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## ARTICLE

# Similar zooplankton responses to low pH and calcium may impair long-term recovery from acidification

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**Abstract**

Throughout much of the 20th century, unprecedented industrial emissions have led to widespread acidification of regions in North America and Europe and, as lake water pH dropped, aquatic ecosystems have experienced dramatic declines in biodiversity. International emission-control agreements have led to sweeping increases in lake pH, however acid-structured zooplankton communities still persist in many lakes. Concomitantly, calcium concentrations have been declining as a legacy of acidification and are approaching or have reached concentrations that could represent a barrier to the re-establishment of zooplankton communities similar to those in non-acidified or circumneutral reference lakes. To understand how declining calcium may influence the re-establishment of zooplankton in acid-damaged lakes we manipulated calcium and pH using a factorial in-lake mesocosm experiment and assessed their individual and combined effects on a regionally diverse zooplankton assemblage. We found that the impacts of low calcium on zooplankton species were similar to those of acidification and, consequently, may prevent the recovery of acid-structured communities. Abundance of the larger bodied and acid-sensitive *Daphnia pulex/pulicaria* increased in high pH treatments, albeit nonsignificantly yet, by the end of our experiment, only two individuals were sampled among our 10 low calcium enclosures. In contrast, small acid-tolerant cladocerans, such as *Daphnia catawba*, *Daphnia ambigua*, and eubosminids maintained significantly higher abundances in low calcium treatments relative to all other treatment combinations. Although we did not detect an effect of calcium on mean body size, the disproportionately high abundance of small cladocerans in low calcium treatments resulted in low calcium communities with higher overall abundance and lower cladoceran evenness. Our results, along with a landscape comparison demonstrating parallel changes in zooplankton relative abundance from 34 historically acidified lakes, suggests that declining calcium will be an important, on-going factor that may limit the recovery of zooplankton, despite regional improvements in lake pH.

**KEYWORDS**

acid rain, calcium decline, crustacean zooplankton, mesocosm experiment, multiple stressors, recovery

## INTRODUCTION

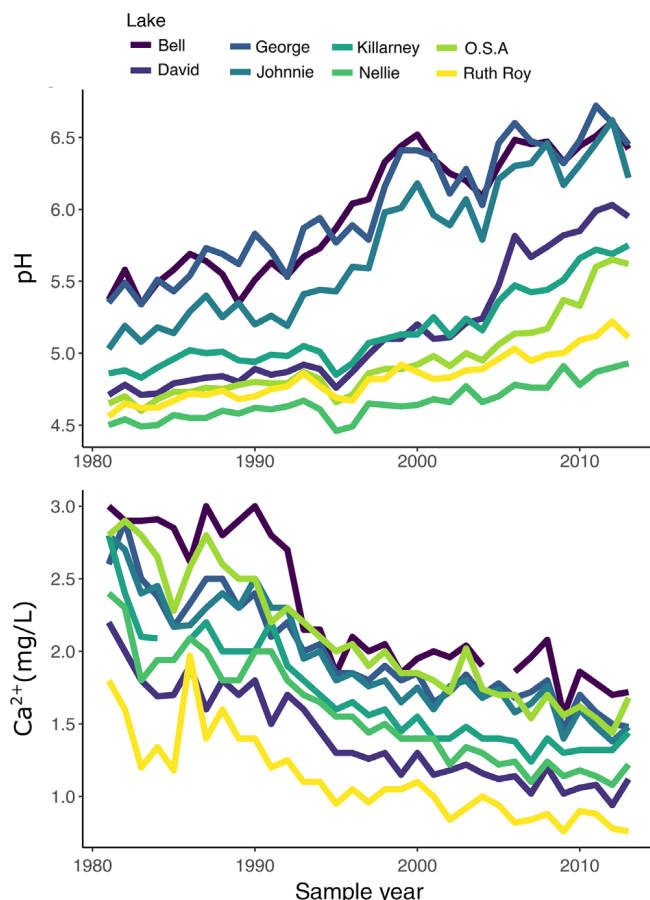
Across North America and Europe, historic acid deposition has led to widespread acidification and subsequent biodiversity loss in terrestrial and aquatic ecosystems. In aquatic ecosystems, biological damage has been particularly severe, resulting in local extirpation of sensitive species in thousands of lakes and streams (Keller & Pitblado, 1986; Ormerod & Edwards, 1987; Schindler et al., 1985). Environmental legislation in North America and Europe has been successful, reducing emissions of acid-producing compounds and had led to a marked improvement in surface water pH (Keller et al., 2003; Skjelkvåle et al., 2005; Stoddard et al., 1999). Despite improvements to surface water pH the recovery of aquatic biota has been slow, and, for zooplankton in particular, many species have failed to re-establish to preacidification or reference lake abundances (Gray & Arnott, 2009; Keller et al., 2019; Palmer et al., 2013; Yan et al., 2004). Several factors have probably contributed to this lag in biological recovery, including: (1) local abiotic conditions of chronic lake acidity (Gray et al., 2012; Keller et al., 2002; Yan et al., 2004) and persistent heavy metal toxicity (Labaj et al., 2015; Valois et al., 2010); (2) biological resistance from local, acid-structured zooplankton communities (Binks et al., 2005; Gray & Arnott, 2012) and predator communities (Arnott et al., 2006; Valois et al., 2010; Yan et al., 1991); and (3) dispersal limitation, inhibiting the arrival and establishment of potential zooplankton colonists (Gray & Arnott, 2011). Additionally, in historically acidified areas, improvements in lake acidity are now being countered by substantial and broad-scale declines in aqueous calcium (Weyhenmeyer et al., 2019), the effects of which are unknown for biological recovery of zooplankton.

Declining calcium concentrations are largely a legacy of broad-scale acidification. Large watershed-wide inputs of wet and dry acid deposition has caused an initial pulse of base cations from soil complexes into streams and lakes that has led to temporary increases in calcium in surface waters during acidification (Likens et al., 1996). After this, more labile pools of calcium were quickly depleted and calcium concentrations began to decline at rates higher than they could be replaced through mineral weathering and atmospheric deposition (Likens et al., 1996; Watmough & Dillon, 2003). Timber harvesting in many of these same watersheds has further exacerbated calcium loss through the removal of calcareous-laden biomass (i.e., trees; Watmough & Aherne, 2008). Widespread calcium decline has been documented in areas of eastern North America and Northern Europe where acidification was prevalent (Clair et al., 2002; Jeziorski et al., 2008; Weyhenmeyer et al., 2019).

Reductions of calcium in previously acidified environments represent yet another challenge for biological

recovery, as there is mounting evidence that freshwater biota are sensitive to low calcium concentrations. Low calcium concentrations reduce population growth rates for some zooplankton species (Arnott et al., 2017; Azan & Arnott, 2018), and shifts in the relative abundance and dominance of cladocerans from soft-water lakes has, in certain cases, been attributed to reductions in calcium concentrations (Desellas et al., 2011; Hessen et al., 1995; Jeziorski et al., 2008, 2014; Tessier & Horwitz, 1990). At the individual level, laboratory and field studies have suggested that low calcium environments can lead to decreases in daphniid body size (Alstad et al., 1999), lower survival and reproduction rates (Ashforth & Yan, 2008; Hessen et al., 2000), reduced movement (Betini et al., 2016), and impaired antipredator defenses (Cox et al., 2018; Riessen et al., 2012). In particular, much attention has been paid to *Daphnia pulex*, as low calcium concentrations can affect their presence, reproduction, molting and population growth rates (Ashforth & Yan, 2008; Azan & Arnott, 2018; Pérez-Fuentetaja & Goodberry, 2016; Prater et al., 2016; Rice et al., 2021; Tessier & Horwitz, 1990) and because of their role as keystone species in aquatic ecosystems (Ives et al., 1999). An ecosystem-level implication of these community, population, and individual effects is that lower grazing rates due to calcium-mediated shifts in zooplankton communities can lead to increased chlorophyll-*a* abundances and trophic cascades (Korosi et al., 2012). Despite evidence that suggested that declining calcium concentrations affect zooplankton, understanding the effect of calcium in relation to pH is not possible due to their correlation within natural environments. Therefore, a manipulative experiment that isolates the relative role of pH and calcium in structuring zooplankton communities is needed to uncouple the direct and indirect effects of low calcium from other factors, and determine whether calcium is a determinant for biological recovery.

Killarney Provincial Park (KPP), located 65 km southwest of Sudbury, Ontario, Canada, is an ideal region to investigate the relative role of calcium and pH in the recovery of zooplankton communities from acidification. During the 1970s, KPP and the Sudbury region was the world's epicenter for experiencing and understanding the effects of severe acid deposition. Lake pH was below 6.0 in 37 of 47 KPP lakes sampled between 1971–1973, with 60% of these lakes falling below a pH of 5 (Gunn et al., 1995; Sprules, 1975). Biological impacts of acidification in KPP were severe; populations of fish and zooplankton were extirpated (Beamish & Harvey, 1972; Sprules, 1975, 1977), species richness of rotifers declined (MacIsaac et al., 1986), and phytoplankton assemblages changed (Kwiatkowski & Roff, 1976). Since the 1970s, pH has



**FIGURE 1** Long-term pH and calcium trends for eight KPP lakes monitored annually between 1981–2013 (Co-operative Freshwater Ecology Unit, Ontario Ministry of Environment, Conservation and Parks, unpublished data)

recovered in many lakes (Figure 1), and 22 out of 47 KPP lakes sampled in 2016 have pH >6 (Suenaga, 2018). Despite notable increases in the pH of KPP lakes, the recovery of crustacean zooplankton communities has lagged chemical recovery (Gray et al., 2012) and now calcium concentrations in many lakes have declined below potentially critical concentrations (57% are below 1.5 mgCa/L and 16% are below 1.0 mgCa/L; Suenaga, 2018).

In this study, we address how calcium concentrations may influence zooplankton in environments where lakes are chemically recovering but not yet biologically recovered. Our study objective was to experimentally disentangle the individual and combined effects of calcium and pH on zooplankton abundance and community metrics for environments characteristic of historically acidified lakes. We then present how these experimental effects compared with landscape-wide changes in KPP zooplankton species from time periods that represent peak acidification and contemporary zooplankton communities.

## MATERIALS AND METHODS

### Site description

Experimental mesocosms were deployed in Lumsden Lake (46°01'N/81°25'W), a small oligotrophic lake in KPP. Located within the La Cloche mountain range, the surrounding bedrock is primarily quartzite (Beamish & Harvey, 1972), with low alkalinity, nutrients, and calcium (Beamish & Harvey, 1972). Lumsden Lake was severely acidified yet pH has increased from 4.4 in 1971 to 5.7 in June of 2013. In 2013, the calcium concentration was 0.9 mg/L compared with 4 mg/L when sampled in 1971 (Beamish & Harvey, 1972). Fish were extirpated from Lumsden Lake during the acidification period and have not recolonized (Snucins & Gunn, 1999). The present-day zooplankton community is depauperate and dominated almost exclusively by *Leptodiptomus minutus*. Other rare species include *Skistodiptomus oregonensis*, *Diacyclops thomasi*, *Diaphanosoma* sp., *Polyphemus pediculus*, *Eubosmina tubicen*, *Mesocyclops edax*, and the predatory dipteran larvae, *Chaoborus* spp. (Appendix S1: Figure S1).

### Experimental design

To assess the influence of pH and calcium concentrations on zooplankton, we conducted a mesocosm experiment in Lumsden Lake from 9 June 2013 to 5 August 2013. We used a full factorial design with calcium (low, high) and pH (low, high) as main factors. The ambient water chemistry of Lumsden Lake was used for our low calcium (mean = 0.93 mg/L, SD = 0.065), low pH (mean = 5.72, SD = 0.094) environment. The low calcium treatment reflects regionally relevant calcium concentrations that have been shown to influence zooplankton survival and reproduction in laboratory and field studies (Ashforth & Yan, 2008; Azan & Arnott, 2018). The low pH treatment was typical of lakes recovering from acidification yet still below biological thresholds for recovery (Holt & Yan, 2003). High calcium concentration (mean = 2.36 mg/L, SD = 0.31) and pH (mean = 6.33, SD = 0.11) represent levels that are not likely to limit survival and reproduction of most zooplankton species, and that are within the range of pH and calcium concentrations observed in KPP lakes (Suenaga, 2018). Each treatment was replicated five times, for a total of 20 enclosures.

Experimental enclosures were constructed from clear, 4 ml polyethylene plastic tubes (Filmtech Plastics, Brampton, Ontario) that were 1 m in diameter, approximately 3.2 m deep, and sealed at the bottom. They were suspended from floating wooden frames that were

oriented in a north–south direction and anchored in 16–20 m of water. Each enclosure was covered in black window screen to minimize accumulation of debris and aerial colonization of organisms. On 6 June enclosures were filled with water from Lumsden Lake that was filtered through 80  $\mu\text{m}$  mesh to remove zooplankton and macroinvertebrates, but to allow most edible phytoplankton to pass through. Treatments were assigned to enclosures in a haphazard manner to ensure that treatments were interspersed. Calcium and pH treatments were established on 8 June by adding 92 ml 0.5 M NaOH to each high pH enclosure and 15.73 g of  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  to each high calcium treatments.

On 9 June, enclosures were stocked with zooplankton collected from three chemically recovered lakes in KPP (Appendix S1: Table S1). The lakes were chosen based on (1)  $\text{pH} > 6.0$ ; (2) within 20 km of Lumsden Lake, ensuring they could be a realistic source for potential dispersers; (3) containing a diverse assemblage of zooplankton, including several *Daphnia* species; and (4) the absence of *Bythotrephes cederstroemii* (formerly *Bythotrephes longimanus*; Korovchinsky & Arnett, 2019), an invasive invertebrate predator that has established in several lakes in the region. For each source lake, a conical 80- $\mu\text{m}$  mesh net with 25-cm diameter opening was vertically towed from 2 m above the sediment–water interface to the lake surface. For each enclosure, 840 L of zooplankton-containing water were filtered from each source lake (33% of enclosure volume). Zooplankton were transported in 1 L Nalgene bottles in coolers and transferred into enclosures after hiking for  $\sim 1$  h to Lumsden Lake.

## Environmental and zooplankton sampling

Zooplankton communities were sampled every 2 weeks between 10 June and 5 August. Two zooplankton samples were taken from each enclosure with 3 m vertical zooplankton tows (conical net; 80- $\mu\text{m}$  mesh, 0.15 m diameter), then were pooled and preserved in 70% ethanol.

Zooplankton samples were enumerated using a Leica MZ16 (Leica Microsystems; Wetzlar, Germany) dissecting microscope. Preserved samples were resuspended in 100 ml of water, homogenized, and then subsampled in 5-ml increments. Sequential subsamples were processed until no new species were detected in three consecutive subsamples. Cladocerans and copepods were identified to species using Ward and Wipple (1966), Smith and Fernando (1978), Witty (2004), and Aliberti et al. (2013). Due to taxonomic similarity *Bosmina freyi* and *Bosmina liederi* were combined as *Bosmina freyi/lieideri*, and *Daphnia pulex* and *Daphnia pulicaria* as *Daphnia pulex/pulicaria*. For samples

on the final day, body length of the first 30 individuals encountered for each species was measured using a calibrated eyepiece micrometer to assess whether treatment effects influenced mean individual size and community size structure. If 30 individuals were not present, all encountered individuals were measured.

For each enclosure, water samples were collected using a 3-m integrated tube sampler to assess Ca, pH and total/edible chlorophyll-*a*. pH was sampled fortnightly beginning on Day 0 of the experiment and was analyzed using a desktop pHm220 laboratory pH meter (Radiometer Analytical, Lyon, France). Calcium concentrations were assessed for 10 June, 8 July and 5 August water samples using atomic absorption at the Dorset Environmental Science Centre (1026 Bellwood Acres Rd, Dorset, ON, Canada, P0A 1E0). For both pH and calcium we have presented the mean and standard deviation of three sampling events. Total chlorophyll-*a* was sampled weekly then measured after passing 300 ml of water through a G4 glass fiber filter with a 1.2- $\mu\text{m}$  pore size (Fisher Scientific; Pittsburgh, Pennsylvania, USA). Edible chlorophyll-*a* was measured after first passing 300 ml of water through a 30- $\mu\text{m}$  mesh, then collecting the seston from the filtrate on a G4 glass fiber filter. Filters were frozen, then chlorophyll-*a* was extracted in methanol for 24 h, and subsequently analyzed using a TD-700 fluorometer (Turner Designs; Sunnyvale, California, USA) following Welschmeyer (1994).

## Statistical analysis

Linear mixed-effects models were used to determine the effect of calcium, pH, and their interaction on species abundances, species richness, species evenness ( $E_{\text{var}}$ ), and total/edible chlorophyll-*a* throughout the experiment. Calcium and pH were fixed categorical factors, while sample date was a fixed continuous variable beginning on Day 14 of our experiment and ending at Day 56 ( $n = 4$  sampling time points; samples collected fortnightly). Models contained a random intercept for enclosure to account for temporal pseudoreplication in our experiment. Every response variable was tested against a full model of each fixed factor and the interaction between pH and calcium while controlling for enclosure effects [i.e.,  $\text{Response} \sim \text{pH} + \text{Ca} + \text{Day} + \text{pH:Ca} + (1|\text{enclosure})$ ]. Model assumptions of normality of residuals and variance heterogeneity were confirmed using histograms of residual values, residual versus fitted plots, correlation tests between residual versus fitted values, and scale-location plots. All response variables were log transformed. Analyses were run in R (R Development Core Team 2014); mixed-effects models were analyzed



using the “lmer” function from the *lme4* package (Bates et al. 2014), marginal pseudo- $R^2$  values were produced from the *MuMin* package to determine the variance explained by our fixed effects (Barton 2014), and *p*-values were calculated for fixed effects using the type III Satterthwaite ANOVA method from the *lmerTest* package (Kuznetsova et al. 2014).

Standard two-factor ANOVAs were used to assess initial differences in Day 0 enclosure communities (zooplankton abundance, richness, evenness; total and edible chlorophyll-*a*), as well as zooplankton body-size structure on the final day of the experiment. A fully parametrized factorial ANOVA was used in both cases to assess whether calcium or pH independently or interactively affected a given response variable. Model assumptions of normality and heterogeneity were tested and confirmed; all data were log transformed.

Adjusted *p*-values using the false-discovery rate (FDR) were applied to all experimental analyses to control type I and type II error rates (Benjamini & Hochberg, 1995). FDR groupings were made for each community-wide analysis (i.e., adjusted *p*-values within all abundance analyses, *n* = 68; all richness analyses, *n* = 12; all evenness analyses, *n* = 12; all body-size analyses, *n* = 28).

To contextualize our experimental results, we assessed changes in zooplankton relative abundance in 34 KPP lakes between 1971–1973 and 2016 using paired *t* tests. Zooplankton species that responded to our experimental treatments were assessed, as well as *D. pulex/pulicaria* due to their prevalence in the acidification and calcium literature. Zooplankton and water chemistry were collected between July and September at the deepest location of the lake for both historic and contemporary survey periods. Zooplankton were sampled from the entire water column using either 25-cm or 30-cm diameter nets with mesh sizes ranging from 75 to 110  $\mu$ m; detailed collection methodology can be found in Sprules (1975) for the 1970s samples and Suenaga (2018) for the 2016 samples. Zooplankton abundances were log transformed for each test based on assessment of normality and variance using histograms and boxplots of relative abundance. All bosminids and eubosminids were grouped together as *Bosmina* spp. because of differences in taxonomic identification between 1972 and 2016 samples.

## RESULTS

### Individual species

We stocked 19 zooplankton species, including 11 cladocerans and eight copepods into our experimental enclosures

(Appendix S1: Table S3). No differences in zooplankton abundance, richness or evenness were observed at the beginning of the experiment (Appendix S1: Table S4). At the end of the experiment, 15 of the original 19 species stocked into our enclosures were detected, with *Daphnia mendotae*, *Daphnia retrocurva*, *Epischura lacustris*, and *Skistodiaptomus oregonensis* being absent in all enclosures by the final day. Abundances of *Sida* spp., *Acanthocyclops vernalis*, and *Leptodiaptomus siciloides* were present at low densities and low occurrence, resulting in models that were unable to converge; these taxa have not been included in subsequent analyses but their densities through time can be found in Appendix S1: Figure S2.

Our high pH treatment led to increased abundance of two daphniid species and *Diaphanosoma* sp., no significant changes in density of the acid-tolerant *D. catawba*, and decreased abundance of *H. glacialis* and *M. edax*, relative to low pH treatments (Table 1). Abundance of *D. pulex/pulicaria* was 43% higher in high pH treatments, although this effect was not significant (Figure 2a). Abundance of *D. ambigua* was 42% higher in high compared with low pH treatments (Figure 2c) and, while *Diaphanosoma* sp. abundance increased over the course of the experiment, densities were 78% higher in high pH compared with low pH treatments (Figure 2h). The abundance of *H. glacialis* declined through time in all treatments but was 19% more abundant in low pH treatments across all sample dates (Figure 2g). *M. edax* abundance was 65% lower in high pH relative to low pH conditions across all treatment sample dates (Figure 2i).

Our low calcium treatments directly affected the abundance of several species. We did not detect a calcium effect for *D. pulex/pulicaria* abundance over the entire course of our experiment (Figure 3a), yet populations in low calcium treatments were nearly extirpated by the end of our experiment with only two individuals sampled among our 10 low calcium enclosures (Appendix S1: Figure S2a). In contrast, abundance of *D. catawba* (Figure 2b) and *D. ambigua* (Figure 2c) were 51% and 67% higher in low calcium compared with high calcium treatments, respectively. Several other cladocerans and calanoid copepodids also thrived under low calcium conditions. *Eubosmina tubicen* (Figure 2d), *H. glacialis* (Figure 2g), and *Diaphanosoma* sp. (Figure 2h) abundances were 88%, 51%, and 80% higher in low calcium than high calcium treatments. While calcium did not elicit a strong response for adult copepod species, calanoid copepodid abundance was 44% higher in low calcium treatments (Figure 2k).

In some cases, our calcium and pH treatments led to interactive effects on species abundance (Table 1). Abundance of the small eubosminid, *E. longispina*, increased

**TABLE 1** Model summaries of all mixed-effects models for species and community/taxonomic groups that were tested using type III Satterthwaite ANOVA

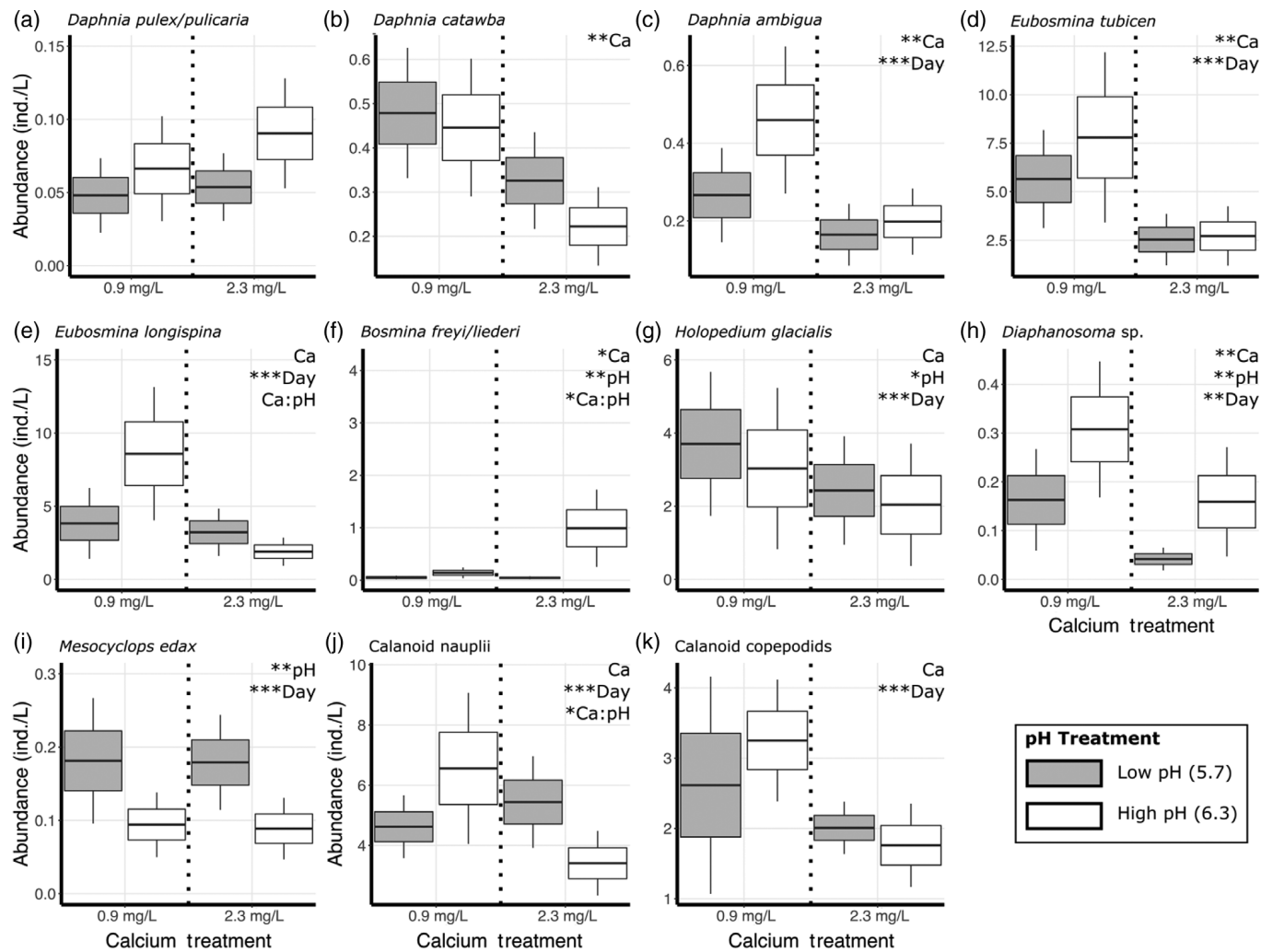
	Calcium				pH				Day				Calcium:pH				
Species and community groups	df	F	p	Adjusted p	df	F	p	Adjusted p	df	F	p	Adjusted p	df	F	p	Adjusted p	R <sup>2</sup>
Abundance																	
<i>Daphnia pulex/pulicaria</i>	1,20	1.10	0.31	0.47	1,20	3.40	0.079	0.15	1,60	1.35	0.25	0.40	1,20	0.40	0.54	0.67	0.08
<i>Daphnia catawba</i>	1,80	10.01	<b>0.0022</b>	<b>0.0088</b>	1,80	1.59	0.21	0.36	1,80	1.08	0.30	0.46	1,80	0.43	0.51	0.67	0.14
<i>Daphnia ambigua</i>	1,80	11.34	<b>0.0012</b>	<b>0.0054</b>	1,80	4.24	0.043	0.10	1,80	17.00	<b>&lt;0.0001</b>	<b>0.0004</b>	1,80	1.74	0.19	0.33	0.30
<i>Eubosmina longispina</i>	1,20	5.95	<b>0.024</b>	0.062	1,20	0.71	0.41	0.60	1,60	42.96	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	1,20	3.28	<b>0.024</b>	0.06	0.41
<i>Eubosmina tubicen</i>	1,80	13.30	<b>0.00047</b>	<b>0.0029</b>	1,80	0.62	0.43	0.58	1,80	38.90	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	1,80	0.23	0.63	0.75	0.41
<i>Bosmina freyi/liederi</i>	1,20	7.77	<b>0.011</b>	<b>0.033</b>	1,20	14.57	<b>0.0011</b>	<b>0.0053</b>	1,60	0.04	0.85	0.88	1,20	8.02	<b>0.010</b>	<b>0.031</b>	0.35
<i>Holopedium glacialis</i>	1,80	5.25	<b>0.025</b>	0.063	1,80	7.67	<b>0.007</b>	<b>0.023</b>	1,80	126.96	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	1,80	0.16	0.69	0.80	0.64
<i>Diaphanosoma</i> spp.	1,80	10.66	<b>0.0016</b>	<b>0.0068</b>	1,80	9.58	<b>0.0027</b>	<b>0.0097</b>	1,80	9.71	<b>0.0026</b>	<b>0.0097</b>	1,80	0.054	0.82	0.87	0.28
<i>Mesocyclops edax</i>	1,80	0.00	0.96	0.96	1,80	11.41	<b>0.0011</b>	<b>0.0053</b>	1,80	16.55	<b>0.00011</b>	<b>0.0007</b>	1,80	0.026	0.87	0.88	0.26
Calanoid nauplii	1,80	4.12	<b>0.046</b>	0.10	1,80	1.13	0.29	0.67	1,80	16.63	<b>0.0001</b>	<b>0.0007</b>	1,80	8.44	<b>0.0047</b>	<b>0.016</b>	0.28
Calanoid copepodid	1,20	5.51	<b>0.03</b>	0.072	1,20	0.39	0.53	0.46	1,60	21.70	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	1,20	3.56	0.070	0.14	0.29
<i>Diacyclops thomasi</i>	1,20	0.19	0.67	0.79	1,20	0.089	0.77	0.84	1,60	122.47	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	1,20	0.04	0.84	0.88	0.60
<i>Leptodiaptomus minutus</i>	1,20	0.71	0.41	0.58	1,20	0.29	0.6	0.73	1,60	0.41	0.52	0.67	1,20	3.35	0.080	0.65	0.07
<i>Orthocyclop modestus</i>	1,20	0.95	0.34	0.50	1,20	1.95	0.18	0.32	1,60	36.59	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	1,20	0.48	0.49	0.15	0.32
Total zooplankton	1,20	16.63	<b>0.00059</b>	<b>0.0033</b>	1,20	0.10	0.75	0.84	1,60	3.58	0.063	0.13	1,20	6.03	<b>0.023</b>	0.06	0.36
Cladocerans	1,20	35.26	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	1,20	0.50	0.49	0.65	1,60	1.43	0.24	0.40	1,20	3.75	0.067	0.14	0.39
Copepods	1,20	3.01	0.10	0.18	1,20	0.08	0.78	0.84	1,60	0.11	0.74	0.84	1,20	3.91	0.060	0.13	0.10
Richness																	
Total zooplankton	1,80	1.76	0.19	0.76	1,80	0.01	0.92	0.98	1,80	0.0004	0.98	0.98	1,80	0.12	0.73	0.98	0.02
Cladocerans	1,20	0.0007	0.98	0.98	1,20	0.05	0.83	0.98	1,60	0.095	0.76	0.98	1,20	0.11	0.74	0.98	0.003
Copepods	1,80	5.15	<b>0.03</b>	0.16	1,80	0.10	0.75	0.98	1,80	5.60	<b>0.019</b>	0.156	1,80	0.20	0.65	0.98	0.12
Evenness																	
Total zooplankton	1,80	2.92	0.090	0.18	1,80	0.64	0.43	0.645	1,80	4.01	<b>0.049</b>	0.1272	1,80	0.0017	0.97	0.99	0.09
Cladocerans	1,80	5.08	<b>0.03</b>	0.13	1,80	0.21	0.65	0.78	1,80	4.40	<b>0.040</b>	0.1272	1,80	0.00	0.99	0.99	0.11
Copepods	1,20	0.23	0.64	0.78	1,20	4.24	0.053	0.1272	1,60	16.43	<b>0.0002</b>	<b>0.0024</b>	1,20	2.70	0.12	0.21	0.23
Body size																	
<i>Daphnia pulex/pulicaria</i>	1,3	0.33	0.61	0.92	1,3	1.81	0.27	0.87	–	–	–	–	1,3	0.089	0.79	0.92	0.43
<i>Daphnia catawba</i>	1,16	0.47	0.50	0.92	1,16	1.19	0.29	0.87	–	–	–	–	1,16	0.025	0.88	0.92	0.10
<i>Daphnia ambigua</i>	1,14	0.03	0.87	0.92	1,14	0.0012	<b>0.97</b>	0.97	–	–	–	–	1,14	4.43	0.054	0.70	0.24
Daphniids	1,16	1.27	0.28	0.87	1,16	0.08	0.78	0.92	–	–	–	–	1,16	1.73	0.21	0.87	0.16
Total zooplankton	1,16	3.06	0.10	0.70	1,16	0.34	0.57	0.92	–	–	–	–	1,16	0.057	0.82	0.92	0.18
Cladocerans	1,16	0.04	0.84	0.92	1,16	3.58	0.077	0.92	–	–	–	–	1,16	0.035	0.85	0.92	0.19
Copepods	1,16	0.72	0.41	0.92	1,16	0.26	0.62	0.70	–	–	–	–	1,16	0.11	0.74	0.92	0.06

Notes: Adjusted *p*-values were calculated using a false-discovery rate adjustment, and bolded *p*-values indicate factors significant at  $\alpha < 0.05$ . Marginal pseudo-R<sup>2</sup> values are provided to describe variance explained for model factors. The effect of day was not included in the body-size model as individuals were only measured on the experiment's final day.

when low calcium co-occurred with higher pH treatments (Figure 2e). Similarly, calanoid nauplii abundance was highest in low calcium and high pH treatments (Figure 2j). For both *E. longispina* and calanoid nauplii abundance, post-hoc Tukey tests identified that low calcium/high pH treatments produced significantly higher

abundances than other treatment combinations. Additionally, *Bosmina freyi/lieri* abundances were significantly higher in the high calcium/high pH treatment compared with all other treatment combinations.

All remaining cladoceran and copepod species (including juvenile cyclopoids) that persisted to the end



**FIGURE 2** (a–k) Mean abundance from Day 14 to Day 56 of the experiment for species that responded to Ca and pH treatments, along with *D. pulex/pulicaria* due to their importance to food webs and their prevalence within the calcium literature. The thick, horizontal black line represents the treatment sample mean, with the bottom and top edges of the box representing the standard error of the mean. Whiskers extending from the box represent the 95% confidence intervals of the mean. Factors written in the top right of each panel represent variable significance associated with FDR-corrected alpha significance levels (no symbol,  $p < 0.1$ ; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ )

of our experiment displayed either no response to experimental treatments, or occurrences in enclosures were too rare to calculate effects (Appendix S1: Figure S2). Individual body size of *Daphnia* was not influenced by pH or calcium (Table 1; Appendix S1: Figure S3).

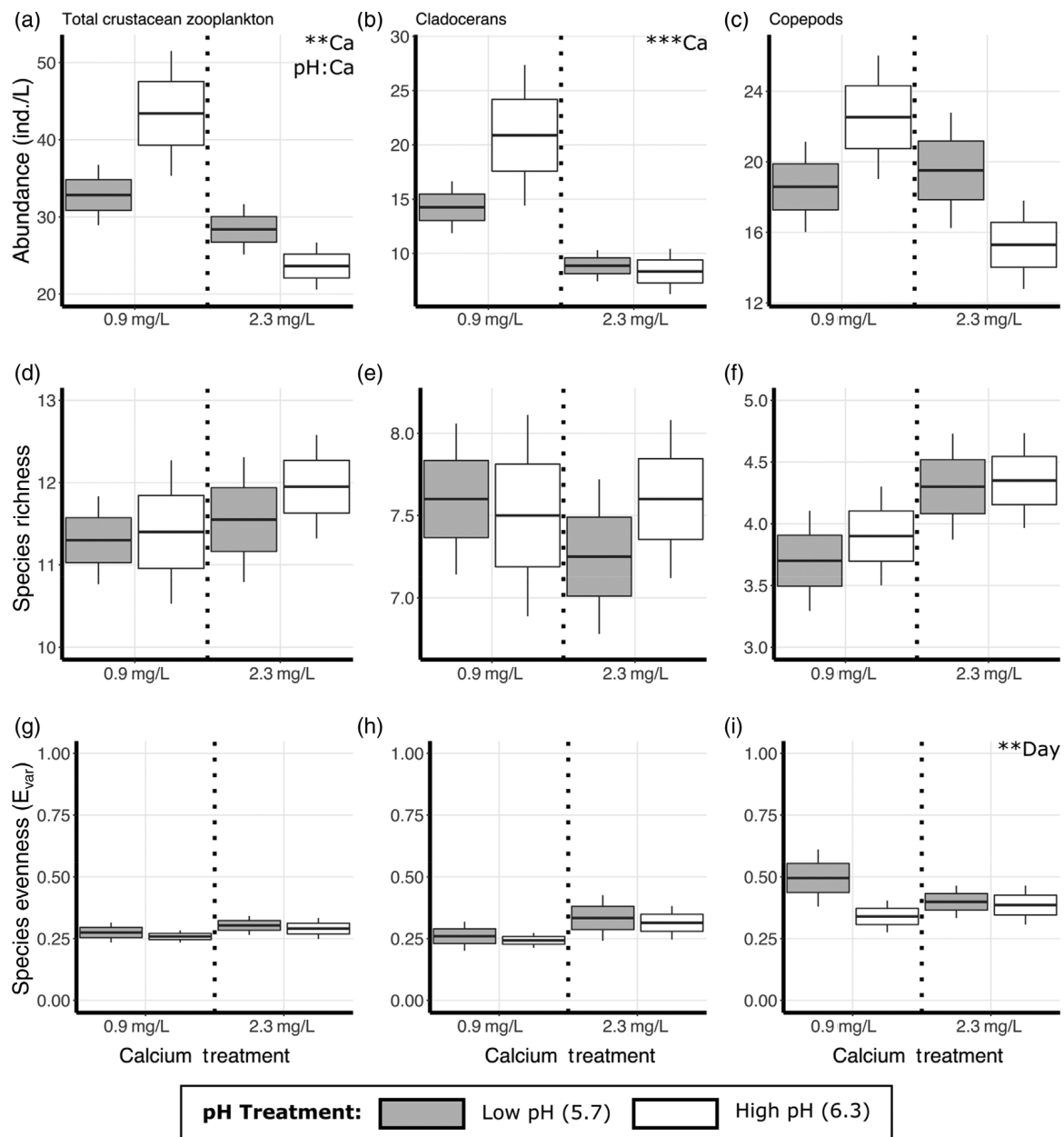
## Taxonomic groups and community metrics

Total zooplankton abundance was 38% higher in low calcium compared with high calcium treatments (Figure 3a). However, the effect of calcium was influenced by a significant interaction with pH. Pairwise comparisons indicated that high pH increased total zooplankton abundance when calcium was low but led to decreased abundance when calcium was high (Figure 3a). Species richness (Figure 3d), species evenness (Figure 3g),

and abundance-weighted zooplankton size were unaffected by our treatments (Table 1; Appendix S1: Figure S3g).

Cladoceran abundance responded to our calcium treatment, but not pH or any interactions therein (Table 1). Elevated eubosminid, *D. catawba*, and *D. ambigua* abundance (Figure 2) led to 68% higher total cladoceran abundance in low calcium compared with high calcium treatments (Table 1, Figure 3b). In contrast, cladoceran species richness was unaffected by treatments, as very few taxa were extirpated in individual treatments (Figure 3e). Species evenness ( $E_{var}$ ) was higher in high calcium treatments (Figure 3h) with low calcium treatments being dominated by a small number of cladoceran species, however when using FDR adjusted  $p$ -values this effect was no longer significant. Neither calcium nor pH treatments affected mean





**FIGURE 3** (a–i) Zooplankton community response to low and high Ca and pH treatments between Days 14–56 of the experiment. The thick, horizontal black line represents the treatment sample mean, with the bottom and top edges of the box representing the standard error of the mean. Whiskers extending from the box represent the 95% confidence intervals of the mean. Factors written in the top right of each panel represent variable significance associated with FDR-corrected alpha significance levels (no symbol,  $p < 0.1$ ; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ )

abundance-weighted daphniid or cladoceran body size (Table 1; Appendix S1: Figure S3d,e, respectively).

Copepod group metrics were mostly unaffected by experimental treatments (Table 1) and total abundances did not show any clear response (Figure 3c). Mean copepod richness was moderately reduced in low calcium (3.75 species) compared with high calcium treatments (4.25 species; Figure 3f), but neither copepod evenness (Figure 3i) or abundance-weighted copepod

size was influenced by calcium, pH, or their interaction (Appendix S1: Figure S3f).

### Chlorophyll-*a*

No differences in food resources were found between treatments at the beginning or throughout our experiment (Appendix S1: Table S4, Figure S5).

## Landscape comparison

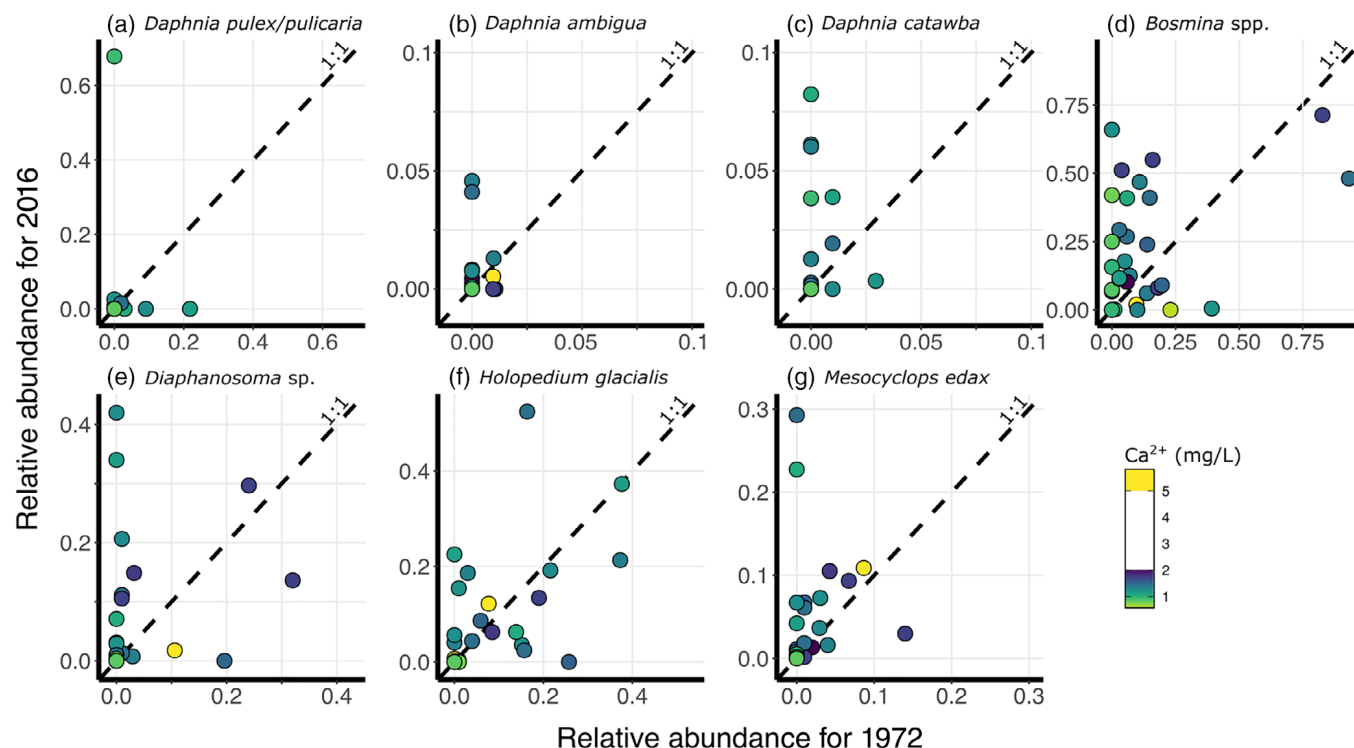
Since 1972, the relative abundance of the acid-sensitive *D. pulex/pulicaria* has mostly decreased in KPP, albeit nonsignificantly because of one outlying lake that experienced a large increase in density (Table 2, Figure 4). The abundance of two acid-tolerant daphniids have largely increased over time; *D. ambigua* has increased albeit not

significantly, and there has been a significant increase in *D. catawba* across the 34 historically acidified KPP lakes (Table 2, Figure 4). Of the other species that responded to our experimental calcium and pH treatments, abundance of bosminids (including *Eubosmina* spp. and *Bosmina freyi/liederi*) have significantly increased between 1972 and 2016, the relative abundance of *H. glacialis* and *Diaphanosoma* sp. has remained unchanged, and there

**TABLE 2** Paired *t* tests that compare historic (1972) and contemporary (2016) relative zooplankton abundance for a set of 34 acidified KPP lakes

Species	Percentage change	df	<i>t</i>	<i>p</i>
<i>D. pulex/pulicaria</i>	3 (−12, 18)	8	0.4	0.7
<i>D. ambigua</i>	0.5 (−0.5, 1.5)	13	1.08	0.3
<i>D. catawba</i>	2.3 (0.12, 4.6)	10	2.35	<b>0.041</b>
<i>Bosmina</i> spp.	7.8 (0.9, 14.8)	30	2.3	<b>0.029</b>
<i>H. glacialis</i>	0.4 (−3.4, 4.2)	27	0.21	0.84
<i>Diaphanosoma</i> sp.	3.5 (−1.0, 8.0)	25	1.60	0.12
<i>M. edax</i>	4 (0.6, 7.6)	23	2.4	<b>0.025</b>

Notes: A positive percentage change represents an increase in mean relative abundance for 2016, and 95% confidence intervals of mean between-year difference are reported within parentheses. Only zooplankton that responded to our experimental treatments and *Daphnia pulex/pulicaria* because of its food web importance and predicted sensitivity, have been included in this landscape analysis.



**FIGURE 4** (a–g) Mean relative abundance for species we detected experimental mesocosm effects from within a set of 34 historically acidified KPP lakes. Relative abundance is compared for samples taken from KPP lakes near peak acidification (1972) and contemporary times (2016). The dashed diagonal line represents a one-to-one relationship of relative abundance between the two time periods; points that fall beneath the one-to-one line indicate higher relative abundances during the acidified 1972 time period, and points that fall above the one-to-one line indicate higher relative abundances during 2016 where lakes have experienced marked declines in acidity. Calcium concentrations were sampled in 2016 and are identified for each lake by color gradient

was a significant increase in *M. edax* relative abundance (Table 2, Figure 4).

## DISCUSSION

Our study suggests that declining calcium concentrations will probably prevent the recovery of several acid-sensitive zooplankton species in thousands of North American and European lakes now recovering from acidification. The response to low calcium treatments for five of the seven taxa that responded to our experimental treatments was in the opposite direction of response to increased pH, and furthermore, changed in a direction consistent with how zooplankton assemblages in KPP have changed following calcium decline (Table 3). For example, the abundance of *D. pulex/pulicaria*, a large-bodied keystone herbivore that declined when lakes acidified (Keller & Pitblado, 1986), increased in our high pH treatment (albeit marginally nonsignificantly), yet at the end of our experiment was nearly extirpated from low calcium environments, consistent with expectations based on the literature (Ashforth & Yan, 2008; Azan & Arnott, 2018; Tessier & Horwitz, 1990; Appendix S1: Figure S2a). In agreement with these results, we found no detectable change in *D. pulex/pulicaria* abundance through time across the KPP landscape despite one lake that experienced an anomalously large increase over time (Figure 4a). Conversely, abundance of smaller bodied species, *D. catawba*, *D. ambigua*, and *Eubosmina tubicen*,

which typically thrive in acidic conditions (Keller & Pitblado, 1986; Sprules, 1975), did not respond statistically to our pH treatment, but experienced significant increases in low calcium treatments. The abundance of *D. catawba* and *Bosmina* spp. (which includes both *Eubosmina* spp. and *Bosmina freyi/lieri*) also increased through time in our lake survey (Table 2). In the experiment and landscape surveys, we detected evidence of potential recovery for two species, *Diaphanosoma* sp. and *D. ambigua*. In both high pH and low calcium treatments the abundance of both species increased, albeit marginally nonsignificantly for *D. ambigua* in high pH treatments after accounting for the FDR (Table 1), while also experiencing slightly higher relative abundances over time across the Killarney lakescape (Table 3). These results suggested that as calcium continues to decline in thousands of waterbodies across the northern hemisphere, it is unlikely there will be a return to pre-acidification zooplankton assemblages.

## Species-specific response

The abundance of two *Daphnia* spp. (*D. catawba* and *D. ambigua*) responded directly to our calcium treatments in ways that suggest acid-structured *Daphnia* assemblages will be maintained despite improvements to lake pH. Low calcium can negatively influence *Daphnia* spp. survival, relative abundance, and population growth rates (Ashforth & Yan, 2008; Pérez-Fuentetaja & Goodberry,

**TABLE 3** A summary of experimental trends for high pH (representing acid-recovered lakes) and low calcium treatments (representing present-day trends of declining calcium), and how they compare with landscape relative abundances in formerly acidified Killarney Provincial Park lakes

Species	Experimental effects; abundance		Landscape effects; relative abundance
	High pH	Low Ca	Killarney survey; relative abundance 2016 compared with 1972
<i>D. pulex/pulicaria</i>	↑	↓	↑
<i>D. catawba</i>	↓	↑**	↑*
<i>D. ambigua</i>	↑	↑**	↑
<i>Bosmina</i> spp. <sup>a</sup>	↓	↑***	↑*
<i>H. glacialis</i>	↓*	↑†	↑
<i>Diaphanosoma</i> sp.	↑**	↑**	↑
<i>M. edax</i>	↓**	↓	↑*

Note: Landscape relative abundances are compared between 1972 (period of intense acidification) and 2016 (period of improved lake pH, dramatically lower calcium), and arrows indicate an (↑) increase or (↓) decrease in abundance. Associated significance levels from FDR adjusted experimental treatments and landscape *t* tests are indicated by †,  $p < 0.10$ ; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

<sup>a</sup>*Bosmina* spp. are grouped in both experimental and landscape groups due to limited taxonomic resolution of the landscape survey. It must be noted, significance of *Bosmina* spp. experimental effects are not controlled for false-discovery rate as individual responses of *E. longispina*, *E. tubicen*, and *Bosmina freyi/lieri* were pooled for this specific analysis only (linear mixed-effects model with type III Satterthwaite ANOVA method: Ca  $F_{1,80} = 13.19$ ,  $p = 0.0005$ ; pH  $F_{1,80} = 0.42$ ,  $p = 0.52$ ; Day  $F_{1,80} = 88.6$ ,  $p < 0.0001$ ; Ca  $\times$  pH  $F_{1,80} = 1.60$ ,  $p = 0.21$ ). For individual experimental effects with appropriate false-discovery rates for *Eubosmina tubicen*, *Eubosmina longispina* and *Bosmina freyi/lieri* see Table 1 and Figure 2.

2016; Prater et al., 2016; Tan & Wang, 2010), with our results suggesting these calcium effects also persisted at relatively high pH. Surprisingly, abundances of *D. pulex/pulicaria* were not strongly affected by either the pH or calcium treatments (Figure 2a), despite being nearly extirpated in low calcium treatments at the end of our experiment (Appendix S1: Figure S2a). *D. pulex/pulicaria* are important grazers and prey items that are typically limited by low surface water pH and calcium concentrations (Ashforth & Yan, 2008; Keller & Pitblado, 1986; Rice et al., 2021; Tessier & Horwitz, 1990); it is possible that low initial experimental abundances limited our ability to detect treatment effects (Figure 2a). While the experimental effects of *D. pulex/pulicaria* were not what we hypothesized, the direction of response for all three *Daphnia* spp. mentioned here were consistent with expectations across the landscape. If it were not for one of our survey lakes having high *D. pulex/pulicaria* relative abundance in 2016 (Clearsilver Lake, KPP), it appears that *D. pulex/pulicaria* abundances are lower now than they were during peak acidification (Figure 4a), and relative abundances of *D. catawba* and *D. ambigua* have increased (Figure 4c,b, respectively; Table 3) in lakes during the period of time when calcium has dramatically declined (Figure 1). Collectively, these results suggested that declining calcium concentrations may prevent broad-scale daphniid assemblage recovery.

Our experiment suggests that the abundance of eubosminids will increase as lake pH improves and calcium declines. Although we did not detect a main effect of pH, abundances of *E. tubicen* were significantly higher in low, compared with high, calcium treatments. Additionally, there was a marginally nonsignificant interaction between pH and calcium after accounting for the FDR in which *E. longispina* abundance was highest when calcium concentrations were low and pH was above 6 (Figure 2d,e), the condition expected for thousands of acid-recovering lakes (i.e. low calcium and pH > 6; Weyhenmeyer et al., 2019). Both *E. tubicen* and *E. longispina* can sustain positive population growth rates at calcium concentrations as low as 0.5 mg/L (Azan & Arnott, 2018), and in historically acidified lakescapes eubosminid assemblages were predominantly associated with very soft water (Carter et al., 1980; Keller & Pitblado, 1986). Additionally, small cladocerans such as bosminids have demonstrated a release from competition when large grazers, such as *D. pulex/pulicaria*, are absent (Vanni, 1986). While our experiment cannot account for possible shifts in zooplankton composition caused by invertebrate or fish predation (Arnott & Vanni, 1993; Valois et al., 2010; Vanni, 1988), our hypothesis that bosminids would be released from competition seems to be supported when comparing our experimental results

to those from the surveys of Killarney lakes. As peak acidification in 1972, *Bosmina* spp. (which includes *Eubosmina* spp. and *Bosmina freyi/lieperi*) has increased in relative abundance, despite improvements to lake pH (Figure 4d); *Bosmina* spp. are now 8% more common across all historically acidified Killarney lakes than they were during peak acidification, and are dominant in the lakes they occupy. In 11 of the 34 lakes studied, *Bosmina* spp. accounted for more than 25% of total individuals within their zooplankton community (Figure 4d).

The abundance of *H. glacialis* decreased in our high pH treatments (Figure 2g), consistent with a negative relationship between abundance and pH in the literature (Frost et al., 2006; Hessen et al., 1995; Keller & Pitblado, 1986). Moreover, in our low calcium and low pH treatments, *H. glacialis* abundance was nearly two times higher than at high calcium and pH, respectively. Because *H. glacialis* is similarly affected by low pH and low calcium, we detected no differences in *H. glacialis* relative abundance across acidified Killarney lakes over the last 40 years (Figure 4f, Table 2). This is in agreement with previous research documenting high abundance at low calcium concentrations (Hessen et al., 1995; Jeziorski et al., 2012, 2014) that are likely to be driven by the loss of large competitors, such as *Daphnia* (Hessen et al., 1995). Together, these results suggested that, despite regional increases in lake pH, *H. glacialis* abundance is likely to remain high as calcium becomes the predominant stressor structuring competitive interactions among larger bodied cladocerans (Hessen et al., 1995).

*Diaphanosoma* sp. increased in both our high pH and low calcium treatments, largely agreeing with literature-based expectations for recovery. In historically acidified lakes, *Diaphanosoma* sp. occurred across a range of pH, but was consistently found at higher densities in lakes with circumneutral pH (Havens et al., 1993; Holt & Yan, 2003). Accordingly, *Diaphanosoma* sp. abundance was nearly 80% higher in our high pH treatments, however low calcium also resulted in ~80% increase in abundance (Figure 2h). Based on these results, we were surprised that relative abundance of *Diaphanosoma* sp. was not statistically different in Killarney lakes between peak acidification and the recent survey. However, trends in our landscape comparison suggested that *Diaphanosoma* sp. relative abundance is increasing in low calcium lakes (Table 3), and that the largest increases are found in lakes with some of the lowest calcium concentrations (Figure 4e). As calcium concentration of historically acidified lakes continues to decline, our experiment and landscape trends suggest that *Diaphanosoma* sp. may be one of the few species that will recover from acidification, as lower calcium concentrations are likely to reinforce high relative abundances (Table 3).

Our experiment indicates that adult species of copepods should not be affected by falling calcium concentrations for lakes where pH is now recovering. *M. edax* was the only copepod that responded to our experimental treatments, with low pH treatments eliciting the highest abundances (Figure 2i). However, these results are different than the landscapewide increase described for *M. edax* relative abundance in lakes where pH has now increased (Figure 4g). *M. edax* abundances were historically low in severely acidified lakes (pH < 5.2; i.e., much lower than our “low”) but thrived in lakes with moderately acidic to circumneutral pH (between pH 5.5–6.4; Sprules, 1975, Keller & Pitblado, 1986, Frost et al., 2006). Additionally, while we did not detect an effect of calcium on *M. edax* abundance in our experiment, there is evidence of reduced population growth rates under low calcium concentrations (Azan & Arnett, 2018). Differences among experimental studies, landscape surveys, and patterns in the literature indicate that there is still uncertainty about what promotes *M. edax* abundance in formerly acidified environments (e.g., potential biotic interactions and physiological tolerance). More research is required to understand how the biocomplexity of formerly acidified environments influences copepod fitness.

Calcium and pH do not explain the extirpation of four species that were stocked from the regional species pool at the beginning of our experiment (*D. mendotae*, *D. retrocurva*, *E. lacustris*, and *S. oregonensis*). Prior to our experiment, all four of these species were cultured in similar-styled enclosures, so it is unlikely that confinement resulted in their disappearance (Gray & Arnett, 2011, 2012). While it is possible that a combination of biotic or abiotic conditions prevented their establishment (e.g., sex ratios, habitat availability, oxythermal and food preferences), more research is needed to determine the interspecific effects that the decline in calcium will have for these and other zooplankton species in acid-recovering regions.

## Community and taxonomic group response

Our experiment showed that total zooplankton abundance, taxonomic group abundance (i.e., cladocerans/copepods), and species evenness ( $E_{var}$ ) are sensitive community metrics for formerly acidified lakes now experiencing calcium declines. The high density of small cladocerans and daphniids in low calcium treatments has led to significantly higher abundance of cladocerans and total zooplankton in low calcium relative to high calcium or pH treatments (Figure 3a,b). The numerical dominance of relatively few cladoceran species in low calcium treatments also led to decreased cladoceran evenness, although this effect was diminished after controlling for

the FDR (Table 1, Figure 3h). While total zooplankton and cladoceran richness typically decline in acidified lakes (Locke, 1992; Sprules, 1975), we did not detect a richness response in our experiments. Additionally, there was no effect of calcium on chlorophyll-*a* concentrations (Appendix S1: Table S4), despite evidence in one study suggesting that changes in zooplankton communities due to low calcium can affect phytoplankton concentrations (Korosi et al., 2012). It may be that our gradients of pH and calcium were too short to elicit extirpations and associated changes in our experimental food web (Ashforth & Yan, 2008; Holt et al., 2003) or that these effects may have only been detected over longer exposures.

Our experiment suggests that calcium decline is not likely to be a driver of copepod community metrics in formerly acidified lakes (Figure 3c,f,i). The lack of a statistical response in abundance metrics is in contrast with studies that have demonstrated that copepod abundance, growth rates, and community dominance can vary across calcium gradients (Azan & Arnett, 2018; Leach et al., 2019; Wærvågen et al., 2002). The strength, direction, and variability of copepod responses to falling calcium concentrations may be structured by biotic interactions. In an experiment in which cladocerans dominated relative community abundance (90% relative abundance), copepod population growth rates remained above zero at calcium concentrations as low as 0.6 mg/L, despite decreased population growth rates, as calcium concentrations were reduced (Azan & Arnett, 2018). Yet, in a region where communities are dominated by copepods, decreases in copepod biomass were closely associated with falling calcium concentrations (Leach et al., 2019). It is possible that the muted copepod response in our experiment could be explained by the relatively even split of copepod and cladoceran relative abundance. While the interaction of calcium and pH was not significant, copepod abundance was highest in the low calcium/high pH treatment (i.e., the current and future scenario for many formerly acidified lakes; Figure 3c), which may have implications for lakes as pH continues to increase and calcium continues to decrease. Wide variation in copepod responses from our experiment and throughout the literature highlights the need for more studies to understand what covariates influence copepod community metrics in low calcium environments.

In our experiment, neither pH or calcium affected individual body size or community-weighted size structure, despite strong responses in species abundances. Studies have suggested that calcium concentrations can affect community body-size structure (Tessier & Horwitz, 1990), and that *Daphnia* body length can decline in low calcium water (Ashforth & Yan, 2008; Hessen et al., 2000). However, the narrowness of our calcium gradient may have precluded a response in individual body size; larger calcium gradients were used in studies in which decreased body size was



observed at low calcium (Ca range 0.5–10 mg/L, Hessen et al., 2000; Ca range 0.1–10 mg/L, Ashforth & Yan, 2008). It is possible that zooplankton communities starting with a higher relative abundance of large cladocerans would experience shifts in community-weighted size structure, but high initial relative abundance of small cladocerans (i.e., *D. catawba*, *D. ambigua*, *Bosmina freyi/lieri*) that were maintained throughout all treatments during the experiment and probably diminished any effect of our treatments on community or taxonomic group size structure. Although body size did not respond to calcium or pH treatments, high abundance of both small cladocerans and copepods in our low calcium/high pH treatments has potential implications for zooplankton productivity. Increased abundance in the treatment that represents the future scenario for many formerly acidified lakes indicates that zooplankton productivity may increase over time because of high reproductive rates of small cladocerans or may decrease because of the prevalence of small-bodied species. This is a phenomenon that surely demands further research attention.

### Landscape drivers of zooplankton communities

The species-specific effects of calcium and pH that we detected in our experiment were generally in agreement with long-term changes across the Killarney lakescape (Table 3), suggesting that declining calcium concentrations are likely to affect zooplankton communities in acid-recovering lakes. While the co-gradient of pH and calcium is the primary environmental driver of cladoceran assemblages in northern regions of continental North America (Labaj et al., 2021), it is likely that regional variation in abiotic conditions and food web structure will also modulate zooplankton responses. For example, in historically acidified Adirondack lakes (New York, USA), the effect of declining calcium on zooplankton was modified by changes in lake “browning”/dissolved organic carbon concentrations (Leach et al., 2019). Multiple stressors of browning and declining calcium led to a dramatic reduction in the dominance of a single acid-tolerant species (*Leptodiantomus minutus*) and not changes in cladoceran taxa such as those that we observed (Leach et al., 2019). Conversely, in Fennoscandian and Sudbury area lakes, effects of declining calcium will probably be modified by biotic interactions of competition and predation. Established acid-structured zooplankton communities may limit the recovery potential for species in which calcium is declining, and it is likely that continued fish and macroinvertebrate predation will limit zooplankton re-establishment similar to that seen in the initial stages of biotic recovery in acid-structured communities (Binks et al.,

2005; Valois et al., 2010; Wærvågen & Nilssen, 2003; Webster et al., 2013). Identifying how declining calcium may be interacting with other regionally distinct stressors will be crucial for the further refinement of realistic recovery targets for historically acidified lakes.

Acidified lakes in Canada, the United States, and Europe have experienced limited improvements in zooplankton community metrics since the 1970s (Angeler & Johnson, 2012; Arseneau et al., 2011; Gray et al., 2012; Malley & Chang, 1994; Vrba et al., 2003) and, although there are a suite of reasons why (Gray & Arnott, 2009), our results suggested that low calcium has probably contributed to the stagnated biological response and will certainly be a factor in biological recovery going forward. Furthermore, we experimentally demonstrated that low calcium may appear as an invisible stressor across acidified landscapes, with low calcium concentrations affecting species abundance in markedly similar ways to low pH (Table 3). The scale of this issue is problematic. Of the thousands of North American and European lakes that were historically acidified, the vast majority are now seeing substantial decreases in calcium concentrations despite associated increases in pH (Weyhenmeyer et al., 2019). As production and biomass of aquatic food webs are steadily structured through predictable density and body-size relationships (i.e., biomass size spectra; Kerr & Dickie, 2001), sustained shifts to less diverse zooplankton assemblages composed of smaller taxa may have profound implications on aquatic ecosystems.

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### CONFLICT OF INTEREST


The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

All data and code (Ross, 2021) associated with this publication are publicly archived in Figshare at <https://doi.org/10.6084/m9.figshare.14706705.v1>. The author requests that users please read “notesForMetadata.rtf” prior to downloading any files.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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