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Effects of calcium and phosphorus limitation on the nutritional ecophysiology of *Daphnia*

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Abstract

Declines in environmental calcium (Ca) and phosphorus (P) concentrations have occurred over the past 30 yrs in lakes across the Canadian Shield in southern Ontario, and these reductions appear to be placing strong constraints on populations of *Daphnia* in this region. Here, we report results from a factorial manipulation of Ca concentrations and food P content under controlled laboratory conditions where we measured resulting changes in daphnid elemental content, individual growth and survival, and life history traits related to population growth. We found significant effects of Ca- and P-limitation on all variables measured; however, dietary P explained a majority of the variation in daphnid nutrient content and growth. Dietary effects of low P [high food carbon (C): P ratios] on individual *Daphnia* life-history traits also translated into significant population level effects. Dietary P also explained relatively more experimental variation in population level responses than Ca concentrations. Experimental Ca concentrations most strongly altered daphnid survival partly due to the use of a lethally low Ca concentration in our experiment. Although recent work examining shifts in zooplankton communities in this region mainly focuses on the effects of Ca-limitation, we show that Ca concentrations and food nutrient content, at levels commonly found on the Canadian Shield, are both likely to strongly alter *Daphnia* life-history and populations dynamics. Our results underscore the need to more fully examine how multielemental limitation (e.g., Ca, N, P) affects consumer physiology and life-history given the plausible translation of these effects on the community structure of lake zooplankton.

Humans are dramatically affecting nutrient supplies in aquatic ecosystems across the world. While human activities usually result in greater nutrient inputs (e.g., Carpenter and Lathrop 2008; Smith and Schindler 2009), reductions in nutrient loading have also been documented and appear capable of dramatically affecting aquatic foodwebs (Mancha and Ruggiu 1998; Sarpe et al. 2014). For instance, lakes located downwind of industrial areas in eastern Canada have been subjected to large alterations in calcium (Ca) concentrations over the last century (Jeziorski et al. 2008; Frisch et al. 2014). Greatly reduced Ca export into lake ecosystems in this region has resulted from recent changes in atmospheric acid deposition and its past legacy effects on lake catchments (Likens et al. 1996). During the same time period, there has been reduced P exports from forested catchments in the same region and reports of declining P concentrations in receiving lakes (Raney and Eimers 2014). Together, surface waters in this area and in other boreal ecosystems in countries such as Norway are experiencing simul-

taneous reductions in Ca and P concentrations (Skjelkvale et al. 1997; Eimers et al. 2009; Palmer et al. 2011). While the effects of reduced Ca concentrations on freshwater zooplankton, such as *Daphnia*, have been heavily studied in lake ecosystems in south eastern Canada (Jeziorski et al. 2015), the relative effects of decreased P loading has received much less attention.

Dissolved Ca concentrations have been proposed as the primary controller of zooplankton assemblages in eastern Canadian Shield lakes (Jeziorski et al. 2008). This conclusion is based on observed imbalances between low Ca concentrations in lake water and the putative requirements for growth and survival of some zooplankton taxa (Jeziorski and Yan 2006). Generally, lakes undergoing long-term Ca reductions have relatively fewer Ca-rich *Daphnia* and more Ca-poor *Holopedium* (Jeziorski et al. 2012). These changes are consistent with *Daphnia*'s sensitivity to low Ca concentrations in laboratory experiments, presumably due to their relatively high body Ca content (Ashforth and Yan 2008). Although links between nutrition and performance are based on the premise that body Ca content of zooplankton provides a reliable index of animal metabolic requirements for Ca, recent work

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suggests that body Ca content may not directly relate to metabolic needs (Tan and Wang 2010). One reason for this is the possibility that other factors, such as dietary P-limitation, might affect consumer growth and thereby alter animal body Ca requirements.

While low Ca concentrations can clearly constrain the performance of Ca-rich *Daphnia* (Ashforth and Yan 2008), whether the nature of these effects depend on food P content is less clear. Daphnids frequently consume P deficient diets (Elser and Hassett 1994; Elser et al. 2000), which produces a number of physiological effects including low growth rates, reduced reproduction, and elevated mortality (Sterner et al. 1993; Frost et al. 2005). Given that slow growth alters *Daphnia* Ca content (Rukke 2002), it is plausible that their body Ca could also vary with their dietary P intake or due to other forms of elemental limitation (C- or N-limitation). For instance, juvenile *Daphnia* have higher relative Ca content compared with larger bodied adults (Hessen and Rukke 2000), so decreases in growth due to nutrient limitation could result in higher Ca consumers. As Ca-phosphates are used in carapace formation (Neville 1975), allometric increases in daphnid Ca : P ratios may result from high surface to volume ratios in smaller slower-growing juveniles. Despite their metabolic and structural connections, the effects of low dietary P content on consumer growth and body Ca content have not been specifically examined. As low Ca and P concentrations co-occur in Canadian Shield lakes (Yan et al. 2008), determining how low environmental supplies of both elements alter consumer body stoichiometry and life-history is essential for understanding long-term changes in zooplankton populations in this region.

Low Ca concentrations can alter *Daphnia* life-history and population growth (Ashforth and Yan 2008), but these responses are also produced by dietary P-limitation. The intrinsic rate of population increase (r) decreases with low Ca and low food P content due to decreased reproductive output (R_0), longer generation times (G_t), and increased age of first reproduction (Hessen et al. 2000; Urabe and Sterner 2001; Ashforth and Yan 2008; Tan and Wang 2010). In addition to population growth parameters, low Ca and P concentrations can significantly decrease age-specific survivorship (l_x) (Urabe and Sterner 2001; Ashforth and Yan 2008). While the independent effects of Ca- and P-limitation on life-history traits and population growth rates have been established no study, to our knowledge, has examined the relative responses of life-history traits and population growth to low supplies of both elements.

Here, we examine the individual and combined effects of Ca- and P-limitation on *Daphnia*. Using results from factorial concentration gradient experiments, we determine how Ca- and P-limitation alters individual daphnid life-history and body stoichiometry responses. We also explore how nutrient-limited growth might affect *Daphnia* populations and discuss implications of our results for understanding

environmental Ca-limitation and its ability to explain declining zooplankton populations in the lakes of southern Ontario.

Methods

Algae and *Daphnia* culturing

We grew an algal food source, *Scenedesmus obliquus* (Canadian Physiological Culture Centre strains 10 purchased as *Scenedesmus acutus*), in multiple semicontinuous culture jars. We manipulated algal P content by altering daily dilution rates and media P concentrations (Frost et al. 2010; Wagner and Frost 2012). Harvested cells were centrifuged ($4066 \times g$) for 20 min and resuspended in N-, P-, and Ca-free COMBO media (Kilham et al. 1998). Elemental content of algae was estimated by first filtering algae onto ashed GF/C glass fiber filters and drying filters at 60°C. We determined P content of algal cells after persulfate digestion followed by molybdate-blue ascorbic acid colorimetry and spectroscopy (APHA 1992). Algal C and N content were determined using an Elemental Analyzer (Vario EL III, Elementar Incorporated, Mt Laurel, New Jersey). We used these concentrations to mix nominal food concentrations for experiments. Additional filters were saved to determine the elemental composition of food after mixing provided to experimental animals.

The *Daphnia pulex* clone used in this experiment was clone # TCO SOM I.1, obtained from Norm Yan (York University, Toronto, Ontario, Canada) and raised in our lab for more than 1 yr in COMBO media. To produce experimental animals, we grew second generation clonal mothers in 400 mL of P-free COMBO media and ensured they were well-fed with P-rich (C : P ratio ~ 80) *S. obliquus*. On the morning of each experiment, we collected < 24 h old neonates that were born in the second to fifth broods of mothers.

Growth and body nutrient composition experiment

We simultaneously manipulated dietary P concentrations and media Ca availability during 6 d growth experiments. Dietary food was replenished every other day for 6 d at a quantity of 4 mg C L^{-1} across a range of food quality (C : P 100, 300, 500, 700 by mol). Media Ca concentrations (0.5 mg L^{-1} , 1 mg L^{-1} , 2 mg L^{-1} , 5 mg L^{-1} , 7 mg L^{-1} , 10 mg L^{-1} , 15 mg L^{-1}) were manipulated by adjusting the amount calcium chloride added to the N- and P-free COMBO. Food C : P ratios and Ca concentrations were chosen because they bracket the ranges expected for Canadian Shield lakes in southern Ontario (Elser and Hassett 1994; Jeziorski et al. 2008). For individual growth experiments, neonates were rinsed several times with N-, P-, and Ca-free COMBO media, and 20 individuals were randomly assigned to experimental 400 mL jars. Samples of animal growth media were saved in vials and later analyzed on an X-ray spectrometer (see below for details; TXRF S2 PicoFox, Bruker Corp, Billerica, Massachusetts). Initial neonate mass ($n = 3$ of 20 pooled individuals) was measured from dried

samples using a microbalance and was included in subsequent mass specific growth rate (MSGR) calculations.

After 6 d of growth, all animals from individual jars of *Daphnia* were placed into aluminum cups ($n = 5$), dried at 60°C for at least 12 h, and weighed on a microbalance to determine average body mass. Growth rates were calculated by subtracting the natural log of the initial neonate mass from the natural log of the final average individual mass and dividing by the duration of the experiment. Body P content was measured on a different set of replicate jars ($n = 5$) using persulfate digestion followed by molybdate-blue ascorbic acid colorimetry and spectrophotometry. Daphnid Ca content was measured by homogenizing all the animals from a final set of replicate jars ($n = 5$) in 900 μL of 10% trace metal free nitric acid with a motorized pestle. An additional 100 μL of 30% hydrogen peroxide was added before digestion tubes were placed in a dry heat block for 24 h at 70°C. For both animal and water Ca samples, an additional 100 μL of gallium (TraceCert-ICP standard Fluka) was added to a final concentration of 0.909 mg/L. Samples were loaded onto quartz discs, and Ca concentrations were determined by comparing them to an internal gallium standard using the TXRF X-ray spectrophotometer (Marguí et al. 2010).

To ensure that growth results were not an artifact of using artificial culture media, we collected water from five Canadian Shield lakes with Ca concentrations varying from 1.36 mg L⁻¹ to 9.44 mg L⁻¹. We filtered this water through 0.7 μm GF/F and 0.2 μm polycarbonate membrane filters to remove the majority of algal and microbial particles. Soluble reactive phosphorus (SRP) content of lake water was determined using P analysis using molybdate-blue analysis as described above. We determined the P-content of our algal food cultures and mixed nominal food concentrations (C : P 100, 300, 500) assuming 100% uptake of this dissolved P by food algae and that algae were 50% C. We used 4 mg C L⁻¹ quantities of lake water-food preparations for growth experiments to ensure that C was not limiting. We conducted 6 d growth experiments and examined *Daphnia* body %P, %Ca, and MSGR as described above.

We also investigated whether the food quality effects on body Ca were specific to P-limitation or to general decreases in growth. To do this, we grew additional animals in groups of 20 as described above under nitrogen limited (C : N 25 by mol, 4 mg C L⁻¹) and low food (0.15 mg C L⁻¹) quantities of *S. obliquus* for 6 d in 400 mL N and P-free media with 10 mg Ca L⁻¹. After 6 d of growth, we saved animals for C, N, P, and Ca body composition and MSGR analysis as described above.

Life-table experiment

Neonates < 24 h old for population measurements were rinsed with N-, P-, and Ca-free COMBO media and individually grown in tubes containing 20 mL (age 0–6 d) or 40 mL (age 7–30 d) of N- and P-free media containing a prescribed Ca concentration (0.1 mg L⁻¹, 0.5 mg L⁻¹, 1 mg L⁻¹, 2 mg L⁻¹,

5 mg L⁻¹, 10 mg L⁻¹). *Daphnia* were fed 4 mg C L⁻¹ of algal food every other day for the first 6 d and then increased to 8 mg C L⁻¹ quantities of their assigned diet (C : P 100, 300, 500, 700) for the remainder of the experiment to avoid C-limitation. The diets were crossed with Ca treatments in a factorial design that yielded a total of 24 treatment combinations with each initially containing 25 replicate individuals. We measured size at first reproduction (Sfr) by measuring the distance from the top of the eye to the base of the tail of individuals using a dissecting microscope ($n = 10$). Daily reproductive output was measured from the first day of reproduction to the conclusion of the experiment and combined with survival data collected daily from the start of the experiment to calculate population level parameters. Life-history traits for Ca 0.1 treatments as these organisms did not survive to reproduce.

Statistical analysis

All data were checked for normality using Shapiro–Wilk tests in Past version 3.05 (<http://folk.uio.no/ohammer/past/>), and all dependent variables violated normality assumptions as a result of the experimental design. Therefore, we first analyzed the data using a nonparametric equivalent of a two-way analysis of variance (ANOVA) to determine whether treatment effects significantly altered daphnid growth and elemental content (Scheirer et al. 1976; Sokal and Rohlf 1995; Dytham 2011). This test is formally referred to as a Kruskal–Wallis test with a Scheirer-Ray-Hare extension, where data are rank transformed prior to conducting a two-way ANOVA (Iman and Conover 1979; Akritas 1990; Ashforth and Yan 2008). Sum of squares (SS) data from ANOVA's were then used to calculate Kruskal–Wallace H statistics by dividing the SS for each variable by the total mean square error for the model [(modelSS + errorSS)/df], and p -values were then calculated using a chi-squared table adjusted for df of individual H -values. We then determined the relative strength of each variable on daphnid nutrient content and growth by estimating variance components using random effect general linear models fitted using restricted maximum likelihood estimates (REML) (Searle et al. 2015).

To investigate connections between consumer MSGR and body stoichiometry, a spearman correlation was first used to establish the interactions between daphnid body Ca and P. Then, we used an ordinary least squares regression to examine relationships between *Daphnia* body Ca : P ratios and MSGR. Visual inspection of *Daphnia* Ca : P/MSGR residual plots revealed a nonrandom distribution of residuals; therefore, we plotted these values over the Ca concentration gradient to further examine the effects of low Ca levels on daphnid body stoichiometry. We also investigated changes in consumers under additional forms of elemental limitation (C and N) and on animals grown in lake water to see whether changes due to diet were P specific and to ensure that Ca effects were not due to our use of artificial growth media.

Table 1. Effects of food quality and Ca concentration on *Daphnia* elemental body composition and growth. Proportion of variance explained by the individual effects of food quality (diet C : P), Ca concentration (media Ca), and their interaction (C : P \times Ca) on *Daphnia* body elemental content (%P, %Ca, and Ca : P ratios) and mass specific growth rates (MSGR). Error, adjusted R^2 , and p -values are reported for general linear models.

	%P	%Ca	Ca : P	MSGR
Diet C : P	80.68	75.93	84.30	58.68
Media Ca	0.78	4.96	4.90	23.05
C : P \times Ca	7.77	9.34	4.45	15.69
Error	10.75	9.78	6.35	2.60
Adj. R^2	0.89	0.90	0.94	0.97
p -value	<0.0001	<0.0001	<0.0001	<0.0001

Daily reproduction and lx data were used to quantify several variables key to controlling population growth. We estimated G_t , R_0 , and r using the Poptools add-in v 3.2 for Microsoft Excel, and Sfr was measured on a subset of daphnids. Standard errors around each parameter were calculated using Jackknife techniques. Significance of individual and interactive effects of Ca and P on population parameters was determined using nonparametric ANOVA analyses, and we further examined the relative strength of treatment effects using variance components analyses as described above. Daily survival data was used to calculate lx, and logistic regression was used to compare log odds survival ratios within a food C : P treatment across the Ca gradient. Cox Regression analysis (Cox 1972) was used to model hazard functions to examine the combined influence of food C : P and media Ca on survival probabilities and to determine how the likelihood of death changes across a treatment (Ashforth and Yan 2008). All statistics unless otherwise stated were performed in SAS (Cary, North Carolina).

Results

Influence of nutrition and growth on *Daphnia* body Ca and P

Over 75% of the variation in daphnid elemental content was explained by dietary P (Table 1), which not only significantly affected organismal P content but also exerted strong control over *Daphnia* body Ca and Ca : P ratios. Media Ca did not significantly affect daphnid elemental content ($p > 0.05$), and no interactions between treatments were found. However, daphnid Ca content was negatively correlated with body P across all treatments (Fig. 1A), and increased MSGR in high P treatments was associated with lower daphnid Ca : P ratios (Fig. 1B). This effect appeared to be independent of specific elemental limitation, as daphnid body Ca : P ratios increased with growth limitation from low food quantity, elevated C : N and C : P ratios, and under low Ca concentrations (Fig. 1C). However, Ca-limitation had a distinct effect on body Ca : P ratios compared with the other

forms of limitation as low Ca treatment values fell below the regression line. Nonrandom distribution of residuals from body Ca : P-growth regressions (Fig. 1D) was largely explained by Ca concentrations $< 2.0 \text{ mg L}^{-1}$.

Effects of Ca- and P-limitation on *Daphnia* growth

Dietary P and media Ca combined to strongly affect organismal MSGR (Table 1). A majority of the difference in daphnid growth explained by the model was linked to dietary P (58%), and maximum growth rates generally decreased at high food C : P ratios (Fig. 2A,B). No significant interactions between treatments were found; however, Ca showed significant individual effects on growth, which differed qualitatively depending on food treatment. For C : P 100 and 700 diets, increased Ca concentrations led to higher but saturating growth rates; however, at C : P 300 and 500, we found increased growth rates up to 2 mg L^{-1} Ca but slower growth at higher Ca concentrations (Fig. 2A). We found similar individual effects between Ca and P in animals grown in natural lake water that varied in Ca concentrations (Fig. 2B). For this experiment, low natural Ca concentrations also affected daphnid body Ca : P/MSGR relationships as animals grown in lake water below 2 mg L^{-1} fell below the regression line for animals grown under Ca replete conditions (Fig. 3).

Effects of Ca and P on *Daphnia* population growth and life-history

Differences in life-history traits explained by variance components were much greater for R_0 and r than for G_t and Sfr, although all traits were significantly affected by both treatments (Table 2). As with individual responses, dietary P explained a majority of variation in all population level traits; however, media Ca also had significant effects on all traits. Maximum life-history trait responses occurred in higher P treatments where R_0 and r increased and Sfr and G_t decreased (Fig. 4) under low C : P diets. Media Ca concentrations had interactive effects with dietary P on all traits except for r ($p = 0.1$), leading to the greatest increases in life-history traits in high nutrient combinations.

Effects of Ca and P on *Daphnia* survival

Unlike individual growth and population responses, Ca limitation was found to be the major driver in survivorship (Fig. 5), with population extinction occurring at extremely low Ca levels (0.1 mg L^{-1}). Logistic regressions indicated increased mortality ($> 1000\times$) with declines in Ca concentrations from 10 mg L^{-1} to 0.1 mg L^{-1} . In comparison, individual effects of P-limitation were much lower with decreases in survivorship by $< 10\times$ across the entire food C : P ratio range. Hazard functions from Cox regressions incorporating main effects of both elements were significant ($p < 0.05$) and matched the findings of logistic regressions. The hazard function of reduced dietary P was very low (1.001; where a value of 1.0 represents no change) meaning that the chance of dying increased only 0.1% between each treatment and only

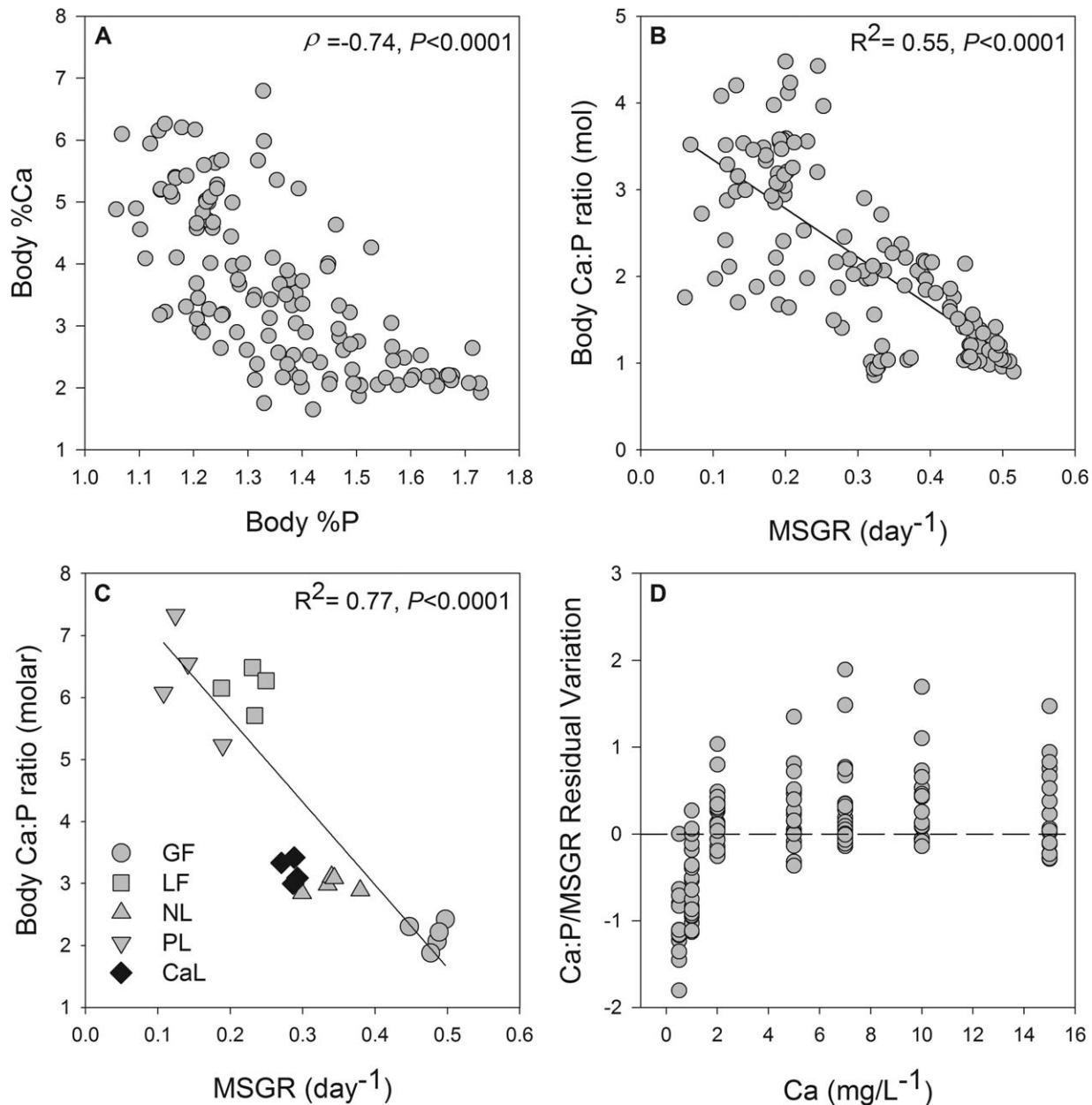


Fig. 1. Relationships between *Daphnia* (A) body %Ca and %P, (B) body Ca : P ratios and MSGR, (C) body Ca : P and MSGR of animals consuming diets with different limiting nutrients (GF nutrient replete, LF low food quantity, NL low nitrogen, PL low P, and CaL low Ca), and (D) residual variation from Ca : P/MSGR regressions in panel (B). The spearman correlation coefficient (ρ) and p -value are reported for panel (A). Adjusted R^2 and p -values for panels (B and C) are given for ordinary least squared regressions.

increased a total of 0.4% from C : P 100 to C : P 700. In comparison, the hazard function for media Ca was very large (0.862) meaning that the odds of survival decreased by 14% when moving down a level in concentration and decreased by a total of 84% going from Ca 10.0 mg L^{-1} to 0.1 mg L^{-1} .

Discussion

We found significant combined effects of dietary P- and environmental Ca-limitation on *D. pulex*. While interactions

between limiting nutrients were found, dietary P-limitation had the overall strongest effects on consumer body Ca : P stoichiometry and life-history. Our results suggest that body Ca content is not a reliable indicator of Ca demand, as body composition is highly variable in response to multiple forms of elemental limitation and to differences in consumer growth. As large differences in supplies of both nutrients exist among aquatic ecosystems in boreal regions around the world, it is probable that P- and Ca-limitation exert a

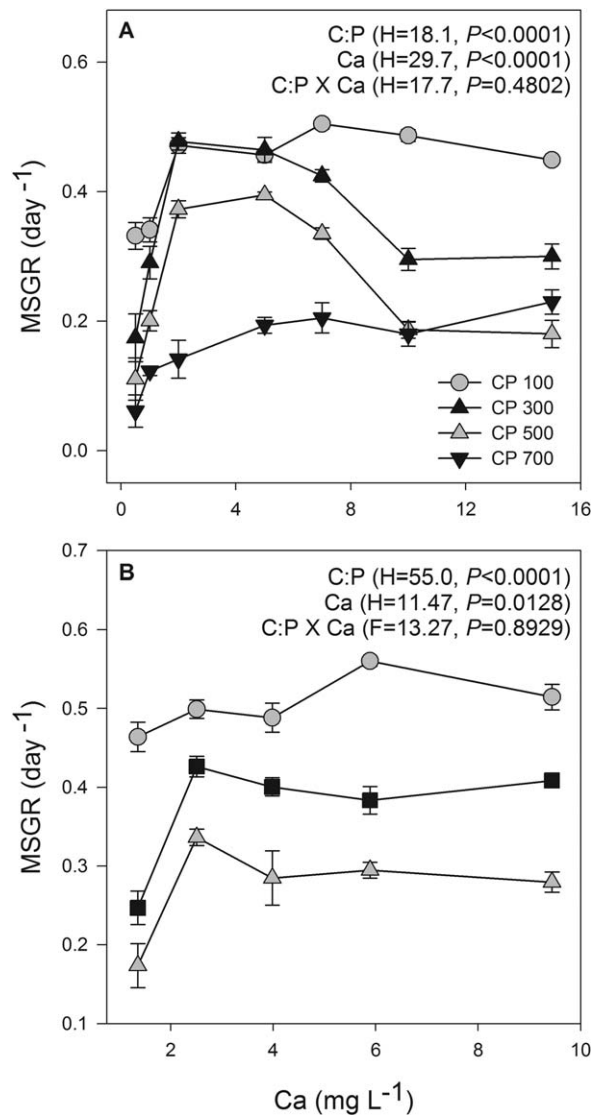


Fig. 2. Effects of food P content on *Daphnia* MSGR across a Ca gradient in (A) artificial media and (B) natural lake water. Mean and standard error are displayed for each data point, and *H* and *p*-values for fixed and interactive effects of dietary C : P and media Ca are reported from two-way nonparametric ANOVA's (Kruskal–Wallis tests with a Scheirer-Ray-Hare extension).

combined effect on consumer nutrient content and life-history in nature.

Elemental limitation resulted in large changes in consumer body stoichiometry and growth rates in animals grown across nutrient gradients. Our results confirm previous daphnid studies showing changes in body Ca and P content in animals grown under low nutrient supplies (He and Wang 2009; Tan and Wang 2009). However, we found that dietary P was the most significant variable controlling daphnid elemental content, explaining the majority of variation in consumer body Ca : P ratios. Additionally, *Daphnia* elemental content was strongly related to organismal MSGR,

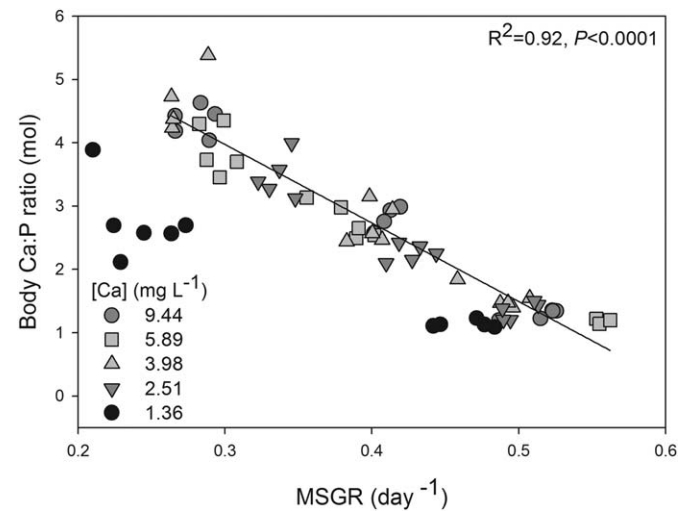


Fig. 3. Relationship between *Daphnia* body Ca : P ratios and MSGR in animals fed different food C : P diets and grown in water from different lakes across a Ca gradient. Black symbols (●) indicate animals grown in Ca concentrations < 2 mg L⁻¹. Adjusted *R*² and *p*-values are shown for ordinary least squared regression of animals grown in Ca concentrations > 2 mg L⁻¹.

Table 2. Effects of food quality and Ca concentration on *Daphnia* life-history and population growth. Proportion of variance explained by the effects of food quality (diet C : P), Ca concentration (media Ca), and their interaction (C : P × Ca) on *Daphnia* life-history and population growth parameters: generation time (*G*_t), reproductive output (*R*₀), size at first reproduction (*S*_{fr}), and intrinsic rate of increase (*r*). Error, adjusted *R*², and *p*-values are reported for general linear models.

	<i>G</i> _t	<i>R</i> ₀	<i>S</i> _{fr}	<i>r</i>
Diet C : P	32.53	73.91	41.86	73.78
Media Ca	12.48	8.05	5.28	10.72
C : P × Ca	7.08	7.47	14.65	6.03
Error	47.91	10.57	38.21	9.47
Adj. <i>R</i> ²	0.52	0.89	0.62	0.91
<i>p</i> -value	<0.0001	<0.0001	<0.0001	<0.0001

where changes in growth were tightly linked to consumer body Ca : P ratios suggesting strong physiological connections between daphnid nutrient content and life-history. Consumers growing quickly require a large amount of P for ribosome synthesis, so their overall P content increases with fast growth and decreases under nutrient limitation (Elser et al. 2003; Hood and Sterner 2010). Body Ca is mostly found in the carapace where it shows an allometric relationship with growth and body size, with smaller individuals having higher specific Ca content (Hessen and Rukke 2000). Overall, decreased growth rate resulted in higher consumer body Ca : P ratios as the relative proportions of consumer P decreased and Ca increased under P-limitation. However, this relationship between growth and nutrient content was

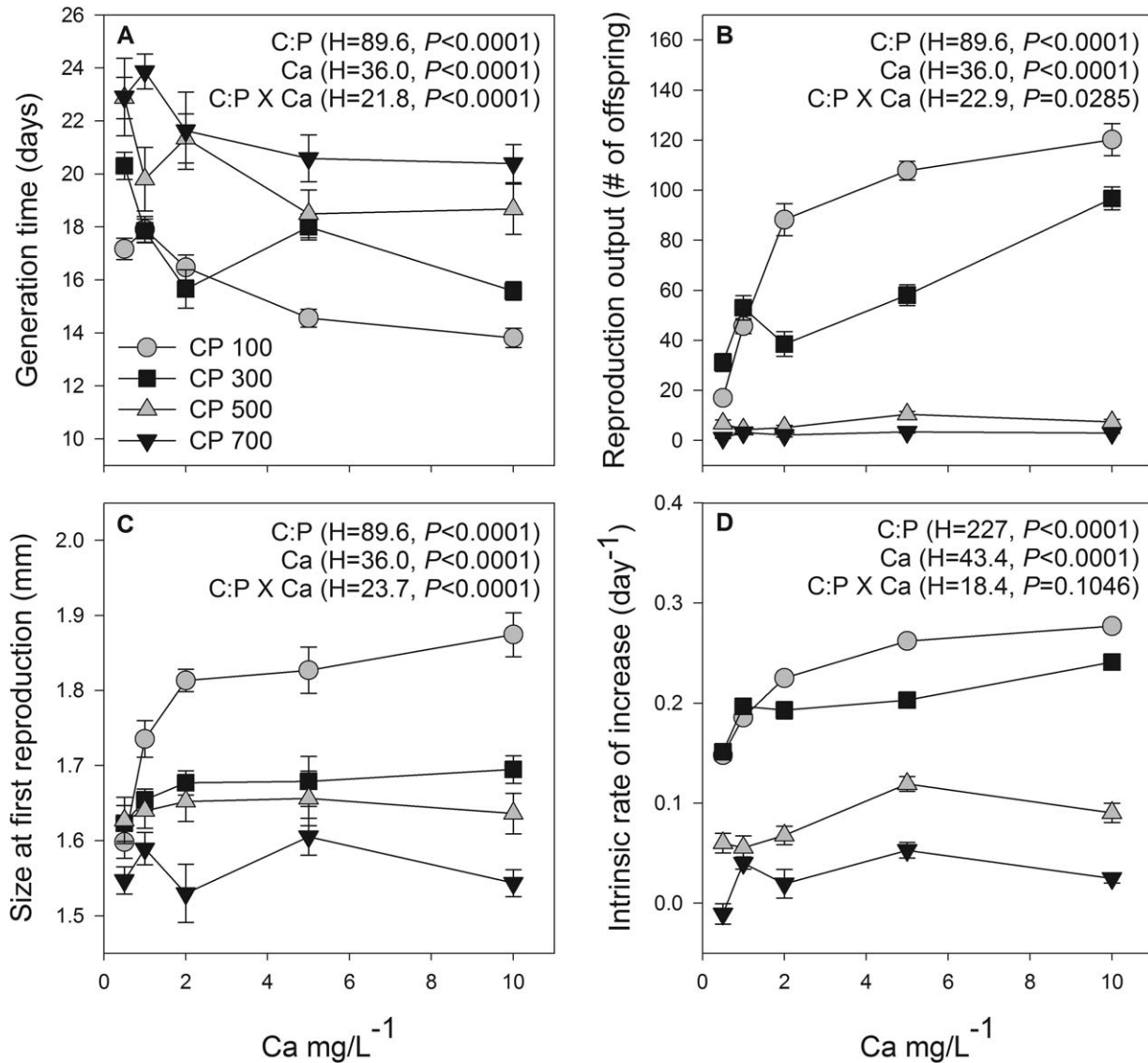


Fig. 4. Effects of food C : P on *Daphnia* life-history traits across a Ca gradient. (A) generation time, (B) reproductive output, (C) size at first reproduction, and (D) intrinsic rate of population increase. Mean and standard error are displayed for each data point, and H and p -values for fixed and interactive effects of dietary C : P and media Ca are reported from two-way nonparametric ANOVA's (Kruskal–Wallis tests with a Scheirer–Ray–Hare extension).

not specific to P-limited growth as it was also found under other forms of nutrient limitation (e.g., food quantity and N content).

Altered daphnid body Ca : P ratios resulted from decreased growth due to dietary C-, N-, and P-limitation and low media Ca concentrations. However, extremely low environmental Ca concentrations had a distinct effect on consumer Ca content as Ca-deprived consumers fell below the regression line of body Ca : P and MSGR across all food treatments. Residual variation of consumer body Ca : P/MSGR relationships plotted across the Ca gradient show that consumer body Ca : P ratios were lower than predicted under low Ca concentrations indicating that severe Ca-limitation strongly alters animal Ca content. This effect was also seen in animals grown in natural

lake water where animals grown in Ca concentrations $< 2 \text{ mg L}^{-1}$ had much lower body Ca : P ratios compared with other animals. Therefore, daphnid body Ca : P was determined not only by growth dilution in nutrient replete conditions but also by physiological responses under Ca-stress, which complicates predictions of species-specific Ca demands that are only based on animal body elemental composition (Wagner et al. 2013). As body Ca appears to be a poor predictor of metabolic requirements for this element, development of physiologically based models incorporating Ca uptake and assimilation efficiencies coupled with growth requirements for Ca could prove highly valuable in future efforts to estimate threshold Ca concentrations for growth and survival. Such threshold Ca values, similar to those used to determine

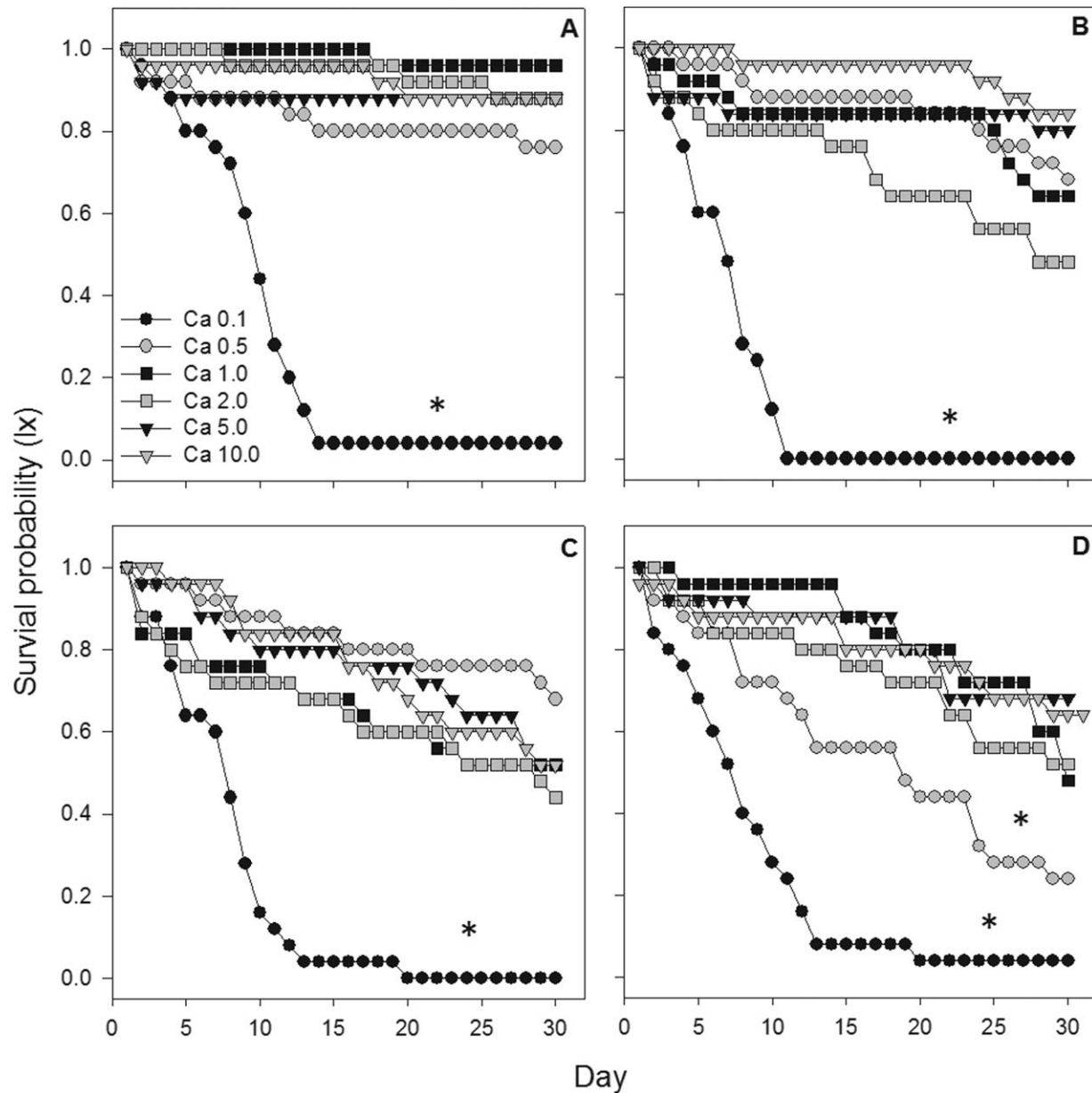


Fig. 5. Effects of Ca concentrations on survivorship (lx) of consumers fed different diets (A) C : P 100, (B) C : P 300, (C) C : P 500, (D) C : P 700. Significant differences in survivorship ($*p < 0.05$) determined by logistic regression of each Ca treatment compared with Ca 10 treatments are indicated for each diet treatment.

threshold elemental ratios for P-limitation (e.g., Frost et al. 2006), could subsequently be used to more fully understand the metabolic demands for Ca in species found across the region. These models, if built to include dietary P requirements, would be useful in better understanding population level effects of co-occurring Ca- and P-limitation in eastern Canadian Shield lakes.

Dietary P content explained the majority of variation in all but one life-history trait in our study, supporting several previous studies showing the importance of elemental food quality for population growth (Urabe and Sterner 2001;

Makino et al. 2002; Andersen et al. 2007). Populations consuming high quality food were found to have the fastest r due to earlier maturity and increased production of offspring. As it is strongly linked to overall fitness, r is important for measuring population performance (Stearns 1992; Roff 2002) and can be used to predict competitive outcomes within and among species under nutrient limited conditions. Although we found significant interactive effects of Ca and P supply, the overall effects of Ca explained far less variation in daphnid life-history traits than individual effects of dietary P. Most variables had weak relationships with

environmental Ca at food C : P ratios above 300, which are commonly found in Canadian Shield lakes (Hecky et al. 1993; Elser and Hassett 1994) and across the globe (Sterner et al. 2008). Although it is possible that our lab grown food may not be directly comparable to food quality present in lakes as our food treatments were solely composed of readily digestible algae, the overwhelming individual and population level effects of P-limitation compared with low Ca indicate that diet quality should be considered as an important factor regulating zooplankton growth and reproduction. While food P content was the most important element controlling population growth traits, it had a relatively weaker effect on daphnid survival indicating that minimal Ca supplies are essential to sustain daphnid populations.

Environmental Ca was found to have the largest influence on the survival probability of *Daphnia* in our experiment, although this effect may have been amplified due to our experimental design and data analysis. Similar to previous studies, we found that the Ca threshold for survival fell between 0.1 mg L⁻¹ and 0.5 mg L⁻¹ (Hessen et al. 2000; Ashforth and Yan 2008). However, these treatment levels were the only ones with significantly worse survival odds than the highest Ca treatment (10 mg Ca L⁻¹). Therefore, the Cox model used to investigate the effects of combined limitation across the whole Ca gradient likely overestimated this treatment effect. As lakes with Ca concentrations around 0.5 mg L⁻¹ seem uncommon in eastern Canada (Jezierski et al. 2008), including these concentrations in our experiments may have led to an over-estimation of the importance of environmental Ca for daphnid survival. Alternately, effects of low food P content may have been under-estimated by not including more severe forms of P-limitation (food C : P ratios > 700), which can occur seasonally in Canadian lakes (Hecky et al. 1993; Elser et al. 1998) having a large influence survival (Sundbom and Vrede 1997; Urabe and Sterner 2001). Despite experimental limitations, our study provides reasonable evidence that Ca- and P-limitation combine to alter population growth and survival in lakes of this region. While we acknowledge that caution should be used when extrapolating the results from controlled laboratory experiments to large regional scales, our results suggest that Ca declines may not be the only nutritional factor affecting zooplankton assemblages in eastern Canadian lakes.

Zooplankton populations vary in abundance and in body elemental composition within and among lakes (Jezierski and Yan 2006; Jezierski et al. 2012), and natural selection on organismal Ca content has been proposed as an important cause of species sorting in regional assemblages. Shifts from high Ca content daphnids to low Ca content cladocerans such as *Bosmina* spp. and *Holopedium glacialis* have been attributed to declines in lake Ca concentrations over the last century (Jezierski et al. 2008). While this explanation infers a tight link between consumer body Ca content, demand, and environmental supply, our study shows that even within

a species these relationships are affected by other forms of nutrient limitation. Therefore, environmental factors other than Ca-limitation may be responsible for intraspecific differences in abundances of high Ca organisms such as *D. pulex*, whose populations do not always respond to low Ca supplies as predicted by the Ca limitation hypothesis (Jezierski et al. 2012). We suggest that some of this unexplained variation in zooplankton abundance and body Ca may be caused by dietary elemental composition, which changes dramatically across growing seasons in lakes (Kreeger et al. 1997).

Like body Ca, P content of daphnids and other taxa vary significantly (Andersen and Hessen 1991), and differences in P demand may also play a role in zooplankton competition in lake communities (Elser et al. 1988). In general, *Daphnia* are much more P-rich than other cladocerans such as *Bosmina* spp. and *Holopedium* spp., and differences in dietary P content have been found to affect the outcome of intra- and inter-specific competition in zooplankton taxa (Elser et al. 1988; Weider et al. 2005). Given the especially P-poor nature of lakes from this region of Canada (Hecky et al. 1993; Elser and Hassett 1994), it is likely that zooplankton frequently encounter dietary P-limitation, due to high seston C : P ratios. Consequently, the singular importance of low environmental Ca concentration as the driver of zooplankton community composition may need to be reconsidered. In particular, more controlled work investigating the ecophysiological effects of Ca- and P-limitation on populations of other zooplankton taxa (e.g., *Holopedium*) found in high numbers in soft water lakes around the world is needed to establish critical levels for lake Ca concentrations and dietary P content to better inform potential management strategies in the future. Our results demonstrate that declining supplies of both P and Ca over the recent past (Yan et al. 2008; Eimers et al. 2009) have likely altered zooplankton community composition in these lakes.

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