Unsaturated fatty acid transfer

A research on fatty acid transfer and bioconverting capabilities in *Pallaseopsis quadrispinosa.*

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Unsaturated fatty acid transfer, an early link in the food web

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Bachelor Thesis, Applied biology
Aeres University of Applied Sciences
Lammi Biological Station, University of Helsinki

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Graduation teacher: Danny Merién

Preface

The report that lies before you was written by me, a bachelor student of applied biology at Aeres university of applied sciences, The Netherlands. I hope you will enjoy reading about this research project as much as I enjoyed working on it.

I would like to thank my supervisors; John Loehr, Sami Taipale and Elina Peltomaa which helped me tremendously. Providing me with information, opportunities to grow and a great project to work on. It was a pleasure to work with them and a great learning experience.

I'm also grateful to all the fellow interns at Lammi biological station. When I needed a hand to sample the lakes, there were always people willing to join me on a boat trip on the beautiful Finnish lakes. The great times traveling, grilling and canoeing made my internship in Finland an extraordinary experience.

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Abstract

Early links in the food web are crucial to sustain higher levels in the food web. Molecules of high importance, for example unsaturated fatty acids, play a key role in aquatic ecosystems. Unsaturated fatty acids are produced by primary producers for instance phytoplankton. Almost all organisms need to obtain these molecules from their diet to function properly. However, some organisms are able to bioconvert precursors of these molecules to the unsaturated fatty acids the organism needs.

This study looks at the transfer of unsaturated fatty acids in an aquatic food web. The link from phytoplankton to the small freshwater crustacean *Pallaseopsis quadrispinosa* was studied.

P. quadrispinosa was fed two different phytoplankton diets for 28 days. A low-quality diet (*Acutodesmus* sp.) and a high-quality diet (*Diatoma tenuis)* the latter containing all the essential fatty acids. Biomarkers were used to track the fatty acids from primary producer (i.e., phytoplankton) to *P. quadrispinosa*. The biomarker used in this study was $\delta^{13}C$, the heavier isotope of carbon. Using a gas chromatograph-mass spectrometer this biomarker was traced.

Within 7 days the fatty acid profile of *P. quadrispinosa* changed to closely resemble the obtained diet. This indicates that fatty acids were transferred largely without change from phytoplankton to *P. quadrispinosa*. *P. quadrispinosa* can only convert 7% of DHA and +- 30% EPA (i.e., two of the essential fatty acids) within 28 days. This is not enough to be physiologically sustainable. Therefore, fatty acids can be used as biomarkers in *P. quadrispinosa.* This study showed that fatty acids can be retained for long periods of time >28 days, when essential fatty acids are not present in the diet.

Samenvatting

Vroege verbindingen in het voedsel web zijn cruciaal voor het voortbestaan van hogere niveaus in het voedsel web. Belangrijke moleculen, bijvoorbeeld onverzadigde vetzuren, spelen een van de belangrijkste rollen in aquatische ecosystemen. Bijna alle organismen moeten deze onverzadigde vetzuren uit hun dieet halen om goed te kunnen functioneren. Echter sommige organismen kunnen voorlopers van onverzadigde vetzuren omzetten naar andere essentiële onverzadigde vetzuren.

Dit onderzoek kijkt naar de overdracht van onverzadigde vetzuren in een aquatisch voedsel web. De link van fytoplankton naar kleine zoetwater kreeftachtige (*Pallaseopsis quadrispinosa)* is onderzocht.

P. quadrispinosa kreeg twee verschillen fytoplankton diëten gedurende 28 dagen. Een dieet van lage kwaliteit (*Acutodesmus*) en een dieet van hoge kwaliteit (*Diatoma tenuis*), de laatste bevat alle benodigde essentiële vetzuren. Biomarkers zijn gebruikt om de vetzuren te volgen van primaire producent (fytoplankton) tot *P. quadrispinosa*. In dit onderzoek is ^{13}C als biomarker gebruikt, het zwaardere isotoop van koolstof. Met een gas chromatograaf-massa spectrometer zijn de biomarkers gevolgd.

Binnen 7 dagen veranderde het vetzuur profiel van *P. quadrispinosa* tot het erg overeenkwam met het geconsumeerde dieet. Dit geeft de indruk dat de vetzuren grotendeels overgebracht waren van fytoplankton tot P. quadrispinosa zonder te veranderen. *P. quadrispinosa* kan 7% van DHA en +- 30% EPA (twee van de essentiële onverzadigde vetzuren) binnen 28 dagen omzetten vanuit voorlopers. Dit betekent dat P. quadrispinosa niet genoeg vetzuren kan omzetten om fysiologisch duurzaam te blijven. Hierom kunnen vetzuren gebruikt worden als biomarker in *P. quadrispinosa.* Dit onderzoek geeft weer dat de vetzuren voor langdurige perioden worden behouden >28 dagen wanneer essentiële vetzuren in het dieet ontbreken.

1. Introduction

Early links in the food web e.g., phytoplankton → amphipods, are crucial to sustain higher levels in the food web e.g., fish. The importance largely comes from the polyunsaturated fatty acids (PUFA, Fig. 1). PUFA are crucial biological compounds, for example as building blocks in many animal hormones and have regulatory roles for animal cell membranes (Arts, Brett, & Kainz, 2009). This makes PUFA one of the most important molecules transferred in plant-animal life. The main producers of fatty acids in large, deep lake and marine ecosystems are phytoplankton which can produce fatty acids (FA) *de novo* (Cagliari et al., 2011; Harwood & Guschina, 2009). Consumers e.g., humans, invertebrates and fish, cannot produce these unsaturated fatty acids themselves or have only limited abilities to transform them from other nutrients (Arts et al., 2009). These FA are therefore called essential fatty acids (EFA) (Parrish, 2009).

To humans, PUFA have positive effects on the prevention of neurodegenerative diseases and immune responses. Signalling molecules e.g., eicosanoids and/or docosanoids are derived from long chain polyunsaturated fatty acids (LCPUFA, i.e., fatty acids with more than 20 carbon atoms, Fig. 1). Examples are prostaglandins, leukotrienes and prostacyclins, which are vital cellular signal molecules and are associated with immune and inflammatory regulation (Lee & Park, 2014).

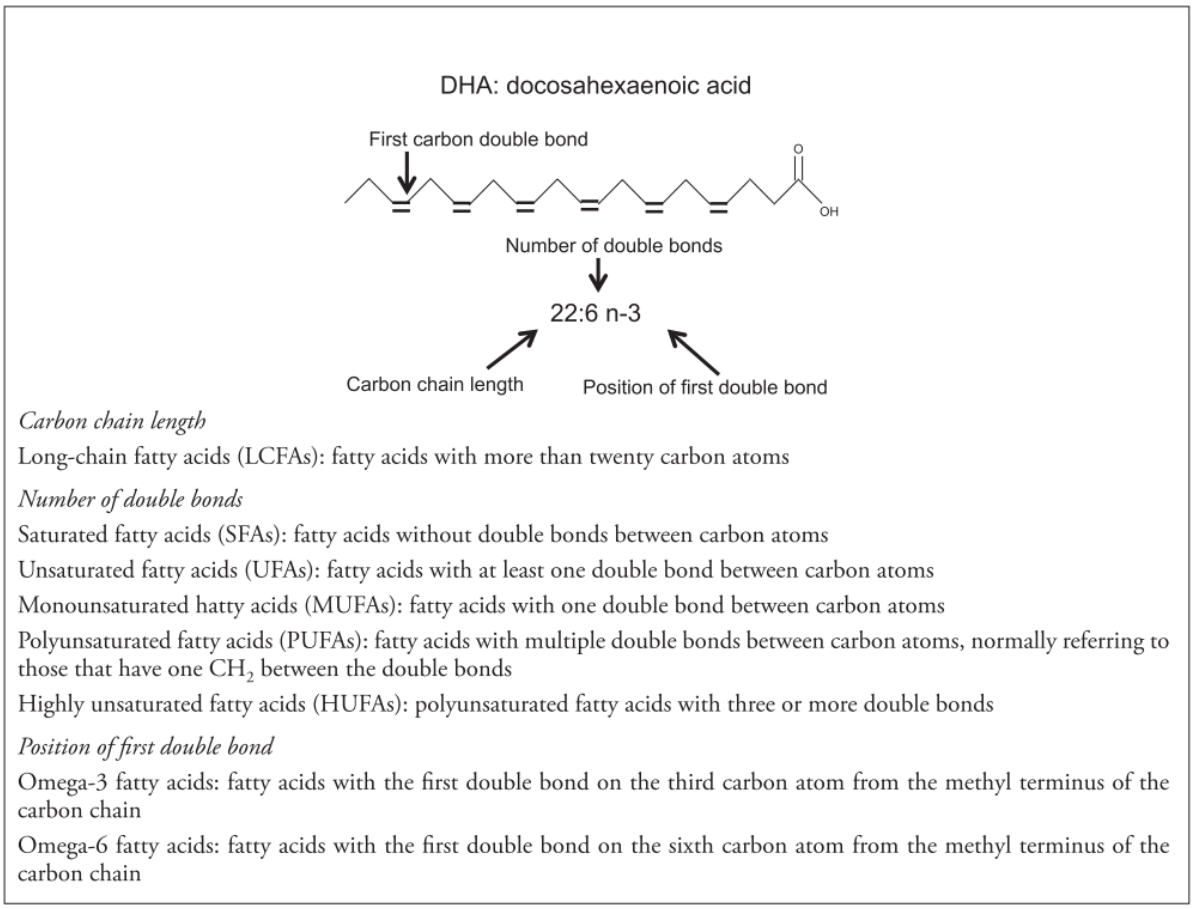

Figure 1. Visualisation and explanation of the different FA; long-chain, saturated, unsaturated, monounsaturated, polyunsaturated fatty acids and the difference between omega 3 and 6 fatty acids. (Twining, Brenna, Hairston, & Flecker, 2016).

Long chain eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) i.e., two of the essential fatty acids, are mainly produced by algae in aquatic environments (Arts, Ackman, & Holub, 2001). The production here is essential to preserve the health of terrestrial organisms including humans (Hixson, Sharma, Kainz, Wacker, & Arts, 2015). Humans obtain these EFA mainly from fish, and are therefore often considered essential dietary nutrients (Peltomaa, Johnson, & Taipale, 2018).

Some organisms can convert precursors of EFA to EFA (DHA, EPA, see Fig. 2) however this is often limited and energy consuming. Humans can only convert α-liolenic acid (ALA; 18:3ω3) to EPA and DHA in small quantities i.e., EPA 8% and DHA 4% of the required amount to sustain themselves (Burdge & Calder, 2005). The rest of the EFA humans need to obtain directly from dietary uptake. There are a number of human health problems which are implicated in relative discrepancies of omega-3 nutrients with excessive actions of omega-6 (Fig. 1) bioactive mediators (Lands, 2012). Associated health problems include asthma, arthritis, cancer proliferation (Cockbain, Toogood, & Hull, 2012), psychiatric disorders, depression, suicide, homicide (Freeman et al., 2006; Hibbeln, Nieminen, Blasbalg, Riggs, & Lands, 2006), heart attacks (Lands, 2003), obesity (Alvheim et al., 2012) etc.

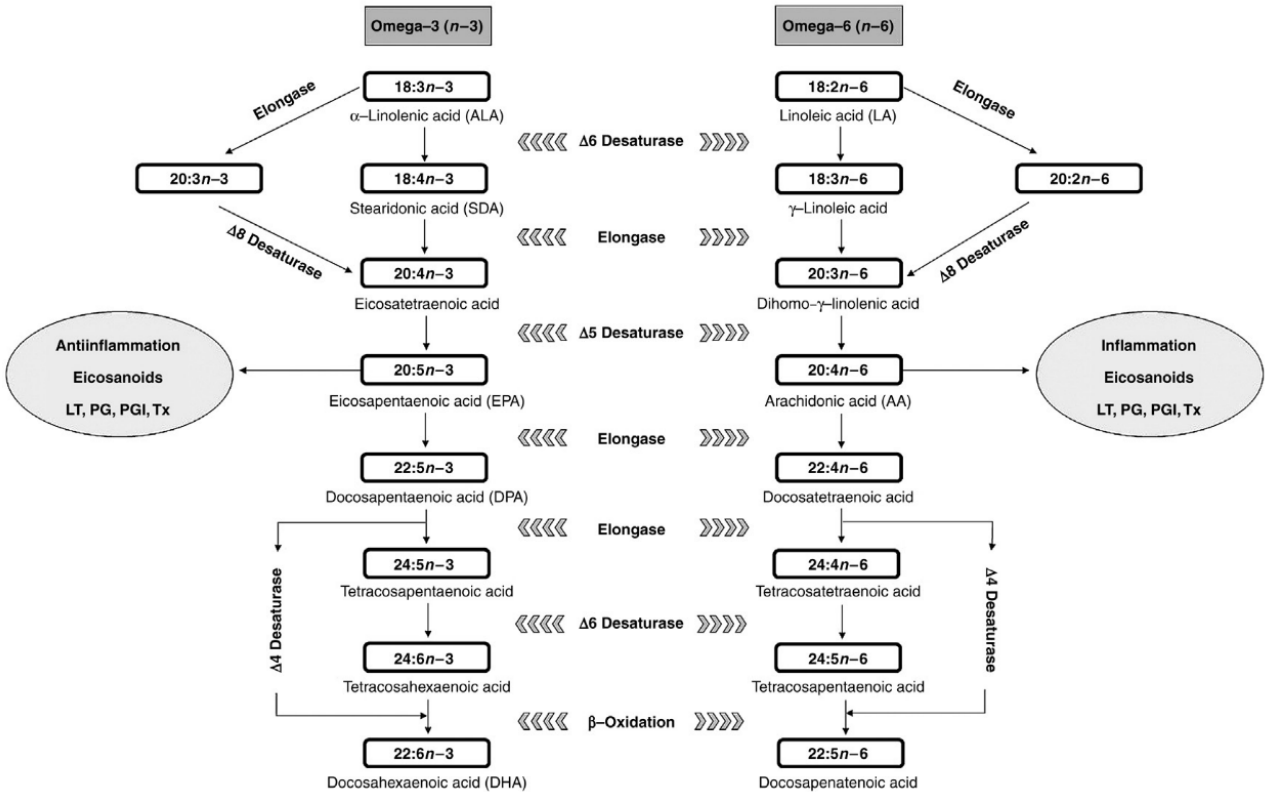

Figure 2. Schematic view of the bio conversion of LPUFA in fish. Shown in the middle column which enzymes convert the FA, note that the same enzymes work for omega-3 as well as omage-6 FA, meaning the two pathways compete with one another (Santos, Carbonera, Vagula, Maruyama, & Visentainer, 2017).

Almost every major aquatic ecosystem has been influenced by humans. These actions have altered the supply of growth limiting nutrients (Smith, 2003). Eutrophic (nutrient rich) and dystrophic (humic with lot of organic material) lakes yield less PUFA in fish i.e. EPA; 20:5:ω3g^{-1} and DHA; 22:6:ω3g^{-1} than oligotrophic (nutrient poor) lakes (Taipale, Hiltunen, Vuorio, & Peltomaa 2016). There are many health implications that discrepancies in EFA can cause. Due to the human interference of aquatic ecosystems, it is crucial to understand these food web interactions. Understanding the food web interactions is important since a large part of the EFA humans obtain originate from aquatic ecosystems.

It is not only important to better understand the impacts humans have on the quality of fish but also to maintain food production and quality for human consumption. For example, Tocher (2010) discussed the need for sustainable feed sources in aquaculture to continue to supply the growing world population. This is especially important since traditional diets in aquaculture are based on fish oil and fishmeal obtained from fisheries, which in the best case have already reached their sustainable limit. Bell, Ghioni and Sargent (1994) argued that dietary fatty acid composition closely resembling freshwater invertebrates might bring health benefits for growth and development in *Salmo salar*. Knowing how fatty acids and lipids are transferred from primary producers to higher levels in the food chain contributes in solving these challenges.

To identify what the diet of an animal is, it is possible to analyse stomach content. However, this is often imprecise and biased to certain parts or tissues which are less digestible (Iverson, Field, Don Bowen, & Blanchard, 2004). On top of this it is often impractical or impossible to analyse stomach content for small organisms e.g., zooplankton. Another, possibly better way of analysing diet is to use FA biomarkers. These biomarkers are commonly used, due to the conservative transfer on higher trophic levels (Dalsgaard, St. John, Kattner, Müller-Navarra, & Hagen, 2003). Conservative transfer is the process in which FA molecules are transferred from one organism to another without change.

Biomarkers can be used to increase insight in food-webs and the transfer of nutrients between predator and prey. The ideal biomarker is not harmful to the organisms, metabolically stable, transferred without change, unique and easy to identify (Dalsgaard et al., 2003). However these ideal biomarkers are rare and researchers often have to work with less ideal biomarkers, such as FA composition (Dalsgaard et al., 2003). An advantage of FA biomarkers is that FA represent time-integrated diet intake, and which FA are most valuable, due to selective retainment in certain tissues (Arts & Wainman, 1998). But there can be substantial variation in FA metabolism between lipid classes, organisms, tissues and FA groups (Taipale, Kainz, & Brett, 2011). In addition FA extraction, identification and quantification methods require considerable training and expertise and in most cases only are able to distinguish algae at class level (Arts & Wainman, 1998). Stable isotope labels (Fig. 3) are used as tracers e.g., for lipids, fatty acids through the food web. When used in addition to biomarkers it is possible to link the identity of the biomarker, activity i.e., labelled isotope assimilation, and biomass i.e., concentration of biomarker (Boschker & Middelburg, 2002).

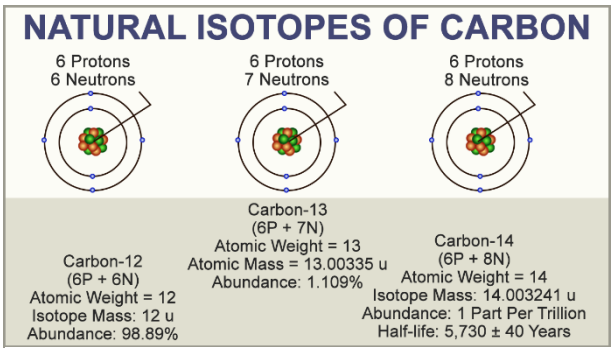

Figure 3. Isotopes, the stable isotope Carbon-13 can be used to track organic molecules. (Phatak, 2018)

The rate at which individual FA turn over can be species specific (Sinclair, 2018). This is often measured in retention time i.e., the time a certain FA stays in the organism before it is used for energy or converted. On top of this FA profiles are often connected to the metabolic condition or reproductive status of the organism, and have rarely be quantified (Dalsgaard et al., 2003). Most ecologists focussed on inorganic nutrients and paid less attention to organic nutrients (Twining et al., 2016). To further study the food web, this study focusses on an early link in the food web. Namely FA transfer between the primary producer of FA (phytoplankton) and the amphipod *Pallaseopsis quadrispinosa.* Amphipods are a potentially large food source for commercially and recreational caught fish: data of Craig (1978) showed that 60% of perch had remains of *Gammarus pulex* in their stomachs. Since amphipods can be considered an important food source it is valuable to understand their role in the food web.

P. quadrispinosa is a glacial relict species meaning they have dispersed to Northern Europe in the last glaciation (Dadswell, 1974; Deerenberg, 1962). The closest relatives of *P. quadrispinosa* can be found in Lake Baikal (Eastern Russia). *P. quadrispinosa* lives in deep oligotrophic to mesotrophic lakes with high oxygen levels (Kolodziejczyk & Niedomagala, 2009). It is a cryophilic species meaning that they typically do best in temperatures below 10 degrees Celsius. *P. quadrispinosa* has an omnivorous benthic diet which consist of, bacteria, phytoplankton, zooplankton, plant remains, detritus, pollen and mineral particles (Hill, 1988). It is an epibenthic species and occasionally moves up in the water column (Kolodziejczyk & Niedomagala, 2009). It inhabits the profundal, littoral and sublittoral zones (Kolodziejczyk & Niedomagala, 2009). Gammaridean amphipods (including *P. quadrispinosa*) themselves are critical food sources for fish and birds (Chapman, 2007).

Recent research on FA transfer showed a rapid shift in FA composition of *Daphnia magna.* In this experiment the diet was changed from a high-quality diet (containing EFA) to a low-quality diet (absent in EFA) (Taipale, Kainz, & Brett, 2011). Results show that analysis of lipid biomarkers provide a strong insight in food source that support their production (Taipale et al., 2011). The ability of an organism to bioconvert FA can alter their FA profile so that it is difficult to analyse their diet source. Bioconversion is the process in which an organism changes a molecule into a different molecule, for example an abundant FA into an EFA which the organisms lacks. It is unknown if *P. quadrispinosa* is able to bioconvert DHA from short chain w-3 PUFA and how long it will retain FA before replacing them. Furthermore Arts and Wainman (1998) argued that the integrative properties of dietary lipids are understudied and might be species specific. In general, only plants are known to synthesize n-3 and n-6 PUFA *de novo*, though there might be some protozoa and invertebrates who can do the same (Dalsgaard et al., 2003). This study aims to discover in more detail what *P. quadrispinosa's* FA profile is and the retention time of FA. Including if FA biomarkers are a good representation of the diet of *P. quadrispinosa* (i.e. if the fatty acids are transferred conservative). This will be done by feeding different diets and looking at the FA retention time (e.g. the time span of which they hold onto FA) and their ability to bioconvert FA. This is of importance to better understand the mechanisms behind a crucial food source i.e., EFA, for humans.

To investigate the differences in processing a high quality and low-quality diet two algae with different FA profiles are used in this experiment, *Diatoma tenuis* and *Acutodesmus*. *Diatoma* produces EPA and DHA whereas *Acutodesmus* cannot produce these EFA (Taipale et al., 2013). *Acutodesmus* has in return higher ALA, Stearidonic acid (SDA; 18:4ω3) and linoleic acid (LIN; 18:2ω6) concentrations (Taipale et al., 2013). It is hypothesised that fatty acid turn-over will be faster when fed a high-quality diet (*Diatoma*) compared to a low-quality diet (*Acutodesmus*). To gain more insight in the utility of FA biomarkers in *P. quadrispinosa* it is necessary to know two facets. Are the obtained FA diets stable or is *P. quadrispinosa* able to bioconvert short-chain PUFA to long-chain PUFA. The hypothesis is that *P. quadrispinosa* is not able to bioconvert DHA from short-chain w-3 PUFA (ALA and SDA) at a physiologically adequately level.

2. Methods

2.1 Phytoplankton culturing and Amphipod sampling

P. quadrispinosa was sampled using plankton dipping nets from the spring Kellolanlähde (Latitude: 61.009188° | Longitude: 25.198°) Hämeenkoski, Finland. Fifty juveniles were roughly selected on size. Size selection took place at the capturing site to prevent excessive accidental capture of adults. The juveniles were transported within 20 minutes in a bucket filled with spring water to the laboratory. Here they were placed in clean treated tap water at 8 degrees Celsius (tap water is of high quality, comparable to spring water). *P. quadrispinosa* was classified as juveniles when signs of maturity e.g. marsupium, ovary or testes are absent (Hyne, 2011), using a Leica S4E microscope at 10x magnification. After the amphipods were classified as juveniles, they were placed back into the bucket in the climate room at 8 degrees Celsius.

Phytoplankton cultures were grown at 18 °C under a 12:12 L-D cycle in MWC growth medium (Guillard & Lorenzen, 1972). When algae cultures became nutrient limited, the cultures were split in half and new MWC medium was added. One week before the beginning of the amphipod feeding experiment a carbon-13 label (5% $NaHCO_3$ of total $NaHCO_3$ in MWC medium) was added to the algae container.

2.2 Experimental setup

The juvenile caught *P. quadrispinosa* were placed in 8 buckets with 8L of treated (chlorine removed) tap water. Lipid content was assessed one day after catching and photographed using a Leica DM750 microscope with a Leica MC170 HD camera attached at 100x magnification. This was done in order to see when lipids are close to depletion (see attachment I). The juveniles were starved for one week and lipid content was assessed using the previously mentioned method.

Every second day all the water from the containers was replaced and the buckets cleaned using alcohol (ETAX A16). Hereafter the containers were thoroughly rinsed with water for preventing the growth of bacteria, which could be a possible food source for *P. quadrispinosa*.

After 7 days lipid content was checked (eye observation through Leica DM750 at 100x magnification). Consequently, the water was replaced with clean water and *P. quadrispinosa* were each placed in their own container which holds 1L of treated tap water. The in MWC medium ^{13}C labelled algae cultures of *Diatoma* and *Acutodesmus* sp. were added to the amphipod containers. Each treatment containing only a low-quality diet (*Acutodesmus*) or a high-quality diet (*Diatoma*). Algae containers were shaken before the transfer and the media with algae in it were transferred to the amphipod containers using a pipette. The amount of added algae depended on the carbon (C) content of the algae. For both species 1500mg C per L was added to the treatment. This to ensure the amphipods got the same amount of nutrients.

Samples, which consist of two *P. quadrispinosa* per treatment will be taken at 0, 7, 14, 21 and 28 days. Samples will immediately be frozen at -80°C and later freeze dried for analysis.

2.3 Fatty acid analysis

Lipids will be extracted from crushed *P. quadrispinosa* using a chloroform:methanol (2:1) mixture. Internal standards will be used; (Nonadecanoic Acid C 19:0 Weight: 0.29915 mg per ml) and (1,2-Dipentadecanoyl-sn-Glycero-3-Phosphatidychlorine C 15:0 Weight: 0.31524 mg per ml) to account for possible losses. Fatty acids will be separated into PLFA (phospholipids) and NLFA (neutral lipids) using Bond Elut LRC-SI cartridges. The resin of the cartridge will be activated using

chloroform:methanol (1:1) mixture 2 x 3ml. Subsequently total lipids will be applied to the cartridge and rinsed through the cartridge under vacuum. This was done using 5 ml chloroform to eluate neutral lipids, 5 ml acetone to eluate glycolipids and 5 ml methanol to eluate phospholipids. Toluene and sulphuric acid:methanol (1:100) were added to the lipid extracts to form fatty acid methyl esters (for further reference of the FA extraction see (Taipale et al., 2011)). ^{13}C values of individual FA were analysed at the Dept of Chemical Ecology and Ecosystem Research, University of Vienna, using a GC-C connected to an isotope ratio mass spectrometer.

2.4 Data analysis

The following fatty acids were analysed (see attachment II), using GCMS (Gas Chromatography-Mass Spectrometry). Data from the GCMS output about FA profiles were used and their retention rate were analysed. Stable isotopes ^{13}C were detected by a stable isotope tracer analysis.

To test the hypothesis "*P. quadrispinosa* is not able to bioconvert DHA from short-chain w-3 PUFA (ALA and SDA) at a physiologically adequately level". FA percentages were analysed using the principal component analysis. This showed the variance between the data and gives an overview of the "grouping" of the data (which data points are similar to each other). Permutational analysis of variance (PERMANOVA) was used to compare the treatments to each other, to visualize which diets and treatments have similar FA profiles.

To test the hypothesis: "fatty acid turn-over will be faster when fed a high-quality diet (*Diatoma tenuis*) compared to a low-quality diet (*Acutodesmus*)" the following data was analysed. The proportion of original FA to newly obtained FA from the diet was calculated for each of the sampling days (0,7,14,21,28). This was done by comparing the actual *P. quadrispinosa* FA profile to hypothetical *P. quadrispinosa* FA profiles. These were obtained by "mixing" the actual *P. quadrispinosa* diets to the two new diets. This in order to discover the proportion of the original diet (what they consume in the wild) that maximized the fit between the observed and predicted profiles. The specific steps of this mixing model are listed in (Taipale et al., 2011).

To get a clear view of the data and the results two analyses (PERMDISP, SIMPER) were performed to visualize this. To observe the integral differences between groups PERMDISP, which shows the homogeneity of multivariate dispersions was used. Using PERMDISP gave an overview of the integral differences which shows how similar individuals in groups are.

SIMPER (analysis of similarity) was used to identify which FA contribute to most of the dissimilarity within treatments.

3. Results

Results were obtained by conducting a controlled experiment. Two treatments were used which consist of a low-quality diet and one high quality diet treatment. The FA sampling was performed over a period of 28 days with measurements every 7 days. Consequently, the obtained data was analysed, and the results are displayed below.

3.1 Fatty acid composition

To illustrate the differences between the two chosen diets for the experiment FA groups were compared. The two diets *Acutodesmus* sp. (low quality) and *Diatoma* (high quality) have different fatty acid profiles. *Acutodesmus* contains ALA + SDA 17,1 +-2,7 % (i.e., C18 w3 PUFA) of total FA (fig. 4: top left). Whereas *Diatoma* contains ALA+SDA only 0,6 +- 0,1% (i.e., C18 w3 PUFA) of total FA, but it contains 32,5 +- 6,3% EPA and 0.9% DHA of total FA (fig. 4: top right). The EFA (DHA, EPA) are absent in *Acutodesmus*. Both EPA and DHA are present in the amphipods after starvation (fig. 4 bottom left). In general omega 3 FA (in particular EPA, DHA) are present in higher quantities in *Diatoma*, making this a high-quality diet. Omega 6 FA is more prevalent in *Acutodesmus sp.* than in *Diatoma* or the wild diet (fig. 4 bottom left).

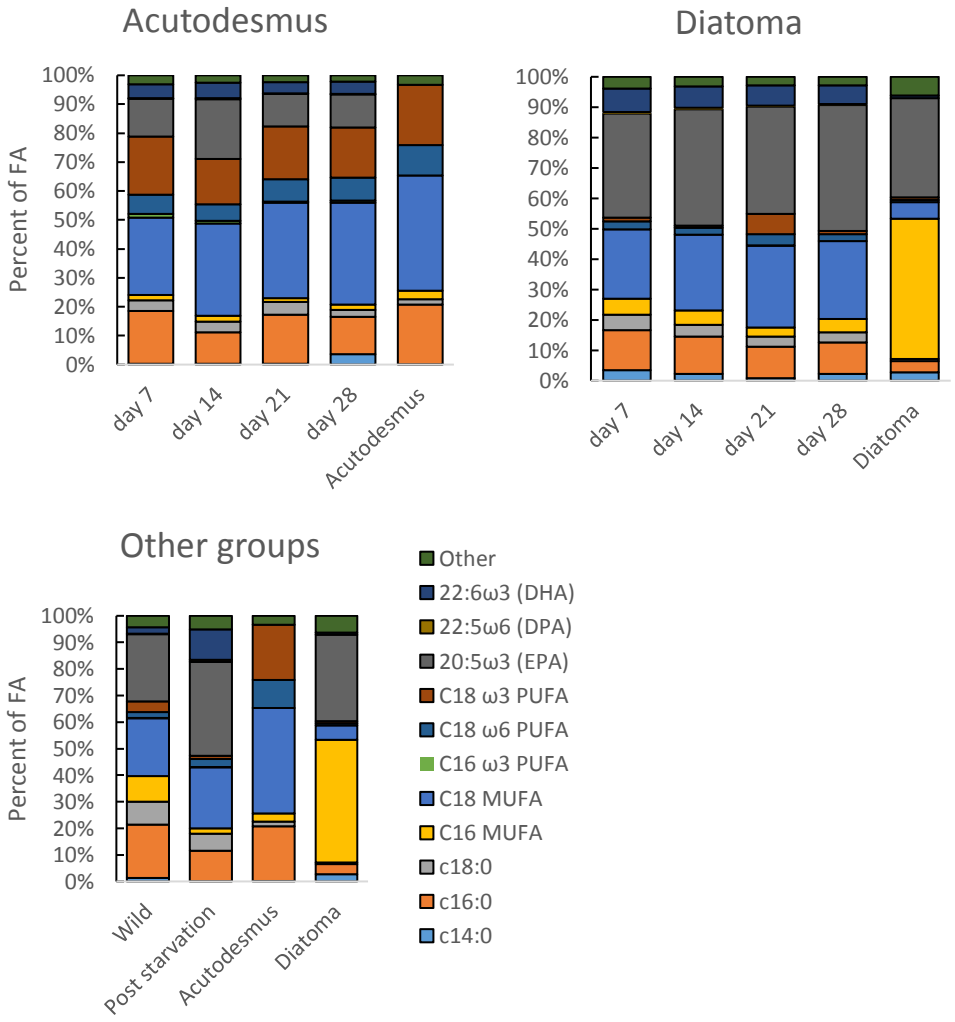

Figure 4. Comparison of the two different diets (Acutodesmus and Diatoma), the FA composition of P. quadrispinosa in the wild and the FA composition of P. quadrispinosa after starvation (initial day 0). The figure shows percentages of different FA groups compared to total FA. PUFA= Polyunsaturated fatty acids, MUFA= Monounsaturated fatty acids, other= remaining FA.

To see if starvation of the amphipods had the intended effect SIMPER analysis was performed to look at the difference within groups (Fig. 5). Initial (after starvation) shows the least variation within the treatments.

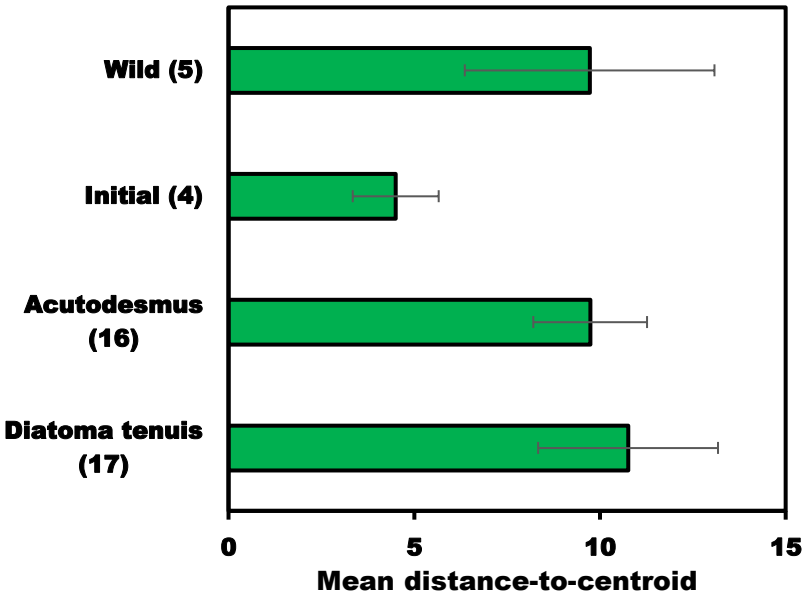

Figure 5. Visualisation of homogeneity of FA profiles within treatment groups using the statistical analysis: SIMPER. Initial FA profile (post starvation) has the highest homogeneity of the four different FA profile groups shown in this figure, meaning there was less variance between the individual samples.

3.2 Principal component analysis

Acutodesmus and *P. quadrispinosa* fed with *Acutodesmus* have a similar FA composition, as well as Diatoma and *P. quadrispinosa* fed with *Diatoma* (Fig. 6). The FA composition of the wild and the starved *P. quadrispinosa* resembles the one of *Diatoma* suggesting that the *Diatoma* diet is closer in FA composition to their wild diet than *Acutodesmus*. *Diatoma* diet contains all of the EFA whereas *Acutodesmus* lacks two (EPA and DHA) (Fig. 4).

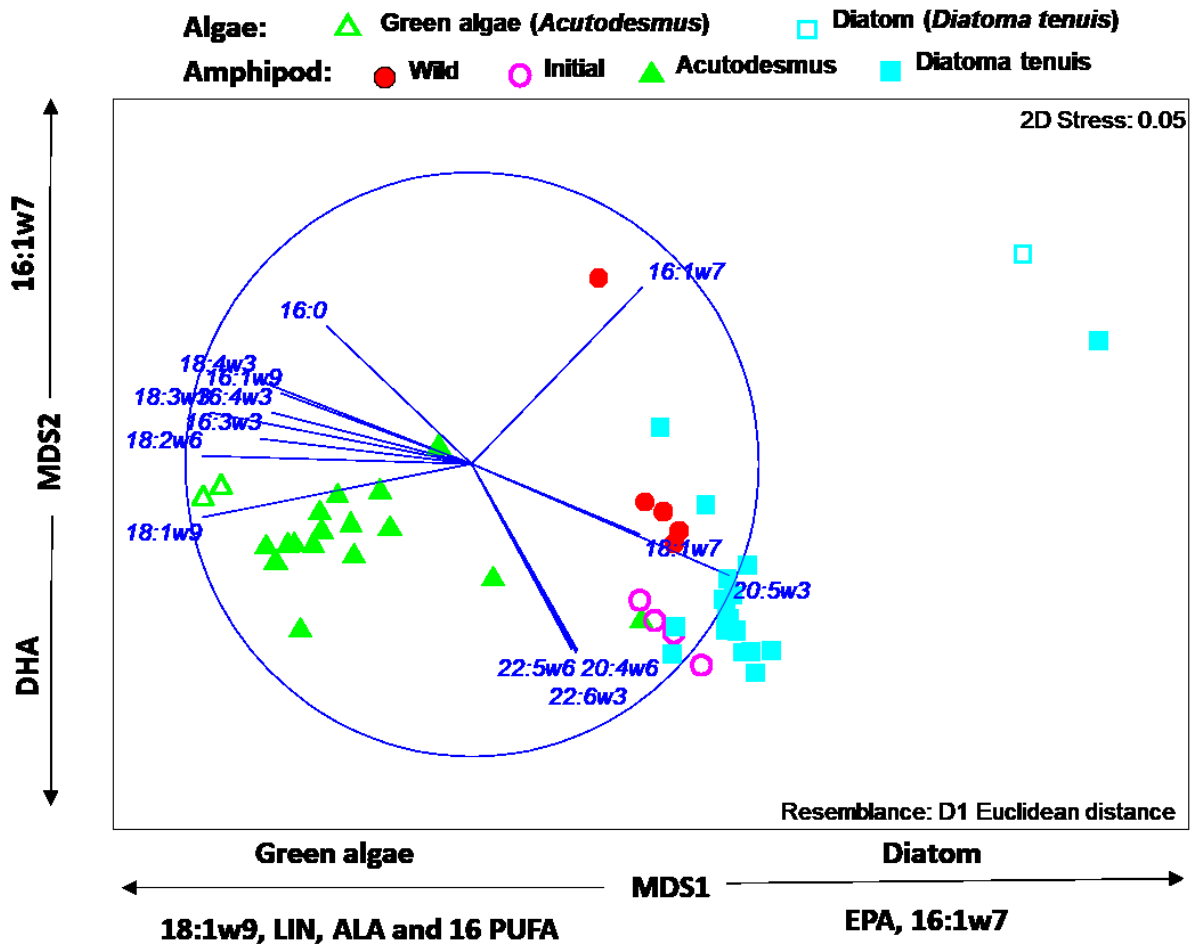

Figure 6. Principal component analysis, visualizes the similarity between treatments based on their FA profiles. Acutodesmus fed P. quadrispinosa lies close to the FA profile of the actual diet, which is the case for Diatoma fed P. quadrispinosa as well. Wild diet and initial (after starvation) FA profiles are similar to Diatoma fed P. quadrispinosa as well as the actual FA profile of Diatoma.

3.3 Shift in FA composition

During the experiment *P. quadrispinosa* obtained FA from their diet and incorporated them into their FA profile. In both treatments a change in FA composition is visible after 7 days. *P. quadrispinosa* fed the *Acutodesmus* diet changed 67% of the FA composition after 7 days. Between day 7, 14 and 21 the FA composition did drop 6%. Between day 21 and 28 FA composition did not change. When fed a high-quality diet (*Diatoma*) FA composition did not change more than 11% of their original diet within 28 days (Fig. 7). After 7 days 10% of the FA composition had changed and fluctuated to 9%- 3% between day 14 and 21 but returned 10% at day 28.

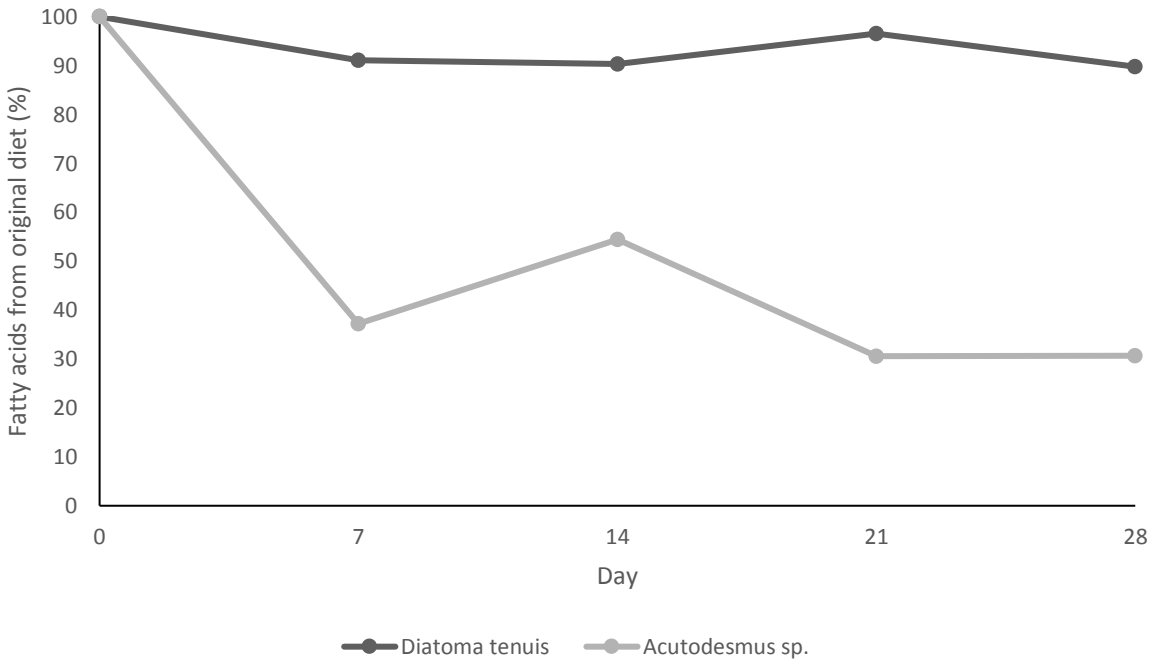

Figure 7. Shift in FA composition when fed Diatoma, Acutodesmus after starvation. Samples days are listed on the X axis, FA of original diet are listed on the Y axis. Day 0 is 100% of original FA profile in the subsequent days the FA profile differs from the original (day 0).

3.4 Stable isotope signature

P. quadrispinosa fed with *Diatoma* turns over LIN completely within 7 days (phospholipid fraction) after consuming the new diet (Fig. 8). DHA, EPA and ALA turn over at a slower rate but keep replacing more of their FA with FA obtained from *Diatoma*. After four weeks (day 28), 79% EPA and 86% DHA in the phospholipid fraction have been replaced by EPA and DHA obtained from the diet.

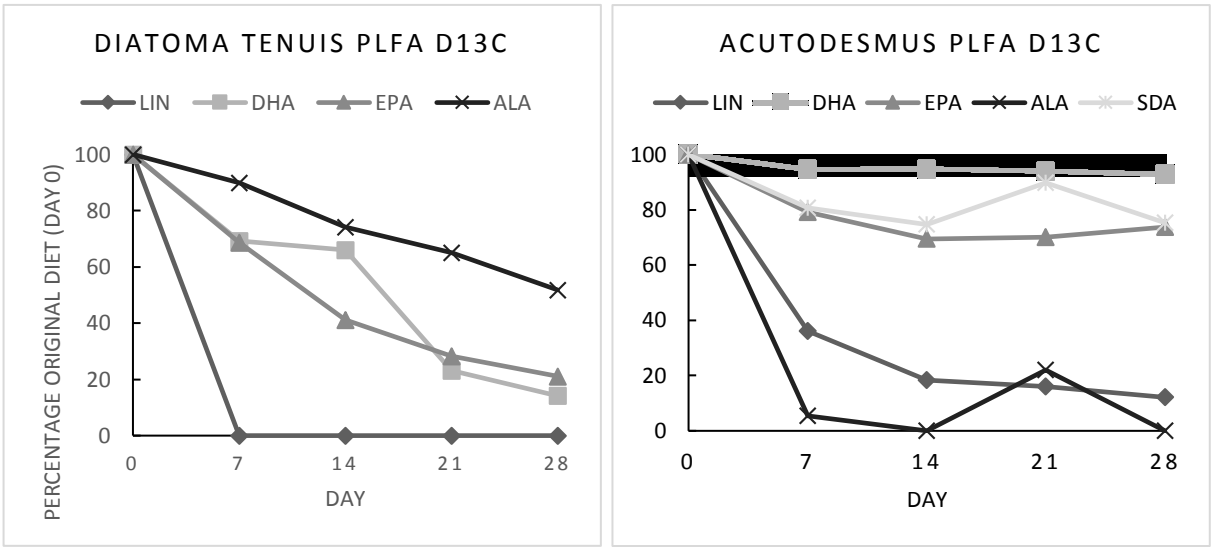

Figure 8. Stable isotope signatures in Phospholipid fraction of FA in P. quadrispinosa fed Diatoma(left) and fed Acutodesmus (right). Visualizes the percentage of original FA retained with 100% at day 0 (start). Left: EPA and DHA is rapidly replaced with ^{13}C labelled EPA and DHA, due to the availability in the Diatoma diet. Right: P. quadrispinosa obtained ^{13}C labelled DHA and EPA at most 7% and 31% respectively. This was not available in the Acutodesmus diet and thus must have been derived from bioconverting.

P. quadrispinosa fed *Acutodesmus* could not obtain EPA and DHA from the diet. EPA and DHA are not rapidly replaced and have the highest percentage turnover in the first 7 days. SDA does not have a high turnover rate, while it is present in the diet. After 21 days 7.2% DHA was replaced and 24.7% EPA was replaced.

LIN and ALA are replaced with labelled LIN and ALA quicker and ALA is replaced completely after 28 days while 12% of LIN is still retained (Fig. 8).

4. Discussion

The aim of this research was to gain more insight in fatty acid metabolism in the amphipod *P. quadrispinosa*. This was done by conducting a controlled experiment. The data analysis showed significant results which are discussed below.

FA profiles of the diets together with the wild *P. quadrispinosa* as well as *P. quadrispinosa* after starvation were analysed. FA profiles differed greatly between *Acutodesmus* and *Diatoma*, EPA and DHA were absent in the *Acutodesmus* diet while present in the *Diatoma* diet (Fig. 4). A principal component analysis was performed to visualize how closely *P. quadrispinosa* did resemble their diet (Fig. 6). The FA composition of *Diatoma*, wild, after starvation and *Diatoma* fed *P. quadrispinosa* all had similar FA profiles. This in contrast to *Acutodesmus* and *P. quadrispinosa* fed *Acutodesmus*. Starving the amphipods for 14 days prior to the feeding experiment helped to increase homogeneity in the FA profiles (Fig. 5).

Within 7 days the FA profile of *P. quadrispinosa* changed to closely resemble their diet (Fig. 7), and the data suggests that it reached a new stable FA composition after 7-14 days. It was observed that *P. quadrispinosa* actively retained EFA when EFA were absent in their diet, this shows the importance of the EFA. In particular the retention of DHA, SDA and EPA when fed with *Acutodesmus* (poor) diet after starvation. When fed the *Diatoma* (high quality) diet the results show that EPA, DHA and SDA are replaced rapidly, and *P. quadrispinosa* continued to replace more during the four-week experiment. This can be explained due to the availability of these EFA in the diet, which makes it unnecessary to retain the "old" EFA, since they can easily be replaced with EFA from the diet. This retention of EFA is also the case for daphnia (Taipale et al., 2011).

EFA turnover is higher when *P. quadrispinosa* is fed a high-quality diet (*Diatoma*), 79% EPA and 86% DHA was replaced after 21 days, as shown in figure 8. When fed the low-quality diet (*Acutodesmus*) the turnover rate is much lower (7% DHA and 24% EPA). This confirms the hypothesis "fatty acid turn-over will be faster when fed a high-quality diet (*Diatoma*) compared to a low-quality diet (*Acutodesmus*), and FA will be replaced fully when fed a high-quality diet in contrast to the low-quality diet".

As hypothesized the results indicate that *P. quadrispinosa* is not able to bioconvert DHA from short-chain w-3 PUFA (ALA and SDA) at physiologically adequate level. the results show that *P. quadrispinosa* can bioconvert FA from precursors (ALA → DPA → EPA → DHA) but the data also suggests that *P. quadrispinosa* cannot do this in high enough quantities to be physiologically sustainable (Fig. 8). Their bioconverting capabilities are shown by the ^{13}C label detected in EPA and DHA in the *Acutodesmus* (poor diet) treatment. In this treatment *P. quadrispinosa* could not obtain EPA and DHA from the diet and had to bio convert them from available precursors (ALA, DPA). Since *P. quadrispinosa* can only convert 7% of DHA and +- 30% EPA from the PLFA fraction within 4 weeks (Fig. 8) this is arguably not enough to be physiologically sustainable. When FA are used as a biomarker in *P. quadrispinosa* it should be noted that slight alterations due to bio converting are possible which should be corrected for. FA biomarkers have been used before in freshwater microalgae (S. Taipale et al., 2013), zooplankton (Desvilettes, Bourdier, Amblard, & Barth, 1997) including many more organisms and is now possible for the crustacean *P. quadrispinosa* as well.

To survive periods with low amounts of food, benthic invertebrates frequently store energy in the form of lipids during periods when food is abundant (Cavaletto & Gardner, 1998). The lipid dynamics also depend on the reproductive strategy of the organisms as well as their activity. Equally so as

when the phytoplankton bloom becomes available to the organisms (e.g. diatom sedimentation). Taipale et al., (2011) showed that daphnia retain EFA and discussed that this could restrain population fluctuations in ecosystems. Due to the buffer of nutrients within the organism, which is available to organisms higher in the food chain. The results suggest that *P. quadrispinosa* can provide the same effect since the amphipod retains FA it has obtained for at least 4 weeks and possibly much longer. In particular EFA are retained at high percentages of the original diet (+- 90% for DHA and +- 70% for EPA) when fed a poor-quality diet.

FA had different retention times depending on the diet, *P. quadrispinosa* fed *Diatoma* rapidly replaced LIN whereas *Acutodesmus* fed *P. quadrispinosa* retained more LIN. LIN is present in higher quantities in *Diatoma* compared to *Acutodesmus*, the abundance of this FA in the diet might explain the high turnover rate.

Future research on fatty acid metabolism in aquatic food webs can be performed in more detail using the fundamental information gained from this research. Retention time of fatty acids in P. quadrispinosa depends on available diet. 79% EPA and 86% DHA turnover when high quality food is available and 7% DHA and 24% EPA when only low quality (lacking essential fatty acids) food is available. It should be noted that due to the slim bioconverting capabilities of *P. quadrispinosa* the difference in fatty acid percentages should be accounted for when using fatty acids as biomarkers.

The controlled experiment went according to the described methods. The method used to get answers on the research questions was the correct method. Results were significant and since the experiment was performed in a mesocosm the light, temperature and food parameters were controlled. Enough replicates were used, though more replicates would be better to have. This was not possible due to the expenses involved in analysing the fatty acid content and ^{13}C label. It should be noted that during the experiment a bacterium was observed on the exoskeleton of *P. quadrispinosa* fed *Diatoma*. This did not seem to have an immediate effect on behaviour or survival rate. But could have a slight effect on the amphipods that was not visible in their behaviour or the data.

5. Conclusion

The goal of this research was to gain more insight in fatty acid metabolism in the amphipod *P. quadrispinosa*. This to increase the knowledge base for future research on aquatic food webs. To fill the gaps in knowledge in this subject an experiment was performed to indicate whether fatty acid can be used as biomarkers in *P. quadrispinosa*. The results show that it is possible to use fatty acid as biomarkers. This is due to their conservative transfer. *P. quadrispinosa* is able to bioconvert small quantities of essential fatty acids, though not enough to be physiologically sustainable.

An important aspect in answering the main question is if *P. quadrispinosa* actively retained essential fatty acids. Results showed that *P. quadrispinosa* did retain essential fatty acids when they were lacking in the diet.

Having answers on these questions is crucial for further research on fatty acid metabolism in *P. quadrispinosa*. Fatty acid biomarkers can be used to gain insight in this part of the food web. Further research is required to better understand the differences between juveniles, adult males and adult females and their seasonal lipid changes. Amphipods might act as one of the first buffers of essential fatty acids in aquatic ecosystems, transferring essential fatty acids to higher levels in the food chain when essential fatty acids from primary producers are not available.

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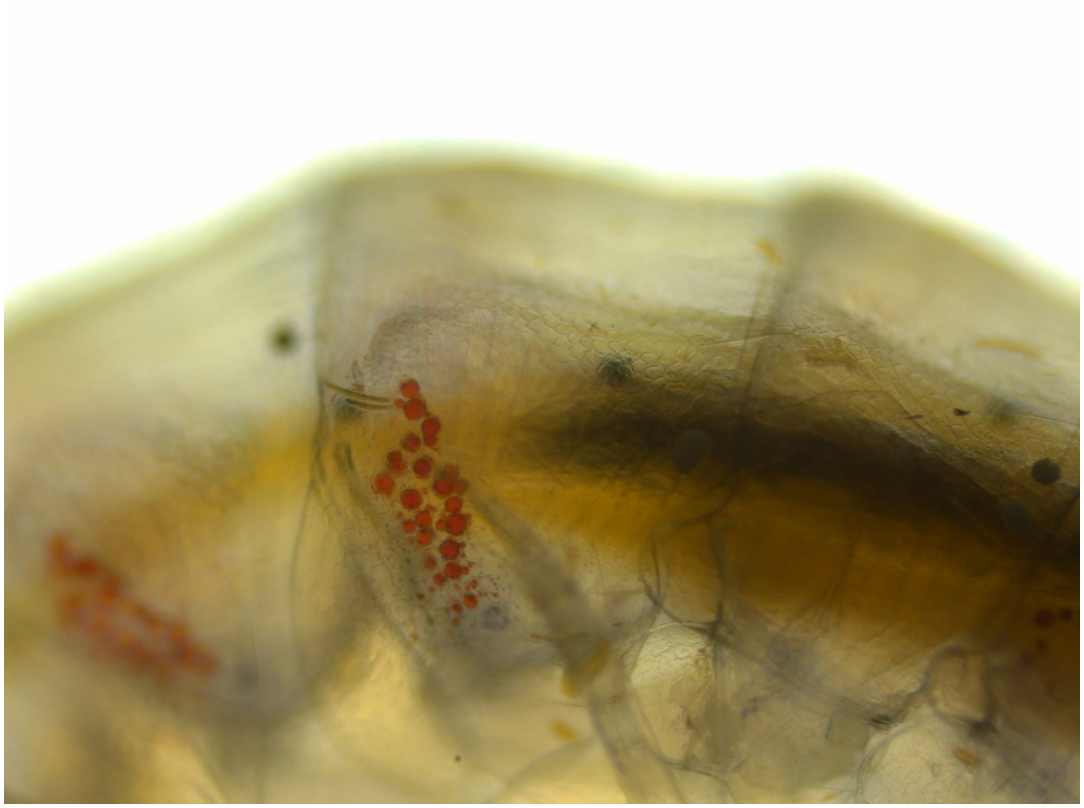
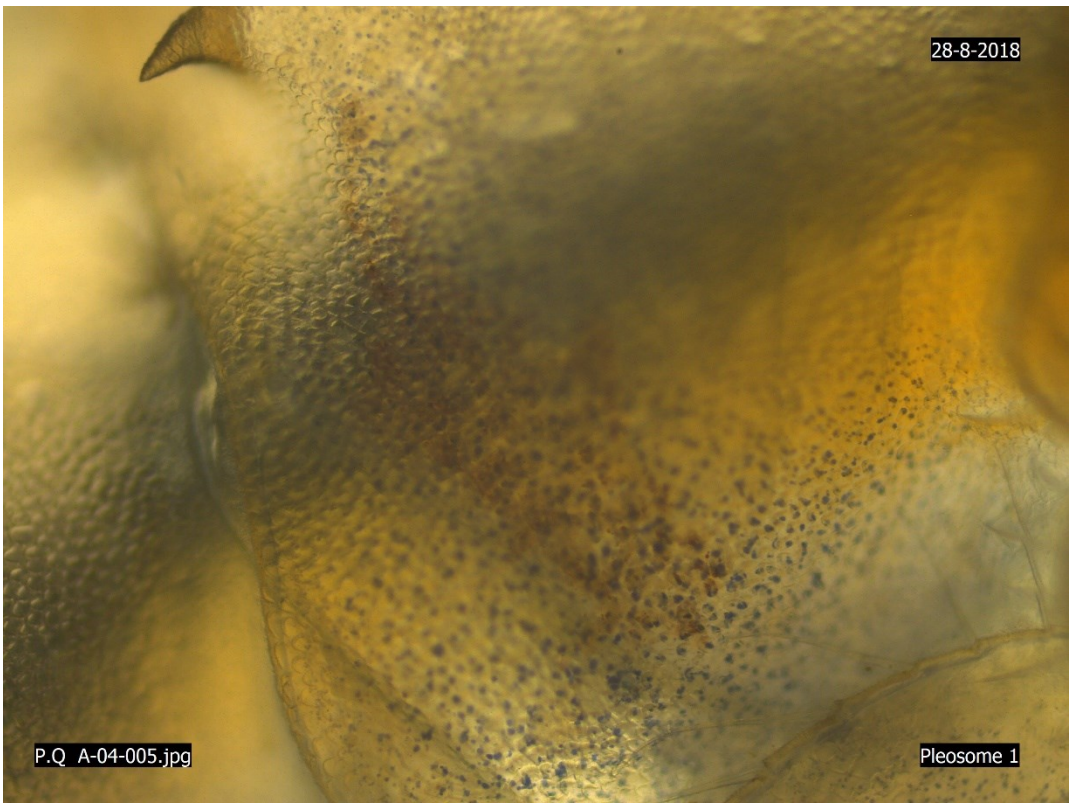
Attachments

I. Lipid droplets

A. Visualisation of Lipid droplets in the pleosome of P. quadrispinosa before starvation. Large drops visible in red, small individual.



B. Visualisation of Lipid droplets in pleosome of P. quadrispinosa after starvation. Lipid drops small and not bright, large individual. Note: top left corner 1 of P. quadrispinosa its spines.



II. Analysed fatty acids

A. List of analysed fatty acids.

14:0	17:1w5	18:0	20:0
16:0	16:2w4	18:1w9	20:4w6
16:1w9	16:3w6	18:1w7	20:5w3
16:1w7	16:3w4	18:2w6	22:0
17:0	16:3w3	18:3w3	22:5w6
17:1w7	16:4w3	18:4w3	22:6w3