Stoichiometric responses to nano ZnO under warming are modified by thermal evolution in *Daphnia magna*

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ABSTRACT

Effects of stressors on body stoichiometry are important as these may cascade through food webs. Contamination and global warming are two key anthropogenic stressors, yet their effects on body stoichiometry have been rarely tested. Further, while thermal evolution may increase the ability to deal with warming, it is unknown how thermal evolution modifies the effect of contaminants under warming. Using resurrection ecology, we studied two *Daphnia magna* subpopulations (old/recent) of which the recent subpopulation evolved a higher heat tolerance. We exposed both subpopulations to a sublethal concentration of nano zinc oxide (nZnO) and 4 °C warming and quantified their effects on body stoichiometry: carbon (C), nitrogen (N), phosphorus (P) contents and their ratios (C:N, C:P, N:P). In the old subpopulation, nZnO only marginally decreased the C content and had no effect on N and P contents and their ratios. In contrast, in the recent subpopulation nZnO strongly increased the body P content (+51%) and reduced the C:P (−34%) and N:P (−34%) ratios at 24 °C but not at 20 °C. Moreover, these stoichiometric changes were not explained by changes of corresponding macromolecules as assumed by theory. Our results indicate that the stoichiometric responses to nZnO in *Daphnia* are temperature-dependent and modified by rapid evolution. The observed changes in body stoichiometry may affect the food quality of this important prey and have the potential to cascade through food webs and shape nutrients cycling.

1. Introduction

Current approaches to assess the risk of contaminants seems ineffective to protect freshwater ecosystems (Malaj et al., 2014; Peters et al., 2013). This could be linked to the presence of unknown pathways of how sublethal contaminants may cascade through food webs and eventually impact ecosystem functions. Stoichiometric responses to stressors may improve the mechanistic understanding of such cascading effects (Sterner and Elser, 2002). Indeed, how stressors shape body stoichiometry may impact the nutrient value of prey for higher trophic levels (Mcfeeters and Frost, 2011). For example, when feeding on P-limited algae the copepod *Acartia tonsa* suffers from a reduced growth (Plum et al., 2015). Furthermore, changes in body stoichiometry may affect key ecosystem functions related to nutrient cycling (Hawlena and Schmitz, 2010a, 2010b). For example, a predatory spider induced a 4% higher body C:N ratio of a grasshopper which on its turn resulted in a 3-fold decrease in the mineralization of plant litter (Hawlena et al., 2012). Body stoichiometry may therefore provide an unexplored organismal trait explaining how contaminants shape ecosystem functions, a key

challenge in ecological risk assessment (Peters et al., 2013; Power, 2010). Yet, whether and how contaminants affect body stoichiometry is largely unknown (but see Janssens et al., 2017).

Many contaminants get more toxic at higher temperatures (Noyes et al., 2009). For example, a review showed that ca. 30% of the studies testing combined effects of contaminants and warming showed a synergistic effect in freshwater ecosystems (Holmstrup et al., 2010). Such synergism can be expected as dealing with thermal stress requires energy thus reduces the available energy to deal with the contaminant (Liess et al., 2016). When a population evolves tolerance to a certain stressor that environmental condition is no longer experienced as a stressor (Van Straalen, 2003). With regard to warming, the acquisition of genetic adaptation to higher temperatures has been demonstrated in several taxa (e.g. Garvin et al., 2015; Gleason and Burton, 2015; Porcelli et al., 2015; for the studied species: Jansen et al., 2017; Yampolsky et al., 2014) and may help organisms to better cope with warming (Geerts et al., 2015; Ljungfeldt et al., 2017). Therefore, thermal evolution may render warming no longer a stressor, and potentially change the interaction between a contaminant and warming.

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A general predictive framework to understand how stressors affect body stoichiometry is the general stress paradigm (GSP, [Hawlena and Schmitz, 2010a](#)). The GSP predicts that under stress, animals will increase their metabolism and allocate resources to defense mechanisms. This is associated with an increased gluconeogenesis whereby carbon (C) rich biomolecules are created partially from the breakdown of nitrogen (N) rich proteins, which reduces the need for phosphorus (P) rich RNA for protein synthesis. This is expected to result in an increased excretion of N and P, and increased body ratios of C:N and C:P ([Hawlena and Schmitz, 2010b](#)). These predicted stoichiometric changes have been shown in response to predation risk ([Hawlena and Schmitz, 2010a](#)) and to warming ([Schmitz, 2013](#)), both stressors that typically increase metabolic rate (predation risk: [Hawlena and Schmitz, 2010b](#); warming: [Gillooly et al., 2001](#)).

The aim of this study is to test the single and combined effects of a nano contaminant (nano zinc oxide, nZnO) and warming on the stoichiometric responses in the water flea *Daphnia magna*, and explore whether thermal evolution can shape these responses. *Daphnia* are keystone species in aquatic food webs ([Miner et al., 2012](#)) and also model species in ecotoxicology ([OECD, 2004](#)). Hence, studying their stoichiometric responses to contaminants may increase our insight in contaminant effects on food web dynamics and ultimately the ecological risk assessment of contaminants. Nano ZnO is one of the most produced nanoparticles with an annual production of 550 tons worldwide and with environmental concentrations up to 17.1 mg kg dry mass in sludge ([Read et al., 2016](#)). Nano ZnO can have various toxic effects on aquatic organisms, such as inducing mortality (e.g. in the amphipod *Melita longidactyla*: [Wong and Leung, 2014](#)), decreasing fertilization success (e.g. in the sea urchin *Tripneustes gratilla*: [Mos et al., 2017](#)), and decreasing growth rate (e.g., in the fish *Oryzias melastigma*: [Wong and Leung, 2014](#); in *D. magna*: [Adam et al., 2014](#)).

To study the impact of evolution, we used resurrection ecology which reconstructs microevolution by comparing within a population the phenotypes of organisms hatched from ancestral eggs with those from descendants hatched from more recent eggs ([Orsini et al., 2013](#)). This approach has been applied to document evolutionary responses of populations to contaminants (e.g. [Kuester et al., 2016](#); [Turko et al., 2016](#)). Here, to study the effect of thermal evolution, we used two *Daphnia* subpopulations (old/recent) of which the recent subpopulation evolved a higher heat tolerance (measured as CT_{max}, [Geerts et al., 2015](#)). We compared the effects of nZnO and warming on *Daphnia* body stoichiometry (C, N and P contents and their ratios) in these two subpopulations. In a companion study ([Zhang et al., 2018](#)), we found that nZnO changed the macromolecules assumed to be important for body stoichiometry by the GSP (C-rich sugar and fat, N-rich proteins, and P-rich RNA) and also found that these changes differed between the two subpopulations. Specifically, nZnO exposure reduced the sugar content at 24 °C in both subpopulations, but the reduction was doubled in the recent subpopulation compare to the old subpopulation. Also nZnO exposure only reduced the RNA:DNA ratio at 24 °C in the old subpopulation. Here, we further tested the effects of nZnO and warming on body stoichiometry of both subpopulations. To advance our insights whether effects of contaminants on body stoichiometry operate through changes in assumed macromolecules, we also investigated the relationship between the body stoichiometry and the macromolecules contents (i.e. C to sugar/fat, N to protein, and P to RNA) ([Sterner and Elser, 2002](#)).

2. Materials and methods

2.1. Study animals and test chemical

Dormant eggs of *D. magna* were hatched from the sediment of Felbrigg Hall Lake (52°54.10'N, 1°15.19'E, UK) where the species has been continuously present since the 1940s ([Davidson et al., 2010](#)). We selected sediment layers corresponding to two periods: 1955–1965

(old) and 1995–2005 (recent). The recent period experienced an increase in average temperature of 1.2 °C and a threefold increase of heat waves compared to the old period ([Met Office, 2012](#)). Paleolimnological data showed that the levels of eutrophication, contamination and macrophyte coverage were highly similar between these two periods ([Sayer et al., 2010a, 2010b](#)). X-ray fluorescence analysis showed the contamination levels (including Zn) in both periods were low and similar (mean $\pm$ SD of Zn in the sediment, 1955–1965: 125.9 $\pm$ 2.0 $\mu\text{g/g}$; 1995–2005: 102.2 $\pm$ 1.7 $\mu\text{g/g}$) (C. Sayer & T. Davidson, unpublished data). The clones make up two subpopulations (hereafter called old and recent subpopulation) of the same population as they show genetic continuity using 43 microsatellite markers ([Geerts et al., 2015](#)).

Both subpopulations of *D. magna* were kept in monoclonal cultures for several years under standard lab conditions (20 °C, photoperiod of 14:10 L:D). All hatched clones were genetically distinct and seven clones were randomly selected from each subpopulation for this experiment. Prior to the exposure experiment, the clones were cultured under standard conditions (20 °C, photoperiod of 14:10 L:D, fed 1×10^5 cells mL^{-1} *S. obliquus* daily, medium refreshed every other day) for several generations (around three months). Cultures were reared in ISO 6341 medium ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$: 0.294 g L^{-1} , $\text{MgSO}_4 \cdot \text{H}_2\text{O}$: 0.123 g L^{-1} , NaHCO_3 : 0.065 g L^{-1} , KCl: 0.006 g L^{-1}) that is recommended for toxicity testing of metals ([OECD, 2004](#)).

A commercial nZnO dispersion (nanoparticles with average size < 35 nm, pH 7.0 $\pm$ 0.1, Sigma-Aldrich, Louis, MO, USA) was used to make the stock solution. Nano ZnO was characterized using a Zeiss EM900 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) (see Fig. S1 in S11). An initial stock solution of 5×10^3 mg L^{-1} Zn was prepared by ultrasonication of nZnO in an ultrasonic bath (Elmasonic S40, Elma®, Germany) for 30 min in Milli-Q water, and then stored at 4 °C under darkness. Each time before making a test solution, the stock solution was ultrasonicated for 30 min to eliminate aggregates.

2.2. Experimental procedure

A full factorial experiment was conducted where each clone was exposed to two nZnO treatments (0 and 86 $\mu\text{g L}^{-1}$, corresponding to 0 and 1.32 μM of Zn) at two temperatures: 20 °C matching the mean summer temperature in ponds inhabited by *Daphnia* in UK ([Van Doorslaer et al., 2010](#)), and 24 °C to simulate the 4 °C warming predicted by 2100 under IPCC (2013) scenario RCP8.5. The chosen concentration of nZnO was 10% of the EC_{50,48h} based on a range-finding experiment using all 14 clones.

The mothers of the experimental *Daphnia* were reared at 20 °C and 24 °C until the second clutch in ISO 6341 medium. Then, sets of 20 juveniles from each clone were placed in a 500 mL vial filled with 450 mL medium and randomly assigned to the nZnO treatments. As our focus was on differences between the two subpopulations, clones were used as replicates at the subpopulation level. So, we had one set of 20 juveniles per clone at each of 4 combinations of nZnO exposure and warming. *Daphnia* were fed daily and the medium was refreshed every other day. Zinc concentrations in unfiltered solution were measured after the medium was freshly renewed using inductively coupled plasma mass spectrometry (Agilent 7700x ICP-MS, Biocompare, USA, detection limit: 0.644 $\mu\text{g L}^{-1}$ Zn). The measured zinc concentrations in the zinc treatment group were 72.01 $\pm$ 2.14 $\mu\text{g L}^{-1}$ (1.11 $\pm$ 0.03 μM , mean $\pm$ SD, n = 5) at 20 °C and 73.24 $\pm$ 2.45 $\mu\text{g L}^{-1}$ at 24 °C (1.13 $\pm$ 0.04 μM , mean $\pm$ SD, n = 5). The measured zinc concentration in the control medium was 3.89 $\pm$ 0.18 $\mu\text{g L}^{-1}$ (0.06 $\pm$ 0.002 μM , mean $\pm$ SD, n = 2). The associated water quality parameters (mean $\pm$ SD, n = 5) across groups were the following: pH: 7.99 $\pm$ 0.03, conductivity: 623.23 $\pm$ 1.55 $\mu\text{S cm}^{-1}$, dissolved oxygen: 8.99 $\pm$ 0.19 mg L^{-1} , hardness: 239.10 $\pm$ 2.75 mg L^{-1} CaCO_3 . The measured temperatures were 20.33 $\pm$ 0.44 °C and 23.80 $\pm$ 0.14 °C, respectively. Ten *Daphnia* per vial were sampled and flash frozen after

they released the second clutch and stored at -80°C . The animals in the cohorts developed under each treatment combination sufficiently synchronously so that we could stop vials when all animals had released their second clutch and while there were no visual signs of the third clutch in the brood pouches.

To measure the body stoichiometry, *Daphnia* were homogenized in 150 μL MilliQ water. Of this homogenate, 40 μL was weighed to the nearest 0.1 μg by drying 60°C for 24 h using pre-weighed tin capsules and analyzed for body C and N contents with an Elemental CN analyzer (Thermo Electron EA 1110, Bremen, Germany). Another 120 μL homogenate was used to measure contents of P content by digesting with 1000 μL ultrapure HNO_3 in glass tubes and heating at 150°C for 20 min. Then the digests were diluted to 10 ml with MilliQ water and measured using inductively coupled plasma mass spectrometry (Agilent 7700x ICP-MS; Biocompare, USA). Four blanks without *Daphnia* homogenate were included as control. We expressed the stoichiometric ratios as molar ratios.

2.3. Statistical analyses

To specifically test for the effects of nZnO and temperature on the stoichiometric variables within each subpopulation, we carried out per subpopulation a set of two-way ANOVAs with nZnO and temperature as main factors. As each clone was tested under all four treatment combinations, we included clone as random factor. We also ran an overarching three-way ANOVA that included subpopulation as a factor (see supporting information). We ran full models with all interactions. Significance levels were not corrected for multiple testing (Moran, 2003). ANOVAs were conducted in the GLM module of STATISTICA v12.0 (Stat-soft, Tulsa, OK, USA). All variables met the ANOVA assumptions except for C:P and N:P, which were log-transformed.

To test for relationships between body stoichiometry and the assumed associated macromolecules, we regressed the three elements against the associated macromolecule: carbon against sugars and fat, nitrogen against proteins, and phosphorus against RNA (contents of sugars, fat and proteins see Zhang et al. (2018), RNA content see supporting information). We did so for each subpopulation separately. Linear regressions were conducted in R version 3.4.1 (R Core Team, 2013).

3. Results

3.1. Effects on body stoichiometry

In the old subpopulation, nZnO only tended ($P = 0.074$) to decrease the body C content (-2.6% , Table 1a, Fig. 1a), but did not affect any other stoichiometric trait (all $P > 0.10$). Neither warming, nor its interaction with nZnO affected stoichiometric traits (Table 2a).

In the recent subpopulation, nZnO tended ($P = 0.056$) to decrease the C:N ratio (-1.4% , Table 1b, Fig. 2b). Moreover, nZnO strongly decreased body C:P (-34.4%) and N:P (-33.2%) at 24°C but not at 20°C (nZnO $\times$ Temperature interactions, Table 1b, Fig. 2d, f). This was because nZnO strongly increased the body P content ($+51.3\%$) at 24°C but not at 20°C (nZnO $\times$ Temperature interactions, Table 1b, Fig. 1f) while it did not change body C and N contents at 24°C (Tukey HSD tests, C content: $P = 0.97$, N content: $P = 0.56$, Fig. 1b, d).

3.2. Relationships between body stoichiometry and macromolecules

We did not detect significant relationships between C, N and P contents and their associated macromolecules in the old subpopulation. The relationships all had a limited strength ($R^2 < 0.10$): carbon vs. sugar ($R^2 = 0.09$), carbon vs. fat ($R^2 = 0.01$), nitrogen vs. proteins ($R^2 = 0.0001$), and phosphorus vs. RNA ($R^2 = 0.003$) (all $P > 0.13$, Fig. 3).

While still moderate, we found positive relationships between the

Table 1

Summary of two-way ANOVAs testing for the effects of nZnO (Zn) and temperature (T) on the stoichiometry of *Daphnia magna* in the (a) old and (b) recent subpopulations. Significant P -values are marked in bold.

(a) Old subpopulation							
Variables		Zn		T		Zn $\times$ T	
	d.f.	F	P	F	P	F	P
%C	1, 18	3.63	0.073	0.06	0.814	0.00	0.965
%N	1, 18	2.56	0.127	0.07	0.800	0.20	0.657
%P	1, 18	0.43	0.520	2.85	0.109	0.11	0.740
C:N	1, 18	0.01	0.925	0.00	0.969	0.50	0.487
C:P	1, 18	1.27	0.274	1.29	0.270	0.37	0.553
N:P	1, 18	1.11	0.306	1.16	0.295	0.27	0.611
(b) Recent subpopulation							
Variables		Zn		T		Zn $\times$ T	
	d.f.	F	P	F	P	F	P
%C	1, 18	1.79	0.197	0.17	0.689	3.87	0.065
%N	1, 18	0.01	0.921	0.18	0.677	3.93	0.063
%P	1, 18	19.18	< 0.001	4.11	0.058	10.26	0.005
C:N	1, 18	3.95	0.062	0.04	0.835	0.66	0.426
C:P	1, 18	18.09	< 0.001	1.62	0.220	5.93	0.025
N:P	1, 18	16.80	0.001	1.89	0.186	5.71	0.028

carbon content and the sugar content ($R^2 = 0.28$, $P = 0.004$), and between the nitrogen content and the protein content ($R^2 = 0.24$, $P = 0.008$) in the recent subpopulation. As in the old subpopulation, there were no significant relationships between the carbon content and the fat content ($R^2 = 0.07$, $P = 0.159$), and between the phosphorus content and the RNA content ($R^2 = 0.003$, $P = 0.769$).

4. Discussion

We detected effects of nZnO on body stoichiometry, yet these did not match the predictions of the general stress paradigm (Hawlena and Schmitz, 2010a). Moreover, when *Daphnia* were exposed to this nano contaminant the effects on body stoichiometry were strongly dependent upon temperature and the subpopulation, indicating thermal evolution shaping the stoichiometric response to nZnO.

4.1. Stoichiometric responses to nZnO in the recent subpopulation

Contrary to the predicted increase in body C:N under stressors by the GSP (Hawlena and Schmitz, 2010a), nZnO marginally decreased the body C:N ratio in the recent subpopulation. The only two studies testing the stoichiometric response to contaminants both used pesticides and also showed no support for the GSP predictions: chlorpyrifos did not change body C:N in damselfly larvae (Janssens et al., 2017), and lindane decreased body C:N in *D. magna* (Ek et al., 2015). These deviations were explained by reductions in both C-rich fat and sugars and in N-rich proteins (Janssens et al., 2017), and a higher incorporation of carbon relative to nitrogen (Ek et al., 2015), respectively. In current study, this marginal decrease could be only partly explained by the associated decrease of the C-rich sugar content, as this decrease in sugar content was only observed at 24°C (Zhang et al., 2018). Furthermore, while we detected the expected positive relationship at the individual level between the sugar content and the C content in this subpopulation, this relationship was of moderate strength ($R^2 = 0.28$).

Exposure to nZnO considerably ($> 30\%$) decreased the body C:P and N:P ratios but only under warming in the recent subpopulation. This was because nZnO only increased the body P content but did not change body C and N contents under warming. This increase in P content was not related to increases in P-rich RNA, which instead

Fig. 1. Body elemental contents (C, N and P) of *Daphnia magna* as a function of nZnO and temperature in the old (a, c, e) and recent subpopulations (b, d, f). Given are least-squares means + 1 SE. The asterisks indicate significant differences between the nZnO treatment levels for a given temperature based on Tukey tests (** $P < 0.01$, *** $P < 0.001$).

decreased under nZnO exposure at 24 °C (Zhang et al., 2018). Moreover, we could not detect a relationship at the individual level between the P content and the RNA content in current study. We instead hypothesize the increase in P content under nZnO exposure was caused by a higher food intake. Food intake indeed strongly determines the body elemental content (Wilder and Jeyasingh, 2016), and algae have been shown to increase the body P content when exposed to similar zinc concentrations within 48 h (within the medium refresh time) through zinc-phosphate precipitation inside the cell (Gao et al., 2016). As the recent subpopulation has evolved a higher heat tolerance (Geerts et al., 2015), it was likely not (or less) stressed by the combination of warming and nZnO compared to the old subpopulation. Indeed, we found that the intrinsic growth rate, the metabolic rate and the energy storage molecules were only affected by warming and nZnO in the old subpopulation but not in the recent subpopulation (Zhang et al., 2018). Moreover, non-stressful warming often has been shown to increase the food intake rate in different taxa (e.g. Iles, 2014; Rall et al., 2012; Sanford, 1999; Vucic-Pestic et al., 2011; for the studied species: Rall et al., 2012; Tsui and Wang, 2004). We thereby hypothesize that the recent subpopulation had a higher food intake rate under warming resulting in the observed strong increase in P content when exposed to nZnO under warming. In addition, also a higher digestive enzyme activity may have contributed to the higher assimilation of P in the recent subpopulation under warming. As this was not the focus of current study, we have no data to directly test these hypotheses, but doing so

would be an important aspect of future mechanistic studies on the combined effects of Zn and warming on body stoichiometry.

4.2. Thermal evolution modified the effects of nZnO on body stoichiometry

Unlike the recent subpopulation, exposure to nZnO only marginally decreased the body C content in the old subpopulation and did not impact other stoichiometric responses, indicating rapid evolution could modify the stoichiometric responses to this nano contaminant. This absence of a clear stoichiometric response to nZnO occurred despite a nZnO-induced reduction in P-rich RNA and in C-rich sugar contents at 24 °C (Zhang et al., 2018). This suggests no coupling between nZnO-induced changes in these macromolecules and the body stoichiometry. Such decoupling was further confirmed by the absence of any significant relationships between the three key elements and the macromolecules in the old subpopulation. Notably, this also indicates that the relationships between carbon and fat contents, and between nitrogen and protein contents evolved: while being not present in the old subpopulation they were detected in the recent subpopulation.

The here observed rapid evolution of the stoichiometric responses to nZnO in terms of %P and body C:P and N:P ratios cannot be explained by an increase in Zn pollution from the old to the recent period. Indeed, the environmental concentrations of zinc in both periods in the sediment of Felbrigg Hall Lake are low and if anything the recent subpopulation experienced an even lower concentration compared to the

Fig. 2. Body stoichiometric molar ratios (C:N, C:P and N:P) of *Daphnia magna* as a function of nZnO and temperature in the old (a, c, e) and recent subpopulations (b, d, f). Given are least-squares means + 1 SE. The asterisks indicate significant differences between the nZnO treatment levels for a given temperature based on Tukey tests (* $P < 0.05$, ** $P < 0.01$).

old period (mean sediment concentrations of Zn, 1955–1965: $125.9 \pm 2.0 \mu\text{g/g}$; 1995–2005: $102.2 \pm 1.7 \mu\text{g/g}$, see methods). Although we cannot completely exclude the possibility that other variables may differ between the two periods, it is highly likely the thermal evolution shaped the changes to nZnO, particularly because the differences were only observed under warming. Indeed, the recent subpopulation evolved a higher heat tolerance in response to increases in both mean temperature and frequency of heat waves in the studied area (Geerts et al., 2015).

4.3. Relevance for ecological risk assessment

The here observed contaminant induced changes in the body stoichiometry of *D. magna* may have important yet overlooked effects that are highly relevant for ecological risk assessment. First, the changed body stoichiometry may alter the nutritional value of this keystone species for higher trophic levels in aquatic food webs. This may lower their growth rate as has, for example, been shown in an algae (resource) and copepod (consumer) system (Plum et al., 2015). It may thus provide new insight to understand how sublethal contaminant may generate indirect effects in natural communities (Relyea and Hoverman, 2006). Second, the stoichiometric responses to contaminant help to explain how contaminants shape ecosystem functions. Contaminants can change ecosystem functions (e.g. primary production and

community respiration) through either direct ways (for example by changing ecosystem properties such as pH) or indirect ways (for example by reducing biodiversity) (Mcmahon et al., 2012). The underlying mechanisms are not clear but are often related with the declined species at different trophic levels (Duffy et al., 2007). Our result indicated contaminants may shift ecosystem functions by affecting nutrient transfer through changing body stoichiometry, and indeed, the changed body stoichiometry has been shown to strongly affect nutrient cycling (Hawlena and Schmitz, 2010a, b).

Studying the effects of contaminants coupling with other stressors such as warming has long been a hot topic in ecotoxicology (Dinh Van et al., 2013; Moe et al., 2013; Sokolova and Lannig, 2008). While recently incorporating evolutionary insights to improve our understanding of ecotoxicology is also gaining increasing attention (Brady et al., 2017; Whitehead et al., 2017). Our study combined these two topics using a resurrection approach and showed the different reactions to contaminant between these two subpopulation under warming, which highlights the importance of considering *in situ* thermal evolution when assessing ecological risks of contaminants in a warming world.

5. Conclusions

Our results provide proof-of-principle that sublethal level of nano

Fig. 3. Relationships between the four key macromolecules (sugars, fat, proteins and RNA) identified in the general stress paradigm and their key element in *Daphnia magna* for the old (circle) and recent (triangle) subpopulations: (a) carbon vs. sugar, (b) carbon vs. fat, (c) nitrogen vs. protein, (d) phosphorus vs. RNA. The dashed/solid lines show the patterns for the old/recent subpopulation. DW refers to dry weight.

contaminant can change the body stoichiometry of a keystone species and that these stoichiometric responses under warming were modified by thermal evolution. Another important finding was that effects of the studied nano contaminant on key stoichiometric elements (carbon, nitrogen and phosphorus) and on key macromolecules were largely independent. This matches the idea that both types of response variables may respond differently to stressors (Wilder and Jeyasingh, 2016). Future work should focus on the detailed mechanisms underlying the here identified stoichiometric responses. Given *Daphnia* plays an important role in nutrient recycling in aquatic food webs (Miner et al., 2012), these strong stoichiometric changes may constitute an overlooked mechanism on how nano contaminants like nZnO may disturb ecosystem functioning, a key challenge in ecological risk assessment (Peters et al., 2013).

Author contributions

CZ and RS conceived the study and wrote the manuscript. CZ performed the experiment and analyzed the data. MJ, ES and LDM all edited the manuscript and all authors gave final approval for publication.

Data accessibility

Data for this study has been stored in figshare and can be accessed via the link: <https://figshare.com/s/178a6b5203bf06ceae33>.

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Competing interests

We declare no competing interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.aquatox.2018.07.005>.

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