

The stable isotopic composition of *Daphnia* and their ephippia

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Experimental assessment of environmental influences on the stable isotopic composition of *Daphnia pulicaria* and their ephippia

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Received: 27 October 2014 – Accepted: 4 December 2014 – Published: 4 February 2015

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Published by Copernicus Publications on behalf of the European Geosciences Union.

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12, 2573–2606, 2015

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Abstract

The stable isotopic composition of fossil resting eggs (ephippia) of *Daphnia* spp. is being used to reconstruct past environmental conditions in lake ecosystems. However, the underlying assumption that the stable isotopic composition of the ephippia reflects the stable isotopic composition of the parent *Daphnia*, of their diet and of the environmental water have yet to be confirmed in a controlled experimental setting. We performed experiments with *Daphnia pulicaria* cultures, which included a control treatment conducted at 12 °C in filtered lake water and with a diet of fresh algae, and three treatments in which we manipulated the stable carbon isotopic composition ($\delta^{13}\text{C}$ value) of the algae, stable oxygen isotopic composition ($\delta^{18}\text{O}$ value) of the water, and the water temperature, respectively. The stable nitrogen isotopic composition ($\delta^{15}\text{N}$ value) of the algae was similar for all treatments. At 12 °C, differences in algal $\delta^{13}\text{C}$ values and in $\delta^{18}\text{O}$ values of water are reflected in those of *Daphnia*. The differences between ephippia and *Daphnia* stable isotope ratios were similar in the different treatments ($\delta^{13}\text{C}$: $+0.2 \pm 0.4\text{‰}$ (SD); $\delta^{15}\text{N}$: $-1.6 \pm 0.4\text{‰}$; $\delta^{18}\text{O}$: $-0.9 \pm 0.4\text{‰}$) indicating that changes in dietary $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values of water are passed on to these fossilizing structures. A higher water temperature (20 °C) resulted in lower $\delta^{13}\text{C}$ values in *Daphnia* and ephippia than in the other treatments with the same food source and in a minor change in the difference between $\delta^{13}\text{C}$ values of ephippia and *Daphnia* (to $-1.3 \pm 0.3\text{‰}$). This may have been due to microbial processes or increased algal respiration rates in the experimental containers, which may not affect *Daphnia* in natural environments. There was no significant difference in the offset between $\delta^{18}\text{O}$ and $\delta^{15}\text{N}$ values of ephippia and *Daphnia* between the 12 °C and 20 °C treatments, but the $\delta^{18}\text{O}$ values of *Daphnia* and ephippia were on average 1.2‰ lower at 20 °C compared with 12 °C. We conclude that the stable isotopic composition of *Daphnia* ephippia provides information on that of the parent *Daphnia* and of the food and water they were exposed to, with small offsets between *Daphnia* and ephippia relative to variations in *Daphnia* stable isotopic composition reported from downcore studies. However, our experiments also indicate

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that temperature may have a minor influence on the $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of *Daphnia* body tissue and ephippia. This aspect deserves attention in further controlled experiments.

1 Introduction

The strong, positive relationships between the stable carbon isotopic composition (expressed as $\delta^{13}\text{C}$ values) of organisms and that of their diet can allow the identification of the autotrophic sources of organic matter at the base of a food web (DeNiro and Epstein, 1978; Vander Zanden and Rasmussen, 1999; McCutchan et al., 2003). Likewise, stable nitrogen isotope ratios (expressed as $\delta^{15}\text{N}$ values) can be used to estimate the trophic position of consumers in food webs (DeNiro and Epstein, 1981; Minagawa and Wada, 1984), and stable oxygen isotope ratios (expressed as $\delta^{18}\text{O}$ values) have been found to reflect those of the water in the environment organisms live in (Hobson, 2008; Soto et al., 2013).

Approaches are continuing to be developed that apply stable isotope ratio analysis to chitinous remains of aquatic invertebrates preserved in lake sediments (Heiri et al., 2012; Leng and Henderson, 2013). For example, the $\delta^{13}\text{C}$ values of fossil head capsules of benthic larvae of non-biting midges (Chironomidae) and of the remains of water fleas of the genus *Daphnia* (Cladocera) have been used to investigate past changes in carbon cycling and energy pathways in lake food webs (Perga, 2011; Wooller et al., 2012; van Hardenbroek et al., 2013; Belle et al., 2014; Frossard et al., 2014). The $\delta^{15}\text{N}$ values of chironomid head capsules and of *Daphnia* resting eggs (ephippia) have also been examined to investigate changes in nitrogen sources in an arctic lake (Griffiths et al., 2010). Past variations in lake water $\delta^{18}\text{O}$ values have been reconstructed by analyzing the $\delta^{18}\text{O}$ values of fossil chironomid head capsules (Wooller et al., 2004; Verbruggen et al., 2010b), and a correspondence has been found between $\delta^{18}\text{O}$ values of lake water and of chironomid head capsules and *Daphnia* ephippia buried in surface sediments (Verbruggen et al., 2011).

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Daphnia can occur in high abundances and often dominate the zooplankton community in lakes (Lampert, 2011). Being first order consumers of algae, bacteria and detritus (Geller and Müller, 1981; Gophen and Geller, 1984; Kamjunke et al., 1999; Lampert, 2011), they form an important link between primary production and the higher orders of the pelagic food web. This makes *Daphnia* particularly suited for ecological investigations of freshwater ecosystems and food webs using stable isotopes. While *Daphnia* usually reproduce parthenogenetically, they may also reproduce sexually. Environmental cues such as food availability, photoperiod and population density (Kleiven et al., 1992; Cáceres and Tessier, 2004) may trigger sexual reproduction, upon which eggs are formed enclosed by rigid sheaths (ephippia). The chitinous ephippia are found abundantly in a wide range of lake sediment types and remain well preserved in sediments hundreds to thousands of years old (Szeroczyńska and Samarja-Korjonen, 2007). Since the chemical composition of chitinous invertebrate remains stays largely unchanged even in fossils more than ten thousand years old (Miller et al., 1993; Verbruggen et al., 2010a), they are believed to retain their isotopic composition after deposition (Heiri et al., 2012). Therefore, ephippia may provide material for reconstructing the past stable isotopic composition of *Daphnia* in lakes, and, consequently, for investigating past conditions in aquatic food webs (e.g. Wooller et al., 2012; van Hardenbroek et al., 2013, 2014).

The use of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of organisms to infer likely organic carbon and nitrogen sources relies heavily on assumptions regarding the difference between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of organisms and their diet ($\Delta^{13}\text{C}$, $\Delta^{15}\text{N}$). There is a need for more controlled laboratory studies investigating $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ (Martínez del Rio et al., 2009), and the relationships between the $\delta^{18}\text{O}$ values of organisms and those of environmental water (Rubenstein and Hobson, 2004). $\Delta^{13}\text{C}$, which is generally assumed to be between 0 and +1‰ for a range of animals, including invertebrates (DeNiro and Epstein, 1978; McCutchan et al., 2003), has been studied for chironomids under controlled laboratory conditions (Goedkoop et al., 2006; Wang et al., 2009; Heiri et al., 2012; Frossard et al., 2013) and ranges from -0.8 to $+1.2$ ‰. For *Daphnia magna*,

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$\Delta^{13}\text{C}$ values range from +1.7 to +3.1‰ (Power et al., 2003). $\Delta^{15}\text{N}$, which is usually assumed to be between +3 and +4‰ (DeNiro and Epstein, 1981; Minagawa and Wada, 1984) ranges from -1.5 to +3.4‰ for chironomids (Goedkoop et al., 2006; Wang et al., 2009; Heiri et al., 2012) and from +1 to +6‰ for *Daphnia* (Adams and Sterner, 2000; Power et al., 2003; Matthews and Mazumder, 2008). Measurements of *Daphnia* and ephippia collected in the field have been used to infer that *Daphnia* exoskeletons have 0.8‰ lower $\delta^{13}\text{C}$ and 7.9‰ lower $\delta^{15}\text{N}$ values than whole *Daphnia* (Perga, 2010) whereas no clear differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between *Daphnia* and ephippia were apparent (Perga, 2011). To date, no controlled experiments investigating the offset between whole body tissue and ephippia have been published for *Daphnia*. Quantifying this offset is essential for further development of palaeoecological approaches based on stable isotope analyses on *Daphnia* remains and for interpreting results from the fossil record. In terms of oxygen, the $\delta^{18}\text{O}$ values of aquatic invertebrates is strongly and positively related to the $\delta^{18}\text{O}$ values of local precipitation and the water in which the invertebrates live (Wang et al., 2009; Nielson and Bowen, 2010; van Hardenbroek et al., 2012; Soto et al., 2013). To our knowledge, no controlled experiments have been performed examining the relationship between $\delta^{18}\text{O}$ values of environmental water and *Daphnia*, or their ephippia.

We present results from an experiment developed to examine the relationships between the $\delta^{13}\text{C}$ values of diet and the $\delta^{18}\text{O}$ values of environmental water, and the $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values of *Daphnia*. The experiment was specifically designed to examine whether offsets in $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values exist between *Daphnia* and their ephippia. Furthermore, we investigated whether the stable isotopic compositions of *Daphnia* and their ephippia are influenced by temperature by performing the experiment at two different temperatures.

2 Methods

2.1 *Daphnia* cultivation

Three ex-ephippial *Daphnia pulicaria* clones (LC PUL 53, 99 and 101; Möst, 2013) from Lower Lake Constance (Switzerland) that showed extensive ephippia production in culture in pre-tests were selected for the experiment. For each clone 20 neonate *Daphnia* (< 48 h old) were grown in 2.5 L batch cultures prior to the experiment. From these batch cultures 7–8 second to third clutch neonates (< 48 h old) were transferred to 180 mL jars, containing 160 mL of filtered lake water (natural abundance or labeled water, according to treatment conditions described below). The lake water was filtered with 0.45 μm glass fiber filters (Sartorius Stedim AG, Switzerland). Initially, *Daphnia* were fed three times per week with fresh algae, concentrated to an equivalent of 1 mg CL^{-1} . After day 21 of the experiment, the amount of food was doubled because the number of *Daphnia* in most jars exceeded 30 individuals. Experimental water was refreshed once per week and ephippia (if present) were retained in the cultures. Due to potentially higher productivity and evaporation, the water was refreshed twice per week in Treatment 4 (20 °C).

2.2 Food and water sources in the experiment

Three weeks before the experiment two 1 L chemostats were started simultaneously to produce the algae (*Acutodesmus obliquus*, Turpin) to be used as food for *Daphnia* in the experiment. The algae were cultivated in “WC”-medium (Guillard, 1975). For one of the chemostats, 45 % of the sodium bicarbonate in the medium (5.67 mg L^{-1} of 12.6 mg L^{-1}) was replaced by sodium bicarbonate containing 99.9 % ^{12}C (Sigma Aldrich, USA), lowering the $\delta^{13}\text{C}$ values of the algae from this chemostat by on average 1.8 ± 1.2 (one standard deviation (1 SD))‰ (see Sect. 3). Once per week, the chemostat-grown algae were harvested, centrifuged (5000 rpm) to remove residual medium, stored at 9 °C in the dark and used to feed the *Daphnia* during the follow-

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ing week. Seven days before the start of the experiment 250 L of lake water were collected from Lake Greifensee (Switzerland) (pH 8.0, TP 0.04 mgL⁻¹, TN 1.6 mgL⁻¹; data provided by the Cantonal Bureau for Waste, Water, Energy and Air (AWEL, Zürich; www.awel.zh.ch)). This water was stored in the dark at 12 °C for the duration of the experiment. 50 L of this water were stored in a separate container and 0.9 mL of water containing 97 % ¹⁸O (Sigma Aldrich, USA) were added to increase the δ¹⁸O value of the water with 5.6 ‰ relative to the unlabeled water (see Sect. 3).

2.3 Experimental design

The experiment consisted of four cultivation treatments: a control treatment in which *Daphnia* were cultivated in untreated, filtered lake water at 12 °C on a diet of fresh chemostat-grown algae (Treatment 1), and treatments with conditions identical to Treatment 1, with the exception of the algae in Treatment 2, which had 1.8 ± 1.2 (1 SD) ‰ lower δ¹³C values. The culturing water in Treatment 3 had δ¹⁸O values that were 5.6 ‰ higher than in the other treatments, and Treatment 4 had a temperature (20 °C) that was higher than the other treatments.

Each treatment consisted of 30 glass jars which were sterilized using an autoclave. Prior to the experiment, each glass jar was assigned to one of three replicate groups (A, B, C). The neonate *Daphnia* were evenly distributed in the jars to ensure that every experimental replicate group contained 10 jars, with 3–4 jars per clone (Fig. 1). All the jars for a given treatment were held in one large tray, and the jars within each treatment were evenly distributed within the trays (Fig. 1). The trays were held in the dark, in temperature controlled incubators.

The experiment was designed to assess the following: (a) the effect of a change in algal δ¹³C values on those of *Daphnia* and their ephippia (Treatment 2), (b) the effect of a change in environmental water δ¹⁸O values on those of *Daphnia* and their ephippia (Treatment 3), (c) the effect of a difference in temperature (i.e. 12 and 20 °C) on the δ¹³C, δ¹⁵N and δ¹⁸O values of *Daphnia* and their ephippia (Treatment 4), and (d) the offset between *Daphnia* and ephippia in terms of their δ¹³C, δ¹⁵N and δ¹⁸O

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values (Treatments 1–4). Statistical analyses were performed with the PAST software package, version 1.97 (Hammer et al., 2001), except for tests used to compare the algae from both chemostats. To account for repeated measures, linear mixed effects models (LME) were applied, fitting a random intercept for each probing date with the `lme` function in the `nlme` package in the R statistical package (R Core team, 2013). Significance was analyzed using an F test.

2.4 Sample collection

After the weekly harvest, a small portion of algae from each chemostat was rinsed with deionized water and centrifuged five times to remove the culturing medium. The concentrated algae were freeze dried and a small aliquot (150–200 μg) was loaded into tin cups (6 \times 4 mm, Lüdi Swiss, Switzerland) to measure the $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of the algae ($\delta^{13}\text{C}_{\text{algae}}$, $\delta^{15}\text{N}_{\text{algae}}$ and $\delta^{18}\text{O}_{\text{algae}}$). In each treatment, one jar was assigned to monitoring variation in $\delta^{18}\text{O}$ values of the water ($\delta^{18}\text{O}_{\text{water}}$). Once per week, before discarding the water, 12 mL were transferred to a 12 mL glass vial with no head space (Labco, UK) and stored in the dark at 7°C. Every second sample was analyzed for $\delta^{18}\text{O}_{\text{water}}$ values. Every third week a sample of the water in the storage barrels was collected, stored and measured for $\delta^{18}\text{O}_{\text{water}}$ values.

The experiment was terminated after 62 days. He and Wang (2006) have demonstrated that *Daphnia* carbon turnover rate is 11–36 % per day, which suggests that after 62 days our *Daphnia* likely had achieved isotopic equilibrium with the experimental diet and water. *Daphnia* and ephippia were harvested and pooled according to treatment (1–4) and replicate group (A, B, C). Adult *Daphnia* were hand-picked from a Bogorov sorting tray (Gannon, 1971) with a fine forceps under a binocular and freeze-dried, after which they were loaded into tin cups (6 \times 4 mm, Lüdi Swiss, Switzerland; ~ 10 to 12 individuals per measurement) for analysis of $\delta^{13}\text{C}_{\text{Daphnia}}$, $\delta^{15}\text{N}_{\text{Daphnia}}$ and $\delta^{18}\text{O}_{\text{Daphnia}}$ values. For each treatment replicate group, three samples were prepared and measured, resulting in 36 measurements for each chemical element. Ephippia were collected and

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(ASIF) at the University of Alaska, Fairbanks. The analytical precisions for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values are expressed as 1 SD from the mean based on the results from multiple ($n = 13$) analyses of a laboratory standard (peptone), and were ± 0.2 and $\pm 0.1\%$, respectively. The $\delta^{18}\text{O}$ values of the water samples were measured on an on-line pyrolysis, thermochemical reactor elemental analyzer (TCEA) (Finnigan ThermoQuest) coupled to a continuous flow (Conflo III) IRMS (Finnigan MAT Delta V) at the ASIF. Analytical precision is expressed as 1 SD from the mean based on the results from multiple ($n = 3$) analyses of a laboratory standard (doubly labeled water; $\pm 0.3\%$). The $\delta^{18}\text{O}$ values of the algae, *Daphnia* and ephippia were measured using the same techniques and instruments as used for the water samples. Analytical precision based on replicate ($n = 12$) laboratory standard measurements (benzoic acid, Fisher Scientific, Lot No 947459) was $\pm 0.4\%$. Stable isotopic compositions are expressed in standard delta (δ) notation in $\%$ relative to V-PDB for $\delta^{13}\text{C}$ values, AIR for $\delta^{15}\text{N}$ values and V-SMOW for $\delta^{18}\text{O}$ values.

3 Results

3.1 Food and water

The $\delta^{13}\text{C}_{\text{algae}}$ values from both chemostats showed some variation with time (Fig. 2). On all sampling dates except the first, the algae cultured on ^{13}C -depleted medium had lower $\delta^{13}\text{C}_{\text{algae}}$ values than the standard algae (Fig. 2). As a consequence, the mean $\delta^{13}\text{C}_{\text{algae}}$ value for the culture grown using ^{13}C -depleted medium ($-20.6 \pm 1.84\%$) was $1.8 \pm 1.2\%$ ($n = 9$) lower than the mean $\delta^{13}\text{C}_{\text{algae}}$ of the standard algae ($-18.8 \pm 2.4\%$), and this difference was statistically significant (LME, $F_{(1,8)} 18.04$, $p < 0.005$). There was no statistically significant difference between the algae cultures in terms of $\delta^{15}\text{N}$ values (standard algae $2.5 \pm 0.3\%$, ^{13}C -depleted algae $2.2 \pm 0.3\%$; $F_{(1,8)} 4.58$, $p > 0.05$), $\delta^{18}\text{O}$ values (standard algae $13.4 \pm 1.0\%$, ^{13}C -depleted algae $14.6 \pm 1.1\%$; $F_{(1,7)} 5.43$, $p >$

0.05), or atomic C:N ratios (standard algae 6.4 ± 1.3 , ^{13}C -depleted algae 6.5 ± 1.3 ; $F_{(1,8)} 0.18$, $p > 0.05$) (Fig. 2).

The addition of ^{18}O -enriched water led to an increase in $\delta^{18}\text{O}_{\text{water}}$ values in the storage barrels by 5.6‰ ($\delta^{18}\text{O}$ value of $-3.4 \pm 0.1\text{‰}$, $n = 3$) relative to the non-labeled water ($\delta^{18}\text{O}$ value of $-9.0 \pm 0.1\text{‰}$, $n = 3$) (Fig. 3). The $\delta^{18}\text{O}_{\text{water}}$ values from the experimental jars in Treatment 1, 2 and 4 were not significantly different (One-way ANOVA, $F_{(2,2)} 30.1$, $p > 0.05$) between the three treatments throughout the experiment, and the mean was $-8.2 \pm 0.5\text{‰}$ ($n = 11$). Water from experimental jars from Treatment 3 had a mean $\delta^{18}\text{O}_{\text{water}}$ value of $-3.3 \pm 0.6\text{‰}$ ($n = 4$). The mean $\delta^{18}\text{O}_{\text{water}}$ values in the storage barrels and the mean $\delta^{18}\text{O}_{\text{water}}$ values in the experimental jars after one week were used to approximate the baseline $\delta^{18}\text{O}_{\text{water}}$ values during cultivation for resolving Eq. (1), by taking the mean of the two values. This resulted in estimates of -8.6‰ for the cultures in non-manipulated lake water at 12°C (Treatment 1 and 2) and -3.4‰ for the cultures in Treatment 3 with ^{18}O -enriched water.

3.2 *Daphnia* stable isotope ratios

The mean $\delta^{13}\text{C}_{\text{Daphnia}}$ value in Treatment 2 (where *Daphnia* were offered ^{13}C -depleted algae) was lower ($-20.2 \pm 0.1\text{‰}$) than in Treatment 1 ($-18.7 \pm 0.1\text{‰}$) and 3 ($-17.9 \pm 0.1\text{‰}$) (Fig. 4). For treatments at 12°C (1–3), the mean $\delta^{13}\text{C}_{\text{Daphnia}}$ value was $0.5 \pm 0.3\text{‰}$ higher than the mean $\delta^{13}\text{C}_{\text{algae}}$ value *Daphnia* were cultured on. The mean $\delta^{13}\text{C}_{\text{Daphnia}}$ value in Treatment 4 (20°C ; $-19.0 \pm 0.1\text{‰}$) was $0.2 \pm 0.1\text{‰}$ lower than the mean $\delta^{13}\text{C}_{\text{algae}}$ value. The results from all treatments in terms of $\delta^{13}\text{C}_{\text{Daphnia}}$ values were significantly different from each other (One-way ANOVA and Tukey post-hoc test; Table 1).

Mean $\delta^{15}\text{N}_{\text{Daphnia}}$ values at 12°C were $5.5 \pm 0.1\text{‰}$ (Treatment 1), $5.7 \pm 0.1\text{‰}$ (Treatment 2) and $6.2 \pm 0.1\text{‰}$ (Treatment 3), and $3.4 \pm 0.3\text{‰}$ higher than the mean $\delta^{15}\text{N}_{\text{algae}}$ value (Fig. 4). At 20°C (Treatment 4), the mean $\delta^{15}\text{N}_{\text{Daphnia}}$ value ($6.5 \pm 0.2\text{‰}$) was

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4.0 ± 0.2‰ higher than the mean $\delta^{15}\text{N}_{\text{algae}}$ value. All treatments, except for Treatment 1 and 2, were significantly different from each other with regards to $\delta^{15}\text{N}_{\text{Daphnia}}$ values (One-way ANOVA and Tukey post-hoc test; Table 1).

Treatment 1 and 2 were both performed at 12 °C and with similar water in terms of $\delta^{18}\text{O}$ values. The mean $\delta^{18}\text{O}_{\text{Daphnia}}$ values in these treatments were 11.7 ± 0.1‰ and 11.0 ± 0.2‰, respectively (Fig. 4). In Treatment 3, where the mean $\delta^{18}\text{O}_{\text{water}}$ value was 5.2‰ higher than in the other treatments, the mean $\delta^{18}\text{O}_{\text{Daphnia}}$ value was 14.6 ± 0.3‰, which was 2.9 and 3.6‰ higher than in Treatment 1 and 2, respectively. In Treatment 4, with $\delta^{18}\text{O}_{\text{water}}$ as in Treatment 1 and 2, but run at higher temperature (20 °C), the mean $\delta^{18}\text{O}_{\text{Daphnia}}$ value (10.2 ± 0.2‰) was 1.5 and 0.8‰ lower than in Treatment 1 and 2, respectively. A significant difference in $\delta^{18}\text{O}_{\text{Daphnia}}$ values was found between all treatments (One-way ANOVA and Tukey post-hoc test; Table 1).

3.3 Ehippia stable isotope ratios

In all treatments ehippia production started between day 27 and day 34 of the experiment. Until day 48 of the experiment, ehippia production was low (on average 1–1.5 ehippia per jar per week), after which production increased to 4.5–6 ehippia per jar per week in Treatment 1, 2, and 3, whereas production in Treatment 4 remained low. Across the replicate treatments (A–C) the production of ehippia was similar with on average 12–13 ehippia per jar at the end of the experiment. The majority of the ehippia were produced by clone LC PUL 99 (55%), whereas LC PUL 101 and 53 were responsible for 23 and 22% of the ehippia production, respectively.

The measurements we performed on untreated ehippia did not reveal a detectable effect of the KOH treatment on the $\delta^{13}\text{C}_{\text{ehippia}}$, $\delta^{15}\text{N}_{\text{ehippia}}$ and $\delta^{18}\text{O}_{\text{ehippia}}$ values (Fig. 5; t tests: $\delta^{13}\text{C}$ t 0.41, $p > 0.05$; $\delta^{15}\text{N}$ t 2.20, $p > 0.05$; $\delta^{18}\text{O}$ t 0.03, $p > 0.05$). The mean $\delta^{13}\text{C}_{\text{ehippia}}$ value was on average 0.2 ± 0.8‰ lower than the mean $\delta^{13}\text{C}_{\text{Daphnia}}$ value, but this difference was not statistically significant (paired t test, t 0.83, $p > 0.05$;

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Fig. 6). However, this value was strongly affected by the results from Treatment 4 (20°C), which yielded unexpected values that will be discussed below. In the three treatments at 12°C $\delta^{13}\text{C}_{\text{ephippia}}$ values were on average $0.2 \pm 0.4\%$ higher than $\delta^{13}\text{C}_{\text{Daphnia}}$, although this difference was again not significant (paired t test, t 1.50, $p > 0.05$). Over all four treatments, $\delta^{15}\text{N}_{\text{ephippia}}$ values were on average $1.6 \pm 0.4\%$ lower than $\delta^{15}\text{N}_{\text{Daphnia}}$ values (paired t test, t 14.01, $p < 5 \times 10^{-8}$), and $\delta^{18}\text{O}_{\text{ephippia}}$ values were on average $0.9 \pm 0.4\%$ lower than $\delta^{18}\text{O}_{\text{Daphnia}}$ values (paired t test, t 5.58, $p < 5 \times 10^{-5}$).

4 Discussion

Statistically significant differences were found between nearly all treatments for all investigated *Daphnia* stable isotope ratios, even in cases where we expected no differences based on the manipulations. For example, Treatment 1 and 3 were identical in terms of $\delta^{13}\text{C}$ values of the food source and temperature and only differed in the $\delta^{18}\text{O}$ values of the water. Treatment 1, 2 and 3 were identical in terms of $\delta^{15}\text{N}$ values of the food source and temperature. Treatment 1 and 2 were similar in terms of $\delta^{18}\text{O}$ values of the water *Daphnia* were cultivated in and temperature. However, the unexpected differences between these treatments were generally small and of the same order of magnitude as the analytical precisions associated with each element (Fig. 4). They may represent the inherent variability associated with stable isotope ratios in organisms (Schimmelmann, 2011). In previous experiments $\delta^{13}\text{C}_{\text{Daphnia}}$ and $\delta^{15}\text{N}_{\text{Daphnia}}$ values have been found to differ as much as 1‰ between identical treatments (Power et al., 2003). Moreover, the stable isotope ratios of the algae showed some variability over the course of the experiment (Fig. 2). Therefore, a slight difference in timing in the buildup of biomass may have led to small differences in *Daphnia* stable isotope ratios. The differences in *Daphnia* stable isotope ratios were much larger when

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comparing treatments with manipulated $\delta^{13}\text{C}_{\text{algae}}$ and $\delta^{18}\text{O}_{\text{water}}$ values to those with non-manipulated algae and water.

4.1 The food experiment: changing $\delta^{13}\text{C}_{\text{algae}}$

Offering *Daphnia* algae with on average 1.8‰ lower $\delta^{13}\text{C}_{\text{algae}}$ values resulted in 1.5 to 2.1‰ lower $\delta^{13}\text{C}_{\text{Daphnia}}$ values. Since the $\delta^{13}\text{C}_{\text{algae}}$ values were variable over time, we cannot reconstruct the exact $\delta^{13}\text{C}$ value of the carbon that *Daphnia* in our different treatments assimilated, and therefore cannot calculate a precise estimate of $\Delta^{13}\text{C}$. Based on the mean $\delta^{13}\text{C}_{\text{algae}}$ value over the duration of the experiment, however, $\Delta^{13}\text{C}$ between *Daphnia* and algae is estimated to be $+0.5 \pm 0.3\text{‰}$ at 12°C . This is in agreement with commonly found $\Delta^{13}\text{C}$ values of 0 to $+1\text{‰}$ for a range of animals, including invertebrates (DeNiro and Epstein, 1978; McCutchan et al., 2003). *D. magna* has been reported to have a $\Delta^{13}\text{C}$ value of $+1.7\text{‰}$ at 12°C on a diet of aquarium food (Power et al., 2003). However, in this study a lipid-correction was applied to infer $\delta^{13}\text{C}$ values based on C:N ratios following a model by McConnaughey and McRoy (1979). This leads to relatively higher $\delta^{13}\text{C}$ values, and the procedure has been criticized, since it potentially provides biased estimates when comparing isotopic ratios of different organisms and tissues (Mintenbeck et al., 2008). Power et al. (2003) did not report the C:N of the food and *Daphnia*, so we cannot back-calculate the $\delta^{13}\text{C}$ values they measured prior to lipid correction.

$\delta^{13}\text{C}_{\text{ephippia}}$ values also reflected the difference in $\delta^{13}\text{C}_{\text{algae}}$ values between the treatments. At 12°C , they were not significantly different from the $\delta^{13}\text{C}_{\text{Daphnia}}$ values (although they were consistently lower at 20°C , see below). This is in line with the findings by Perga (2011), who found that the $\delta^{13}\text{C}$ value of ephippia deposited in sediment traps was slightly, but not significantly higher than the $\delta^{13}\text{C}$ value of *Daphnia* collected in the same lake. This suggests that $\delta^{13}\text{C}_{\text{ephippia}}$ values are a reliable indicator for changes in $\delta^{13}\text{C}_{\text{Daphnia}}$ values, and consequently for variations in $\delta^{13}\text{C}$ values of *Daphnia* diet:

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at 12 °C $\delta^{13}\text{C}_{\text{ephippia}}$ was $0.7 \pm 0.2\text{‰}$ higher than the mean $\delta^{13}\text{C}_{\text{algae}}$. The absence of a clear offset in $\delta^{13}\text{C}$ values between whole *Daphnia* and *Daphnia* ephippia at 12 °C is in contrast to the difference found between whole *Daphnia* and *Daphnia* exoskeletons (0.8‰ ; Perga, 2010) and chironomid body tissue and chironomid head capsules (~ 1‰; Heiri et al., 2012; Frossard et al., 2013).

4.2 $\delta^{15}\text{N}$ values of *Daphnia* and ephippia

At 12 °C, the observed $\Delta^{15}\text{N}$ was $+3.4 \pm 0.3\text{‰}$, which agrees well with $\Delta^{15}\text{N}$ values referred to in the literature (+3 to +4‰, DeNiro and Epstein, 1981; Minagawa and Wada, 1984). A range of $\Delta^{15}\text{N}$ values for *Daphnia* have been reported. *D. pulicaria* reared on a diet of frozen algae pellets had a $\Delta^{15}\text{N}$ of +1.4‰ (Matthews and Mazumder, 2008). This is lower than the $\Delta^{15}\text{N}$ we found. According to Matthews and Mazumder (2008), the low $\Delta^{15}\text{N}$ they observed may be explained by the observation that a diet consisting of detritus (dead algae) is associated with considerably (~ 2.5‰) lower $\Delta^{15}\text{N}$ values than one consisting of living plant matter (Vanderklift and Ponsard, 2003). Our observed $\Delta^{15}\text{N}$ for *D. pulicaria* is within the range of reported *D. magna* $\Delta^{15}\text{N}$ values (+1 to +6‰ ; Adams and Sterner, 2000; Power et al., 2003).

$\delta^{15}\text{N}_{\text{ephippia}}$ values were lower ($1.6 \pm 0.4\text{‰}$) than $\delta^{15}\text{N}_{\text{Daphnia}}$ values. In contrast, Perga (2011) found $\delta^{15}\text{N}_{\text{ephippia}}$ values to be slightly, but not significantly lower than $\delta^{15}\text{N}_{\text{Daphnia}}$ values in the field. Together with Perga's (2011) results, our data provide an indication that $\delta^{15}\text{N}_{\text{ephippia}}$ values are indicative of $\delta^{15}\text{N}$ values of *Daphnia* and their diet, with only relatively minor offsets between food, *Daphnia* and ephippia. For chironomids, differences of similar magnitude between whole body $\delta^{15}\text{N}$ values and head capsule $\delta^{15}\text{N}$ values (-1 to +1‰) were observed over a large range of $\delta^{15}\text{N}$ values (2.5–15‰; Heiri et al., 2012). Therefore, it seems likely that differences between *Daphnia* and ephippia $\delta^{15}\text{N}$ values may also be similar across this $\delta^{15}\text{N}$ range.

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4.3 The water experiment: changing $\delta^{18}\text{O}_{\text{water}}$ values

$\delta^{18}\text{O}_{\text{water}}$ values were 5.2‰ higher in Treatment 3 than in Treatment 1 and 2, and the mean $\delta^{18}\text{O}_{\text{Daphnia}}$ values in Treatment 3 were 2.9‰ higher than in Treatment 1 and 3.6‰ higher than in Treatment 2. This implies that, as expected, differences in $\delta^{18}\text{O}_{\text{Daphnia}}$ values reflect differences in $\delta^{18}\text{O}_{\text{water}}$, yet that, as in other invertebrates, only part of the oxygen incorporated by the *Daphnia* originated from the water. Wang et al. (2009) reported that 69 % of the oxygen in chironomid larvae stemmed from the water in their environment. Soto et al. (2013) estimated that 84 % of the oxygen in protein isolated from chironomids came from the water in their environment, and Nielson and Bowen (2010) reported that 69 % of the oxygen in chitin from brine shrimp came from water in their environment. Based on Eq. (1), we estimate that in our experiment 56–69 % of the oxygen in *Daphnia* came from the water, based on Treatment 1 and 2, respectively. These estimates are similar to the values reported by Wang et al. (2009), and Nielson and Bowen (2010).

$\delta^{18}\text{O}_{\text{ephippia}}$ values closely reflected differences in $\delta^{18}\text{O}_{\text{Daphnia}}$: they were on average 0.9 ± 0.4 ‰ lower than $\delta^{18}\text{O}_{\text{Daphnia}}$ values. This suggests that $\delta^{18}\text{O}_{\text{ephippia}}$ may be used as an indicator of $\delta^{18}\text{O}_{\text{Daphnia}}$, which in turn can be expected to be related to lake water $\delta^{18}\text{O}$ values. This is in agreement with the correspondence between surface sediment $\delta^{18}\text{O}_{\text{ephippia}}$ values and lake water $\delta^{18}\text{O}$ values found in a field survey of a number of European lakes (Verbruggen et al., 2011).

4.4 The temperature experiment

Power et al. (2003) reported an increase of 0.1‰ in $\Delta^{13}\text{C}$ values for *D. magna* with a temperature increase from 12 to 20 °C (and +1.4‰ when temperature increased from 12 to 26 °C). Therefore, we expected $\Delta^{13}\text{C}$ values for *Daphnia* in Treatment 4 (20 °C) to be similar to or slightly higher than in the other treatments (12 °C). $\Delta^{13}\text{C}$ values were clearly lower, however, in Treatment 4 (-0.2 ± 0.1 ‰) than in the other treatments

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($+0.5 \pm 0.3\%$). While we cannot exclude a negative relation between temperature and $\Delta^{13}\text{C}$ values for *Daphnia*, we choose to treat this result with caution due to the discrepancy with the positive $\Delta^{13}\text{C}$ values as reported in other studies (DeNiro and Epstein, 1978; McCutchan et al., 2003; Power et al., 2003). A higher lipid content of *Daphnia* may potentially lead to lower $\delta^{13}\text{C}_{\text{Daphnia}}$ values (McCutchan et al., 2003). However, the C : N ratios of *Daphnia* in Treatment 4 were slightly lower (but not significantly different; *t* test, *t* 1.18 *p* > 0.05) than those of *Daphnia* in Treatment 1, which does not agree with a higher lipid content in *Daphnia* from Treatment 4 (Smyntek et al., 2007). Alternatively, ^{13}C -depletion of algal biomass during dark respiration may have affected the $\delta^{13}\text{C}_{\text{algae}}$ in Treatment 4 disproportionately due to the higher temperature. Degens et al. (1968) found that $\delta^{13}\text{C}$ values of the alga *Dunaliella teriolecta* were 4‰ lower after three days in darkness. The rate of respiration by algae depends on temperature and can be 2 to 4 times higher at 20 °C than at 12 °C (e.g. Vona et al., 2004). Microbial activity in the experimental jars could have been affected by temperature and could have also influenced our results. Additionally, if *Daphnia* in Treatment 4 had a different timing of growth compared to Treatment 1, as can be expected, they may have been assimilating carbon from algae with different $\delta^{13}\text{C}_{\text{algae}}$ values during the main phase of their growth compared to the other treatments, since $\delta^{13}\text{C}_{\text{algae}}$ values were relatively low in the beginning and at the end of the experiment (Fig. 2). $\delta^{13}\text{C}_{\text{ephippia}}$ values were also lower in Treatment 4, and $1.3 \pm 0.3\%$ lower than $\delta^{13}\text{C}_{\text{Daphnia}}$ values. For the same reasons as outlined above, it remains unclear whether this observation is the consequence of a fundamental change in the offset between $\delta^{13}\text{C}_{\text{Daphnia}}$ and $\delta^{13}\text{C}_{\text{ephippia}}$ with temperature or whether it is affected by variations in $\delta^{13}\text{C}_{\text{algae}}$ and algal respiration rates or differences in *Daphnia* growth rates between our treatments. Controlled experiments over a range of temperature values analyzing not only $\delta^{13}\text{C}_{\text{Daphnia}}$ and $\delta^{13}\text{C}_{\text{ephippia}}$ values, but also $\delta^{13}\text{C}$ values of respired CO_2 and microbial biomass would be desirable to further explore this issue. Although the results of Treatment 4 indicate that the difference between $\delta^{13}\text{C}_{\text{ephippia}}$ and $\delta^{13}\text{C}_{\text{Daphnia}}$ values may be more variable

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than indicated by the cultivations at 12 °C, the offset is still relatively small compared to the variation in $\delta^{13}\text{C}_{\text{ephippia}}$ values in lake sediments (up to 10‰; e.g. Wooller et al., 2012).

$\Delta^{15}\text{N}$ between *Daphnia* and algae was $+4.0 \pm 0.2\text{‰}$ at 20 °C, 0.6‰ higher than at 12 °C. A small increase (0.4‰) in $\Delta^{15}\text{N}$ at this temperature range has also been reported for *D. magna* (Power et al., 2003). Power et al. (2003) found a decrease of 2.7‰ in $\Delta^{15}\text{N}$ values for *D. magna* between 20 and 26 °C, however, and Barnes et al. (2007) found a decrease of 0.6‰ in $\Delta^{15}\text{N}$ values for sea bass with a temperature increase from 11 to 16 °C. Previously observed $\Delta^{15}\text{N}$ values in field studies of aquatic food webs (Vander Zanden and Rasmussen, 2001), and specifically in experimental studies of *Daphnia* (Adams and Sterner, 2000; Matthews and Mazumder, 2008) are in some cases lower than +3 to +4‰. A potential effect of temperature on $\Delta^{15}\text{N}$ values for *Daphnia* which, based on presently available observations, may amount to 2.7‰ at temperatures above 20 °C (Power et al., 2003) therefore deserves future attention. The offset between $\delta^{15}\text{N}_{\text{Daphnia}}$ and $\delta^{15}\text{N}_{\text{ephippia}}$ in our experiment was, however, not significantly different (*t* test, *t* 0.26 *p* > 0.05) between Treatment 1 (control, 12 °C) and 4 (20 °C).

The effect of temperature on oxygen isotope fractionation during the formation of chitin by aquatic organisms has not been examined previously. Schimmelmann and DeNiro (1986) analyzed the $\delta^{18}\text{O}$ values of chitin of marine crustaceans collected along a temperature gradient of 10 °C and van Hardenbroek et al. (2012) studied the $\delta^{18}\text{O}$ values of aquatic beetles in museum specimens selected to represent a temperature gradient across North America. Both studies concluded that the temperature effect on oxygen isotope fractionation during chitin formation (if any) was smaller than the variability due to minor differences in local environmental conditions. In this study we had a close control on the environmental conditions and source water $\delta^{18}\text{O}$ values and we found that $\delta^{18}\text{O}_{\text{Daphnia}}$ was slightly (0.8–1.5‰) lower with an increase of temperature by 8 °C but otherwise similar conditions. This may indicate an effect of temperature on oxygen isotope fractionation by *Daphnia*. We do note, however, that

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the potential temperature effect on oxygen isotope fractionation by *Daphnia* observed in our experiment was relatively small, and resulted from a large difference in temperature. Therefore, $\delta^{18}\text{O}_{\text{Daphnia}}$ values most likely primarily reflect environmental water $\delta^{18}\text{O}$ values. The offset between $\delta^{18}\text{O}_{\text{ephippia}}$ and $\delta^{18}\text{O}_{\text{Daphnia}}$ in Treatment 4 (20°C) was not significantly different, however (t test, t 0.09, $p > 0.05$), from that in Treatment 1 (control, 12°C). This suggests that, in contrast to the difference between $\delta^{18}\text{O}_{\text{water}}$ and $\delta^{18}\text{O}_{\text{Daphnia}}$, this offset is not affected by temperature in the investigated temperature range (12–20°C). Verbruggen et al. (2011) measured the $\delta^{18}\text{O}$ values of recently deposited ephippia from surface sediments in lakes along a geographical gradient in Europe. They found a strong correlation between $\delta^{18}\text{O}_{\text{ephippia}}$ values and lake water $\delta^{18}\text{O}$ values. In their dataset, the $\delta^{18}\text{O}$ values of lake water increased by $\sim 4.8\text{‰}$ with a temperature increase of 8°C, whereas $\delta^{18}\text{O}_{\text{ephippia}}$ values increased by only $\sim 3\text{‰}$ over this temperature gradient, a difference of $\sim 1.8\text{‰}$. This difference is of a similar order of magnitude as the 0.8–1.5‰ lower $\delta^{18}\text{O}_{\text{Daphnia}}$ values we found with an 8°C temperature rise. The data of Verbruggen et al. (2011) and our experimental data would therefore be in agreement with a slight temperature effect on the fractionation of ^{18}O between lake water and *Daphnia* biomass. However, other mechanisms, such as a change in timing of *Daphnia* ephippia production with temperature and variations in $\delta^{18}\text{O}$ values of food across the examined temperature gradient could also explain varying offsets between $\delta^{18}\text{O}_{\text{water}}$ and $\delta^{18}\text{O}_{\text{Daphnia}}$ at different temperatures in the study of Verbruggen et al. (2011). Moreover, Verbruggen et al. (2011) reported air temperature, and differences in air temperature at lakes do not necessarily lead to similar differences in lake water temperatures.

4.5 Implications for palaeoecological studies

In general, we found that the stable isotopic composition of ephippia closely reflected the stable isotopic composition of *Daphnia*. The offsets were consistent within treatments and between most treatments (Fig. 6), and the ephippia stable isotope ratios

responded to the manipulations in $\delta^{13}\text{C}_{\text{algae}}$ and $\delta^{18}\text{O}_{\text{water}}$ we performed. Studies investigating the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of fossil *Daphnia* ephippia have recorded shifts up to 5–10‰ in $\delta^{13}\text{C}$ values (Wooller et al., 2012; Frossard et al., 2014) and 3‰ in $\delta^{15}\text{N}$ values (Griffiths et al., 2010). Shifts of 2–3‰ in $\delta^{18}\text{O}$ values have been reported for fossil chironomid head capsules (Wooller et al., 2004; Verbruggen et al., 2010b). In our experiment, the SD of the offset between *Daphnia* and ephippia stable isotope ratios was much smaller than the reported shifts in stable isotope ratios of fossil remains: $\pm 0.4\%$ for $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ ($\pm 0.8\%$ for $\delta^{13}\text{C}$ when including Treatment 4 at 20°C). If our findings are representative of the offset in stable isotope ratios between *Daphnia* and their ephippia in nature, they indicate that reported shifts in stable isotope ratios of fossil ephippia can reliably be interpreted as indicating past variations in *Daphnia* stable isotope ratios. These in turn can be expected to reflect past changes in isotopic composition of *Daphnia* diet and/or the $\delta^{18}\text{O}$ of the water they lived in. Although we only cultured *Daphnia* at two different temperatures, we found indications that temperature may have affected $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$, and the relationship between $\delta^{18}\text{O}_{\text{water}}$ and $\delta^{18}\text{O}_{\text{Daphnia}}$ values in an experimental setting. Future experiments could be conducted at a range of temperatures to examine such potential influences of temperature.

Acknowledgements. We thank Christine Dambone-Boesch and Esther Keller for their help in maintaining the algae cultures, and Päivi Rinta for feeding the *Daphnia* and refreshing the water on occasion. We also thank the Cantonal Bureau for Waste, Water, Energy and Air (AWEL, Zürich) for providing data on the water chemistry of Lake Greifensee. This research was supported by the European Research Council under the European Union's Seventh Framework Programme (FP/2007-2013)/ERC Grant Agreement n. 239858 (RECONMET) and by a grant of the Swiss Science Foundation (CR3213_125211 to P. Spaak).

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Table 1. Results of one-way ANOVA and post-hoc Tukey tests for statistical differences between the four (1–4) treatments (one-way ANOVA) and between pairs of treatments (Tukey test) for $\delta^{13}\text{C}_{\text{Daphnia}}$, $\delta^{15}\text{N}_{\text{Daphnia}}$ and $\delta^{18}\text{O}_{\text{Daphnia}}$ values. The results of the Tukey test are presented below the F and p values for the one-way ANOVA, showing Q values (lower left part of matrix) and p values (upper right).

<i>Daphnia</i> $\delta^{13}\text{C}$ values				<i>Daphnia</i> $\delta^{15}\text{N}$ values				<i>Daphnia</i> $\delta^{18}\text{O}$ values			
$F_{(2,3)} 303.8 p < 1 \times 10^{-8}$				$F_{(2,3)} 52.1 p < 1 \times 10^{-5}$				$F_{(2,3)} 255.3 p < 1 \times 10^{-8}$			
1	2	3	4	1	2	3	4	1	2	3	4
1	0.0002	0.0003	0.005	1	0.648	0.0006	0.0002	1	0.02	0.0002	0.0003
2	28.16	0.0002	0.0002	2	1.686	0.002	0.0003	2	5.646	0.0002	0.01
3	13.62	41.78	0.0002	3	10.16	8.476	0.03	3	24.6	30.25	0.0002
4	6.968	21.19	20.58	4	15.32	13.63	5.154	4	11.88	6.234	36.48

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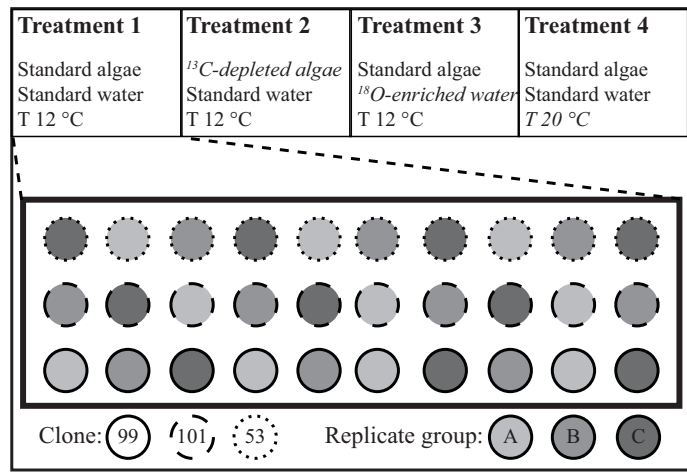


Figure 1. Schematic representation of the experimental design used for culturing *Daphnia*. The top panel shows the four treatments and the bottom shows the setup of each treatment exemplified for Treatment 1. For each treatment, three replicate groups (A, B and C) and *Daphnia pulicaria* clones were distributed evenly among 30 experimental glass jars in a large tray. This was done so that each treatment replicate consisted of 10 jars with 3–4 jars for each clone.

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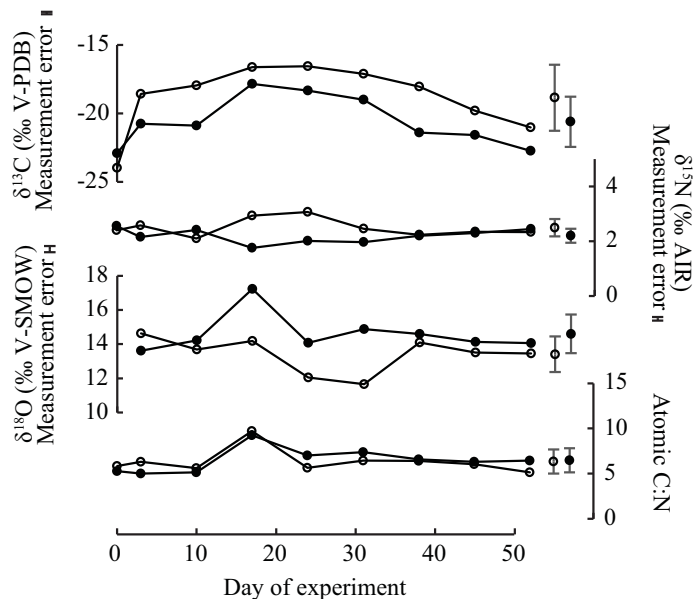


Figure 2. $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{18}\text{O}$ values and atomic C:N ratios of the algae harvested from both chemostats during the experiment. Open circles represent the standard algae, and the closed circles represent the algae that were cultured on a medium with the addition of ^{13}C -depleted bicarbonate. The data points and error bars on the right side of the plots indicate average values and 1 SD, respectively.

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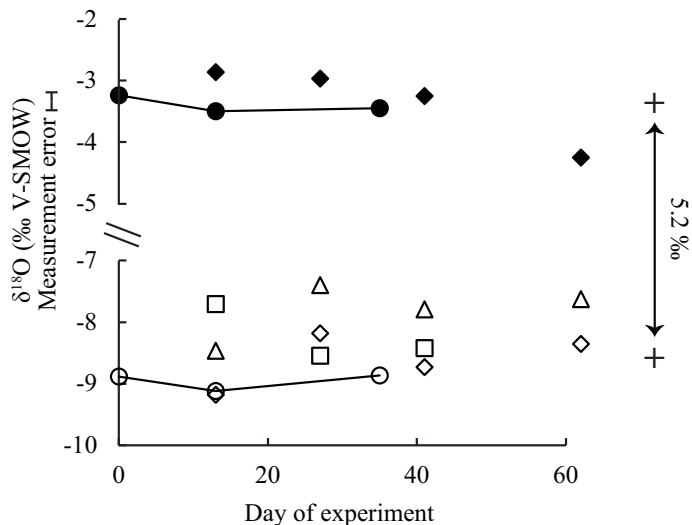
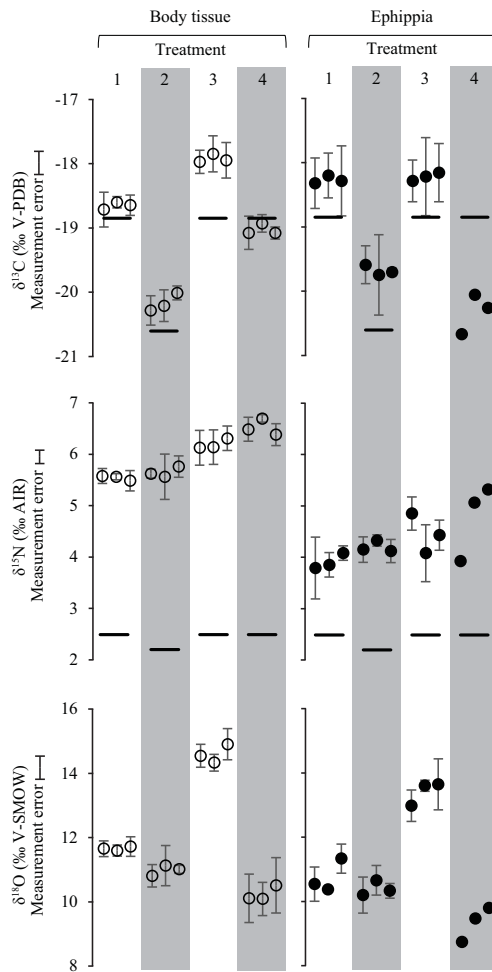


Figure 3. $\delta^{18}\text{O}$ values of the water in the storage barrels for the standard water (open circles) and the artificially ^{18}O -enriched water (closed circles) sampled on day 0, 13 and 35, and the $\delta^{18}\text{O}$ values of the water sampled from the experimental jars before water was exchanged for Treatment 1 (open diamonds, control), Treatment 2 (open triangles, ^{13}C -depleted algae), and Treatment 3 (closed diamonds, ^{18}O -enriched water) sampled on day 13, 27, 41 and 62, and Treatment 4 (open squares, 20°C) sampled on day 13, 27 and 41. The plus symbols (+) on the right side indicate the mean of the mean experimental jar values and the mean storage barrel values for the standard water and the ^{18}O -enriched water, respectively.

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Figure 4. $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of *Daphnia* body tissue (left, open circles) and ephippia (right, closed circles) for Treatment 1 (control), 2 (^{13}C -depleted algae), 3 (^{18}O -enriched water) and 4 (elevated temperature). Each data point represents one of the treatment replicate groups and consists of three measurements, of which the SD is indicated by the error bars (only one measurement per replicate treatment group was available for ephippia in Treatment 4). The black horizontal lines in the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ plots represent the average value of the algae used in that treatment.

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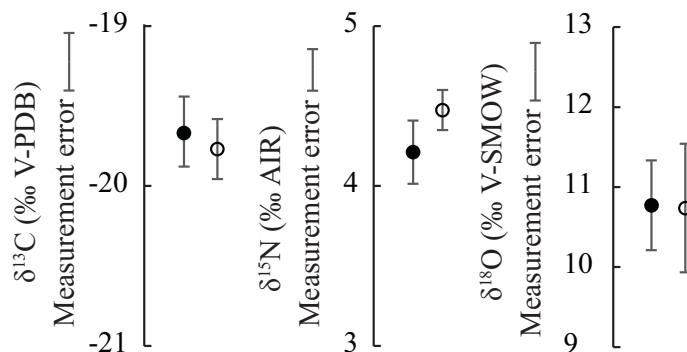


Figure 5. $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of *Daphnia* ephippia treated with 10% KOH for 2 h (closed circles, $n = 9$) and those of ephippia that were not chemically treated (open circles, $n = 3$). The values for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ are from Treatment 2, those for $\delta^{18}\text{O}$ from Treatment 1. Error bars indicate SDs.

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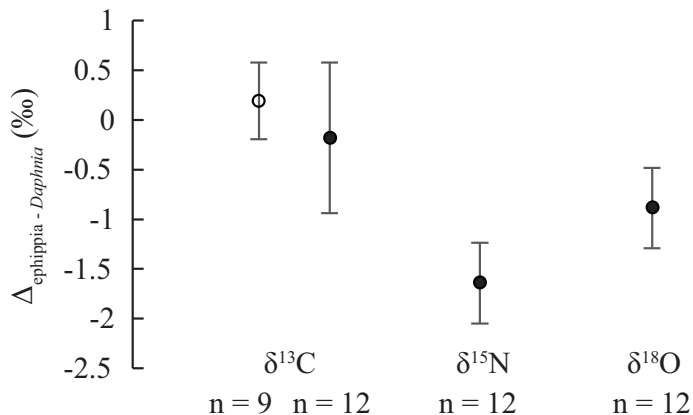


Figure 6. The difference in $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values between ephippia and *Daphnia* for all four treatments (closed circles). The open circle gives the offset for the three treatments at 12 °C excluding Treatment 4 (20 °C), which yielded unexpected results for $\delta^{13}\text{C}$ (see text). Error bars indicate SDs.