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**Biochemical adaptation by the tropical copepods *Apocyclops royi* (Lindberg 1940) and *Pseudodiaptomus annandalei* (Sewell 1919) to a PUFA-poor brackish water habitat**

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ABSTRACT

The cyclopoid *Apocyclops royi* and the calanoid *Pseudodiaptomus annandalei* are two tropical copepods suspected of the capability to biosynthesize the physiologically important n-3 polyunsaturated fatty acids (n-3 PUFA) eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA). We demonstrated this suspected ability using 13C18 α-linolenic acid (C18:3n-3, ALA) fed to the copepods through liposomes and a subsequent fatty acid (FA) analysis by GC-MS at three different time points, 0, 24 and 48h. Two different diets were applied post liposome-exposure, baker’s yeast *Saccharomyces cerevisiae* and the microalgae *Dunaliella tertiolecta.*  For both copepods, further elongated and desaturated 13C n-3 PUFA were found at all time points. At T48h, *A. royi* and *P. annandalei* contained 13C-labelled DHA contents of 1.3 ± 0.2 and 0.7 ± 0.3 µg 13C-FA · mg Ccopepod-1 when fed baker’s yeast, respectively, and 1.2 ± 0.1 and 1.6 ± 0.5 µg 13C-FA · mg Ccopepod-1 when fed *D. tertiolecta*, respectively, with significant differences observed only between *P. annandalei* diet treatments. The 13C-labelled EPA content of *A. royi* and *P. annandalei* at T48h was 0.6 ± 0.4 and 0.7 ± 0.4 µg 13C-FA · mg Ccopepod-1 when fed baker’s yeast and 0.8 ± 0.2 and 0.3 ± 0.1 µg 13C-FA · mg Ccopepod-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 PUFA from the precursor ALA in comparatively large quantities. This ability enables these two species to inhabit habitats characterized by PUFA-poor particulate material.

KEY WORDS: Copepod, DHA, Biosynthesis, Isotope-labelling

1. INTRODUCTION

In aquatic food webs certain phytoplankton are the biggest contributors to the production of polyunsaturated fatty acids (PUFA, ≥ 2 double bonds) (Taipale et al. 2013, Jónasdóttir 2019), including the physiologically important fatty acids (FA), 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 PUFA.

In recent years, several studies have challenged the general perception that only primary producers can produce long chain PUFA (LC-PUFA, ≥ 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 PUFA 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-PUFA, 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 of n-3 PUFA biosynthesis at present and how environment 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 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*.* (Norsker & Støttrup 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* (Lee et al. 2006a), and *Apocyclops royi* (Pan et al. 2017, Nielsen et al. 2019) are all to various degree suggested to possess this particular trait. Bell et al. (2007) concluded that the trace amounts of D5-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 (Colombo et al. 2016, Dalsgaard 2003). Furthermore, high-latitude copepods develop lipid-storages 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 (Lee et al. 2006b). In contrast, subtropical and tropical copepods do not develop such large lipid storages as food is somewhat constant throughout the year, and the high temperatures cause high 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 (Colombo et al. 2016, Dalsgaard 2003). 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 (Pan et al. 2017, Rayner et al. 2017, Nielsen et al. 2019). The two species of copepods examined here are found as the dominating species in Southern Taiwanese artificial aquaculture ponds (Blanda et al. 2015, Su et al. 2005), 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 two 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 microalgae 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) presented a DHA content of 5.4 % of total FAs in *P. annandalei* when fed for several months on *Tetraselmis chuii,* a microalgae lacking DHA, and Blanda et al. ( 2017) presented 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 the two copepods dominance in the aquaculture ponds (Blanda et al. 2015, Su et al. 2005), we believe that these two 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 has 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 produced by the copepods themselves. So far, isotope-tracing experiments has 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*.* Their results demonstrated production of DHA by *P. parvus* and *C. finmarchicus* albeit in negligible amounts. This, however, 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 hypothesize that both *A. royi* and *P. annandalei* are capable of biosynthesizing DHA from ALA. Hence, in the present study, we pursue to demonstrate this capability through an isotope labelling experiment using 13C18-ALA fed as liposomes to *A. royi* and *P. annandalei*, a setup inspired from Bell et al. (2007)*.* Additionally, we aim at a quantification of this biosynthesis, compare it to the few other reported copepods capabilities, and explain this crucial trait as a biochemical adaptation to a PUFA-poor habitat.

2. METHODS

2.1 *Stock cultures*

The marine microalgae *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 oC, with aeration, and continuous 50-65 µmol PAR photons m-2s-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. When retrieved to Roskilde University, Denmark, the copepods were cultivated in 50 L tanks in a walk-in climate room under dark conditions. The cultures were kept in an aerated salinity 20 mixture of 0.2 µm UV filtrated seawater and demineralized water at 25 o C. The copepods were fed *D. tertiolecta* for > 3 generations (> 5 weeks) every other day *ad libitum* as evidenced with residual algae in the cultivation tanks immediately before feeding.

*2.2. Liposome preparation and labeling*

For later enabling us to document the copepods biosynthesis of LC-PUFAs, liposomes were used as carriers in order to deliver the isotope marked FA 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 in Bell et al. (2007) with a few adjustments. The company VWR International, Denmark supplied all chemicals except for the marked FAs. Approximately 2.02 µmol of 13C18-ALA methyl ester (633 µg) (Cambridge Isotope Laboratories, UK) 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 was 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 salinity 20 seawater was added. The contents were stirred vigorously, and liposome droplets were formed. The liposome size distribution was measured on a Beckman Coulter – Multisizer 4e (Brea, California, USA), 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 according to 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 *C. finmarchicus*. To optimize the intake of liposomes, preliminary observations were made on the coloration of the copepods as an estimation of intake with differing liposome densities and differing starvation periods. Because of these observations, the copepods of the current study were fed a density of > 200.000 liposomes mL-1, which is an estimated factor 7 higher than the density in 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 they were not 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 four replicates per time point T0h, T24h, and T48h, where the samples were terminated for FA analysis. In each replicate were placed 50 and 30 individuals of *A. royi* and *P. annandalei,* respectively. 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, T0h was terminated, and to the remaining beakers, 3.5 mg suspended baker’s yeast was added, 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, T24h and T48h 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 oC 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 an 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 13C-labelled FAs (13C-FA) in the copepods were estimated, and the relative conversion calculated as percentages of specific 13C-FAs of total amount of 13C-FAs. Furthermore, to compare the two different-sized copepods capability, a carbon-specific content was calculated as µg 13C-FA · mg Ccopepod-1. The carbon content of *A. royi* and *P. annandalei* were calculated with the carbon-length regression C = 1.24·10-6·L2.259 (T.A. Rayner and B.W. Hansen, unpubl.) and C = 2.19·10-9·L3.136 (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. Fatty acid methyl ester analysis*

The samples were freeze dried for 24 h in a Christ-Alpha 1-2 (Osterode am Harz, Germany) equipped with a vacuum pump. The vials were added 3 mL of a 2:1 (v:v) chloroform:methanol solution in accordance with Folch et al. (1957). An internal standard (C23:0) was added to the vials and they were then stored at -20 oC for 24 h. Approximately 1.7 mL of the solution was transferred to 2 mL GC-vials and dried under a gentle nitrogen stream on a heating block at 60 oC. The vials were added 1000 µL of a 66:85:15 (v:v:v) solution of toluene:methanol:acetyl chloride. The vials were capped and left on a heating block at 95 oC for 2 h for transesterification. After de-capping, 500 µL 5 % NaHCO3 (w/v) prepared with a few minutes of nitrogen bubble-through was added to remove excess acid from the organic phase. The solution was mixed and left to phase- separate before transferring the organic phase to a new GC vial. The content of the original vials was washed with 500 µL heptane twice and the new organic phases were transferred to the new GC vial. The vials were then dried under a gentle nitrogen stream on a heating block at 60 oC before 500 µL of chloroform was added. The vials were capped and kept at -20 oC until analysis.

The samples, now derivatized into fatty acid methyl esters (FAMEs), were hereafter analysed on an Agilent GC 6890 N (Wiesental, Waghäusel, Germany) with an Agilent J&W DB-23 column (60 m x 250 µm x 0.25 µm) with He as a carrier gas. Initial temperature was 50 oC and increased in a rate of 25 °C min-1 until reaching 200 oC, and kept constant for 10 min. Hereafter, the temperature increased at a rate of 5 oC min-1 until 250 oC, and kept constant for 3 min. The GC was connected to an Agilent Mass Selective 5975 detector (Wiesental, Waghäusel, Germany) 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 indicative of poly-unsaturation, and thus the position of the unsaturated ion is so far down the carbon-chain that the 13C- 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), predicting an n-3 methyl end (C8H12) at m/z = 108, and therefore a fully 13C-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. 13C-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 (116:108R). For every sample, the mass of every isotope-labelled n-3 PUFA (m13C-FA) were calculated as in the following equation:

(1)

Where m12C-FA is the measured mass of the corresponding non-labelled FA.

*2.5. Statistics*

All mean values in the text are presented with ± S.D. Significant differences in 13C-FA content were tested with time, copepod species, and diet as independent variables in a three-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 fecal 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 after the addition of baker’s yeast (~ 33 – 50 %), but a much lower mortality when fed *Dunaliella tertiolecta* (~ 10 %)*.* Unfortunately, the mortality was not measured but an estimation was applied as 33% mortality at T24h and 50% at T48h for *P. annandalei* fed baker’s yeast. Furthermore, a conservative estimation was applied of mortality at T24h and T48h of *P. annandalei* fed *D. tertiolecta* (both 15%).

13C-ALA was metabolized and the n-3 LC-PUFA derivatives were found in all treatments (Table 1). The lowest total amount of 13C-FA was found at T48h in *A. royi* fed *D. tertiolecta* after initial liposome exposure (90 ± 32 ng 13C-FA ind-1), and the highest total amount was found at T0h in *P. annandalei* fed *D. tertiolecta* (1,520 ± 367 ng 13C-FA ind-1). Due to the body size-differences in *A. royi* and *P. annandalei,* the total amounts of 13C-FAs were normalized between species as total µg 13C-FA · mg Ccopepod-1. The carbon-specific 13C-FA contents were similar at T0h in both *A. royi* and *P. annandalei* fed baker’s yeast (166.9 ± 103.2 and 161.1 ± 73.0 µg 13C-FA · mg Ccopepod-1, respectively), but differed when fed *D. tertiolecta* (263.7 ± 91.7 and 589.0 ± 142.2 µg 13C-FA · mg Ccopepod-1, respectively), despite T0h being experimentally the same. For all treatments except *P. annandalei* fed baker’s yeast, total contents of 13C-FAsdecreased over time.

The predominant 13C-FA in all treatments were ALA with a relative content of > 85 % of total 13C-FAs measured. The second-most prominent 13C-FA was ETE, with the highest relative contents found at T48h in *A. royi* fed *D. tertiolecta* (6.0 ± 2.1 % of total 13C-FAs)*.* EPA and DHA were found at all the time points in all treatments, increasing in relative content with time. The highest relative content of DHA was found at T48h in *P. annandalei* fed *D. tertiolecta* (3.1 ± 1.3 % of total 13C-FAs), while the highest relative content of EPA was found at T48h in *A. royi* fed *D. tertiolecta* (1.6 ± 0.5 % of total 13C-FAs), see Table 1.

The carbon-specific contents of the different 13C-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 x species and species x 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 x species and species x diet but was in addition affected by the interaction of all three factors, time x species x 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 x species, time x diet, and species x 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 x diet and time x species x 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.*

At T48h 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* were 1.7 ± 0.8 and 0.6 ± 0.6 µg 13C-FA · mg Ccopepod-1 when fed baker’s yeast, respectively, and 1.5 ± 0.3 and 0.2 ± 0.04 µg 13C-FA · mg Ccopepod-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 13C-FA · mg Ccopepod-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 13C-FA · mg Ccopepod-1, respectively) and *P. annandalei* fed *D. tertiolecta* (2.8 ± 0.27 µg 13C-FA · mg Ccopepod-1) (p < 0.05), but the latter three did not differ from each other (p > 0.05). The EPA content of *A. royi* and *P. annandalei* were 0.6 ± 0.4 and 0.7 ± 0.4 µg 13C-FA · mg Ccopepod-1, respectively when fed baker’s yeast, and 0.8 ± 0.2 and 0.3 ± 0.1 µg 13C-FA · mg Ccopepod-1 when fed *D. tertiolecta,* respectively, and only the latter twodiffered 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 13C-FA · mg Ccopepod-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 13C-FA · mg Ccopepod-1, respectively) (p > 0.05), but the latter two 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* were able to biosynthesize the physiologically important FAs 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 (Bell et al. 2007, De troch et al. 2012, Moreno et al. 1979, Werbrouck et al. 2017).

*A. royi* and *P. annandalei* were fed 13C18-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 was observed in *P. annandalei* when fed baker’s yeast (> 30 %), whereas lower mortality was observed in *P. annandalei* when fed *D. tertiolecta* (< 10 %). 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 24h starvation and general handling could result in high mortality. Because of the high mortality of *P. annandalei* when fed baker’s yeast, it was concluded that for future experiments the post-liposome diet should be *D. tertiolecta.* However, retention of the 13C-marked FAs over time was higher when fed baker’s yeast (~ 100 - 400 total µg 13C FA · mg Ccopepod-1 at T48h) than when fed *D. tertiolecta* (~ 50 total µg 13C FA · mg Ccopepod-1 at T48h), 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 13C-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 T48h, the content of each of the biosynthesized 13C-FAs in *A. royi* did not differ between those fed baker’s yeast and those fed *D. tertiolecta* (p > 0.05)*.* This suggest that over time *A. royi* was able to biosynthesize equally well when fed the two diets. However, for *P. annandalei* it 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 T48h*.* 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 of baker’s yeast lack of ALA. Kabeya et al. (2018) presented 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 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 T48h 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 are 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 13C-DHA · mg Ccopepod-1 at T48h). Admittedly, 1000 ng 13C-DHA · mg Ccopepod-1 could be considered only trace amounts as *A. royi* has previously been reported to contain ~ 65 ng DHA ind-1 (~ 40 µg DHA mg Ccopepod-1, based on the carbon-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 mg Ccopepod-1 (Rayner et al. 2017). Furthermore, an average daily increase in 13C-DHA was estimated to be 366 and 620 ng 13C DHA mg Ccopepod-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) presented the low DHA content of the available seston in the Taiwanese aquaculture ponds in which *A. royi* and *P. annandalei* proliferate. The lowest DHA content was found in July-August, 1.2 µg DHA mg C-1, and the highest was found in October , 2.7 µg DHA mg C-1 (Blanda et al. 2017). An estimated ingestion rate for *A. royi* is 1.12 mg C mg Ccopepod-1 d-1, based on a maximum ingestion of ~ 40.000 cells ind-1 d-1 of *Rhodomonas salina* at 20 psu (Greve et al. in press), 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 mg Ccopepod-1 d-1. At these ingestion rates, *A. royi* would consume 1.3 mg DHA mg Ccopepod-1 d-1 in July-August and 3.0 mg DHA mg Ccopepod-1 d-1 in October, while *P. annandalei* would consume 1.8 and 4.0 mg DHA mg Ccopepod-1 d-1 in July-August and October, respectively. The biosynthesized 13C-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 the PUFA-lacking baker’s yeast for more than two years. Thus, the copepods reproduce despite the lack of dietary DHA. Further research on the EFA-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 D5-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 oC.An estimated carbon-content, 221 µg C ind-1 for adult females reared in a laboratory at 8 oC (Campbell et al. 2001), yields a carbon-specific content of just 0.065 ng D5-DHA · mg Ccopepod-1  for that particular large oceanic copepod. Therefore, *A. royi* and *P. annandalei* produced a factor of 104 more carbon-specific DHA than *C. finmarchicus.* The experimental setup of the present study was altered 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 13C-FA and 0.035 ± 0.00 % of total DFA for *P. annandalei* fed *D. tertiolecta* at T48h and *C. finmarchicus* at T96h, respectively) illustrates that differences in liposome consumption alone cannot explain the differences in carbon-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 14C-ALA (10.4 % · 0.10 µM · 2.4 L·280.4 µg/µmol = 7 µg) after 10 h of exposure and a conversion of 0.6 % of the 14C-ALA into DHA, equaling 42 ng 14C-DHA per sample. Sample size was ~ 1 g (ww) copepods, which according to the dw:ww conversion factor of 0.20 (Postel et al. 2000) and carbon 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 14C-ALA · mg Ccopepod-1 and a DHA content of 0.42 ng 14C-FA · mg Ccopepod-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* still 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* an 13C-enriched bacteria lacking DHA for 9 days, and reported a 13C-DHA content of 0.15 ng C · ind-1 (~ 0.19 ng 13C-DHA · ind-1) and a carbon content of 430 ± 20 ng · C ind-1. This equals a carbon specific 13C-DHA content of 440 ng 13C-FA · mg Ccopepod-1. In a similar study by Werbrouck et al. (2017) the harpacticoid copepod *Platychelipus littoralis* was fed 13C-enriched *D. tertiolecta* for 6 days after 3 days of starvation at three different temperatures (4, 15, and 24 oC). The highest content of 13C-DHA was observed in copepods cultured at 24 oC (~800 ng FA · mg Cassimilated-1) with a total assimilated content of 0.11 µg C ind-1 (= 0.88 ng DHA ind-1). The carbon content was reported as 1.6 µg C ind-1, resulting in a carbon specific 13C-FA content of 550 ng 13C-FA · mg Ccopepod-1. As the isotope-labelling technique and the exposure time of the two 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 13C-FA · mg Ccopepod-1 in *M. littorale* and 550 ng 13C-FA · mg Ccopepod-1 in *P. littoralis* is a lot more similar to the contents found in *A. royi* and *P. annandalei* (~1000 ng 13C-FA · mg Ccopepod-1) compared to those of *C. finmarchicus* (0.065 ng D5-DHA · mg Ccopepod-1) and *P. parvus* (0.420 ng 14C-FA · mg Ccopepod-1). This fits well with the general perception that harpacticoids are likely to be proficient in n-3 LC-PUFA biosynthesis because of them characteristically feeding 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 copepods ability to biosynthesize DHA is through FA profile comparison of copepods fed monoalgal diets with differing n-3 PUFA profiles. The FA profile have been analyzed for several copepod species while cultured on *D. tertiolecta*: *Amonardia* sp*.* (Nanton & Castell 1999), *A. royi* (Nielsen et al. 2019, BLH Nielsen, unpublished), *P. annandalei* (BLH Nielsen, unpublished), *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 indicatives of differing capabilities of n-3 LC-PUFA biosynthesis, such as *T. discipes* had a significantly lower content of DHA when fed *D. tertiolecta* (3.7 ± 2.2 ng · µg Ccopepod-1) compared to when fed the n-3 LC-PUFA-rich microalgae *Rhodomonas salina* (31.0 ± 12.5 ng · µg Ccopepod-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 two 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 and 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 · µg Ccopepod-1 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* (Nosker & Støttrup 1994)*.* The FA contents of that study unfortunately were only analyzed 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 differing 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 our own, unpublished results *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 algae that contains high contents of EPA (Rayner et al. 2017). This difference illustrates that the dietary FAs has an effect on the relative contents 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 in n-3 LC-PUFA biosynthesizing 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 the experiments of the current study, but which biosynthetic pathway they utilize is still ambiguous. However, 13C-labelled SDA, which is produced by the desaturation of ALA at the C6-C7 position, was present in both copepods and therefore suggests they both possess a Δ6 desaturase. Similarly, they both likely contain a Δ5 desaturase as 13C-labelled EPA, which is produced by the desaturation of C20:4n-3 at the C5-C6 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 13C-FAs with chain-length of C20 (ETE and EPA) and the 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 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) (Bell & Tocher 2009, Sprecher 2000), which was hypothesized to be the pathway utilized by *A. royi* by Nielsen et al. (2019). 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 C4-C5 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 with an altogether 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 C8-C9 positions of ETE into C20:4n-3, is still ambiguous. However, for both *A. royi* and *P. annandalei,* the most abundant 13C-FA after ALA was ETE (> 45% of total produced 13C-FA at T48h), 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 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 were 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 thought able to biosynthesize 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, Blanda et al. 2015, Blanda et al. 2017, Su et al. 2007). *Amonardia* sp. was collected from the Northwest Arm, Nova Scotia (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 (Lee et al. 2006a). *T. discipes* was collected in the brackish Kiel Bight, Germany (Arndt & Sommer 2014). The precise origin of *Eucyclops serrulatus* and *T. holothuriae* was not specified in the respective studies (Desvilettes et al. 1997, Norsker & Støttrup 1994). But *E. serrulatus* was reared from a lake and is mostly found in temperate areas (Alekseev et al. 2011), and *T. holothuriae* was cultured at temperate conditions (i.e. at 18 oC) (Norsker & Støttrup 1994) and is a marine epibenthic copepod. All 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 storages 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, polar copepods lipid storage and overwintering strategy has proven to be an adequate adaption and may thus have down regulated 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, as a recent study by Helenius et al. (2020) presented 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 the copepod habitats is needed to better understand the dynamics of the adaptation towards n-3 LC-PUFA biosynthesis.

*4.3. Closing remarks*

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

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FIGURES AND TABLES

Fig. 1: *Apocyclops royi* A) starved for 24 h and B) fed Nile Red stained liposomes.

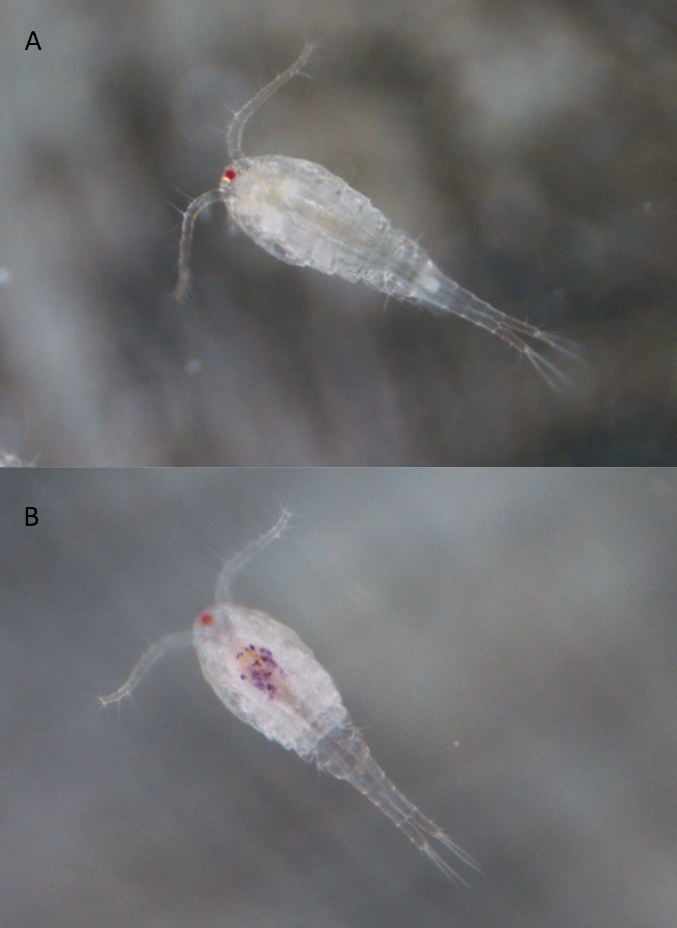


Fig. 2: Comparisons of the carbon-specific contents of the isotope-labelled n-3 LC-PUFAs (13C-FAs) stearidonic acid (C18:4n-3, SDA), eicosatrienoic acid (C20:3n-3, ETE), eicosapentaenoic acid (C20:5n-3, EPA), and docosahexaenoic acid (C22:6n-3, DHA) in *A. royi* and *P. annandalei* fed baker’s yeast and *D. 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 T0h, T24h, T48h. The columns represent means ± S.D. (n = 4). Significant differences were tested by three-way ANOVA and Tukey’s test for all datasets. Significant differences are represented with letters (P < 0.05).

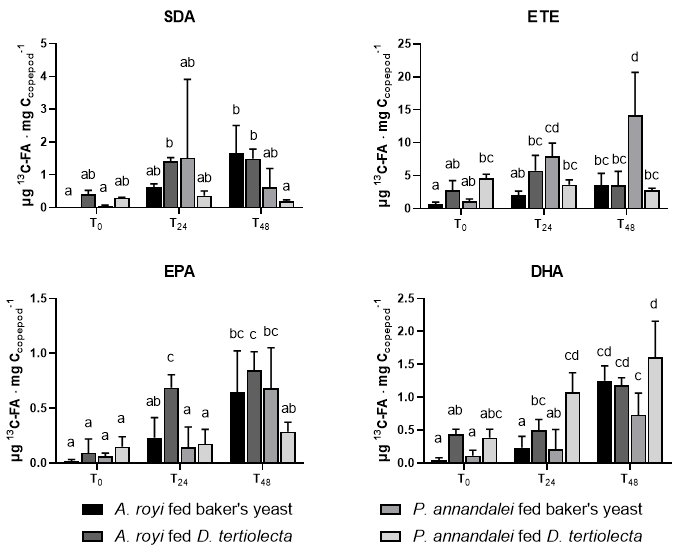


Table 1: The relative distribution of the isotope labelled fatty acids in the copepods after 0, 24, and 48 h after initial incubation with liposomes presented as mean ± S.D. (n = 4). The masses of the isotope labelled FAs are not presented with the additional weight of 13C. Total ng isotope labelled 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 13C-FA ind-1** | **Total µg 13C-FA · mg Ccopepod-1** |  |  | *13C-labelled fatty acids (% of total)* | | |  |
|  | **ALA** | **SDA** | **ETE** | **EPA** | **DHA** |
| **Baker's yeast** | *A. royi* | 50 | *0* | 274 ± 169 | 166.9 ± 103.2 |  | 99.51 ± 0.26 | n.d. | 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 |
|  |  |  |  |  |  |  |  |  |  |  |  |
|  | *P. 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 |
|  |  |  |  |  |  |  |  |  |  |  |  |
| ***D. 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 |

Table 2: The result of three-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 *A. royi* and *P. annandalei* fed baker’s yeast and *D. tertiolecta* with copepod species, diet, and time as independent variables.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Source of variation** | ***df*** | **MS** | **F ratio** | **P-value** |
| **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 x Species | 2 | 0.37 | 4.92 | 0.01 |
| Time x Diet | 2 | 0.18 | 2.37 | 0.11 |
| Species x Diet | 1 | 0.41 | 5.42 | 0.03 |
| Time x Species x 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 x Species | 2 | 0.05 | 0.39 | 0.68 |
| Time x Diet | 2 | 2.36 | 20.40 | <0.0001 |
| Species x Diet | 1 | 1.96 | 16.95 | <0.001 |
| Time x Species x 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 x Species | 2 | 0.08 | 5.14 | 0.01 |
| Time x Diet | 2 | 0.06 | 3.59 | 0.04 |
| Species x Diet | 1 | 0.15 | 9.19 | 0.005 |
| Time x Species x 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 x Species | 2 | 0.04 | 2.26 | 0.12 |
| Time x Diet | 2 | 0.04 | 1.96 | 0.16 |
| Species x Diet | 1 | 0.16 | 8.70 | 0.006 |
| Time x Species x Diet | 2 | 0.09 | 4.77 | 0.01 |