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ORIGINAL ARTICLE

Interactive effects of copper and calcium in *Daphnia pulex*

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ABSTRACT

Many freshwater habitats around the world suffered dramatic water chemistry changes over the last century mostly due to anthropogenic activities, including an overall reduction in pH due to high sulfur emissions and unsustainable forestry practices. One consequence of this change in water chemistry is a drop in available calcium concentration, which creates problems for aquatic organisms that rely on dissolved calcium to build their exo- or endoskeletons and reinforce their carapace during regular molts. *Daphnia* populations in shield lakes in northern Ontario are also exposed to other stressors, including copper, which persists at high concentrations in many of these freshwater lakes and ponds due to mining and other human activities. Copper toxicity on animals is influenced by the availability of other competing ions, such as calcium. Using our newly developed high throughput toxicity screening system, we show that mortality of *Daphnia pulex* increases with exposure to low calcium (0.05 mg L^{-1}) and high copper (300 µg L^{-1}). When these two stressors were combined, we found that copper was less toxic at high calcium concentrations, indicating a protective effect of calcium against copper toxicity. We then established basic calcium uptake kinetics in *D. pulex* using radioactive tracer ^{45}Ca and provide evidence that copper, at environmentally relevant concentrations, competes with calcium uptake based on K_m and V_{max}. Our data show that both calcium decline and copper increase in aquatic ecosystems may negatively impact natural Daphnia populations, and that interactions between these two metals may occur in natural environments that result in fitness consequences for zooplankton.

Key words: *Daphnia pulex*; competition; ion uptake; calcium; copper.

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INTRODUCTION

Anthropogenic activities have resulted in drastic water-chemistry shifts of boreal lakes and watersheds in North America and Europe over the last century. These include but are not limited to acidification as a consequence of high sulfur emissions (SO_2) (Neary and Dillon, 1988; Keller *et al.,* 1999a, 1999b; Keller *et al.,* 2001; Keller, 2009). The deposition of strong acids in the water represents a major stressor in these freshwater habitats leading to the loss of fish and zooplankton (Neary and Dillon, 1988; Schindler, 1988; Gray *et al.,* 2012). Although industrial SO_2 emissions were reduced beginning in the 1980s and many lakes recovered to a less acidic pH, many lakes have not returned to their original state (Keller *et al.,* 1999a, 1999b; Keller *et al.,* 2001; Jeffries *et al.,* 2003; Keller, 2009).

One direct consequence of acidification is the reduction of available calcium to freshwater organisms (Jeziorski and Yan, 2006; Keller, 2009). Calcium is an essential element for normal cellular function and structural support in animals and plants alike. *Daphnia*, keystone species in many freshwater habitats, have especially high demands for dissolved calcium due to regularly occurring molts that replace their carapace, an essential mechanism allowing cladocerans and other crustaceans to grow in size (Ebert, 2005). Extensive growth and consequently high calcium demands occur during juvenile stages. Hence, calcium decline in lakes has been shown to impose limitations on *Daphnia* growth and development (Cairns and Yan, 2009; Giardini *et al.,* 2015a; Prater *et al.,* 2016). These limitations can also be expressed in the form of trade-offs where metabolic resources previously devoted to specific life-history traits or processes are redirected towards other processes (Ebert, 1992; Alstad *et al.,* 1999; Ashforth and Yan, 2008; Flatt and Heyland, 2013; Giardini *et al.,* 2015a).

One major challenge for *Daphnia* specifically, and other crustaceans in general is the need to transfer calcium from the external environment into tissues, while maintaining consistent cytosolic and blood calcium concentrations (Neufeld and Cameron, 1993). This results in a concentration gradient where the calcium concentration of the blood or circulatory system is higher than the concentration in the water. Consequently, these organisms must spend energy to actively take up and regulate calcium. This requires active transporters such as the plasma membrane Ca^{2+} ATPase along with facilitative uptake using Na^+/Ca^{2+} exchangers (Wheatly and Ayers, 1995; Chavez-Crooker *et al.,* 2002; Fig. 1 for more details). Crustaceans inhabiting hard fresh water or marine environments may not need to invest as

much metabolic energy to achieve calcium homeostasis compared to those inhabiting soft water environments (Neufeld and Cameron, 1993; Glover and Wood, 2005). Hence, reduction of available calcium can create stressful conditions for crustaceans (Jeziorski *et al.,* 2008a; Giardini *et al.,* 2015b; Prater *et al.,* 2016). A suite of other factors such as pharmaceuticals, elevated temperature, oxygen availability etc., can further amplify the consequences of such stressful situations for aquatic organisms. Thus, it is important to study the fitness consequences of stressors in combination, especially since it is difficult to predict if and to what degree the effect will be additive (sum of the individual effects), synergistic (greater than the sum of the individual effects), or antagonistic (lesser than the sum of the individual effects) (Folt *et al.,* 1999; Altshuler *et al.,* 2011).

At µg L^{-1} concentrations copper has been shown to be toxic to a diversity of aquatic organisms and, similarly to other metals, it has been shown to inhibit the activity of membrane-located carrier proteins as well as interact with sodium and calcium transport processes (Wheatly and Ayers, 1995; Chavez-Crooker *et al.,* 2002; Tellis *et al.,* 2014). For example, copper has been shown to inhibit sodium uptake in mussels (Giacomin *et al.,* 2013) and there is now evidence that copper disrupts calcium homeostasis in developing sea urchins (Tellis *et al.,* 2014). Furthermore, other metals have been shown to inhibit calcium uptake in fish (Hogstrand *et al.,* 1994). Copper toxicity can be suppressed when ambient concentrations of competing ions are high (Tan and Wang, 2009, 2012); in other words, certain ions can have protective effects against copper toxicity. More specifically, the metal ion can be in competition with other ions for the ligand binding site. For example, high calcium concentrations protect fish from copper toxicity (Pagenkopf, 1983; Lauren and Mcdonald, 1986; De Vera and Pocsidio, 1998; Chen *et al.,* 2012), and there is also evidence of this effect in *Daphnia magna* (De Schamphelaere and Janssen, 2002). Alternatively, protective effects could be the result of biomechanical processes whereby the protective ion alters the physical properties of the biotic ligand tissue (Markich and Jeffree, 1994; Di Toro *et al.,* 2001).

Many low-calcium lakes are contaminated with copper and their calcium levels are continuing to decline, creating a multiple stressor scenario. In particular, there are environmental concerns in regions such as Sudbury, Ontario, where copper has heavily contaminated water and sediments of shield lakes due to mining and in which calcium decline is also prevalent (Keller *et al.,* 1999b; Taylor *et al.,* 2016). Bioaccumulation of copper in invertebrates and fish has been documented (Winner, 1985; Chen *et al.,* 2012; Giacomin *et al.,* 2013). Furthermore, sublethal toxic effects of copper have been shown to act on subsequent generations in *Daphnia* (Fernandez-Gonzalez *et al.,* 2011). The effect that copper may have on calcium uptake has not been investigated for many freshwater planktonic organisms, and *Daphnia pulex* offers the ideal opportunity to gain detailed insight into the potential interaction of copper and calcium and its relevant to natural condi-

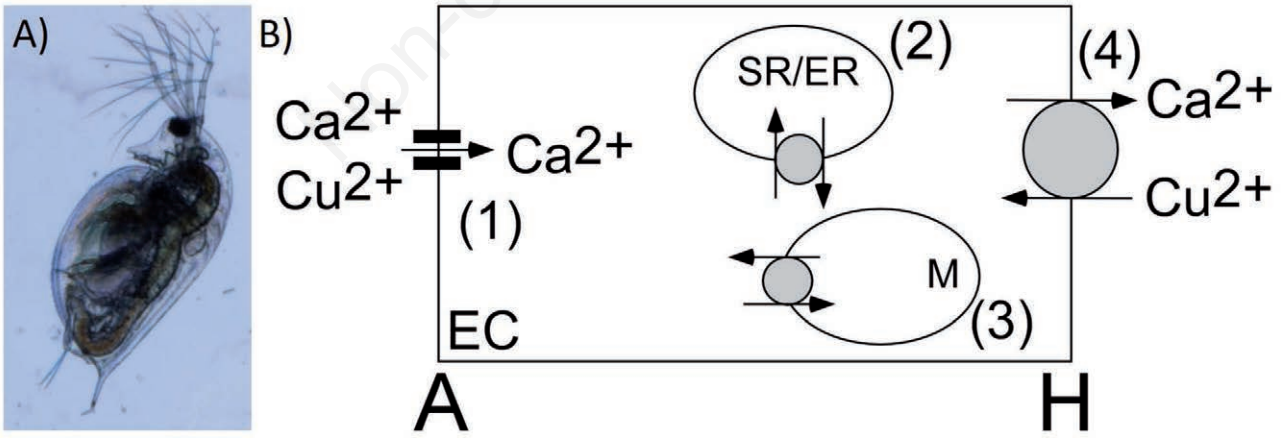

Fig. 1. A) Representative *D. pulex* specimen used in this experiment. B) The current working model for the apical (A) to basolateral (hemolymph – H) transcellular Ca^{2+} transport and potential sites of interactions with Cu^{2+} transport. 1) Ca^{2+} enters the cell *via* carrier mediated facilitated diffusion or simple diffusion. 2) ER (endoplasmic reticulum) and SR (sarcoplasmic reticulum) are important Ca^{2+} storage sites inside the cell. Various energy dependent processes are involved in this Ca^{2+} transport. 3) Mitochondria (M) are involved in longer term Ca^{2+} storage and transport into mitochondria involves ATPases while extrusion may involve antiporter mechanisms. 4) Basolateral (Hemolymph) Ca^{2+} transport requires an array of energy dependent processes (Ca pumps, PMCA - calmodulin-dependent plasma membrane Ca^{2+} ATPase, NCX - high capacity Na^{+}/Ca^{2+} exchanger). Theoretically, Cu^{2+} can interfere or facilitate Ca^{2+} transport in all four locations. Knowledge about the basic Ca^{2+} uptake kinetics in the presence and absence of Ca^{2+} is required.

tions. *D. pulex* is broadly distributed in Canadian shield lakes with variable calcium concentrations (Jeziorski *et al.,* 2008b). The two major objectives for this study were to: 1) determine the independent and combined effects of calcium and copper concentrations on survivorship of juvenile *D. pulex* using a novel high throughput mortality screening system and, 2) investigate the calcium uptake kinetics with and without sublethal copper concentrations in juvenile *D. pulex*. Juveniles were used in all experiments due to their higher calcium demands than adults (Cairns and Yan, 2009; Giardini *et al.,* 2015a), which should make them more vulnerable to reduced environmental calcium.

METHODS

Animal husbandry

Daphnia pulex *culture maintenance*

A *D. pulex* clone was obtained from the Great Lakes Institute for Environmental Research (GLIER) where it was maintained in a single large aquarium. For the duration of this project, animals were kept in the Hagen Aqualab or the Summerlee Science Complex at the University of Guelph. In both locations, animals were maintained at 21°C (±2°C), with a 16:8 hour (light:dark) photoperiod under cool full spectrum lighting (940 lumen, 15 Watts). Animals were kept in 1800 mL of soft COMBO medium (Kilham *et al.,* 1998) in 2 L beakers and fed 4.2 x 10^5 cells mL^{-1} of *Pseudokirchneriella subcapitata* twice per week. Four beakers were maintained at all times, and 90% water changes were performed via reverse filtration biweekly. Fifty milliliter falcon tubes were modified by replacing the cap with a mesh screen and inserting plastic tubing in the base, thereby creating a reverse filtration syphon. During water changes, animals were pooled and then re-divided amongst the four beakers. Glassware was routinely autoclaved after each waterchange.

Algae culture maintenance: Algae cultures of *Pseudokirchneriella subcapitata* were grown in 1.5 L flasks filled with soft COMBO medium (Giardini, 2014). Culturing flasks were sealed off from the environment with parafilm (Bemis Company Inc.), and the media were oxygenated using standard air bubblers, sterilized Nalgene air filters (0.22 µm), and plastic tubing. Cell counts of the algae cultures were conducted using a Neubauer hemocytometer every 2-3 days of growth until the desired density of 30 x 10^6 cells mL^{-1} was achieved. Ethanol and flame-sterilized pipettes were used for culture sampling and any open culture was surrounded with 3 portable Bunsen burners to create an upward air current and prevent culture contamination. Upon harvesting, cultures were stored at 4°C until they were fed to *D. pulex* cultures. *Daphnia* were only fed algae that was stored less than 3 months.

Experimental design

Flame atomic spectrophotometry

We tested calcium and copper concentrations using Flame atomic spectrophotometry (FAS) in order to test for potential changes in the actual concentration of these ions in COMBO medium due to chelating effects. All samples were analyzed at the School of Environmental Sciences Analytical Laboratory at the University of Guelph with a Varian SPECTRAA 220 flame atomic absorption spectrometer (Giardini, 2014).

Survivorship experiments

We developed a novel screening system (Fig. 2) that allows the assessment of survivorship in *Daphnia* with high temporal accuracy and low number of individuals. Survivorship was assessed for a copper gradient, a calcium gradient, and a combined gradient. In each experiment, 4 different treatments were each assigned to a row of 6 replicate wells in a 24-well culture plate (Costar® Corning). The volume of each well was 1.5 mL. Replicate well plates were analyzed in sequence for each experiment. For the copper experiments, the treatments were 0, 100, 200, and 300 µg L^{-1}, all in soft COMBO medium with a calcium concentration of 10 mg L^{-1}. Note that in this case COMBO medium was made without the addition of calcium (no calcium COMBO). For the combined experiments, the treatments were 1) 6.4 mg L^{-1} Ca, 2) 6.4

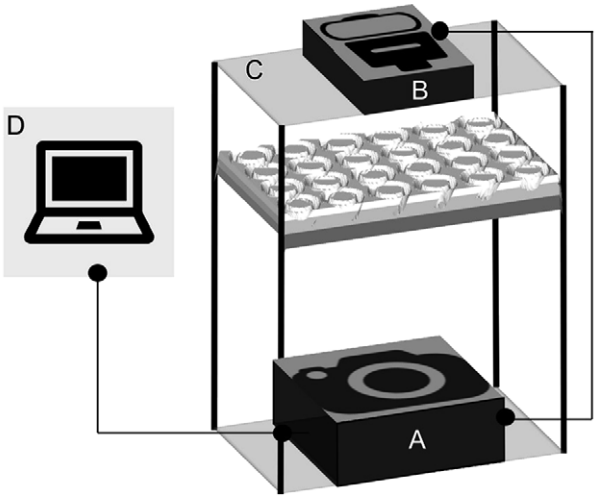

Fig. 2. Schematic diagram of the Toxicology Screen Device (TSD). The TSD consists of: A) a camera capable of capturing high resolution images in at least 5-min intervals; B) an LED flashlight connected and synchronized with the camera's shutter system; C) a diffuser plate; D) a workstation or laptop running software for the processing of capture images. We used this device to assess survival in *D. pulex*.

mg L^{-1} Ca + 300 µg L^{-1} Cu, 3) 0.0512 mg L^{-1} Ca, and 4) 0.0512 mg L^{-1} Ca + 300 µg L^{-1} Cu. Note that all concentrations were selected in accordance with environmentally relevant levels (Jeziorski *et al.,* 2008b; Giardini *et al.,* 2015b; Taylor *et al.,* 2016). For copper treatments, we added $CuSO_4$ (Sigma-Aldrich) solution to COMBO medium. For calcium treatments, we added 32 mg $L^{-1}$of CaCl solution to no calcium COMBO medium and performed serial dilutions with no calcium COMBO. Juvenile *D. pulex* of similar size were selected and pooled into a dish. From this, each individual was randomly added to 1mL of its specific treatment in a 24-well culture plate. Juveniles in wells were recorded from above using time-lapse photography. The experimental setup made use of the Toxicology Screen Device (TSD), a novel semi-automatic technique to monitor individual *Daphnia* in multi-well plates (Fig. 2). Every 24 hours, water changes were performed for each well. Time to death (TTD) was determined by reviewing photographic data with 10 minute accuracy. Animals that did not move over a minimum of three photographs were considered to be dead. TTD was used to calculate relative survivorship in percent. The experiment was terminated when all Daphnia were dead to a maximum of 7 days.

Calcium kinetics experiments

The purpose of the calcium gradient uptake experiments was to establish Michaelis-Menten parameters from an appropriate range of ambient calcium concentrations in the presence and absence of copper. Uptake of the radioisotope ^{45}Ca ($CaCl_2$) was measured in a series of incubation experiments. For all uptake experiments, 10 *D. pulex* juveniles were selected and randomly assigned to scintillation vials filled with 10 mL of ^{45}Ca solution in no calcium COMBO. After incubation, 0.5 mL of the incubation fluid (IF) from each vial was sampled in order to monitor background levels of radiation. This served as a technical control to ensure that all IF samples were equivalent. Juveniles within each vial were washed 3 times for 5 minutes each with non-radioactive COMBO medium after which 0.5 mL of the final wash solution (FW) was sampled to ensure there was no remaining residual radiation. This served as a technical control to ensure that the washes removed all radiation from the IF. Juveniles were then digested for 8 hours with 30% HNO_3 at 70°C. After cooling vials to 20°C, 5 mL of scintillation fluid (Giardini, 2014) was added and vials were incubated for a minimum of 8 hours. Samples were then counted in a Beckman LS6500 scintillation counter.

Time-series uptake experiment

The purpose of the time series experiment was to establish the timing of calcium equilibration across membranes in juvenile *D. pulex*. Juveniles were incubated in COMBO medium with 10 mg L^{-1} ^{45}Ca for 1-8 hours. Five replicates were measured in the scintillation counter each hour.

Experiment 1: Calcium uptake kinetics

We tested calcium uptake kinetics parameters for 0.0512, 0.256, 1.28, 6.4, and 32 mg L^{-1} of ambient calcium. These calcium concentrations were intended to cover an environmentally relevant range, including concentrations that are known to limit survival, reproduction and calcification in *D. pulex* (Taylor *et al.* 2016). Replicates were done sequentially with one hour spacing to allow processing of samples.

Experiment 2: Copper effect on calcium uptake kinetics

In the second experiment we tested calcium uptake parameters in the presence and absence of copper. We chose 100 µg L^{-1} (1.57 µmol L^{-1}) Cu as this is an environmentally relevant concentration that is known to be toxic to *D. pulex* juveniles (Taylor *et al.,* 2016). In a first run we tested the same calcium concentration used in Experiment 1. For the copper exposure, *D. pulex* juveniles were pre-incubated in 100 µg L^{-1} Cu for twelve hours in scintillation vials prior to ^{45}Ca exposure. Based on the changes of calcium uptake we observed, we modified the calcium gradient to 0.289, 1.156, 4.625, 18.5, and 74 mg L^{-1} in subsequent experiments. For both trials, Cu was added to stock calcium solutions containing ^{45}Ca, and then allocated to vials containing *D. pulex*. Non-radioactive medium was removed by pipetting from vials containing *D. pulex* before stock radioactive solutions were introduced.

Statistical analyses

Survivorship

Survivorship data was analyzed using IBM SPSS Statistics 22. In order to compare survivorship data across separate plate runs, time to death (TTD in minutes) data from survivorship experiments were normalized to values between 0 and 1 using equation 1:

$$\text{Normalized } (e_i)=(e_i - E_{min})/(E_{max} - E_{min}) \qquad \text{(eq. 1)}$$

In eq. 1 e_i is TTD for a given juvenile, E_{min} is the minimum TTD, and E_{max} is the maximum TTD. This data was then expressed as a percentage. By normalizing the data in this way, interindividual variation could be minimized. Additionally, a nested ANOVA was used to test for an effect between replicate plates within each experimental design, using $P<0.3$ (Underwood, 1997) to express homogeneity between plates. This was used as a justification to treat each well within the plates as the unit of replication. Linear regression was performed on the cop-

per gradient and calcium gradient experiments. A Univariate ANOVA was performed to test for differences between treatments for each of the survivorship experiments. Bonferroni post-hoc analyses were applied to each survivorship experiment, and a simple contrast was done in order to reveal any differences between treatment groups. Differences were considered significant when $P<0.05$.

Calcium uptake

Michaelis-Menten parameters: K_m and V_{max}, were estimated for each calcium uptake experiment. This was done in SPSS via a nonlinear regression test using the model from equation 2:

$$(Vmax*[Ca])/(Km+[Ca]) \quad \text{(eq. 2)}$$

In eq. 2, [Ca] is nominate non-radioactive calcium concentration of the incubation fluid. Raw data outputs from the scintillation counter were in units of counts per minute (CPM). Raw data was converted to inward flux of Ca^{2+} (V_i) in order to account for radioactivity of the incubation fluid in each vial. V_i was calculated using equation 3:

$$V_i=V_{cpm}/(IF_{cpm}/[Ca]) \quad \text{(eq. 3)}$$

In eq. 3, V_{cpm} is the CPM of a given vial of 10 juveniles, and IF_{cpm} is the CPM of a given 0.5 mL sample of incubation fluid. V_i is expressed in units of CPM 4 hours^{-1}, since all incubations over a [Ca]-gradient were 4 hours in duration.

Copper effects on calcium uptake

Mean K_m and V_{max} was calculated for copper and control treatments from Michaelis-Menten kinetic models of each replicate. These means were compared between treatments using independent samples t-tests. Differences were considered significant when $p<0.05$, and means are presented as ± SE where SE is one standard error.

RESULTS

Flame atomic spectrophotometry

The measured concentration of calcium is the same as the intended concentration at low concentration, but is less than the intended concentration at 16 and 32 mg L^{-1}, with and without copper (Fig. 3A, Supplementary Tabs. 1 and 2). Copper concentrations are less than intended concentrations at >0.1 mg L^{-1} (Fig. 3B) and do not change with changing calcium concentration (Fig. 3B). Neither of these results should compromise the integrity of the experiments, as all other concentrations within the tested

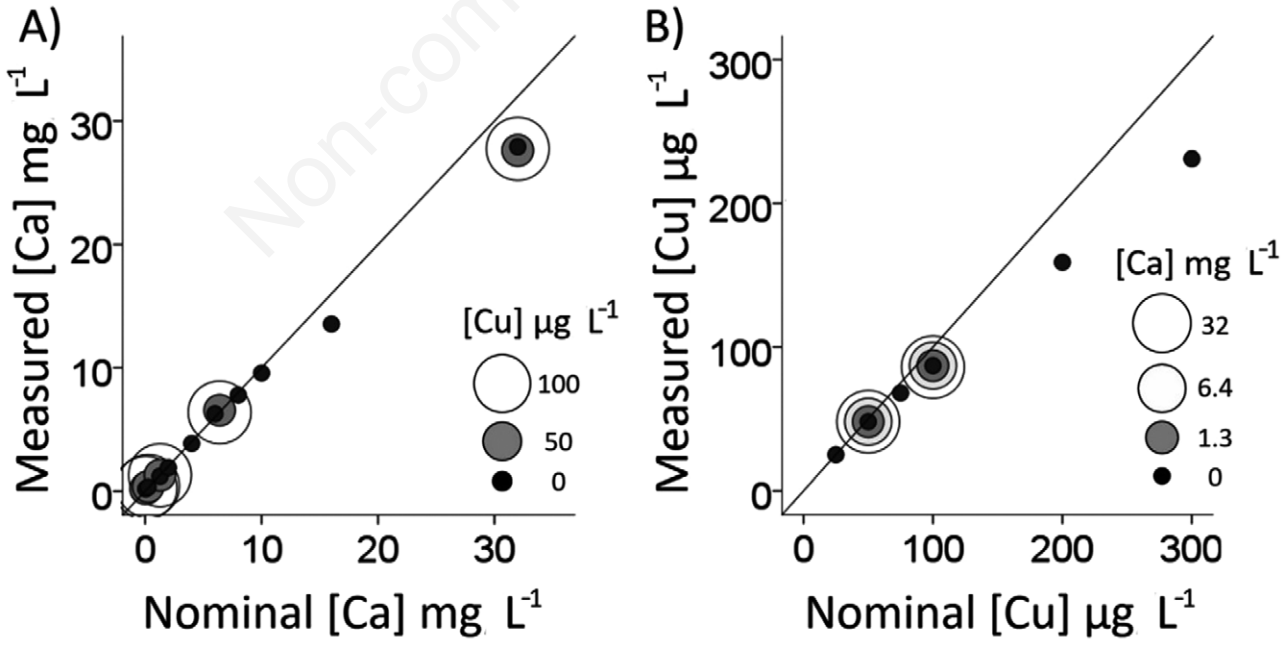

Fig. 3. Measured calcium and copper concentrations are lower than nominal concentrations in *Daphnia* growth medium. In order to test the effect of metal chelators such as EDTA, which is commonly used in COMBO medium, on the actual concentration of calcium (A) and copper (B) in the growth medium, we compared the nominal concentration to the actual concentration measured using atomic absorption spectroscopy (AAS).

ranges are very close to the intended concentrations. Thus, we report the intended calcium and copper concentrations used in the experiments.

Survivorship experiments

Dissolved copper in the COMBO medium had a negative effect on survivorship at the highest concentration (Fig. 4A). Nested ANOVA did not reveal a plate effect (F=0.734, p=0.784, df1=20, df2=120) and consequently, replicate wells within plates were treated as independent replicates (Underwood, 1997; Heyland and Hodin, 2004). ANOVA comparisons between copper concentrations resulted in significant differences between treatments (F=3.779, P=0.012, df1=3, df2=143), with significantly lower survivorship at 300 µg L^{-1} than 0 µg L^{-1}. Linear regression resulted in a significant negative relationship between copper concentration and mean survivorship (Beta= -0.257, R^2= 0.066, P=0.002). Bonferroni *post-hoc* comparisons between 0 and 300 µg L^{-1} copper showed significantly higher survival in 0 µg L^{-1} (mean difference=0.220±0.066; P=0.006), but there was no significant difference between 0 µg L^{-1} and either 100 µg L^{-1} (mean difference=0.101±0.066; P=0.757) or 200 µg L^{-1} Cu (mean difference=0.096±0.066; P=0.871).

Lower dissolved calcium had a negative effect on survival (Fig. 4B). Nested ANOVA resulted in a significant plate effect (F=1.380, P=0.224, df1=8, df2=60) and therefore plates were treated as independent replicates. ANOVA showed significant differences between treatments (F=3.538, P=0.019, df1=3, df2=71). Linear regression resulted in a significant positive relationship between calcium concentration and survival (Beta=0.360, R^2=0.130, P=0.002). Bonferroni post-hoc comparisons between 6.4 and 0.0512 mg L^{-1} Ca showed significantly higher survival in 6.4 mg L^{-1} (mean difference=0.250±0.082; p=0.019), but there was no significant difference between 6.4 and 1.28 mg L^{-1} (mean difference=0.052±0.082; P=1.00) or 0.256 mg L^{-1} Ca (mean difference=0.125±0.082; P=0.779).

The survivorship experiment with combined calcium and copper concentrations showed an interactive effect between calcium and copper (Fig. 4C) based on the two-factor ANOVA using calcium and copper as separate factors (F=15.870, P<0.010, df1=1, and df2=47). Post-hoc comparisons between treatment groups using Bonferroni correction for multiple comparisons showed that survival in low copper/low calcium was significantly lower compared to all other treatments (low copper/high calcium: mean difference=0.402±0.060, P<0.010; high copper/low calcium: mean difference=0.225±0.060, P<0.010; high copper/high calcium: mean difference=0.287±0.060, P<0.010). We also found that survival in low copper/high calcium was significantly higher compared to high copper/low calcium/ (mean difference=0.176±0.060, P=0.027) but not high copper/high calcium (mean difference=0.115±0.060, P=0.362). There was no difference in survival between low and high calcium at high copper concentration (mean difference=0.061±0.060, P=1.00).

Note that the calcium concentration in the copper dose response is 10 mg L^{-1}, while the calcium concentration in the combined experiment is 0.05 mg L^{-1} and 6.4 mg L^{-1}, respectively. This difference may in part explain the difference in survival between these two experiments.

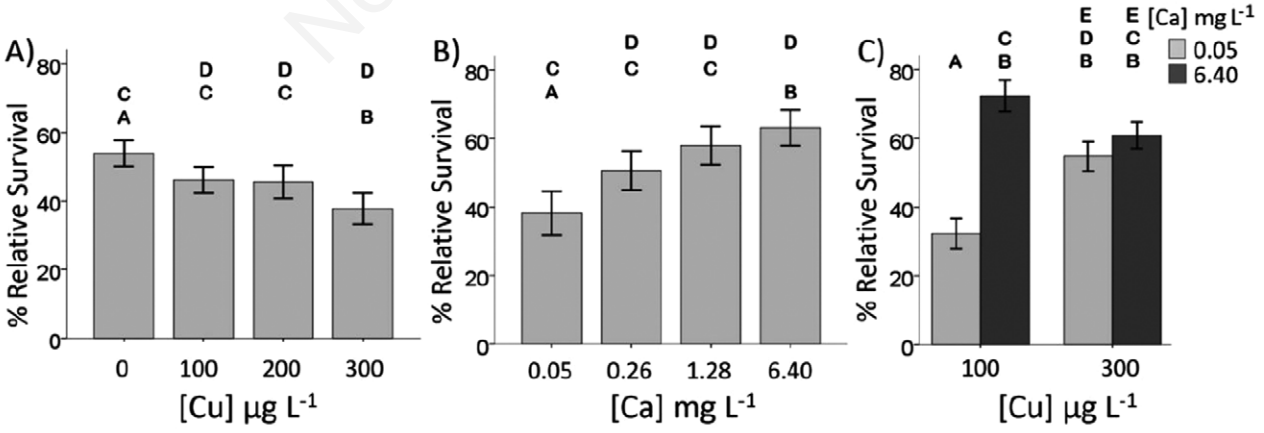

Fig. 4. Increasing copper and decreasing calcium concentrations lead to reduced survival of *D. pulex*. We used a novel automated scoring system to assess toxicity in *D. pulex*. Relative survival was calculated from the time to death (TTD) of individual *Daphnia* with 10-min precision. A) Relative survival of *D. pulex* with increasing copper concentration. B) Relative survival of *D. pulex* with decreasing calcium concentration. C) Interaction between copper and calcium; decreased survival at low copper and calcium concentration supports a competitive interaction between copper and calcium uptake mechanisms. Horizontal bars indicate significance <0.05%.

Calcium kinetics experiments

Time-series experiment

The purpose of this experiment was to determine the duration of incubations for the calcium gradient experiment (below). In COMBO medium with standard calcium (10 mg L^{-1}), tissue calcium in juvenile *Daphnia* increased with each hour until the 6 hour time-point (Fig. 5A). Four hours was within the range of optimal uptake rate and was determined to be long enough to allow sufficient time to process samples (Fig. 5A).

Calcium gradient experiments

In order to assess basic uptake kinetics of calcium in *D. pulex* we performed a calcium gradient experiment with 10 juveniles per vial. When fit to a Michaelis-Menten model, this experiment resulted in an asymptotic curve with mean V_{max}=1.15±0.20 and mean K_m=7.21±2.6 mg L^{-1} (Fig. 5B). The calcium gradient experiment with 0 Cu resulted in mean V_{max}=1.57±0.34 and mean Km=3.04±0.93 mg L^{-1}. The mean Vmax=1.93±0.55 and mean Km=10.75±6.68 mg L^{-1} in the 1.57 µmol L^{-1}100 µg L^{-1} Cu treatment (Fig. 5C). There was no difference in V_{max} (p=0.345) between the 0 and 100 µg L^{-1} Cu groups (Tab. 1), but K_m was significantly lower in 0 µg L^{-1} Cu compared to 100 µg L^{-1} Cu (P=0.044).

DISCUSSION

The assessment of survivorship curves in *Daphnia* and other aquatic organisms can be difficult as it requires large numbers of individuals and it may be difficult to recover dead organisms in the water column with precision (Epa, 2002). Alternatively, specimens can be exposed in individual vials for extensive time periods, but this can increase the investment in space and time (Cairns and Yan, 2009; Prater *et al.,* 2016). Moreover, both approaches provide limited temporal resolution as the exact time of death can generally be determined with very coarse accuracy (6-12h). As part of this study, we developed a mortality screening system and employed it to assess survivorship in *D. pulex* in calcium and copper treatments. We conclude that this system provides a useful tool for life-history experiments involving *D. pulex* and other aquatic zooplankton. Processing the survival data was done manually by examining the photographs and recording the time point at which each individual ceased to move for the remainder of the experiment. In future trials, this could be done by creating an image stack and instructing the computer to analyze the data by tracking the image automatically. This would increase the efficiency of running these experiments so that the only human involvement is setting up the plates and doing the water changes. Also,

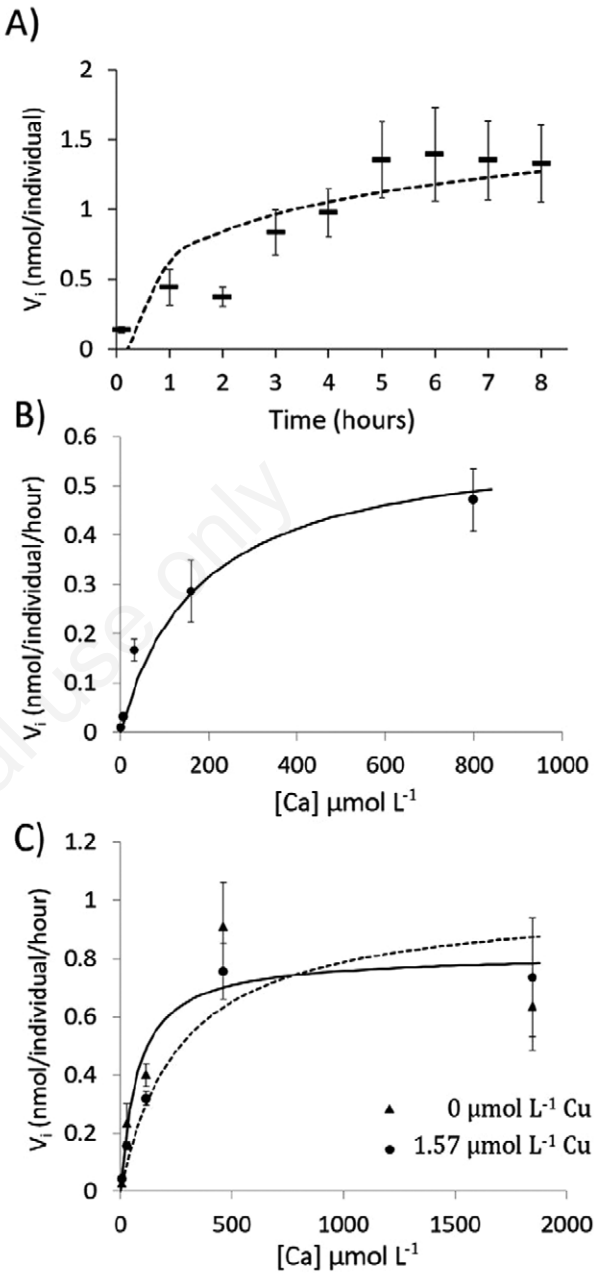

Fig. 5. Copper competitively inhibits calcium uptake at low calcium levels. A) Calcium accumulation over time in *D. pulex* juveniles. B) Calcium uptake kinetics for juvenile *D. pulex*; we calculated mean V_{max}=0.599±0.104 nmol/individual/hour and mean K_m=180±65.1 µmol $L^{-1}Ca^{2+}$. The solid line represents the Michaelis-Menten model estimated from this data set; we used four replicates per treatment; vertical bars represent ±1 SE. B) Calcium uptake kinetics in the presence and absence of copper; we calculated mean V_{max}=0.978±0.226 nmol individual 1 $hour^{-1}$ and mean K_m=43.2±24.4 µmol L^{-1} Ca^{2+} in 0 µmol L^{-1} Cu, and mean V_{max}=1.19±0.267 nmol $individual^{-1}$ $hour^{-1}$ and mean K_m= 71.6±36.6 µmol L^{-1} Ca^{2+} in 1.57 µmol L^{-1} Cu; the solid and dashed lines represent the Michaelis-Menten models estimated from the 0 µmol L^{-1} Cu and 1.57 µmol L^{-1} Cu data sets, respectively; there were four replicates per treatment; vertical bars represent ±1 SE.

the design can be modified in a number of ways to accommodate a greater sample size of individuals per trial. This would be possible by using a wider angle camera and setting up multiple plates within the field of view and/or by setting up more cameras that are synchronized to shoot simultaneously. The time-lapse method also allows studies of other observable life-history traits, such as timing of reproduction and molting, body morphometrics, movement patterns and clutch size. It is worth noting, however, that the survival data reported using this approach are not directly comparable to survival data usually reported in toxicity studies. It is therefore critical to assess the overall patterns in experiments.

Using this novel toxicity screening system, we found that low calcium and high copper concentration reduce survival when applied in isolation. When applied in combination however, survival is highest at the low copper/high calcium concentration and lowest at the low copper/low calcium concentration. In contrast, we did not find any difference in survival between the calcium concentrations at the high copper concentration. These results indicate an interaction between these two metals. Specifically, survivorship in the low copper treatment increased significantly with the addition of calcium, indicating a potential protective effect of calcium against copper toxicity. Conversely, high copper is somewhat toxic, so survivorship decreases from the low copper/high calcium treatment, and given that, calcium has less of an impact. This hypothesis is supported by our calcium uptake experiments, in which Vmax and Km are higher at the high copper concentration, and by previously published data in fish and *D. magna*, where a protective effect of calcium against copper toxicity was observed (Pagenkopf, 1983; Lauren and Mcdonald, 1986; De Vera and Pocsidio, 1998; De Schamphelaere and Janssen, 2002; Chen *et al.,* 2012). The protective effect of calcium against copper toxicity has broader implications for the natural environment. Specifically, it emphasizes that organisms may experience reduced copper stress when calcium is present at higher concentrations in the environment, a finding we were able to partially confirm with our laboratory experiments. Still, the exact nature of this interaction in *D. pulex* and other *Daphnia* species requires further research.

Calcium uptake curves presented as part of this study followed expected Michaelis-Menten kinetics, typical of active uptake and facilitated diffusion. Baseline calcium uptake has been measured in other *Daphnia* species using the same method described here (Tan and Wang, 2009), also yielding results that demonstrate active uptake. This is consistent with other studies involving calcium uptake in freshwater crustaceans (Greenaway, 1974; Neufeld and Cameron, 1993; Wheatly and Ayers, 1995). Exact mechanisms of calcium transport are not fully understood, however the current working model is well described for crustaceans, although it is primarily based on studies involving relatively large malacostracan crustaceans such as crayfish, crabs, and lobsters (Wheatly and Ayers, 1995; Wheatly, 1999; Wheatly *et al.,* 2002; Ahearn *et al.,* 2004). Typically, calcium undergoes passive and facilitated diffusion along with active transport from the apical surface to the basolateral side of the cell membrane by a number of channels and (Wheatly and Ayers, 1995; Wheatly, 1999; Ahearn *et al.,* 2004). Passive diffusion only occurs across the apical cell membrane, as cytosolic calcium concentrations are <40 mg L^{-1}, which is lower than any aquatic environment that crustaceans can inhabit (Wheatly *et al.,* 2002). However, transporting cytosolic calcium across the basolateral cell membrane into the hemolymph ($[Ca^{2+}]$=200 to 480 mg L^{-1}) requires active transport fueled by ATP (Wheatly *et al.,* 2002). There are also a number of calcium transporters on organelles that aid in the movement and storage of calcium which become especially important as requirements shift during the molt cycle. In particular, there is a uniporter on the mitochondrion that may transport both calcium and copper into the organelle, which means that calcium transport may be inhibited by copper in the cytoplasm (Chavez-Crooker *et al.,* 2002). Additionally, antiporters identified on the membrane of the organelle are suspected to be inhibited by copper from transporting calcium out of the mitochondria (Chavez-Crooker *et al.,* 2002). Also, epithelial copper uptake has been identified to occur via a sodium antiporter that can substitute for calcium as the exiting cation (Chavez-Crooker *et al.,* 2002). Therefore, it is possible that copper could disrupt calcium transport and storage dynamics in complex ways. Perhaps if copper levels are too high outside the cell, large amounts of calcium become displaced and leave the tissues altogether. In our calcium uptake experiments, decreased K_m in the copper treatment compared to the control suggests competitive inhibition with calcium at the biotic ligand (Hogstrand *et al.,* 1994). Inhibition could be occurring at calcium stor-

Tab. 1. Comparison of mean ± 1 SE V_{max} and K_m between 0 and 1.57 µmol L^{-1}Cu treatments from the extended calcium gradient experiment (Fig. 4). T-tests showed no significant difference in V_{max} (p=0.345) between 0 and 1.57 µmol L^{-1} Cu groups, however K_m was significantly lower in the 0 µmol L^{-1} Cu group compared to the 1.57 µmol L^{-1} Cu group (P=0.044), n=4.

Treatment (µmol L^{-1} Cu)	V_{max} (nmol individual^{-1} hour^{-1})	K_m (µmol L^{-1} Ca^{2+})
0	0.816±0.175	3.04±0.93
1.57	1.00±0.285	10.75±6.68

age sites such as the mitochondria or *via* cation exchange at the cell membrane (Fig. 1), however the possibility that inhibition occurs when copper occupies transporter sites on the cell, or even alters the physical properties of tissues involved in calcium uptake cannot yet be ruled out.

While other experiments suggest that copper can disrupt sodium uptake in some taxa, and increased sodium concentration protects against copper toxicity (Lauren and Mcdonald, 1986; Niyogi and Wood, 2004; Giacomin *et al.,* 2013), evidence for competitive inhibition of calcium uptake by copper in daphniids has not previously been published. Most relevant to this study however, is the evidence that calcium protects against copper toxicity in *D. magna* (De Schamphelaere and Janssen, 2002).

Our data show that when calcium uptake becomes saturated at high calcium concentrations 1.57 µmol L^{-1} (100 µg L^{-1}) copper has no impact on calcium uptake. This suggests that the inhibition observed at lower calcium concentrations is indeed competitive. A decrease in V_{max} in the presence of copper would have suggested a non-competitive inhibition (Hogstrand *et al.,* 1994). The competitive interactions between copper and calcium observed in our experiments have potentially significant ecological implications for daphniids living in shield lakes. In lakes or microhabitats of watersheds with copper contamination, calcium decline may have a more severe effect on *Daphnia* populations than previously thought. Failing to take up enough calcium may not only result in higher mortality, but can also cause a number of sublethal effects such as decreased reproduction or incomplete calcification (Ashforth and Yan, 2008; Agra *et al.,* 2010; Giardini *et al.,* 2015b). This in turn can lead to decreases in body mass (Alstad *et al.,* 1999), which is also reflected in *D. pulex* sedimentary remains from lakes {Jeziorski, 2012 #289}. Since timing of reproductive maturation is related to body size {Ebert, 1992 #258}, low calcium results in a delay of sexual maturation, which contributes to decreased reproduction. Based on our data, these may be some of the consequences of low calcium and high copper levels in lakes. However, *D. pulex* should not experience decreases in calcium uptake in lakes that are contaminated with high levels of copper but maintain high calcium concentrations. In these settings, calcium acts as a buffer from copper interference of uptake mechanisms.

CONCLUSIONS

The purpose of this study was to determine the effects of sublethal copper concentrations on calcium uptake in *D. pulex* juveniles. Copper competitively inhibits calcium uptake only at low calcium levels when copper concentration is low, which may have implications for naturally occurring populations living in low-calcium lakes that are also contaminated with copper. This study also adds to our understanding of calcium transport mechanisms in crustaceans as the evidence presented here supports the idea that copper competes with calcium uptake, which may specifically occur at the mitochondria where calcium can be stored. It would be of interest to test calcium uptake in the presence of copper in other life-stages of *Daphnia* as well as other taxa, and to address how exposure to sublethal copper concentrations contributes to greater variability among individuals in regards to calcium uptake.

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