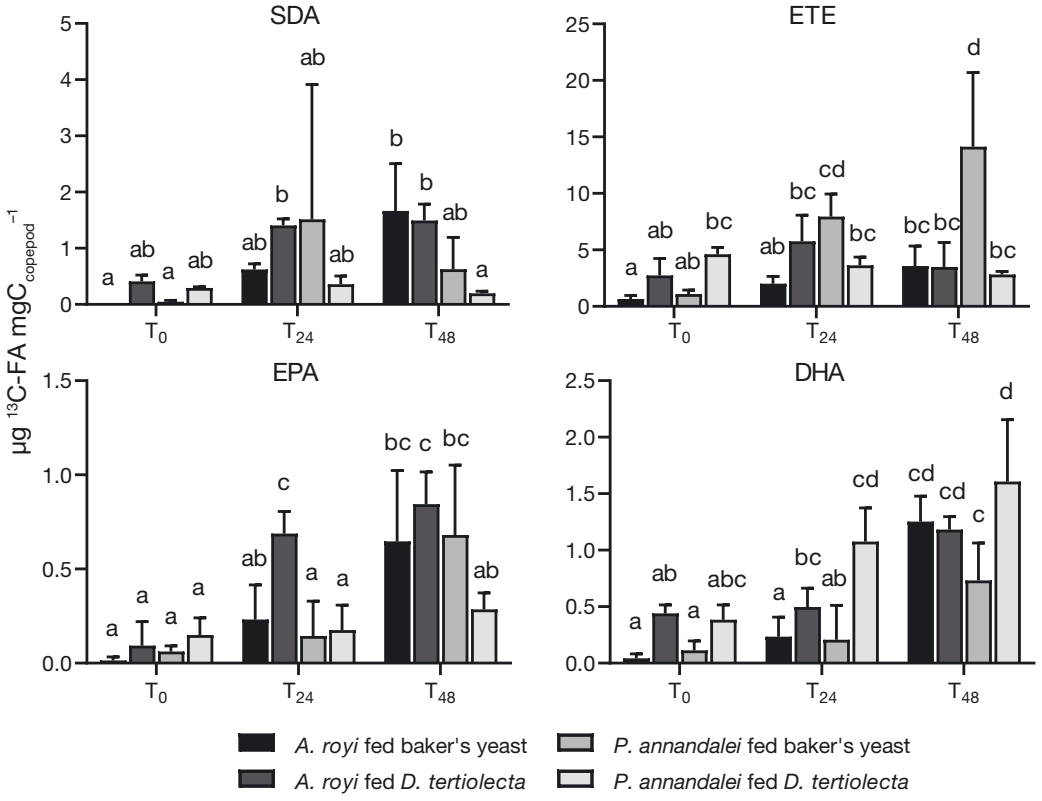

Fig. 2. Comparisons of the carbon-specific contents of the isotope-labelled n-3 polyunsaturated fatty acids (^{13}C-FAs) stearidonic acid (C18:4n-3, SDA), eicosatrienoic acid (C20:3n-3, ETE), eicosapentaenoic acid (C20:5n-3, EPA), and docosahexaenoic acid (C22:6n-3, DHA) in *Apocyclops royi* and *Pseudodiaptomus annandalei* fed baker's yeast and *Dunaliella tertiolecta* after liposome exposure. Samples of 50 individuals of *A. royi* and 30 individuals of *P. annandalei* were collected in quadruplicates at the time points T_{0h}, T_{24h}, and T_{48h}. The columns represent means ± SD (n = 4). Significant differences were tested by 3-way ANOVA and Tukey's test for all datasets. Different letters between columns represent significant differences ($p < 0.05$)

At T_{48h} there were no significant differences in the content of SDA, ETE, EPA, and DHA between *A. royi* treatments ($p > 0.05$), but there were significant differences between *P. annandalei* treatments for ETE and DHA ($p < 0.05$) and between *A. royi* and *P. annandalei* treatments. The SDA content of *A. royi* and *P. annandalei* was 1.7 ± 0.8 and 0.6 ± 0.6 µg ^{13}C-FA mgC$_{copepod}^{-1}$ when fed baker's yeast, respectively, and 1.5 ± 0.3 and 0.2 ± 0.04 µg ^{13}C-FA mgC$_{copepod}^{-1}$ when fed *D. tertiolecta*, respectively. Of these, *P. annandalei* fed *D. tertiolecta* was significantly different from both *A. royi* treatments ($p < 0.05$), but otherwise the SDA contents did not differ between the species and their dietary treatments. The ETE content of *P. annandalei* fed baker's yeast (14.1 ± 6.5 µg ^{13}C-FA mgC$_{copepod}^{-1}$) was significantly higher than both of the *A. royi* fed baker's yeast and *D. tertiolecta* (3.6 ± 1.8 and 3.5 ± 2.2 µg ^{13}C-FA mgC$_{copepod}^{-1}$, respectively) and *P. annandalei* fed *D. tertiolecta* (2.8 ± 0.27 µg ^{13}C-FA mgC$_{copepod}^{-1}$) ($p < 0.05$), but the latter 3 did not differ from each other ($p > 0.05$). The EPA content of *A. royi* and *P. annandalei* was 0.6 ± 0.4 and 0.7 ± 0.4 µg ^{13}C-FA mgC$_{copepod}^{-1}$, respectively, when fed baker's yeast, and 0.8 ± 0.2 and 0.3 ± 0.1 µg ^{13}C-FA mgC$_{copepod}^{-1}$ when fed *D. tertiolecta*, respectively, and only the latter 2 differed significantly from each other ($p < 0.05$). The DHA content of *A. royi* fed baker's yeast and *D. tertiolecta* (1.3 ± 0.2 and 1.2 ± 0.1 µg ^{13}C-FA mgC$_{copepod}^{-1}$, respectively) did not significantly differ from that of *P. annandalei* fed baker's yeast and *D. tertiolecta* (0.7 ± 0.3 and 1.6 ± 0.5 µg ^{13}C-FA mgC$_{copepod}^{-1}$, respectively) ($p > 0.05$), but the latter 2 did differ significantly from each other ($p < 0.05$).

4. DISCUSSION

The purpose of the current study was to determine if and to which extent the tropical copepods *Apocyclops royi* and *Pseudodiaptomus annandalei* are able to biosynthesize the physiologically important FAs

Table 2. Results of 3-way ANOVA on the content of isotope-labelled stearidonic acid (C18:4n-3, SDA), eicosatrienoic acid (C20:3n-3, ETE), eicosapentaenoic acid (C20:5n-3, EPA), and docosahexaenoic acid (C22:6n-3, DHA) in *Apocyclops royi* and *Pseudodiaptomus annandalei* fed baker's yeast and *Dunaliella tertiolecta*, with copepod species, diet, and time as independent variables

Source of variation	df	MS	*F*-ratio	p
SDA				
Time	2	1.02	13.64	<0.0001
Species	1	0.95	12.34	0.001
Diet	1	0.03	0.43	0.52
Time × Species	2	0.37	4.92	0.01
Time × Diet	2	0.18	2.37	0.11
Species × Diet	1	0.41	5.42	0.03
Time × Species × Diet	2	0.11	1.44	0.25
ETE				
Time	2	2.11	18.23	<0.0001
Species	1	2.16	18.65	<0.0001
Diet	1	0.12	1.00	0.33
Time × Species	2	0.05	0.39	0.68
Time × Diet	2	2.36	20.40	<0.0001
Species × Diet	1	1.96	16.95	<0.001
Time × Species × Diet	2	0.80	6.95	0.003
EPA				
Time	2	0.60	37.58	<0.0001
Species	1	0.15	9.60	0.004
Diet	1	0.05	2.96	0.09
Time × Species	2	0.08	5.14	0.01
Time × Diet	2	0.06	3.59	0.04
Species × Diet	1	0.15	9.19	0.005
Time × Species × Diet	2	0.04	2.59	0.09
DHA				
Time	2	1.31	72.54	<0.0001
Species	1	0.01	0.73	0.40
Diet	1	0.92	50.94	<0.0001
Time × Species	2	0.04	2.26	0.12
Time × Diet	2	0.04	1.96	0.16
Species × Diet	1	0.16	8.70	0.006
Time × Species × Diet	2	0.09	4.77	0.01

EPA and DHA from ALA. We hypothesized that *A. royi* and *P. annandalei* would show a high production of n-3 LC-PUFAs compared to their congeners in previous, similarly conducted studies (Moreno et al. 1979, Bell et al. 2007, De Troch et al. 2012, Werbrouck et al. 2017).

A. royi and *P. annandalei* were fed $^{13}C_{18}$-ALA through liposomes for 24 h and subsequently fed baker's yeast or *Dunaliella tertiolecta* for an additional 48 h. Mortality was not observed in *A. royi,* but a high mortality (>30%) was observed in *P. annandalei* when fed baker's yeast, whereas lower mortality (<10%) was observed in *P. annandalei* when fed *D. tertiolecta*. As mortality in *P. annandalei* was higher than in *A. royi* when fed both diets, it is likely that *P. annandalei* had a poorer tolerance towards the liposomes or the general handling during the experiments. Furthermore, *P. annandalei* fed baker's yeast had a higher mortality than when fed *D. tertiolecta*, suggesting that baker's yeast had an additional effect on the mortality of *P. annandalei.* Baker's yeast was fed in excess, and visible formations of yeast cells were present at the bottom of the beakers. Farhadian et al. (2008) suggested that excessive addition of yeast will result in worsening water quality and will affect survival of copepods, which could be the case for *P. annandalei*. Furthermore, another calanoid copepod, *Acartia tonsa*, has previously been documented to desist feeding if the feed particles were nutritiously inadequate, and not resume feeding when returned to nutritious feed sources (Støttrup & Jensen 1990). The same could be true for *P. annandalei* in both the case of the liposomes and the baker's yeast, and the lack of nutrition combined with the stress of the prior 24 h starvation and general handling could result in high mortality. Because of the high mortality of *P. annandalei* when fed baker's yeast, we concluded that for future experiments the post-liposome diet should be *D. tertiolecta.* However, retention of the ^{13}C-marked FAs over time was higher when fed baker's yeast (~100–400 total µg ^{13}C-FA $mgC_{copepod}^{-1}$ at T_{48h}) than when fed *D. tertiolecta* (~50 total µg ^{13}C-FA $mgC_{copepod}^{-1}$ at T_{48h}), which was the initial argument for the use of baker's yeast as the post-liposome diet, as the lack of dietary non-labelled ALA could limit the turnover of the ^{13}C-FAs. Therefore, baker's yeast should be considered a viable option as post-liposome diets for copepods that thrive on it.

4.1. n-3 LC-PUFA biosynthesis proficiency

At T_{48h}, the content of each of the biosynthesized ^{13}C-FAs in *A. royi* did not differ between those fed baker's yeast and those fed *D. tertiolecta* ($p > 0.05$). This suggests that over time, *A. royi* was able to biosynthesize equally well when fed the 2 diets. However, for *P. annandalei* ^{13}C-FA contents varied, with a significantly higher content of ETE in those fed baker's yeast ($p < 0.05$), and a significantly higher content of DHA in those fed *D. tertiolecta* ($p < 0.05$) at T_{48h}. This suggests that *P. annandalei* allocated its energy differently when fed baker's yeast than when fed *D. tertiolecta.* This is not surprising, as *P. annandalei* was obviously stressed when fed baker's yeast, which could both be a result of worsening water quality but could also be because baker's yeast lacks

ALA. Kabeya et al. (2018) reported that cyclopoid, harpacticoid, and siphonostomatoid copepods are able to biosynthesize ALA from C18:1n-9 and C18:2n-6, while calanoid copepods do not possess such an ability. This could account for the differences in strategy between *A. royi* and *P. annandalei* when fed baker's yeast, as *P. annandalei* may not have had sufficient energy to biosynthesize DHA in similar quantities as when fed *D. tertiolecta.* However, at T_{48h} there was no statistical difference in DHA content between *A. royi* and *P. annandalei* when they both were fed *D. tertiolecta,* suggesting that their capability to produce DHA is similar when fed an ALA-rich diet. However, significant differences were found in SDA, ETE, and EPA between *A. royi* and *P. annandalei,* which suggests that their strategies may differ according to their own specific needs.

Both copepods fed both diets successfully produced DHA (~1000 ng ^{13}C-DHA $mgC_{copepod}^{-1}$ at T_{48h}). Admittedly, 1000 ng ^{13}C-DHA $mgC_{copepod}^{-1}$ could be considered only trace amounts, as *A. royi* has previously been reported to contain ~65 ng DHA $ind.^{-1}$ (~40 µg DHA $mgC_{copepod}^{-1}$, based on the C estimate of 1.6 µg C $ind.^{-1}$) when fed the DHA-lacking *D. tertiolecta* (Nielsen et al. 2019), and *P. annandalei* has previously been reported to contain ~19 µg DHA $mgC_{copepod}^{-1}$ (Rayner et al. 2017). Furthermore, an average daily increase in ^{13}C-DHA was estimated to be 366 and 620 ng ^{13}C DHA $mgC_{copepod}^{-1}$ d^{-1} for *A. royi* and *P. annandalei* fed *D. tertiolecta*, respectively, i.e. <4% of their total DHA content. However, Blanda et al. (2017) reported low DHA contents of the available seston in the Taiwanese aquaculture ponds in which *A. royi* and *P. annandalei* proliferate. The lowest DHA content (1.2 µg DHA mg C^{-1}) was found in July–August, and the highest (2.7 µg DHA mg C^{-1}) was found in October (Blanda et al. 2017). An estimated ingestion rate for *A. royi* is 1.12 mg C $mgC_{copepod}^{-1}$ d^{-1}, based on a maximum ingestion of ~40 000 cells $ind.^{-1}$ d^{-1} of *Rhodomonas salina* at 20 psu (Gréve et al. 2020), 46 pg C $cell^{-1}$ (Nielsen et al. 2019), and 1.6 µg C $ind.^{-1}$. For *P. annandalei*, with a specific growth rate of 0.5 (Blanda et al. 2017), and an estimated growth efficiency of 0.33, an estimated ingestion rate would be 150% of body C, i.e. 1.48 mg C $mgC_{copepod}^{-1}$ d^{-1}. At these ingestion rates, *A. royi* would consume 1.3 mg DHA $mgC_{copepod}^{-1}$ d^{-1} in July–August and 3.0 mg DHA mg $C_{copepod}^{-1}$ d^{-1} in October, while *P. annandalei* would consume 1.8 and 4.0 mg DHA mg $C_{copepod}^{-1}$ d^{-1} in July–August and October, respectively. The biosynthesized ^{13}C-DHA would therefore contribute an additional 12–27% DHA for *A. royi* and 15–35% for *P. annandalei*. These amounts could be considered ecologically relevant quantities. Furthermore, the results in the present study have not been corrected for the total amount of FAs present in the copepods, hence the actual total DHA produced is higher than the presented values. This is also evident, as one could hypothesize that less than a 100% DHA substitution should allow survival but prevent reproduction. However, this does not seem to be the case. At our laboratory we have kept *A. royi* and *P. annandalei* in continuous cultures while fed on the DHA-lacking *D. tertiolecta* for >10 generations, and *A. royi* has successfully been cultured on PUFA-lacking baker's yeast for more than 2 yr. Thus, the copepods reproduce despite the lack of dietary DHA. Further research on the FA-requirements for reproduction in copepods could elucidate whether *A. royi* and *P. annandalei* are able to biosynthesize adequate amounts to cover their own physiological functions.

To further illustrate the n-3 LC-PUFA biosynthetic capabilities of *A. royi* and *P. annandalei*, a comparison to other copepods suspected of having the capability could be made. Bell et al. (2007) inspired the present experimental setup, and they presented in *Calanus finmarchicus* a relative content of 0.035% DHA of total D_5-labelled FAs (DFAs) and a total content of 41.2 ± 9.4 ng DFA $ind.^{-1}$ Bell et al. (2007) tested only female *C. finmarchicus* and kept them at 8°C. An estimated carbon content of 221 µg C $ind.^{-1}$ for adult females reared in a laboratory at 8°C (Campbell et al. 2001), yields a C-specific content of just 0.065 ng D_5-DHA $mgC_{copepod}^{-1}$ for that particular large oceanic copepod. Therefore, *A. royi* and *P. annandalei* produced a factor of 10^4 more C-specific DHA than *C. finmarchicus.* The experimental setup of the present study was modified from Bell et al. (2007), with the specific goal of an optimized consumption of the isotope-labelled ALA, which could explain the large differences in DHA production. However, the difference in relative conversion of ALA into DHA (3.09 ± 1.32% of total ^{13}C-FA and 0.035 ± 0.00% of total DFA for *P. annandalei* fed *D. tertiolecta* at T_{48h} and *C. finmarchicus* at T_{96h}, respectively) illustrates that differences in liposome consumption alone cannot explain the differences in C-specific DHA contents. Isotope-labelling experiments have been done for one other calanoid copepod, *Paracalanus parvus* (Moreno et al. 1979). Moreno et al. (1979) reported a 10.4% incorporation of the added ^{14}C-ALA (10.4% × 0.10 µM × 2.4 l × 280.4 µg $µmol^{-1}$ = 7 µg) after 10 h of exposure and a conversion of 0.6% of the ^{14}C-ALA into DHA, equaling 42 ng ^{14}C-DHA per sample. Sample size

was ~1 g (wet weight, WW) copepods, which according to the dry weight (DW):WW conversion factor of 0.20 (Postel et al. 2000) and C content:DW conversion factor of 0.50 for marine calanoid copepods (Ventura 2006) equals a sample size of ~100 mg C. Therefore, *P. parvus* was reported to have an isotope-labelled uptake of 70 ng ^{14}C-ALA mgC$_{copepod}^{-1}$ and a DHA content of 0.42 ng ^{14}C-FA mgC$_{copepod}^{-1}$. While *P. parvus* produced roughly 7 times more DHA after 10 h than *C. finmarchicus* did after 96 h, *P. annandalei* and *A. royi* produced a lot more DHA. The vast difference in DHA productivity between *P. annandalei* and *C. finmarchicus* and *P. parvus* illustrates that *P. annandalei* easily surpasses the biosynthetic capabilities of its congeners.

Other studies have utilized isotope labelling of the diet rather than introducing pre-made isotope-labelled FAs through liposomes (De Troch et al. 2012, Werbrouck et al. 2017). De Troch et al. (2012) fed the harpacticoid copepod *Microarthridion littorale* ^{13}C-enriched bacteria lacking DHA for 9 d, and reported a ^{13}C-DHA content of 0.15 ng C ind.$^{-1}$ (~0.19 ng ^{13}C-DHA ind.$^{-1}$) and a carbon content of 430 ± 20 ng C ind^{-1}. This equals a carbon-specific ^{13}C-DHA content of 440 ng ^{13}C-FA mgC$_{copepod}^{-1}$. In a similar study by Werbrouck et al. (2017), the harpacticoid copepod *Platychelipus littoralis* was fed ^{13}C-enriched *D. tertiolecta* for 6 d after 3 d of starvation at 3 different temperatures (4, 15, and 24°C). The highest content of ^{13}C-DHA was observed in copepods cultured at 24°C (~800 ng FA mgC$_{assimilated}^{-1}$), with a total assimilated content of 0.11 µg C ind.$^{-1}$ (= 0.088 ng DHA ind.$^{-1}$). The C content was reported as 1.6 µg C ind.$^{-1}$, resulting in a carbon-specific ^{13}C-FA content of 55 ng ^{13}C-FA mg C$_{copepod}^{-1}$. As the isotope-labelling technique and the exposure time of the 2 experiments by De Troch et al. (2012) and Werbrouck et al. (2017) differed from those of the current study, direct comparison is difficult. However, the DHA content of 440 ng ^{13}C-FA mgC$_{copepod}^{-1}$ in *M. littorale* and 55 ng ^{13}C-FA mgC$_{copepod}^{-1}$ in *P. littoralis* is more similar to the contents found in *A. royi* and *P. annandalei* (~1000 ng ^{13}C-FA mgC$_{copepod}^{-1}$) compared to those of *C. finmarchicus* (0.065 ng D_5-DHA mgC$_{copepod}^{-1}$) and *P. parvus* (0.420 ng ^{14}C-FA mgC$_{copepod}^{-1}$). This fits well with the general perception that harpacticoids are likely to be proficient in n-3 LC-PUFA biosynthesis because they characteristically feed on PUFA-poor detritus (Anderson & Pond 2000). Rayner et al. (2017) hypothesized that the same is true for *P. annandalei.*

A less direct approach to determine the ability of copepods to biosynthesize DHA is through FA profile comparison of copepods fed monoalgal diets with differing n-3 PUFA profiles. FA profiles have been analysed for several copepod species while cultured on *D. tertiolecta: Amonardia* sp. (Nanton & Castell 1999), *A. royi* (Nielsen et al. 2019, 2020), *P. annandalei* (Nielsen et al. 2020), *P. littoralis* (Werbrouck et al. 2017), *Tachidius discipes, Tisbe* sp. (Arndt & Sommer 2014), and *Tisbe holothuriae* (Norsker & Støttrup 1994). Of these, only *A. royi, P. annandalei,* and *P. littoralis* had a DHA content of >20% of total FAs, and while it may be tempting to conclude that they therefore have a higher capability of biosynthesizing n-3 LC-PUFAs than the other copepods, it is a premature assessment without comparative quantitative studies. However, there are indications of differing capabilities of n-3 LC-PUFA biosynthesis; for example, *T. discipes* had a significantly lower content of DHA when fed *D. tertiolecta* (3.7 ± 2.2 ng µgC$_{copepod}^{-1}$) compared to when fed the n-3 LC-PUFA-rich microalga *Rhodomonas salina* (31.0 ± 12.5 ng µgC$_{copepod}^{-1}$) ($p < 0.05$) (Arndt & Sommer 2014), while *A. royi* had a not significantly different DHA content of 63.7 ± 11.2 and 97.8 ± 26.2 ng ind.$^{-1}$ ($p > 0.05$), when fed the same 2 diets, respectively (Nielsen et al. 2019). This may suggest that *A. royi* has a higher capability of n-3 LC-PUFA biosynthesis than *T. discipes*. In the same study by Arndt & Sommer (2014), *Tisbe* sp. showed a similar capability as *A. royi* with a not significantly different DHA content of 11.4 ± 4.3 and 18.2 ± 5.1 ng µgC$_{copepod}^{-1}$ when fed *D. tertiolecta* and *R. salina*, respectively ($p > 0.5$). *T. holothuriae*, however, had a differing DHA content of 15.0 and 40.6 ng ind.$^{-1}$ when fed *D. tertiolecta* and *R. salina* (Norsker & Støttrup 1994). The FA contents of that study unfortunately were only analysed in duplicates, so statistical analysis could not be provided, but the differing contents of *T. holothuriae* and *Tisbe* sp. may suggest differing capabilities within the same genus of copepods. *Amonardia* sp. had a not significantly different DHA content of 13.6 ± 3.3 and 16.4 ± 3.8% of total FAs when fed *D. tertiolecta* and the DHA-rich *Isochrysis galbana*, respectively ($p > 0.05$) (Nanton & Castell 1999). However, in Nielsen et al. 2020, *P. annandalei* contained 29.8 ± 7.1% DHA of total FAs when fed *D. tertiolecta*, which is a rather large amount compared to the 5.4% DHA of total FAs when fed *Tetraselmis chuii*, a DHA-lacking alga that contains high contents of EPA (Rayner et al. 2017). This difference illustrates that dietary FAs have an effect on the relative content of FAs, and that conclusions based solely on relative FA content should be made with caution.

A. royi and *P. annandalei* seem to be more proficient at biosynthesizing n-3 LC-PUFAs than most other reported copepods; however, physiological needs of different species of copepods may differ greatly. A comparative study with isotope labelling and a fecundity analysis of different species of copepods could elucidate whether differing biosynthetic proficiency is correlated to differing physiological needs.

4.2. n-3 PUFA biosynthetic pathway

Unequivocally, *P. annandalei* and *A. royi* produced n-3 LC-PUFAs in our experiments, but which biosynthetic pathway they utilized is still ambiguous. However, ^{13}C-labelled SDA, which is produced by the desaturation of ALA at the C_6-C_7 position, was present in both copepods and therefore suggests they both possess a Δ6 desaturase. Similarly, they both likely contain a Δ5 desaturase, as ^{13}C-labelled EPA, which is produced by the desaturation of C20:4n-3 at the C_5-C_6 position, was found in both copepods. This corresponds with the transcriptomes annotated as encoding for Δ6 and Δ5 desaturases reported in *A. royi* by Nielsen et al. (2019). The presence of the ^{13}C-FAs with chain length of C20 (ETE and EPA) and C22 (DHA) suggests the presence of an Elovl5-like enzyme, as Elovl5 is associated with the elongation of C18 and C20 to C22. The combination of an Elovl5-like enzyme and Δ6 and Δ5 desaturases can account for the production of EPA (ALA → SDA → C20:4n-3 → EPA). Furthermore, an Elovl5-like enzyme would be able to produce C22:5n-3 by elongation of EPA. Monroig & Kabeya (2018) reported that an Elovl2-like enzyme (associated with the elongation of C20 and C22 to C24) could be identified in the genome of the copepod *Caligus rogercresseyi.* This suggests that some copepods may be able to utilize the 'Sprecher Shunt', i.e. the elongation, Δ6 desaturation, and subsequent β-oxidation of C22:5n-3 to DHA (C22:5n-3 → C24:5n-3 → C24:6n-3 → C22:6n-3) (Sprecher 2000, Bell & Tocher 2009), which was hypothesized by Nielsen et al. (2019) to be the pathway utilized by *A. royi*. However, as C24:5n-3 and C24:6n-3 were not included in the FAME standards in the current study, this pathway and the utilization of an Elovl2-like enzyme cannot currently be confirmed. While the alternative pathway is to utilize a Δ4 desaturase to directly desaturate the C_4-C_5 positions of C22:5n-3 into DHA, this too cannot currently be confirmed without a functional analysis of desaturases, as a transcript encoding specifically for Δ4 desaturase was not found in *A. royi* by Nielsen et al. (2019). Of course, the lack of detection in the transcriptome analysis is not equivalent to an absence of the enzyme or an absence of activity by other desaturases with multiple functionalities. Likewise, the presence or absence of a Δ8 desaturase activity, i.e. the desaturation of the C_8-C_9 positions of ETE into C20:4n-3, is still ambiguous. However, for both *A. royi* and *P. annandalei,* the most abundant ^{13}C-FA after ALA was ETE (>45% of total produced ^{13}C-FA at T_{48h}), which was also the case for *C. finmarchicus*, *Calanoides acutus* (Bell et al. 2007), and *P. parvus* (Moreno et al. 1979). This may suggest that the high contents of ETE (which is produced by elongation of ALA) are accumulations as a result of a lack of, or perhaps a rate-limiting, Δ8 desaturase activity. However, this should either be tested with a functional analysis of enzymes, or an isotope-labelling experiment where the copepods are fed isotope-labelled ETE. Overall, more research is needed to elucidate the n-3 PUFA biosynthetic pathway in copepods.

4.3. n-3 LC-PUFA biosynthesis as an adaptation mechanism

As n-3 LC-PUFA biosynthesis has been demonstrated across orders of copepods, it is likely that most copepods possess the genes for biosynthesizing DHA from ALA, but that the genes have been up- or down-regulated in various species. Additionally, the copepods listed in this study that are thought to be capable of biosynthesizing DHA come from very different habitats. *A. royi* and *P. annandalei* are both found in tropical estuaries, freshwater areas, and brackish aquaculture ponds (Chang & Lei 1993, Su et al. 2005, Blanda et al. 2015, 2017). *Amonardia* sp. was collected from the Northwest Arm, Nova Scotia, Canada (Nanton & Castell 1999). *M. littorale* and *P. littoralis* were extracted from the temperate Paulina silty intertidal flat in the southwest Netherlands (De Troch et al. 2012, Werbrouck et al. 2017). *P. parvus* was reared from the temperate inshore Argentinian sea (Moreno et al. 1979). *Paracyclopina nana* was reared from a brackish lagoon, Hwajinpo Lake, Korea (K. Lee et al. 2006). *T. discipes* was collected in the brackish Kiel Bight, Germany (Arndt & Sommer 2014). The precise origin of *T. holothuriae* and *Eucyclops serrulatus* and was not specified in the respective studies (Norsker & Støttrup 1994, Desvilettes et al. 1997), but *E. serrulatus* was reared from a lake and is mostly found in temperate areas (Alekseev & Defaye 2011), and *T. holothuriae* was cultured at temperate

conditions (i.e. at 18°C) (Norsker & Støttrup 1994) and is a marine epibenthic copepod. All of these studies have raised direct and indirect arguments that these copepods are able to biosynthesize n-3 LC-PUFAs, but a lack of biochemical descriptions of food items from their habitat makes it difficult to ascertain a pattern of emergence of this biochemical trait. Thus, it is likely that n-3 LC-PUFA biosynthesis is an inherent ability of copepods that, under the right circumstances (i.e. environmental pressure), would be activated. For instance, without large lipid stores and with a high metabolic rate, tropical copepods have perhaps been more likely to adapt to the low-PUFA environments by upregulating their n-3 LC-PUFA biosynthesis-related genes. Meanwhile, the lipid storage and overwintering strategy of polar copepods has proven to be an adequate adaption and may thus have downregulated the relevant FA-modifying genes, as their biosynthesis would be unnecessarily energy consuming. However, because of the rather small data pool on the subject and lack of local ecological evidence, it might be too simplistic to speculate that warm-water copepods are more likely to possess the ability to biosynthesize n-3 LC-PUFAs, especially given that Helenius et al. (2020) recently reported that the North Atlantic to Arctic *C. finmarchicus* was able to biosynthesize EPA from C18:5n-3. A larger and more comprehensive comparative study of either genetic analysis or a quantitative analysis like the one presented in the current study combined with an ecological analysis of copepod habitats is needed to better understand the dynamics of the adaptation towards n-3 LC-PUFA biosynthesis.

4.4. Closing remarks

We have provided evidence for the capability of *A. royi* and *P. annandalei* to biosynthesize DHA from ALA, and that our target copepods, which originated from a PUFA-poor habitat, exhibit a comparatively high capability compared to the prevailing reports about copepods. This may reflect a biochemical adaptation promoted by the harsh environmental characteristics of their habitat. Thus it is relevant to investigate whether this trait lies hidden as a widespread potential mechanism in all copepods that can be activated under the right environmental circumstances.

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