REVIEW 1

We thank the reviewer for examining our manuscript and pointing out areas that could be improved. We replied to several of Reviewer 1's general comments in an earlier response (*Biogeosciences* Discussions 12, C359-363). Here we provide a point-by-point reply to the remaining comments from Reviewer 1.

General comments.

<u>Reviewer 1 comment</u>: As stated at the end of the introduction, this paper was intended 'to experimentally examine whether offsets in δ 13C, δ 15N and δ 18O values exist between Daphnia and their ephippia'. If this a question of interest for those who are working on past changes in Daphnia isotope composition using ephippia recovered from sediment archives. This is however a very small community and I do not think that this paper will touch a large readership.

<u>Author Reply</u>: As outlined in detail in our earlier reply (pages C362-C363) we do not agree with this assessment.

<u>Reviewer 1 comment</u>: It does not really connect either to biogeosciences, and, as a matter of fact, there is very few references to any biological or geochemical processes within the whole paper (although it could be relevant to specify the working hypotheses in the introduction, i.e. according to which physiological hypotheses ephippia should exhibit isotope composition that would be differ from those of the whole carapaces/body).

<u>Author Reply</u>: We outlined in detail in our earlier reply (pages C360-361) that the topic of our article fits well within the scope of topics covered and described for the journal *Biogeosciences*. It is correct that we did not address in detail the biochemical processes that may influence a potential offset in stable isotopic composition between *Daphnia* and their ephippia. This is because it was not the aim of this manuscript to review these processes. However, we agree with the reviewer that some examples of how the isotopic composition of different tissue types can differ within organisms would help the reader to understand the relevance of our work. In addition to the example of *Daphnia* exoskeleton versus *Daphnia* whole body tissue already mentioned in the manuscript, we will also briefly discuss examples of culturing experiments showing offsets between the isotopic composition of whole body and chitinous structures for chironomids and cephalopods.

We note that Reviewer 2 supports publication of our manuscript in Biogeosciences.

<u>Author comment to editor</u>: On page 4, line 17-32, text was added to provide further examples where the stable isotopic composition of chitinous structures differed from that of the whole organism.

<u>Reviewer 1 comment</u>: Besides, the experiment that has been performed is of very limited scale, at a point that unexpected results (such as those obtained for δ 13C at 20°C or very different Daphnia δ 15N in spite for similar δ 15N of the food sources) remain hard to explain

<u>Author Reply</u>: As a pioneering study, our experiment was designed to be broad and provide both the first laboratory based assessment of the effects of variable isotopic composition food and water on the C and O stable isotopes of the ephippia, as well as of the potential effects of temperature on C, N, and O stable isotopes of *Daphnia* ephippia. However, even controlling for the factors we describe in our manuscript required considerable resources and we were only able to investigate two temperature values and conduct experiments with two different δ^{13} C values for algae and two different δ^{18} O values for water. Nevertheless, the experiment allowed us to draw clear conclusions on the isotopic offsets between *Daphnia* and ephippia, the relevance of lake water δ^{18} O values for determining *Daphnia* and ephippia δ^{18} O values, and the response in *Daphnia* and ephippia δ^{13} C values to changes in those of the diet.

The apparent temperature effect on δ^{13} C values was indeed unexpected and could not be explained based on the data we produced, which has led us to conclude that this may be due to e.g. microbial activity or increased algal respiration rates in the cultures. We provide, however, a thorough discussion on what may have been the cause and indicate what can be done in future experiments to further investigate the effect of temperature on δ^{13} C values of *Daphnia* and ephippia. Our results for N and O stable isotopes appear unaffected by these processes and therefore provide first indications in respect to how δ^{15} N and δ^{18} O values of *Daphnia* ephippia respond to changing temperatures.

The δ^{15} N results are not unexpected as the reviewer implies: With an isotopically identical food source, all *Daphnia* had δ^{15} N values within a 1 ‰ range. This range is not larger than reported in similar experiments (Power et al., 2003; Matthews and Mazumder, 2008).

<u>Reviewer 1 comment</u>: Because the range of tested conditions is narrow, the study does not provide any novelty as compared to previous papers on that topic, exception maybe for δ 180.

Author Reply: We do not agree with this comment. Our experiments provide the first study assessing oxygen isotopic offsets between *Daphnia* and environmental water, and carbon, oxygen and nitrogen isotopic offsets between *Daphnia* tissue and *Daphnia* ephippia under controlled laboratory conditions, as well as an assessment of how consistent these offsets are under two different temperature conditions, and for different isotopic compositions of food (for C) and lake water (for O). Earlier experiments were constrained to C and N isotopes and did not include any measurements on ephippia. To our knowledge, this represents the first experimental study on the relationship between δ^{18} O values of lake water, body tissue and fossilizing remains for any aquatic or terrestrial invertebrate group that produces chitinous fossils. Our results are therefore relevant for a further development of isotopic analysis on *Daphnia* remains for environmental reconstruction, but also of wider relevance for the field of invertebrate palaeoecology. We discuss in detail in our previous reply to the reviewer (page C362) how our results differ from earlier studies. The statement that our study does not provide any novelty is, in our opinion, therefore incorrect.

<u>Reviewer 1 comment</u>: Actually, the very annoying point of this paper is that everything is done to inflate and oversell the real content of the paper and the reviewer feels he is getting duped. The title is somewhat catchy, but 'environmental influences' actually refers to (i) test of two δ 13C food values, which differ by less than 1.8 per mil, (ii) two temperature conditions, one of which leading to conditions that 'may not affect Daphnia in their natural environment' and (ii) two δ 18O water values. Even if the experimental setting was ideal, it would have been only two conditions for each factor, and this would not be enough to be called 'environmental conditions'.

<u>Author Reply</u>: When submitting the manuscript we selected a title that was wide enough to cover all the manipulations we did in our experiments (temperature, isotopic

composition of food and water). We suspect this title led the reviewer to expect an investigation of environmental influences on modern *Daphnia* stable isotope ratios, whereas our main aim was investigating whether the stable isotopic composition of (fossil) ephippia is indicative of that of (once living) *Daphnia*, and consequently of changing conditions in the environment the *Daphnia* lived in. We maintain that we clearly set our aims and goals in the abstract, introduction and discussion (see first reply pages C361-262). We realize, based on the reviewer's comments, that the title may be misleading for some readers. We will therefore change the title to "The stable isotopic composition of *Daphnia* ephippia reflects changes in δ^{13} C and δ^{18} O values of food and water".

<u>Author comment to editor</u>. The title of the manuscript has been changed to: The stable isotopic composition of Daphnia ephippia reflects changes in δ^{13} C and δ^{18} O values of food and water

<u>Reviewer 1 comment</u>: It is even more dubious that the experimental design was not perfect. If the point was to test whether food δ 13C affect the isotopic offset, we would expect that a much larger range in δ 13C values for the food sources. My guess is that much more labelled sodium bicarbonate would have required in the algal growth medium to create such a range of δ 13C but this is understandable flaw because this can be usually difficult to anticipate. The experiment had been already conducted by the time that authors realized that labelling was too small to really serve the working hypothesis. In a sense, it is interesting to see that even such a small range of δ 13C values is detectable at the level of ephippia isotope composition, but this is not the way this is presented in the paper.

Author Reply: It is correct that we aimed for a larger difference in algal δ^{13} C values. However, as the reviewer also indicates, this is difficult to achieve without extensive pretesting. Our experiment is "well behaved" in the sense that a relatively minor shift in algal δ^{13} C values (1.8 ‰) leads to a similar shift in *Daphnia* soft tissue (1.5 to 2.1 ‰) and ephippia (1.5 ‰), whereas offsets between *Daphnia* and ephippia δ^{13} C values remain minor and not statistically significant at the temperature for which algae with different δ^{13} C values were provided. Our results therefore confirm the expected behaviour of δ^{13} C values of *Daphnia* and their ephippia to changes in food source δ^{13} C values. This is exactly what the experiment has been set up to investigate. We agree that the small difference in algal δ^{13} C values would have been a problem if we would have received unexpected results, e.g. if a manipulation of algal δ^{13} C values would not have led to a corresponding shift in δ^{13} C values of *Daphnia* and their ephippia in our manuscript that we covered the full range of δ^{13} C values expected for *Daphnia* in nature.

<u>Reviewer 1 comment</u>: To remediate to the narrowness of the potential readership, authors try to increase the perspective of the experiment by relating to the need for the community of isotope ecologists to quantify trophic fractionation factors (p2577, from I 20). Yet, because the experiment has not been initially designed for such purposes, it does not provide any more information than those that have been specifically conducted some time ago (Impact of temperature by Power, 2003; food composition by Matthews and mazumder, 2008).

<u>Author Reply</u>: We strongly disagree with this assessment and replied in detail to this comment in our earlier reply (pages C361-C362).

<u>Reviewer 1 comment</u>: To conclude, authors have targetted a high-level, generalist journal but this experiment, even if everything had worked perfectly, does not have the potential to reach such a readership. Inflating artificially the purpose of the paper is not enough to fool the reader on the actual contribution of the research (may be just enough to upset him/her). This study has been designed for a very specific purpose, and therefore should be published in a very specialized journal. The experiment itself has been performed rigourously, and even though it has a small scale and produced sometimes unexplained results, I am very confident it could be published in the adequate journal (JOPL ?).

<u>Author Reply</u>: We are pleased to see that the reviewer recognizes the strengths of the study. However, we disagree with the reviewer's assessment regarding the target journal. We reply in detail to this comment in reply #1 (pages C360-361).

Specific comments.

<u>Reviewer 1 comment</u>: Overall, the language is very understandable and the paper is clear. However, I found that the graphical representations of the results (fig 2 & 3) were not legible, and hampered the understanding. Fig 1 is not necessary, the text is clear enough. Fig. 5 also can be removed, as it presents very straightforward results.

<u>Author Reply</u>: We agree that Figure 1 and 5 are not strictly necessary and will remove them in the final revision. We will revise Figures 2 and 3 to make them easier to read.

<u>Author comment to editor</u>: Figures 1 and 5 were removed. The lines belonging to the open circles in Figure 1 (previously Figure 2) are now dashed. In Figure 3 (previously Figure 2), the Y-axis is now continuous and the line belonging to the open symbols is now dashed.

<u>Reviewer 1 comment</u>: Table 1: Significance detection in multiple paired comparisons requires accounting for Bonferroni's corrections.

<u>Author Reply</u>: We have applied Bonferroni corrections to the analyses presented in Table 1. The results confirm our previous analyses and do not alter our interpretation. In two cases (comparing $\delta^{15}N$ values between Treatment 3 and 4 and $\delta^{18}O$ values between Treatment 1 and 2) the comparisons are now not significant whereas previously they were marked as significant. However, Treatment 3 and 4 are not discussed in terms of $\delta^{15}N$ values and Treatment 1 and 2 not in terms of $\delta^{18}O$ values because the $\delta^{15}N$ values of the food were the same in Treatment 3 and 4, and the $\delta^{18}O$ values of the water were the same in Treatment 1 and 2. We agree that a Bonferroni correction is appropriate, and have applied it to the results shown in Table 1.

<u>Author comment to editor</u>: A sentence was added in the methods sections (p. 7, lines 13-15) stating that we took into account Bonferroni corrections for multiple comparisons. Since Treatment 1 and 2 no longer differ significantly in terms of δ^{18} O values, a sentence on page 11 was removed. This sentence only served as one of three examples of us finding a significant difference where none was expected.

<u>Reviewer 1 comment</u>: Three different clones were used and they apparently did not contribute equally to ephippia production. Any clone effect on the isotope results?

<u>Author Reply</u>: We chose to work with three clones because this would: 1) give the experiment more resilience in case of unexpected developments (e.g., if a particular clone would not perform well under the experimental conditions), and 2) to avoid the risk of working with a specific clone that exhibits different offsets between *Daphnia* and ephippia than most other clones (in case there is indeed a clone effect). Unfortunately, the amount of ephippia produced was just enough to meet the degree of replication we wanted to achieve, and it was not possible to investigate a clone effect. Therefore, we cannot make statements on this matter.

References.

Matthews, B. and Mazumder, A.: Detecting trophic-level variation in consumer assemblages, Freshwater Biol., 53, 1942–1953, doi:10.1111/j.1365-2427.2008.02018.x, 2008.

Power, M., Guiguer, K. R. R. A., and Barton, D. R.: Effects of temperature on isotopic enrichment in Daphnia magna: implications for aquatic food-web studies, Rapid Commun. Mass Sp., 17, 1619–1625, doi:10.1002/rcm.1094, 2003.

REVIEW 2

We thank the reviewer for assessing our manuscript and providing us with comments.

General Comments.

<u>Reviewer 2 comment</u>: Stratigraphic variability in the isotopic composition of endogenic and biogenic components preserved in lacustrine sediments can provide a valuable insight into palaeoenvironmental conditions. Recently attention has shifted towards the development of proxies capable of recording information of their biochemical heritage. Although, the chitinous remains of aquatic invertebrates are one of the most abundant components preserved in lacustrine sediments, they have received relatively little attention as a tool for inferring past environmental conditions. The adoption of this approach has been hampered by the absence of empirical data exploring the relationship between environmental parameters, the isotopic composition of the remains and the offsets between living organism and fossilising structures.

In this manuscript the results from a series of controlled laboratory experiments investigating the influence of diet, habitat water and temperature on the isotopic composition of Daphnia and their chitinous fossilizing structures are presented. Although the range of variables covered in the investigation are rather limited (from personal experience I appreciate the amount of work that is required to successfully conduct a laboratory calibration study of this nature) this study represents a fledgling step towards improving our understanding of this proxy in the reconstruction of a wide range of past environmental conditions.

<u>Author reply</u>: We are glad to see that Reviewer 2 recognizes the importance of this manuscript in the context of the development of an emerging proxy.

<u>Reviewer 2 comment</u>: I believe that this research has been conducted in a rigorous manner and that the findings may be of interest to the palaeo-biogeosciences community therefore support its publication in this journal. However, I also acknowledge that the manuscript may be more accessible to a more relevant readership in a publication specifically aimed at communities interested in palaeoenvironmental reconstructions.

<u>Author reply</u>: We are glad to see that the reviewer supports publication of our manuscript in *Biogeosciences*. *Biogeosciences* is an open access journal which is well indexed and widely read by palaeoecologists and palaeolimnologists. We therefore believe the article will be easy to find also for specialists working in lake sediment records and palaeoenvironmental reconstruction.

Specific Comments.

<u>Reviewer 2 comment</u>: I think there should be a caveat early on in the manuscript or in the discussion acknowledging, despite their obvious merits, the limitations of laboratory studies (e.g. unable to truly simulate the complex interactions operating in nature).

<u>Author reply</u>: We agree and will add text on this topic in the discussion of the revised manuscript.

<u>Author comment to editor</u>: We have added a line mentioning this caveat in the concluding section of the discussion (p.17, line 17-23).

<u>Reviewer 2 comment</u>: I found it difficult to differentiate between the open and closed circles in Figure 2. Perhaps one line could be dashed and the other solid?

<u>Author reply</u>: We thank Reviewer 2 for the suggestion, we will implement this in the revised manuscript.

<u>Author comment to editor</u>: The lines belonging to the open circles are now dashed in Figure 1 (Figure 2 in the previous version of the manuscript).

<u>Reviewer 2 comment</u>: Methods: The stock water solution was stored at 12°C. Was this water allowed to acclimatised before refreshing in Treatment 4? Do you have any concerns regarding temperature stability with performing replacements twice a week in Treatment 4?

<u>Author reply</u>: On the days of water renewal the first step was to filter the water (at room temperature) before other preparations were made. Therefore, the fresh water was kept at room temperature for ~4 hours (and up to 7 hours on days where all treatments were refreshed; the 12°C samples were refreshed first on those days) before refreshing the water in the 20°C treatment. We therefore have no concerns regarding the temperature of the fresh water in Treatment 4. We will provide more information in the revised manuscript on the amount of time the water was allowed to acclimatize before replacement.

<u>Author comment to editor</u>: On p. 6, line 16-18 we included a statement indicating that the water was allowed to warm up before refreshing in the 20°C treatment.

<u>Reviewer 2 comment</u>: Although the evaluation of the influence of temperature on the stable isotope ratios in chitinous remains is novel, and much needed, it's frustrating that only two temperatures were looked at in this study. One of which, it could be argued, is largely irrelevant in the context of palaeoclimate reconstructions (i.e. 20° C). Was there a specific reason why 12°C and 20°C was chosen as study temperatures? Furthermore, was temperature (either water or air) accurately measured throughout the duration of the experiment? I know from personal experience that maintaining controlled temperatures can very difficult, even in supposedly controlled environments. I found that the original temperature controlled cabinets I was using in my experimentation varied by as much as $\pm 5^{\circ}$ C throughout the duration of a culture!

<u>Author reply</u>: We agree that it would have been useful to examine a range of temperature values instead of two temperatures only. However, due to logistic reasons we had to limit ourselves to two temperature values. Our experiment did not only focus on the effects of temperature on the C, N, and O stable isotopic composition of *Daphnia* ephippia and on the offset between *Daphnia* tissue and ephippia. It was also designed to simultaneously examine the effects of variable isotopic composition of food and water on the C and O stable isotopic composition of *Daphnia* ephippia. As the reviewer indicates the experiments presented in our manuscript already represent a considerable amount of work and measurements. Further expanding the experiment with more treatments was simply not feasible with our available resources. We agree that future investigations with more detailed attention to specific aspects, such as temperature, are needed for *Daphnia*, but also for other organisms producing chitinous microfossils that are analysed in palaeoecological studies focusing on stable isotopes.

Our main concern regarding temperature was that the difference between the two temperature values should be relatively large (in our experiments 8°C) to ensure that any

potential effect on the offset between ephippia and *Daphnia* δ^{18} O values due to temperature would become apparent. 20°C is on the higher end of temperature values that *Daphnia* are exposed to in nature. However, we do not agree with the statement that a temperature of 20°C is irrelevant in the context of palaeoclimate reconstruction. The temperature in the epilimnion of lakes in temperate climates regularly exceeds 20°C during late summer and early fall. In shallow unstratified lakes the entire water column may exceed 20°C during this period. Since in many lakes *Daphnia* ephippia are also produced during late summer and early fall, temperatures of 20°C are therefore not irrelevant in a palaeoclimate context, especially since past climates in many parts of the world include not just periods of cooler temperature than at present, but also warmer intervals (e.g. in Europe in the early Holocene). Moreover, the *D. pulicaria* clones used in this study originate from Lower Lake Constance, where temperatures above 20°C in the upper 10 m of the water column are quite common during summer months (see e.g. green reports on www.igkb.org).

In previous experiments, using the same incubators, it was established that the temperature of the culturing waters can be maintained at a stable level. The temperature never deviated more than 1-2°C from the target temperature when lights (i.e. an extra heat source) were used to simulate a diurnal cycle, whereas in our experiments the lights were never on.

<u>Reviewer 2 comment</u>: Results: It is encouraging to note that there is no statistical difference between δ 18Owater in the "cold" and "warm" treatments. From what I could infer from your results it looks like the Δ 18O between stock and culture waters was pretty small, suggesting minimal evaporative enrichment. Is this a correct inference?

Author reply: Yes, this is correct, the difference was on average 0.6 ‰.

<u>Reviewer 2 comment</u>: I had been considering an elaborate condensing unit to combat the effects of evaporation in my own experimentation but in the end opted for a similar solution to you. However, δ 18Oalgae (Figure 2) jumped by approximately 4 per mill from day 10 to 20, any idea what caused this?

<u>Author reply</u>: We'd like to first point out, for clarity, that the algae were not cultured in the lake water we used in the experiment. The stock solution used for this purpose was based on distilled water with added nutrients. The algae indeed show a peak in δ^{18} O values around day 17 of the experiment. We also noticed that a peak is visible in the δ^{13} C values and C:N ratio of the algae around this time, which may be indicative of a peak in the growth rate of the algae in the cultures. It may be that the peak in algae δ^{18} O is related to a peak in oxygen production and/or consumption in the chemostats, although we cannot demonstrate this based on the available data.

<u>Reviewer 2 comment</u>: Mean isotope values are presented in this section, could you clarify how many samples were measured for each value (n=..).

<u>Author reply</u>: The means presented are based on the results of three replicate treatments, and for each replicate treatment three replicate measurements were undertaken. Therefore, every mean represents 9 measurements. We will clarify this in the text of the revised manuscript.

Author comment to editor: This has been clarified on p. 10 line 9-10.

<u>Reviewer 2 comment</u>: Discussion: In the discussion section the authors state that the unexpected isotopic differences between similar treatments may represent inherent variability in individuals measured. This argument would certainly be valid in nature but given the controlled laboratory conditions in this investigation I suspect that analytical uncertainties and/or variability in the isotopic composition of diet, to be the primary sources of the unexpected variability observed between the similar treatments.

<u>Author reply</u>: The *Daphnia* were given algae from the same stock in the different treatments for which no differences where expected. However, the period of maximum growth may have differed between treatments. Since the isotopic composition of algae did not remain entirely constant over the duration of the experiment, we therefore agree that the isotopic composition of the diet may have played a role for explaining these unexpected differences. In the submitted version of our manuscript we discuss this on p 2586, line 22-25. In the revised version of our manuscript we will move this section up so that it will immediately follow the statement on the inherent variability of individuals measured.

However, the isotopic composition of *Daphnia* was measured 9 times for each isotope in each treatment, and the minor observed differences were very consistent within the treatments. We therefore think that the analytical precision is unlikely to be the cause for the observed variability between the treatments.

<u>Author comment to editor</u>: The statement regarding the differences in timing of maximum growth being a potential cause of the minor differences found between all treatments was moved to lines 1-4 of page 12 and slightly adjusted to connect to the sentence before it.

<u>Reviewer 2 comment</u>: I think the conclusion that the isotopic composition of eppihia reflect Daphina is fair but I feel more emphasis should be placed on the fact that relationship between the two appears not be completely straightforward with further laboratory and field based calibration studies required to accurately determine the fractionations involved during the incorporation of environmental isotopic signatures into both the living Daphina and their fossilizing structures.

In particular, given the results presented in this study greater attention must now be paid to the influence of temperature. My own experimentation with chitinous remains also supports the presence of temperature dependant fractionations, however as with this investigation the magnitude of this influence is similar to analytical uncertainties

<u>Author reply</u>: We agree. Based on the reviewer's comments we will emphasize in the section *Implications for palaeoecological studies* that further studies in the laboratory and in the field are necessary to determine the fractionations involved during the incorporation of environmental isotopic signatures into *Daphnia* ephippia, and especially to further constrain the effects of temperature on the isotopic composition of *Daphnia* and their fossilizing structures.

<u>Author comment to editor</u>: On page 18, in the section running from line line 6-18 we have added text emphasizing the need for further work on the offset between the stable isotopic composition of aquatic invertebrates, their chitinous fossilizing structures and their food and water sources, and that specific attention is needed with regards to potential temperature effects.

Experimental assessment of environmental influences on the The stable isotopic composition of Daphnia pulicaria and their ephippia reflects changes in δ^{13} C and δ^{18} O values of food and water

J. Schilder¹, C. Tellenbach^{2,3}, M. Möst^{2,3}, P. Spaak^{2,3}, M. van Hardenbroek^{1,4}, M. J. Wooller^{5,6} and O. Heiri¹

[1]{Institute of Plant Sciences and Oeschger Centre for Climate Change Research, University of Bern, Altenbergrain 21, 3013 Bern, Switzerland}

[2]{Department of Aquatic Ecology, Eawag, Überlandstrasse 133, CH-8600 Dübendorf, Switzerland}

[3] {Institute of Integrative Biology, ETH Zurich, Zurich, Switzerland}

[4]{Geography and Environment, University of Southampton, Southampton SO17 1BJ, United KingdomUK}

[5]{University of Alaska, School of Fisheries and Ocean Sciences &, Fairbanks, AK 99775-7220, USA}

[6]{Alaska Stable Isotope Facility, Water and Environmental Research Center, Institute of Northern Engineering 99775, <u>Fairbanks, AK 99775-7220</u>, USA}

Correspondence to: J. Schilder (j.c.schilder@gmail.com)

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 (δ^{13} C value) of the algae, stable oxygen isotopic composition (δ^{18} O value) of the water, and the water temperature, respectively. The stable nitrogen isotopic composition (δ^{15} N value) of the algae was similar for all treatments. At 12 °C, differences in algal δ^{13} C values and in δ^{18} 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 (δ^{13} C: +0.2 ± 0.4 ‰ (standard deviation); δ^{15} N: -1.6 ± 0.4 ‰; δ^{18} O: -0.9 ± 0.4 ‰) indicating that changes in dietary δ^{13} C values and in δ^{18} O values of water are passed on to these fossilizing structures. A higher water temperature (20 °C) resulted in lower δ^{13} 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 δ^{13} C values of ephippia and *Daphnia* (to -1.3 ± 0.3 ‰). 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

Formatiert: Schriftart: Nicht Kursiv

Formatiert: Englisch (USA)
Formatiert: Untertitel
Formatiert: Niederländisch (Niederland
Formatiert: Schriftart: (Standard) Times
New Roman, Niederländisch (Niederland

Formatiert: Standard

between δ^{18} O and δ^{15} N values of ephippia and *Daphnia* between the 12 °C and 20 °C treatments, but the δ^{18} 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 that temperature may have a minor influence on the δ^{13} C, δ^{15} N and δ^{18} 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 δ^{13} 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 δ^{15} 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 δ^{18} 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 (<u>Heiri et al., 2012</u>; Leng and Henderson, 2013; <u>Heiri et al., 2012</u>). For example, the δ^{13} C values of fossil head capsules of benthic larvae of nonbiting 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 δ^{15} 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 δ^{18} O values have been reconstructed by analyzing the δ^{18} O values of fossil chironomid head capsules of chironomid head capsules and of chironomid head capsules (Wooller et al., 2004; Verbruggen et al., 2010b), and a correspondence has been found between δ^{18} O values of lake water and of chironomid head capsules and *Daphnia* ephippia buried in surface sediments (Verbruggen et al., 2011).

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).: Schilder et al., 2015).

The use of δ^{13} C and δ^{15} N values of organisms to infer likely organic carbon and nitrogen sources relies heavily on assumptions regarding the difference between δ^{13} C and δ^{15} N values of organisms and their diet (Δ^{13} C, Δ^{15} N). There is a need for more controlled laboratory studies investigating Δ^{13} C and Δ^{15} N (Martínez del Rio et al., 2009), and the relationships between the δ^{18} O values of organisms and those of environmental water (Rubenstein and Hobson, 2004). Δ¹³C, 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, Δ^{13} C values range from +1.7 to +3.1 & (Power et al., 2003). Δ^{15} 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 δ¹³C and 7.9 ‰ lower δ¹⁵N values than whole Daphnia (Perga, 2010) whereas no clear differences in δ¹³C and δ¹⁵N 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 δ^{18} O values of aquatic lacustrine invertebrates is are strongly and positively related to the δ^{18} O values of local precipitation and the water in which the invertebrates live (Wang et al., 2009; Nielson and Bowen, 2010; Verbruggen et al. 2011; van Hardenbroek et al., 2012; Soto et al., 2013)-To our knowledge, no controlled experiments), although laboratory studies have been performed examiningshown that the relationship between oxygen isotopic composition of the diet can also affect invertebrate δ^{18} O values of environmental water (Wang et al., 2009; Nielson and Daphnia, or their ephippia.Bowen, 2010).

There can be distinct offsets in isotopic composition between whole body tissue and chitinous structures of invertebrates. Culturing experiments comparing cephalopod soft tissue and their chitinous mouthparts have shown that their chitinous structures can have $\delta^{15}N$ values 3 to 4 % lower than soft body tissue (Hobson and Cherel, 2006). Heiri et al. (2012) demonstrated that offsets of up to 2 ‰ between chironomid body tissue and chitinous head capsule δ^{13} C and δ^{15} N values are possible. For Daphnia, field studies suggest that (non ephippial) exoskeleton parts can have 0.8 ‰ lower δ^{13} C and 7.9 ‰ lower δ^{15} N values than whole *Daphnia* (Perga, 2010), while no clear differences in δ^{13} C and δ^{15} N values between *Daphnia* and ephippia have been reported in the only available study which examined this offset for Daphnia and free ephippia collected in a vertical net trawl in Lake Geneva, Switzerland (Perga, 2011). For vertebrates, differences in stable C and N isotopic composition between tissue types have been related to differences in contents of specific compounds (e.g. relative abundance of lipids, carbohydrates and protein or of different amino acids; e.g. DeNiro and Epstein 1978; Pinnegar and Polunin, 1999). Differences in biochemical composition also provide a potential explanation for the observed differences in $\delta^{13}C$ and $\delta^{15}N$ values between whole body tissue and chitinous structures of aquatic invertebrates. For oxygen and hydrogen, studies examining the offsets between the stable isotopic composition of the whole body tissue of lacustrine invertebrates and their chitinous structures are still lacking.

To date, no controlled experiments investigating the offset between δ^{13} C, δ^{15} N and δ^{18} O values of whole body tissue and ephippia have been published for *Daphnia*. Similarly, no laboratory

experiments have been performed examining the relationship between δ^{18} O values of environmental water and *Daphnia*, or their ephippia. Quantifying these offsets and relationships is essential for further development of palaeoecological approaches based on stable isotope analyses on *Daphnia* remains and for interpreting results from the fossil record.

We present results from an experiment developed to examine the relationships between the δ^{13} C values of diet and the δ^{18} O values of environmental water, and the δ^{13} C and δ^{18} O values of *Daphnia*. The experiment was specifically designed to examine whether offsets in δ^{13} C, δ^{15} N and δ^{18} 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 μ m 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 C l⁻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 % ¹²C (Sigma Aldrich, USA), lowering the δ^{13} C values of the algae from this chemostat by on average 1.8 ± 1.2 (one standard deviation (1 SD)) ‰ (see results). 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 following 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 mg l⁻¹, TN 1.6 mg l⁻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 δ^{18} O value of the water with 5.6 ‰ relative to the unlabeled water (see results). Before refreshing the water in Treatment 4, the water was allowed to equilibrate with ambient laboratory air temperature (20 °C).

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 δ^{13} C values. The culturing water in Treatment 3 had δ^{18} O 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 to 4 jars per clone (Figure 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 (Figure 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 δ^{13} C values on those of *Daphnia* and their ephippia (Treatment 2), b) the effect of a change in environmental water δ^{18} O values on those of *Daphnia* and their ephippia (Treatment 3), c) the effect of a difference in temperature (i.e. 12 °C and 20 °C) on the δ^{13} C, δ^{15} N and δ^{18} O values of *Daphnia* and their ephippia (Treatment 4), and d) the offset between *Daphnia* and ephippia in terms of their δ^{13} C, δ^{15} N and δ^{18} O values (Treatment 4), and d) the offset between *Daphnia* and ephippia in terms of their δ^{13} C, δ^{15} N and δ^{18} O 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 Ime function in the nIme package in the R statistical package (R Core team, 2013). Significance was analyzed using an F-test. <u>A Bonferroni</u> correction was applied to the multiple (6) comparisons of the stable isotopic composition of *Daphnia* between the treatments (Tukey post-hoc tests).

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 to 200 µg) was loaded into tin cups (6 x 4 mm, Lüdi Swiss, Switzerland) to measure the δ^{13} C, δ^{15} N and δ^{18} O values of the algae ($\delta^{13}C_{algae}, \delta^{15}N_{algae}$ and $\delta^{18}O_{algae}$). In each treatment, one jar was assigned to monitoring variation in δ^{18} O values of the water ($\delta^{13}O_{water}$). 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 $\delta^{18}O_{water}$ values. Every third week a sample of the water in the storage barrels was collected, stored and measured for $\delta^{18}O_{water}$ values.

The experiment was terminated after 62 days. He and Wang (2006) have demonstrated that *Daphnia* carbon turnover rate is 11 to 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 x 4 mm, Lüdi Swiss, Switzerland; ~10 to 12 individuals per measurement) for analysis of $\delta^{13}C_{Daphnia}$, $\delta^{15}N_{Daphnia}$ and $\delta^{18}O_{Daphnia}$ values. For each treatment replicate group, three samples were prepared and measured, resulting in 36 measurements for each chemical element. Ephippia were collected and treated in 10 % KOH for 2 hours to remove any algal matter and egg yolk. Replicate measurements (3 each for C, N and O) of ephippia not treated with KOH were prepared to assess any influence of this treatment on the

isotopic compositions of ephippia. The ephippia were loaded into pre-weighed tin cups (6 x 4 mm, Lüdi Swiss, Switzerland): ~10 to 15 for $\delta^{13}C_{ephippia}$ and $\delta^{15}N_{ephippia}$ analysis, and 15 to 20 for $\delta^{18}O_{ephippia}$ analysis. Three samples were prepared and measured for each treatment replicate group, except for Treatment 4, which yielded only sufficient numbers of ephippia to measure once per treatment replicate group.

2.5 Assessing the source of oxygen in Daphnia

Following Wang et al. (2009), our experimental setup was used to approximate the proportional contribution of oxygen in the *Daphnia* stemming from the environmental water relative to that from the diet, using the following equation:

$$p = \frac{\left(\delta^{18}O_{Daphnia(A)} - \delta^{18}O_{Daphnia(B)}\right)}{\left(\delta^{18}O_{water(A)} - \delta^{18}O_{water(B)}\right)}$$
(1)

2.6 Stable isotope mass spectrometry

The δ^{13} C and δ^{15} N values of the algae, *Daphnia* and ephippia were measured on a Costech ESC 4010 elemental analyzer interfaced via a ThermoConflo III to a Thermo Delta V isotope ratio mass spectrometer (IRMS) at the Alaska Stable Isotope Facility (ASIF) at the University of Alaska, Fairbanks. The analytical precisions for δ^{13} C and δ^{15} 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 ± 0.1 ‰, respectively. The δ^{18} 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; ± 0.3 ‰). The δ^{18} 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 ± 0.4 ‰. Stable isotopeisotopic compositions are expressed in standard delta (δ) notation in ‰

3 Results

3.1 Food and water

The $\delta^{13}C_{algae}$ values from both chemostats showed some variation with time (Figure $2\underline{1}$). On all sampling dates except the first, the algae cultured on ¹³C-depleted medium had lower $\delta^{13}C_{algae}$ values than the standard algae (Figure $2\underline{1}$). As a consequence, the mean $\delta^{13}C_{algae}$ value for the culture grown using ¹³C-depleted medium (-20.6 ± 1.84 ‰) was $1.8 \pm 1.2 ‰$ (n = 9) lower than the mean $\delta^{13}C_{algae}$ of the standard algae (-18.8 ± 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 δ^{15} N values (standard algae 2.5 ± 0.3 ‰, ¹³C-depleted algae 2.2 ± 0.3 ‰; F_(1,8) 4.58, p>0.05), δ^{18} O values (standard algae 13.4 ± 1.0 ‰, ¹³C-depleted algae 14.6 ± 1.1 ‰; F_(1,7) 5.43, p>0.05), or atomic C:N ratios (standard algae 6.4 ± 1.3, ¹³C-depleted algae 6.5 ± 1.3; F_(1,8) 0.18, p>0.05) (Figure 2<u>1</u>).

The addition of ¹⁸O-enriched water led to an increase in $\delta^{18}O_{water}$ values in the storage barrels by 5.6 ‰ ($\delta^{18}O$ value of -3.4 ± 0.1 ‰, n = 3) relative to the non-labeled water ($\delta^{18}O$ value of -9.0 ± 0.1 ‰ n = 3) (Figure 32). The $\delta^{18}O_{water}$ 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 ± 0.5 ‰ (n = 11). Water from experimental jars from Treatment 3 had a mean $\delta^{18}O_{water}$ values of -3.3 ± 0.6 ‰ (n = 4). The mean $\delta^{18}O_{water}$ values in the storage barrels and the mean $\delta^{18}O_{water}$ values in the experimental jars after one week were used to approximate the baseline $\delta^{18}O_{water}$ values during cultivation for resolving Equation 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 ¹⁸O-enriched water.

3.2 Daphnia stable isotope ratios

Mean stable isotope values for *Daphnia* are based on 9 measurements (three measurements for each of the three replicates per treatment). The mean $\delta^{13}C_{Daphnia}$ value in Treatment 2 (where *Daphnia* were offered ¹³C-depleted algae) was lower (-20.2 ± 0.1 ‰) than in Treatment 1 (-18.7 ± 0.1 ‰) and 3 (-17.9 ± 0.1 ‰) (Figure 4<u>3</u>). For treatments at 12 °C (1-3), the mean $\delta^{13}C_{Daphnia}$ value was 0.5 ± 0.3 ‰ higher than the mean $\delta^{13}C_{algae}$ value *Daphnia* were cultured on. The mean $\delta^{13}C_{Daphnia}$ value in Treatment 4 (20 °C; -19.0 ± 0.1 ‰) was 0.2 ± 0.1 ‰ lower than the mean $\delta^{13}C_{algae}$ value. The results from all treatments in terms of $\delta^{13}C_{Daphnia}$ values were significantly different from each other (Oneway ANOVA and Tukey post-hoc test; Table 1)

Mean $\delta^{15}N_{Daphnia}$ values at 12 °C were 5.5 \pm 0.1 ‰ (Treatment 1), 5.7 \pm 0.1 ‰ (Treatment 2) and 6.2 \pm 0.1 ‰ (Treatment 3), and 3.4 \pm 0.3 ‰ higher than the mean $\delta^{15}N_{algae}$ value (Figure 4<u>3</u>). At 20 °C (Treatment 4), the mean $\delta^{15}N_{Daphnia}$ value (6.5 \pm 0.2 ‰) was 4.0 \pm 0.2 ‰ higher than the mean $\delta^{15}N_{algae}$ value. All treatments, except for Treatment 1 and 2, were significantly different from each other with regards to $\delta^{15}N_{Daphnia}$ 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 δ^{18} O values. The mean $\delta^{18}O_{Daphnia}$ values in these treatments were 11.7 ± 0.1 ‰ and 11.0 ± 0.2 ‰, respectively (Figure 4<u>3</u>). In Treatment 3, where the mean $\delta^{18}O_{water}$ value was 5.2 ‰ higher than in the other treatments, the mean $\delta^{18}O_{Daphnia}$ 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 $\delta^{18}O_{water}$ as in Treatment 1 and 2, but run at higher temperature (20 °C), the mean $\delta^{18}O_{Daphnia}$ value (10.2 ± 0.2 ‰) was 1.5 and 0.8 ‰ lower than in Treatment 1 and 2, respectively. A significant difference in $\delta^{18}O_{Daphnia}$ values was found between all treatments (One-way ANOVA and Tukey post-hoc test; Table 1).

3.3 Ephippia stable isotope ratios

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

The measurements we performed on untreated ephippia did not reveal a detectable effect of the KOH treatment on the $\delta^{13}C_{ephippia}$, $\delta^{15}N_{ephippia}$ and $\delta^{18}O_{ephippia}$ values (Figure 5; t-tests: $\delta^{13}C \pm 0.41$, p>0.05; $\delta^{15}N \pm 2.20$, p>0.05; $\delta^{18}O \pm 0.03$, p>0.05). The mean $\delta^{13}C_{ephippia}$ value was on average 0.2 ± 0.8 ‰ lower than the mean $\delta^{13}C_{Daphnia}$ value, but this difference was not statistically significant (paired t-test, t 0.83, p>0.05; Figure 64). 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 $\delta^{13}C_{ephippia}$ values were on average 0.2 ± 0.4 ‰ higher than $\delta^{13}C_{Daphnia}$, although this difference was again not significant (paired t-test, t 1.50, p>0.05). Over all four treatments, $\delta^{15}N_{ephippia}$ values were on average 1.6 ± 0.4 ‰ lower than $\delta^{13}N_{Daphnia}$ values (paired t-test, t 14.01, p<5·10⁻⁸), and $\delta^{18}O_{ephippia}$ values were on average 0.9 ± 0.4 ‰ lower than $\delta^{18}O_{Daphnia}$ values (paired t-test, t 5.58, p<5·10⁻⁵).

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 δ^{13} C values of the food source and temperature and only differed in the δ^{18} O values of the water-, and Treatment 1, 2 and 3 were identical in terms of δ^{15} N values of the food source and temperature. Treatment 1 and 2 were similar in terms of 6¹⁸O 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 (Figure 43). They may represent the inherent variability associated with stable isotope ratios in organisms (Schimmelmann, 2011). Alternatively, since in previous experiments 6¹²Comparis and 6¹⁵Normalize 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 (Figure 2). Therefore, 1), a slight difference in timing in the buildup of biomass may have led to small differences in Daphnia stable isotope ratios. In previous experiments $\delta^{13}C_{Daphnia}$ and $\delta^{15}N_{Daphnia}$ values have been found to differ as much as 1 ‰ between identical treatments (Power et al., 2003). The differences in Daphnia stable isotope ratios were much larger when comparing treatments with manipulated $\delta^{13}C_{algae}$ and $\delta^{18}O_{water}$ values to those with non-manipulated algae and water.

4.1 The food experiment: Changing $\delta^{13}C_{algae}$

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

 $δ^{13}$ C_{ephippia} values also reflected the difference in $δ^{13}$ C_{algae} values between the treatments. At 12 °C, they were not significantly different from the $δ^{13}$ C_{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 $δ^{13}$ C value of ephippia deposited collected in sediment trapsthe field was slightly, but not significantly higher than the $δ^{13}$ C value of Daphnia collected in the same lakenet trawls. This suggests that $δ^{13}$ C ephippia values are a reliable indicator for changes in $δ^{13}$ C cophippia was 0.7 ± 0.2 ‰ higher than the mean $δ^{13}$ C values of Daphnia diet: at 12 °C $δ^{13}$ C ephippia was 0.7 ± 0.2 ‰ higher than the mean $δ^{13}$ C algae. The absence of a clear offset in δ^{13} C values 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 $\delta^{^{15}}N$ values of Daphnia and ephippia

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

 $δ^{15}N_{ephippia}$ values were lower (1.6 ± 0.4 ‰) than $δ^{15}N_{Daphnia}$ values. In contrast, Perga (2011) found $δ^{15}N_{ephippia}$ values to be slightly, but not significantly lower than $δ^{15}N_{Daphnia}$ values in the field. Together with Perga's (2011) results, our data provide an indication that $δ^{15}N_{ephippia}$ values are indicative of $δ^{15}N$ 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 $δ^{15}N$ values and head capsule $δ^{15}N$ values (-1 to +1 ‰) were observed over a large range of $δ^{15}N$ values (2.5 to 15 ‰; Heiri et al., 2012). Therefore, it seems likely that differences between *Daphnia* and ephippia $δ^{15}N$ values may also be similar across this $δ^{15}N$ range.

4.3 The water experiment: Changing $\delta^{18}O_{water}$ values

 $δ^{18}O_{water}$ values were 5.2 ‰ higher in Treatment 3 than in Treatment 1 and 2, and the mean $δ^{18}O_{Daphnia}$ 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 $δ^{18}O_{Daphnia}$ values reflect differences in $δ^{18}O_{water}$, 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 equation (1), we estimate that in our experiment 56 to 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).

 $\delta^{18}O_{ephippia}$ values closely reflected differences in $\delta^{18}O_{Daphnia}$: They were on average 0.9 ± 0.4 ‰ lower than $\delta^{18}O_{Daphnia}$ values. This suggests that $\delta^{18}O_{ephippia}$ may be used as an indicator of $\delta^{18}O_{Daphnia}$, which in turn can be expected to be related to lake water $\delta^{18}O$ values. This is in agreement with the correspondence between surface sediment $\delta^{18}O_{ephippia}$ values and lake water $\delta^{18}O$ 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 Δ^{13} C values for *D. magna* with a temperature increase from 12 °C to 20 °C (and +1.4 ‰ when temperature increased from 12 °C to 26 °C). Therefore, we expected Δ^{13} C values for *Daphnia* in Treatment 4 (20 °C) to be similar to or slightly higher than in the other treatments (12 °C). Δ^{13} C values were clearly lower, however, in Treatment 4 $(-0.2 \pm 0.1 \%)$ than in the other treatments $(+0.5 \pm 0.3 \%)$. While we cannot exclude a negative relation between temperature and Δ^{13} C values for *Daphnia*, we choose to treat this result with caution due to the discrepancy with the positive Δ^{13} 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 $\delta^{13}C_{Daphnia}$ 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, ¹³C-depletion of algal biomass during dark respiration may have affected the $\delta^{13}C_{algae}$ in Treatment 4 disproportionally due to the higher temperature. Degens et al. (1968) found that δ^{13} 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 $\delta^{13}C_{algae}$ values during the main phase of their growth compared to the other treatments, since $\delta^{13}C_{algae}$ values were relatively low in the beginning and at the end of the experiment (Figure 21). $\delta^{13}C_{ephippia}$ values were also lower in Treatment 4, and 1.3 ± 0.3 ‰ lower than $\delta^{13}C_{Daphnia}$ 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 $\delta^{13}C_{Daphnia}$ and $\delta^{13}C_{ephippia}$ with temperature or whether it is affected by variations in $\delta^{13}C_{algae}$ and algal respiration rates or differences in Daphnia growth rates between our treatments. Controlled experiments over a range of temperature values analyzing not only $\delta^{13}C_{Daphnia}$ and $\delta^{13}C_{ephippia}$ values, but also $\delta^{13}C$ values of respired CO₂ and microbial biomass would be desirable to further explore this issue. Although the results of Treatment 4 indicate that the difference between $\delta^{13}C_{ephippia}$ and $\delta^{13}C_{Daphnia}$ values may be more variable than indicated by the cultivations at 12 °C, the offset is still relatively small compared to the variation in $\delta^{13}C_{ephippia}$ values in lake sediments sediment records (up to 10 %; e.g. Wooller et al., 2012).

 Δ^{15} N between *Daphnia* and algae was +4.0 ± 0.2 ‰ at 20 °C, 0.6 ‰ higher than at 12 °C. A small increase (0.4 ‰) in Δ^{15} 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 Δ^{15} N values for *D. magna* between 20 °C and 26 °C, however, and Barnes et al. (2007) found a decrease of 0.6 ‰ in Δ^{15} N values for sea bass with a temperature increase from 11 °C to 16 °C. Previously observed Δ^{15} 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 Δ^{15} 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 δ^{15} N_{Daphnia} and δ^{15} N_{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-in experimental studies. Schimmelmann and DeNiro (1986) analyzed the δ^{18} O values of chitin of marine crustaceans collected along a temperature gradient of 10 °C and van Hardenbroek et al. (2012) studied the δ^{18} 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 δ^{18} O values and we found that $\delta^{18}O_{Daphnia}$ was slightly (0.8 to 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 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, $\delta^{18}O_{\text{Daphnia}}$ values most likely primarily reflect environmental water δ^{18} O values. The offset between $\delta^{18}O_{ephippia}$ and $\delta^{18}O_{Daphnia}$ 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 $\delta^{18}O_{water}$ and $\delta^{18}O_{Daphnia}$, this offset is not affected by temperature in the investigated temperature range (12 °C to 20 °C). Verbruggen et al. (2011) measured the δ^{18} O values of recently deposited ephippia from surface sediments in lakes along a geographical gradient in Europe. They found a strong correlation between $\delta^{18}O_{ephippia}$ values and lake water δ^{18} O values. In their dataset, the δ^{18} O values of lake water increased by ~4.8 ‰ with a temperature increase of 8 °C, whereas $\delta^{18}O_{ephippia}$ values increased by only ~3 ‰ over this temperature gradient, a difference of ~1.8 ‰. This difference is of a similar order of magnitude as the 0.8 to 1.5 ‰ lower $\delta^{18}O_{Daphnia}$ 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 ¹⁸O between lake water and Daphnia biomass. However, other mechanisms, such as a change in timing of Daphnia ephippia production with temperature and variations in δ^{18} O values of food across the examined temperature gradient could also explain varying offsets between $\delta^{18}O_{water}$ and $\delta^{18}O_{Daphnia}$ 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 (Figure 64), and the ephippia stable isotope ratios responded to the manipulations in $\delta^{13}C_{algae}$ and $\delta^{18}O_{water}$ we performed. Studies investigating the $\delta^{13}C$ and $\delta^{15}N$ values of fossil *Daphnia* ephippia have recorded shifts up to 5 to 10 ‰ in $\delta^{13}C$ values (Wooller et al., 2012; Frossard et al., 2014) and 3 ‰ in $\delta^{15}N$ values (Griffiths et al., 2010). Shifts of 2 to 3 ‰ in $\delta^{13}O$ values have been reported for fossil chironomid head capsules (Wooller et al., 2004; Verbruggen et al., 2010b). In our experiment, the standard deviation of the offset between *Daphnia* and ephippia stable isotope ratios was much smaller than the reported shifts in stable isotope ratios of fossil remains: ± 0.4 ‰ for $\delta^{13}C$,

 δ^{15} N and δ^{18} O (± 0.8 ‰ for δ^{13} 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 δ^{18} O of the water they lived in. While experiments offer the possibility to strongly control the food sources and growth conditions for Daphnia, they cannot cover the full range of environments and interactions found in nature. Eurther studies in the field, in the fossil record and in an experimental setting are therefore needed to confirm the findings we present here and improve our understanding of the relationship between the stable isotopic composition of food, ambient water and chitinous fossilizing structures produced by Daphnia and other invertebrates. Although we only cultured Daphnia at two different temperatures, we found indications that temperature may have affected $\Delta^{13}C$ and $\Delta^{15}N$, and the relationship between $\delta^{18}O_{water}$ and $\delta^{18}O_{Daphnia}$ values in an experimental setting. Future experiments could be conducted at a range of temperatures to examine such potential influencesefforts focused on constraining the effect of temperature- on these offsets and relationships are therefore particularly needed.

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. <u>We thank two anonymous referees for their valuable comments</u> on an earlier version of this manuscript. 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.S.).

References

Adams, T. S. and Sterner, R. W.: The effect of dietary nitrogen content on trophic level ¹⁵N enrichment, Limnol. Oceanogr., 45, 601–607, 2000.

Barnes, C., Sweeting, C. J., Jennings, S., Barry, J. T. and Polunin, N. V. C.: Effect of temperature and ration size on carbon and nitrogen stable isotope trophic fractionation, Funct. Ecol., 21, 356–362, 2007.

Belle, S., Parent, C., Frossard, V., Verneaux, V., Millet, L., Chronopoulou, P-M., Sabatier, P., and Magny, M.: Temporal changes in the contribution of methane-oxidizing bacteria to the biomass of chironomid larvae determined using stable carbon isotopes and ancient DNA, J. Paleolimnol., 52, 215-228, doi:10.1007/s10933-014-9789-z, 2014.

Journal of paleolimnology 52 (3) p. 215-228

Cáceres, C. E. and Tessier, A. J.: Incidence of diapause varies among populations of *Daphnia pulicaria*, Oecologia, 141(3), 425–31, doi:10.1007/s00442-004-1657-5, 2004.

Degens, E. T., Guillard, R. R. L., Sackett, W. M. and Hellebust, J. A.: Metabolic fractionation of carbon isotopes in marine plankton—I. Temperature and respiration experiments, Deep Sea Res. Oceanogr. Abstr., 15, 1–9, 1968.

DeNiro, M. and Epstein, S.: Influence of diet on the distribution of nitrogen isotopes in animals, Geochim. Cosmochim. Acta, 45, 341–351, 1981.

DeNiro, M. J. and Epstein, S.: Influence of diet on the distribution of carbon isotopes in animals, Geochim. Cosmochim. Acta, 42, 495–506, 1978.

Frossard, V., Belle, S., Verneaux, V., Millet, L. and Magny, M.: A study of the δ^{13} C offset between chironomid larvae and their exuvial head capsules: implications for palaeoecology, J. Paleolimnol., 50, 379–386, doi:10.1007/s10933-013-9732-8, 2013.

Frossard, V., Verneaux, V., Millet, L., Jenny, J.-P., Arnaud, F., Magny, M. and Perga, M.-E.: Reconstructing long-term changes (150 years) in the carbon cycle of a clear-water lake based on the stable carbon isotope composition (δ^{13} C) of chironomid and cladoceran subfossil remains, Freshw. Biol., 59, 789–802, doi:10.1111/fwb.12304, 2014.

Gannon, J. E.: Two counting cells for the enumeration of zooplankton micro-crustacea, Trans. Am. Microsc. Soc., 90, 486–490, 1971.

Geller, W. and Müller, H.: The filtration apparatus of Cladocera: Filter mesh-sizes and their implications on food selectivity, Oecologia, 49, 316–321, 1981.

Goedkoop, W., Åkerblom, N. and Demandt, M. H.: Trophic fractionation of carbon and nitrogen stable isotopes in *Chironomus riparius* reared on food of aquatic and terrestrial origin, Freshw. Biol., 51, 878–886, doi:10.1111/j.1365-2427.2006.01539.x, 2006.

Gophen, M. and Geller, W.: Filter mesh size and food particle uptake by *Daphnia*, Oecologia, 64, 408–412, 1984.

Griffiths, K., Michelutti, N., Blais, J. M., Kimpe, L. E. and Smol, J. P.: Comparing nitrogen isotopic signals between bulk sediments and invertebrate remains in High Arctic seabird-influenced ponds, J. Paleolimnol., 44, 405–412, doi:10.1007/s10933-009-9354-3, 2010.

Guillard, R. L.: Cultures of phytoplankton for feeding of marine invertebrates, in Culture of marine invertebrate animals conference, edited by W. L. Smith and M. H. Chanley, p. 338, Plenum Press, New York., 1975.

Hammer, Ø., Harper, D. A. T. and Ryan, P. D.: PAST: paleontological Statistics software package for education and data analysis, Paleontol. Electron., 4, 9, 2001.

He, X. and Wang, W.-X.: Releases of ingested phytoplankton carbon by *Daphnia magna*, Freshw. Biol., 51, 649–665, doi:10.1111/j.1365-2427.2006.01519.x, 2006.

Heiri, O., Schilder, J. and Hardenbroek, M. van: Stable isotopic analysis of fossil chironomids as an approach to environmental reconstruction: state of development and future challenges, Fauna Nor., 31, 7–18, doi:10.5324/fn.v31i0.1436., 2012.

Hobson, K. A.: Applying Isotopic Methods to Tracking Animal Movements, in Tracking animal migration with stable isotopes, vol. 7961, edited by K. A. Hobson and L. I. Wassenaar, pp. 45–78, Academic Press, Waltham., 2008.

Hobson, K. A. and Cherel, Y.: Isotopic reconstruction of marine food webs using cephalopod beaks: new insight from captively raised *Sepia officinalis*, Can. J. Zool., 84, 766-770, doi:10.1139/Z06-049, 2006.

Kamjunke, N., Benndorf, A., Wilbert, C., Opitz, M., Kranich, J., Bollenbach, M. and Benndorf, J.: Bacteria ingestion by *Daphnia galeata* in a biomanipulated reservoir: a mechanism stabilizing biomanipulation?, Hydrobiologia, 403, 109–121, doi:10.1023/A:1003722318598, 1999.

Kleiven, O. T., Larsson, P. and Hobæk, A.: Sexual reproduction in *Daphnia magna* requires three stimuli, Oikos, 65, 197–206, 1992.

Lampert, W.: *Daphnia*: Development of a model organism, in Excellence in ecology, p. 250, International ecology institute, Oldendorf/Luhe., 2011.

Leng, M. J. and Henderson A. C. G.: Recent advances in isotopes as palaeolimnological proxies, J. Paleolimnol., 49, 481-496, 2013.

Martínez del Rio, C., Wolf, N., Carleton, S. A. and Gannes, L. Z.: Isotopic ecology ten years after a call for more laboratory experiments, Biol. Rev. Camb. Philos. Soc., 84, 91–111, doi:10.1111/j.1469-185X.2008.00064.x, 2009.

Matthews, B. and Mazumder, A.: Detecting trophic-level variation in consumer assemblages, Freshw. Biol., 53, 1942–1953, doi:10.1111/j.1365-2427.2008.02018.x, 2008.

McConnaughey, T. and McRoy, C. P.: Food-Web structure and the fractionation of Carbon isotopes in the bering sea, Mar. Biol., 53, 257–262, doi:10.1007/BF00952434, 1979.

McCutchan, J. H., Lewis, W. M., Kendall, C. and Mcgrath, C. C.: Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur, Oikos, 102, 378–390, 2003.

Miller, R. F., Voss-Foucart, M.-F., Toussaint, C. and Jeuniaux, C.: Chitin preservation in quaternary Coleoptera: preliminary results, Palaeogeogr. Palaeoclimatol. Palaeoecol., 103, 133–140, 1993.

Minagawa, M. and Wada, E.: Stepwise enrichment of ¹⁵N along food chains: Further evidence and the relation between δ^{15} N and animal age, Geochim. Cosmochim. Acta, 48, 1135–1140, 1984.

Mintenbeck, K., Brey, T., Jacob, U., Knust, R. and Struck, U.: How to account for the lipid effect on carbon stable-isotope ratio (δ^{13} C): sample treatment effects and model bias, J. Fish Biol., 72, 815–830, doi:10.1111/j.1095-8649.2007.01754.x, 2008.

Möst, M.: Environmental change and its impact on hybridising *Daphnia* species complexes , ETH, Zürich., 2013.

Nielson, K. E. and Bowen, G. J.: Hydrogen and oxygen in brine shrimp chitin reflect environmental water and dietary isotopic composition, Geochim. Cosmochim. Acta, 74, 1812–1822, doi:10.1016/j.gca.2009.12.025, 2010.

Perga, M.-E.: Potential of δ^{13} C and δ^{15} N of cladoceran subfossil exoskeletons for paleo-ecological studies, J. Paleolimnol., 44, 387–395, doi:10.1007/s10933-009-9340-9, 2010.

Perga, M.-E.: Taphonomic and early diagenetic effects on the C and N stable isotope composition of cladoceran remains: implications for paleoecological studies, J. Paleolimnol., 46, 203–213, doi:10.1007/s10933-011-9532-y, 2011.

<u>Pinnegar, J. K. and Polunin, N. V. C.: Differential fractionation of δ^{13} C and δ^{15} N among fish tissues: implications for the study of trophic interactions, Funct. Ecol., 13, 225-231, doi:10.1046/j.1365-2435.1999.00301.x, 1999.</u>

Power, M., Guiguer, K. R. R. A. and Barton, D. R.: Effects of temperature on isotopic enrichment in *Daphnia magna*: implications for aquatic food-web studies., Rapid Commun. Mass Spectrom., 17, 1619–25, doi:10.1002/rcm.1094, 2003.

R Core Team: R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, 2013.

Rubenstein, D. R. and Hobson, K. A.: From birds to butterflies: animal movement patterns and stable isotopes, Trends Ecol. Evol., 19, 256–63, doi:10.1016/j.tree.2004.03.017, 2004.

Schilder, J. Bastviken, D., van Hardenbroek, M., Leuenberger, M., Rinta, P., Stötter, T. And Heiri, O.: The stable carbon isotopic composition of *Daphnia* ephippia in small, temperate lakes reflects in-lake methane availability, Limnol. Oceanogr., doi:10.1002/lno.10079, 2015.

Schimmelmann, A.: Carbon, nitrogen and oxygen stable isotope ratios in chitin, in Chitin: formation and diagenesis, edited by N. S. Gupta, pp. 81–103, Springer, New York., 2011.

Schimmelmann, A. and DeNiro, M. J.: Stable isotopic studies on chitin. III. The D/H and ¹⁸O/¹⁶O ratios in arthropod chitin, Geochim. Cosmochim. Acta, 50, 1485–1496, 1986.

Smyntek, P. M., Teece, M. A., Schulz, K. L. and Thackeray, S. J.: A standard protocol for stable isotope analysis of zooplankton in aquatic food web research using mass balance correction models, Limnol. Oceanogr., 52, 2135–2146, doi:10.4319/lo.2007.52.5.2135, 2007.

Soto, D. X., Wassenaar, L. I. and Hobson, K. A.: Stable hydrogen and oxygen isotopes in aquatic food webs are tracers of diet and provenance, edited by D. Raubenheimer, Funct. Ecol., 27, 535–543, doi:10.1111/1365-2435.12054, 2013.

Szeroczyńska. Krystyna and Sarmaja-Korjonen, K.: Atlas of subfossil cladocera from central and northern Europe, Friends of the Lower Vistula society, Świecie., 2007.

Vanderklift, M. A. and Ponsard, S.: Sources of variation in consumer-diet δ^{15} N enrichment: a metaanalysis., Oecologia, 136, 169–82, doi:10.1007/s00442-003-1270-z, 2003. Vander Zanden, M. J. and Rasmussen, J. B.: Primary consumer δ^{13} C and δ^{15} N and the trophic position of aquatic consumers, Ecology, 80, 1395–1404, 1999.

Vander Zanden, M. J. and Rasmussen, J. B.: Variation in δ^{15} N and δ^{13} C trophic fractionation: Implications for aquatic food web studies, Limnol. Oceanogr., 46, 2061–2066, 2001.

Van Hardenbroek, M., Gröcke, D. R., Sauer, P. E. and Elias, S. A.: North American transect of stable hydrogen and oxygen isotopes in water beetles from a museum collection, J. Paleolimnol., 48, 461–470, doi:10.1007/s10933-012-9623-4, 2012.

Van Hardenbroek, M., Heiri, O., Parmentier, F. J. W., Bastviken, D., Ilyashuk, B. P., Wiklund, J. A., Hall, R. I. and Lotter, A. F.: Evidence for past variations in methane availability in a Siberian thermokarst lake based on δ^{13} C of chitinous invertebrate remains, Quat. Sci. Rev., 66, 74–84, doi:10.1016/j.quascirev.2012.04.009, 2013.

Van Hardenbroek, M., Lotter, A. F., Bastviken, D., Andersen, T. J. and Heiri, O.: Taxon-specific δ^{13} C analysis of chitinous invertebrate remains in sediments from Strandsjön, Sweden, J. Paleolimnol., 52, 95–105, doi:10.1007/s10933-014-9780-8, 2014.

Verbruggen, F., Heiri, O., Reichart, G. J., Blaga, C. and Lotter, A. F.: Stable oxygen isotopes in chironomid and cladoceran remains as indicators for lake-water δ^{18} O, Limnol. Oceanogr., 56, 2071–2079, doi:10.4319/lo.2011.56.6.2071, 2011.

Verbruggen, F., Heiri, O., Reichart, G.-J., De Leeuw, J. W., Nierop, K. G. J. and Lotter, A. F.: Effects of chemical pretreatments on δ^{18} O measurements, chemical composition, and morphology of chironomid head capsules, J. Paleolimnol., 43, 857–872, 2010a.

Verbruggen, F., Heiri, O., Reichart, G.-J. and Lotter, A. F.: Chironomid δ^{18} O as a proxy for past lake water δ^{18} O: a Lateglacial record from Rotsee (Switzerland), Quat. Sci. Rev., 29, 2271–2279, doi:10.1016/j.quascirev.2010.05.030, 2010b.

Vona, V., Di Martino Rigano, V., Lobosco, O., Carfagna, S., Esposito, S. and Rigano, C.: Temperature responses of growth, photosynthesis, respiration and NADH: nitrate reductase in cryophilic and mesophilic algae, New Phytol., 163, 325–331, doi:10.1111/j.1469-8137.2004.01098.x, 2004.

Wang, Y. V, O'Brien, D. M., Jenson, J., Francis, D. and Wooller, M. J.: The influence of diet and water on the stable oxygen and hydrogen isotope composition of Chironomidae (Diptera) with paleoecological implications, Oecologia, 160, 225–33, doi:10.1007/s00442-009-1303-3, 2009.

Wooller, M. J., Francis, D., Fogel, M. L., Miller, G. H., Walker, I. R. and Wolfe, A. P.: Quantitative paleotemperature estimates from ¹⁸O of chironomid head capsules preserved in arctic lake sediments, J. Paleolimnol., 31, 267–274, 2004.

Wooller, M. J., Pohlman, J. W., Gaglioti, B. V, Langdon, P., Jones, M., Walter Anthony, K. M., Becker, K. W., Hinrichs, K.-U. and Elvert, M.: Reconstruction of past methane availability in an Arctic Alaska wetland indicates climate influenced methane release during the past ~12,000 years, J. Paleolimnol., 48, 27–42, doi:10.1007/s10933-012-9591-8, 2012.

Table 1. Results of One-way ANOVA and post-hoc Tukeythe tests for statistical differences between the four (1-4) treatments (One-way ANOVA) and between pairs of treatments (Tukey test) for $\delta^{13}C_{Daphnia}$, $\delta^{15}N_{Daphnia}$ and $\delta^{18}O_{Daphnia}$ 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 <u>after</u> <u>Bonferroni correction</u> (upper right).

		Daphnia δ ¹³ C values F _(2.3) 303.8 p<1·10 ⁻⁸				Daphnia δ ¹⁵ N values F _(2.3) 52.1 p<1·10 ⁻⁵					<i>Daphnia</i> δ ¹⁸ O values				
												F _(2.3) 2	55.3 p<1∙	Formatierte Tabel	
	1	2	3	4		1	2	3	4		1	2	3	4	
1		<u><</u> 0. 0002 <u>002</u>	<u><</u> 0. 0003 <u>002</u>	<u><</u> 0. 005 0 5	1		<u>≥</u> 0. 64 <u>89</u>	<u><</u> 0. 0006 <u>005</u>	<u><</u> 0. 0002 <u>002</u>	1		<u>≥</u> 0. 0 <u>21</u>	<u><</u> 0. 0002 <u>002</u>	<u><</u> 0. 0003 <u>005</u>	
2	28. 16		<u><</u> 0. 0002 <u>002</u>	<u><</u> 0. 0002 <u>002</u>	2	1.6 86		<u>≤0.0020</u> <u>1</u>	<u><</u> 0. 0003 <u>002</u>	2	5.6 46		<u><</u> 0. 0002 <u>002</u>	<u>>0.0105</u>	
3	13. 62	41.78		<u><</u> 0. 0002 <u>002</u>	3	10. 16	8.476		<u>≥0.031</u>	3	24. 6	30.2 5		<u><</u> 0. 0002 <u>002</u>	
4	6.9 68	21.19	20.58		4	15. 32	13.63	5.154		4	11. 88	6.23 4	36.48		

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 to 4 jars for each clone.

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

Figure 32. δ^{18} O values of the water in the storage barrels for the standard water (open circles, dashed line) and the artificially ¹⁸O-enriched water (closed circles, solid line) sampled on day 0, 13 and 35, and the δ^{18} O values of the water sampled from the experimental jars before water was exchanged for Treatment 1 (open diamonds, control), Treatment 2 (open triangles, ¹³C-depleted algae), and Treatment 3 (closed diamonds, ¹⁸O-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 ¹⁸O-enriched water, respectively.

Figure 4<u>3</u>. δ^{13} C, δ^{15} N and δ^{18} O values of *Daphnia* body tissue (left, open circles) and ephippia (right, closed circles) for Treatment 1 (control), 2 (¹³C-depleted algae), 3 (¹⁸O-enriched water) and 4 (elevated temperature). Each data point represents one of the treatment replicate groups and consists of three measurements, of which the standard deviation 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 δ^{13} C and δ^{15} N plots represent the average value of the algae used in that treatment.

Formatiert: Englisch (USA)

Formatiert: Englisch (USA)

Figure 5. δ^{43} C, δ^{45} N and δ^{48} 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 δ^{43} C and δ^{45} N are from Treatment 2, those for δ^{43} O from Treatment 1. Error bars indicate standard deviations.

Figure 6.

<u>Figure 4.</u> The difference in δ^{13} C, δ^{15} N and δ^{18} 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 δ^{13} C (see text). Error bars indicate standard deviations.