

Intra-specific Variation in Sensitivity to Low Calcium in *Daphnia pulex*

By

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Abstract

Freshwater systems experience many stressors that can affect aquatic communities; one such stressor is calcium decline. Cation depletion of surrounding soils and watersheds of many North American and Scandinavian lakes is in part due to the legacy effect of logging and acid deposition. An important freshwater grazer, *Daphnia*, is especially sensitive to calcium decline as it has a heavily calcified chitin carapace that is frequently moulted, resulting in the need to uptake large amounts of calcium from the surrounding water. Studies have documented variation among populations of *Daphnia* spp. in response to predators, food concentrations, and cyanobacteria, however only one study has assessed intra-specific variation in *Daphnia* spp. to calcium decline. We predicted that calcium sensitivity of individual *Daphnia* iso-female lines would depend on the lake calcium concentration of their lake of origin, if the historical exposure to low calcium allowed for a fitness advantage (higher reproduction and survival). Alternatively, variation in calcium tolerance among iso-female lines may exist independent of historical exposure. We tested the calcium sensitivity of 10 *Daphnia pulex* sensu lato iso-female lines in a life-history experiment under low food and soft water conditions that are associated with the Muskoka, Canada region. *Daphnia* were exposed to one of five calcium concentrations (0.7, 1.0, 1.5, 2.5, 7mg Ca L⁻¹) for a 21-day period and monitored daily for survival and reproduction and one size measurement was taken at day six. We found that the calcium sensitivity of *Daphnia* varied among iso-female lines for three responses; total reproduction, size at day six, and population growth rate, but this variation was not explained by source-lake calcium. Overall, our results highlight the existence of intra-specific variation in *Daphnia* sensitivity to calcium decline, and the

need for future experiments to include multiple genotypes to fully understand the impacts this stressor has on *Daphnia* communities.

Co-Authorship

Chapter 2 was co-authored with Dr. Shelley E Arnott.

Author contributions: Dr. Shelley E Arnott and Melane S Overhill conceived and designed the experiment. Melanie S Overhill and Rebecca Schmidt executed the experiment and collected the data. Melanie S Overhill performed data analysis and wrote the paper, with input from Dr. Shelley E Arnott.

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List of Abbreviations

AIC – Akaike information criterion

ANOD – Analysis of deviance

ANOVA – Analysis of variance

BBM – Bold Basal Medium

Ca – Calcium

CaSO₄ – Calcium sulfate

Chisq – Chi-squared

DESC – Dorset Environmental Science Centre

DOC – Dissolved organic carbon

DW – Dry weight

FLAMES – Field Laboratory for the Assessment of Multiple Ecological Stressors

GLMs – Generalized linear models

H⁺ – Hydrogen ions

Ldh – Lactase dehydrogenase

MAM – Minimum adequate model

NO_x – Nitrate

P – Phosphorus

QUBS – Queen's University Biological Station

SE – Standard error

SD – Standard deviation

SO₄ – Sulfate

Chapter 1

General Introduction

Freshwater systems face many challenges that can alter community structure and biodiversity (Dudgeon et al. 2006). These challenges do not impose only negative effects on individuals/communities, they can also facilitate organisms' performances. As an example, nutrient additions into freshwater lakes can cause an increase in primary productivity, however if nutrient additions are too high it can cause algae blooms, which can cause low oxygen and reduce biodiversity (Dokulil and Teubner 2011). Globally, aquatic communities are faced with many stressors. For example, urbanization causes aquatic habitat loss and increased extinct rates of local aquatic plant life (Kozłowski and Bondallaz 2013). Increased global temperature associated with climate change is expected to have direct and indirect effects on aquatic biodiversity, species range limits, and aquatic food webs (as reviewed by Wrona et al. 2006). As an example, in a mesocosm study performed by Beisner et al. (1997), increased temperature caused destabilizing effects on the predator-prey dynamic irrespective of community type, causing the herbivore, *Daphnia pulex*, to become extirpated. In Canada, specifically on the Precambrian Shield, aquatic communities also face local and regional stressors such as acidification, invasive species (Palmer and Yan 2013), lake-shore development, and nutrient loading (Palmer et al. 2011).

Calcium Decline

An emerging stressor across the Northern Hemisphere is the regional decline of lake-water calcium (Stoddard et al. 1999, Keller et al. 2001, Hessen et al. 2016). For example, in lakes in southern Norway, calcium concentrations declined, on average, 11%

per decade or $0.023 \text{ mg Ca L}^{-1} \text{ year}^{-1}$ from 1983-2013 (Hessen et al. 2016). A similar trend was found in south-central Ontario lakes with calcium declining, on average, 13% from 1985-2005 (Jeziorski et al. 2008). Lake-water calcium is primarily determined by the size of the exchangeable cation pool in the soil beds surrounding lakes (Houle et al. 2006); therefore, the depletion of the calcium into lakes is attributed to reduced cation pools in surrounding soil causing reduced leaching of cations into the lakes (Aherne et al. 2003).

The reduction of cation pools in soils has been attributed to three main factors: reduced atmospheric deposition of calcium and reduced mineral weathering (Likens et al. 1996, Stoddard et al. 1999), timber harvesting (Federer et al. 1989, Watmough and Dillon 2003, Watmough et al. 2003, Watmough and Aherne 2008), and long term effects from acid deposition (Likens et al. 1996, Stoddard et al. 1999, Aherne et al. 2003). Cation pools in soils are balanced by inputs and outputs, with atmospheric deposition and mineral weathering being inputs. Atmospheric deposition of cations has decreased over the last few decades, in part due to reduced SO_4 availability to create salts (e.g. calcium sulphate CaSO_4) which would deposit out of the atmosphere (Hedin et al. 1994). This results in a lower amount of calcium being added to the exchangeable cation pool in the soil (Likens et al. 1996, Stoddard et al. 1999). Historical increases in leaching (due to acid deposition) and increases in timber harvesting over the last few decades have caused both historical and current outputs from cation pools to exceed the inputs, reducing the total available amount of cations in the soil (Likens et al. 1996).

Timber harvesting removes cations from soil catchments when trees are removed, as large amounts of calcium are stored in the above ground biomass of trees (Watmough

and Dillon 2003). This removal of vegetation also disrupts natural ion exchange in this system, as in undisturbed systems decomposed organic matter would return ions to the soils after tree death (Hyman et al. 1998). As well as direct removal from the system, the planting of new forests increases uptake of calcium from the soil as younger trees are better able to mobilize calcium (Hamburg et al. 2003).

Leaching associated with acid deposition is the largest contributor to the removal of cations from soil catchments (Likens et al. 1996, Stoddard et al 1999, Aherne et al. 2003). Acid deposition accelerated the loss of calcium from the soil by providing hydrogen ions (H^+), which react and displace cations that are otherwise absorbed to the soil, and through the addition of sulfate (SO_4) and nitrate (NO_x) ions, cations remain dissolved in soil water and are easily washed away (i.e., leached into lakes) (Lawrence and Huntington 1999). The increased deposition of SO_4 and NO_x post-industrialization to the late 1980s caused increased leaching of base cations into streams and lakes reducing the total base cation pool in the surrounding soil (Likens et al. 1996). Even with regulations for reduced emissions that were put in place in the late 1980s (Stoddard et al. 1999), acid deposition had already depleted soil base cations and reduced the buffering capacity of many soils (Aherne et al. 2003). Although calcium leaching into lakes was hypothesized to increase after the initial surge from acid deposition, the cation pool in the soil was depleted (Aherne et al. 2003). As the size of the exchangeable cation pool in the soil is the biggest predictor of lake-water calcium (Houle et al. 2006), the historic depletion of the soil base cation pool is caused reduced cation leaching into lakes (Aherne et al. 2003), lowering calcium concentrations of lakes over the last few decades (Hessen

et al. 1995, Keller et al. 2001, Jeziorski et al. 2008, Palmer et al. 2011, Jeziorski et al. 2012a, Hessen et al. 2016).

Community Implications of Calcium Decline

Changes in lake-water cation chemistry have occurred in association with changes in community structure (Hessen et al. 1995, Keller et al. 2001, Jeziorski et al. 2012a, Jeziorski et al. 2012b, Jeziorski et al. 2015). For example, crayfish abundances have declined following changes in lake-water cation chemistry (Edwards et al. 2009), and changes in cladoceran community structure have been observed (Jeziorski et al. 2008, Jeziorski et al. 2012 a, Jeziorski et al. 2015, Korosi et al. 2012, Redmond et al. 2016). Calcium is an important aspect of cellular function (Wheatly 1999) and for crustaceans, who regularly moult, is an essential mineral for growth and the re-hardening of their carapaces (Greenaway 1985).

Freshwater crustacean species are sensitive to changes in lake-water calcium (as reviewed by Cairns and Yan 2009). Crayfish, *Orconectes* sp., for example, have been suggested to have a lower tolerance level of 2.6mg Ca L⁻¹ (Capelli and Magnuson 1983), and their abundance in some areas has been declining over the last few decades (Edwards et al. 2009). Also a gammarid, *Gammarus lacustris*, has been found to have lower threshold limit between 1-6mg Ca L⁻¹ (Økland and Økland 1985, Rukke 2002a). In the Muskoka-Haliburton region of south-central Ontario, 68% of 287 sampled lakes were found to have calcium concentrations below 2.6mg Ca L⁻¹ and 93% were below the 6mg Ca L⁻¹ threshold (CAISN, unpublished data). Population growth rates of several zooplankton species are influenced by aqueous calcium concentration (Arnott et al. 2017, Azan and Arnott *in press*), with *Daphnia pulex* exhibiting declining population growth

rates as calcium concentration was decreased in a community mesocosm experiment (Azan and Arnott *in press*). With calcium decline affecting multiple members of the crustacean community, ambient calcium concentrations have been shown to be a determinant in species richness and community composition (Wærvågen et al. 2002, Strecker et al. 2008).

Zooplankton community composition has shifted in association with calcium decline (DeSellas et al. 2011, Shapiera et al. 2012, Jeziorski et al. 2015) and additional changes are expected as calcium continues to decline. In the Muskoka region of south-central Ontario, low calcium lakes have changed from being *Daphnia*-dominated communities pre-industrialization to *Holopedium*-dominated communities (Jeziorski et al. 2015), with many low calcium/high pH lakes absent of *Daphnia* spp. altogether (Jeziorski et al. 2012b). In contrast, declines in *Daphnia* abundances in Nova Scotia were coincident with increases in *Bosmina* abundances (Korosi et al. 2012). In Dickie Lake, in the Muskoka region, the *Daphnia* community shifted over time (Shapiera et al. 2012), with *D. catawba* suspected to be more dominant in present years, as it is thought to be insensitive to low calcium (Cairns 2010, Jeziorski et al. 2012b); however Azan and Arnott (*in press*) found that *D. catawba* growth rates were also reduced below 2.4mg Ca L⁻¹ in a community mesocosm experiment. Many studies that assess community-level impacts of calcium decline are either lake (Hessen et al. 1995, Cairns 2010) and/or paleolimnological surveys (DeSellas et al. 2008, DeSellas et al. 2011, Jeziorski et al. 2012b, Shapiera et al. 2012, Jeziorski et al. 2015), which can only detect correlations between calcium decline and communities changes; however, there are many other environmental gradients in lake systems which could be the driver of community changes

observed in association with calcium decline. In one of the few community mesocosm experiments performed assessing the growth rates of many zooplankton taxa, calcium decline was found to decrease the population growth rates of several species (Arnott et al. 2017, Azan and Arnott *in press*). Interestingly, *Holopedium*, which was suggested to be replacing *Daphnia* through time, based on long-term lake data (Jeziorski et al. 2015), had decreasing population growth rates as calcium concentrations declined in an experimental mesocosm (Azan et al. 2017). However, when the presence of a predator of *Daphnia* was added, *Bythotrephes longimanus*, *Holopedium* abundances increased in low calcium (Azan and Arnott 2017), possibly due to reduced competition at low calcium. However, these results should be interpreted with caution because overall, *Holopedium* exhibited negative growth rates at all calcium concentrations in this study. Disentangling the effects this particular stressor has on individual zooplankton species in realistic community conditions, but also alone, is important to understand the impacts this stressor will have in the future.

***Daphnia* and Calcium**

Like many other crustaceans, *Daphnia* rely heavily on ambient aqueous calcium from the surrounding environment to fulfill their calcification demands (Porcella et al. 1969, Greenaway 1985, Cowgill et al. 1986). They are an ideal organism to study the potential effects of calcium decline as they are expected to be sensitive to changes in lake-water calcium because of their high calcium demand. *Daphnia* are small filter feeders which reproduce parthenogenetically, meaning females produce offspring without fertilization by a male; offspring, called neonates, are therefore genetically identical to their mother (Green 1956, Allan 1976, Barker and Herbert 1985). Their outer carapace is

composed of a heavily calcified chitin (Porcella et al. 1969, Greenaway 1985, Cowgill et al. 1986) which is frequently moulted (Porcella et al. 1969, Cowgill et al. 1986).

Moulting can occur as frequently as every 2-3 days at maturity with between 1-14 neonates being released with each moult (varies depending on species and environmental conditions; Green 1956). With minimal calcium being stored with each moult (<10%, Alstad et al. 1999), daphniids obtain the required calcium for carapace regrowth from the surrounding water (Porcella et al. 1969, Greenaway 1985, Cowgill et al. 1986). *Daphnia* also have a high percent calcium per dry weight (2.5%-7.75% Ca/DW) when compared to other crustacean taxa (Waervagen et al. 2002, Jeziorski and Yan 2006), making them the focus of many laboratory studies and lake surveys related to calcium decline (Hessen and Rukke 2000, Rukke 2002b, DeSellas et al. 2008, Jeziorski et al. 2008, DeSellas et al. 2011, Korosi et al. 2012, Shapiera et al. 2012, Redmond et al. 2016).

Laboratory studies have been a common method for assessing the effects that calcium has on *Daphnia* in a highly controlled setting. Survival thresholds have been suggested between 0 and 2mg Ca L⁻¹ for *D. galeata* (Rukke 2002b) and between 0.1 and 0.5mg Ca L⁻¹ for *D. magna* (Hessen et al. 2000) and *D. pulex* (Ashforth and Yan 2008); with reproduction requiring higher concentrations (>1.5mg Ca L⁻¹ for *D. pulex*; Ashforth and Yan 2008). Juveniles may also require more calcium as their calcium content is 10-20% higher than that of adults and they have higher influx rates of calcium than adults (Tan and Wang 2009). The calcium content of daphniids was also suggested to vary based on the calcium environment that daphniids were grown in (Tan and Wang 2009), however this trend was not observed in the field surveys (Cairns 2010). The formation of neck spines, which are predator defenses to increase body size to evade a gape limited

predator *Chaoborus*, have also been shown to be hindered in *D. pulex* under low calcium conditions (Riessen et al. 2012) also possibly reducing survival.

Field surveys have shown the presence of *Daphnia* spp. has been linked to the calcium concentration of the water (Cairns 2010). Field thresholds (lake calcium concentrations that populations were found) for survival are suggested to be much higher (1.26-1.65mg Ca L⁻¹; Cairns 2010) than previously suggested laboratory thresholds (Hessen et al. 2000, Rukke 2002b) as other natural stressors could be influencing their calcium tolerance. Cairns (2010) proposed that body size and calcium content may play a role in calcium tolerance. However, although calcium content is normally higher in larger species (Jeziorski and Yan 2006), and therefore we would expect higher calcium demands from these species, she found that the relatively small *D. retrocurva* had a higher calcium demand than other larger bodied daphniids (Cairns 2010), and that *D. catawba* and *D. mendotae*, which have similar calcium content (Jeziorski and Yan 2006), showed different sensitivities to low calcium conditions in the field (Cairns 2010). Tan and Wang (2010) found similar results, as body calcium content was not a good predictor of calcium sensitivity in four cladocerans when tested across a five calcium concentration gradient. This suggests that body calcium content is not the driving factor in determining if species are sensitive to calcium decline, and that calcium thresholds in a field settings may be influenced by more than just their calcium tolerance alone.

Calcium decline in combination with other stressors has increased negative effects on the survival and reproduction of *Daphnia* (Hessen et al. 2000, Rukke 2002b, Hopper et al. 2008, He and Wang 2009, Jesus et al. 2014, Giardini et al. 2015). When in low calcium conditions and exposed to increases in UV light (Hessen and Rukke 2000), low-

food (Ashforth and Yan 2008, Perez-Fuentetaja and Goodberry 2016), and increased temperature (Ashforth and Yan 2008), survival and reproduction are reduced compared to only low calcium conditions. As an example, it has been hypothesized that increases in ingestion rates could explain restored calcium balance found in experimental individuals (Muysen et al. 2008). Muysen et al. (2008) suggested this could allow daphniids to compensate for low calcium conditions with a higher quantity of food, or allow daphniids to obtain small amounts of calcium from dietary sources; however, support for this hypothesis is weak as this was not explicitly assessed in their experiment and other studies have shown *Daphnia* obtain the majority of their calcium requirements from the surrounding water (Porcella et al. 1969, Greenaway 1985, Cowgill et al. 1986). However, if adequate food concentrations give an advantage in low calcium concentrations, when in low food and low calcium conditions, this type of safety mechanism to protect against low calcium cannot be performed and daphniids could display reductions in reproduction (Ashforth and Yan 2008, Perez-Fuentetaja and Goodberry 2016). With many of these stressors affecting *Daphnia* in wild conditions the abundance of these organisms could be greatly affected. To fully understand the impacts that this stressor has we must first test calcium sensitivity in controlled but realistic settings.

Many laboratory studies share a few commonly practiced methodologies that may provide an incomplete picture of daphniid tolerance to calcium decline. Firstly, many studies do not address maternal effects which have been suggested to alter daphniids individual performance when mothers are not exposed to similar conditions as experimental individuals (Sakwinska 2004, Giardini et al. 2015, Mikulski and Pijanowska 2017). Performing a multi-generational study would allow experimental individuals to be

without any residual effects from their mothers. Secondly, many laboratory studies looking at calcium sensitivity use hard water medias, such as Elendt M7 (Elendt and Bias 1990) or COMBO (Kilham et al. 1998), and high food conditions (Rukke 2002*b*, Muysen et al. 2008), which may be unrealistic for daphniids that inhabit oligotrophic, soft-water lakes (e.g., Hessen et al 2000, Rukke 2002*b*, Jesus et al 2014, Prater et al. 2016). For example, experiments range in food concentrations from 0.9mgC L⁻¹ to 4mgC L⁻¹ (Rukke 2002*b*, Tan and Wang 2009, Tan and Wang 2010, Prater et al. 2016) and a conductivity between 221-450µS cm⁻¹ (Elendt M7, Rukke 2002*b*; COMBO, Martha Celis-Salagado unpublished data) while the average summer conditions in the Muskoka region are much lower at 0.44mgC L⁻¹ (Brown and Yan 2015) and conductivity ~38.7µS cm⁻¹ (CAISN unpublished data). As we know that calcium tolerance can be affected by other stressors (Hessen and Rukke 2000, Ashforth and Yan 2008, Perez-Fuentetaja and Goodberry 2016), choosing experimental media and food levels that resemble conditions in which the *Daphnia* spp. are commonly found will allow for assessment of tolerance levels that are better applicable to a region of interest.

Even with some of the shortcomings mentioned in previous studies, daphniids have exhibited variation in their response to environmental stressors. As mentioned previously, food concentrations can affect the ability of *Daphnia* to tolerate low calcium conditions, with low food hindering survival and reproduction at calcium levels even above previously found reproductive thresholds of 1.5mg Ca L⁻¹ (Ashforth and Yan 2008; Perez-Fuentetaja and Goodberry 2016, Prater et al. 2016). However, even within a single genotype, the response to low food can vary, with identical individuals exposed to the same environmental conditions exhibiting large ranges in responses (Olijnyk and Nelson

2013, Cressler et al. 2017). This trend has also been found when *Daphnia* were exposed to metals (Baird et al 1990, DeMille et al. 2016), predators (Boeing et al. 2006), and cyanobacteria (Repka 1997, Schwarzenberge et al. 2012). Variation has been found among clones from different lakes (Repka 1997) and clones taken from the same lake (Jiang et al. 2013). Responses were also found to be more similar in daphniids taken from the same lake system, compared to daphniids from different lakes (Repka 1997). If variation exists within species and across populations to these other environmental variables, we must assume that *Daphnia* will also vary in response to calcium decline.

The presence of intra-specific variation to calcium decline in *Daphnia* has only been tested on a single species (Rukke 2002b). One population of *Daphnia galeata* was collected from two separate lakes in Northern Scandinavia, with average calcium concentrations being 2-3mg Ca L⁻¹ and >10mg Ca L⁻¹ (Rukke 2002b). When exposed for 7 days to a three level calcium concentration gradient, the two populations had significant differences in survival, with daphniids from the high calcium source lake (>10 mg Ca L⁻¹) having higher survival in the lowest calcium concentration (0mg Ca L⁻¹) than their low calcium source lake counterpart (Rukke 2002b). The presence of variation within populations/genotypes has been suggested to allow selection to act on and favour adapted types (Loreau et al. 2003). If variation is present in *Daphnia* populations or genotypes then they may be able to adapt to the changing environmental conditions, this may allow for persistence of *Daphnia* even with continued calcium decline. However, with this being tested on only two populations of one species, this poses the question ‘does intra-specific variation to calcium decline exist in *Daphnia*?’

To fully understand the consequences of calcium decline we must understand the effect it has across and within zooplankton species. With calcium predicted to continue declining in many lakes (Keller et al. 2001, Aherne et al. 2003, Jeziorski et al. 2008, Palmer et al. 2011), we must address the gap in knowledge about intra-specific variation and make it applicable to a landscape. This question can be answered in two ways: Is there variation among clones taken from separate lakes (i.e., comparing across a landscape); and is there variation among clones within a lake (i.e., comparing within a population). In this thesis we will address the former gap in knowledge. Understanding the variation in *Daphnia* clones across a landscape will help us better understand the effects this abiotic factor has on zooplankton communities and the biotic changes that may occur in many North American and Scandinavian freshwater lakes as a result.

Thesis Objectives

The objective of this thesis was to see how calcium decline affects individuals within a species when exposed to typical conditions found in Muskoka region lakes. To achieve this goal we examined variation in calcium sensitivity among *Daphnia* individuals collected from different lakes, in a controlled laboratory setting with food concentrations and water chemistry conditions commonly found in lakes of the Muskoka region (an area known to be declining in calcium; Palmer et al. 2011, Jeziroski et al. 2012a). *Daphnia pulex* sensu lato (Cristescu et al. 2012) was chosen as our experimental species as it is commonly found in this region and is expected to be sensitive to low calcium. The main objectives of this thesis were:

- 1) Determine survival and reproductive thresholds of *Daphnia pulex*, under low food and ion concentrations.

- 2) Determine if within species (intra-specific) variation exists in sensitivity of *Daphnia pulex* to low calcium under more realistic laboratory conditions.

Chapter 2

Intra-specific variation in sensitivity to low calcium in *Daphnia pulex*

Introduction

Freshwater systems face many challenges, from invasive species (Dextrase and Mandrak 2006, Palmer and Yan 2013) to chemical pollution (Jones et al. 2017), to climate change (Wrona et al. 2006, Woodward et al. 2010, Hulme 2017). Organisms that inhabit these systems must be able to adapt to survive and flourish. Changes in water chemistry, including increases of heavy metals (Gagneten and Paggi 2009) and reductions in pH, that were associated with metal smelting and the burning of sulphur-rich coal, caused decreases in zooplankton species abundance (Keller et al. 1990), loss of species, and consequently changes in community composition. Many lakes in northern North America and Europe were affected by acid deposition (Stoddard et al. 1999), and because of the underlying bedrock in areas like the Precambrian shield, which reduces the buffering capacity of soils (Aherne et al. 2003), acid deposition affected the water chemistry of many freshwater lakes (Neary and Dillon 1988, Stoddard et al. 1999, Keller et al. 2001, Jeziorski et al. 2008). A recent and concerning challenge that affects aquatic organisms, particularly in regions that experienced regional acid deposition, is widespread reductions in base cations (Likens et al. 1996, Stoddard et al. 1999, Aherne et al. 2003, Watmough et al. 2003), and specifically decreases in calcium (Keller et al. 2001, Jeziorski et al. 2008, Jeziorski et al. 2015).

Calcium decline has been documented across many lakes in the Northern Hemisphere over the past few decades (Stoddard et al. 1999, Keller et al. 2001, Jeziorski et al. 2008, Palmer et al. 2011, Futter et al. 2014, Garmo et al. 2014, Hessen et al. 2016)

and is attributed to the loss of base cation pools from the surrounding soil. As the size of the exchangeable cation pool in the soil is the biggest predictor of lake water calcium (Houle et al. 2006), reducing base cations from soils in watersheds reduces cation loading into lakes. Increased leaching of cations from watersheds into lakes, due to acid deposition (Likens et al. 1996), is one of the largest historical outputs of cations from the soil; post-industrialization increases in the deposition of SO₄ and NO_x, caused cations from soils to be mobilized and leached out of the soils into lakes (Stoddard et al. 1999). Although during this time lakes would have experienced an initial surge of calcium from the surrounding soils, base cations pools became depleted in the soils and have reduced the total available cation stores to be leached into lakes in the future (Aherne et al. 2003). Along with the effects of acid deposition, lakes also experienced reduced atmospheric deposition of calcium salts over the last few decades (Hedin et al. 1994, Likens et al. 1996). Forest harvesting also removed calcium from soils, through direct removal of biomass, forest regrowth practices, and soil erosion (Federer et al. 1989, Watmough and Dillon 2003, Watmough et al. 2003, Watmough and Aherne 2008).

In the Muskoka region of south-central Ontario, calcium concentrations of many lakes have been falling over the last few decades (Jeziorski et al. 2008, Palmer et al. 2011, Jeziorski et al. 2015). A survey of 770 lakes in Ontario revealed that 62% the lakes had calcium concentrations below 2mg Ca L⁻¹ (Jeziorski et al. 2008). Along with calcium concentration decreases, changes in zooplankton community structure have been observed (Jeziorski et al. 2008, Jeziorski et al. 2012a). Paleolimnological studies have documented shifts from *Daphnia*-dominated cladoceran communities to *Bosmina*-dominated communities (Jeziorski et al. 2008, DeSellas et al. 2011). Lake surveys have

demonstrated increases in *Holopedium* relative abundance through time (Jeziorski et al. 2015) and along declining calcium gradients across lake landscapes (Hessen et al. 1995). A new study by Azan and Arnott (*in press*) found decreases in population growth rates of not only *Daphnia* spp., but also *Bosmina* and copepods to low calcium in a community mesocosm experiment; with decreases in population growth rates of species (e.g. *Daphnia catawba*) that were thought to be calcium insensitive (Cairns 2010, Jeziorski et al. 2015). The effect of calcium decline on zooplankton is important as they are an essential part of freshwater systems being primary consumers and transferring energy up the food chain. To fully understand the effects that calcium decline has on communities across the landscape, it is important to understand intra-specific variation of effects on single species.

Daphnia is a zooplankton herbivore that is important as the primary prey of choice for many planktivorous fish and invertebrates (Leibold 1989, Cry and Curtis 1999) and rely heavily on aqueous calcium to grow, reproduce, and survive (Greenaway 1985). *Daphnia* is commonly found in the Muskoka region of south-central Ontario (Cairns 2010, Korosi et al. 2010, DeSellas et al. 2011, Jeziorski et al. 2012a, Redmond et al. 2016) and some species may be sensitive to calcium decline as they have high calcium body content (Jeziorski and Yan 2006) owing to a calcium chitin carapace which is frequently moulted (He and Wang 2009). *Daphnia* must be able to replace up to 90% of their total body calcium after each moult (Alstad et al. 1999), with the calcium being acquired from the surrounding water (Porcella et al. 1969, Cowgill et al. 1986). When calcium concentration in the surrounding water is low, daphniids experience reduced

reproduction and/or survival (Hessen and Rukke 2000, Hessen et al. 2000, Ashforth and Yan 2008, Hooper et al. 2008, Perez-Fuentetaja and Goodberry 2016, Prater et al. 2016).

Many laboratory studies have been performed accessing calcium tolerances in different species (Hessen and Rukke 2000, Hessen et al. 2000, Rukke 2002*b*, Ashforth and Yan 2008, Perez-Fuentetaja and Goodberry 2016) and across life stages (Tan and Wang 2009). Thresholds for survival can vary among species being between 0.1 and 0.5mg Ca L⁻¹ for *Daphnia magna* (Hessen et al. 2000) and *Daphnia pulex* (Ashforth and Yan 2008), and between 0 and 2mg Ca L⁻¹ for *Daphnia galeata* (Rukke 2002*b*). Reproduction in *D. pulex* has been found to require higher calcium concentrations above 1.5mg Ca L⁻¹ (Ashforth and Yan 2008).

Most laboratory studies examining the relationship between calcium concentration and *Daphnia* life-history traits have been conducted with single laboratory-cultured clones (e.g. Hessen and Rukke 2000, Ashforth and Yan 2008, He and Wang 2009, Jesus et al. 2014, Prater et al. 2016). Only one study has explicitly examined intra-specific variation to calcium concentrations in *Daphnia* (Rukke 2002*b*). As *Daphnia* have shown adaptive abilities to other environmental gradients (Weider and Hebert 1987, Agra et al. 2011, Geerts et al. 2014), if *Daphnia* were locally-adapted to low calcium concentrations we would expect higher tolerances to low calcium in the *Daphnia* from low calcium lakes. Instead, Rukke (2002*b*) found that when testing two populations of *D. galeata* in low calcium conditions, the population from a lake with a high calcium concentration (>10mg Ca L⁻¹) had higher survival than its low calcium source lake (2-3mg Ca L⁻¹) counterpart. Despite these surprising results, a key outcome of the study was to document that variation in response to calcium can exist within a single species (Rukke 2002*b*).

With intra-specific variation in *Daphnia* life-history responses has also been found when testing multiple daphniid genotypes to food gradients (Olijnyk and Nelson 2013), metals (Baird et al. 1990, DeMille et al. 2016), cyanobacteria (Repka 1997, Jiang et al. 2013), and predator kairomones (Boeing et al. 2006), we need to explore the presence of intra-specific variation to calcium decline.

Another important consideration in laboratory studies is the source of daphniids for experiments. Rukke (2002*b*) used clonal lines that were recently collected from nature. As in many laboratory studies, experimental daphniid lines can be housed for years in the laboratory prior to experiments (e.g. Ashforth and Yan 2008, Muysen et al. 2008, He and Wang 2009, Tan and Wang 2009, Prater et al. 2016), with daphniids possibly being selected and adapted to laboratory conditions over that time. Although completing experiments in the laboratory is necessary to control for abiotic factors, using clones that are selected for high survival in the lab may not best represent the populations found in the wild.

Along with the use of single genotypes and long lived laboratory clones, many studies also used hard water media to conduct their experiments (e.g., Hessen and Rukke 2000, Prater et al. 2016). Two commonly used hard water media are Elendt M7 media (Elendt and Bias 1990; used by Hessen and Rukke 2000, Hessen et al. 2000, Rukke 2002*b*, He and Wang 2009, Tan and Wang 2009) and COMBO media (Kilham et al. 1998; used by Olijnyk and Nelson 2013, Prater et al. 2016); with average conductivity being between 221-273 $\mu\text{S cm}^{-1}$ for Elendt M7 (Rukke 2002*b*) and ~445 $\mu\text{S cm}^{-1}$ for COMBO (Martha Celis-Salagado, unpublished data). When testing daphniids against a stressor, controlling for every other environmental aspect is important, using hard water

media allows researchers to eliminate all other stressors and see the effects of the one in question. However, using a hard water media has shown increased reproduction and growth (Jesus et al. 2014), and when testing a potential stressor could allow for daphniids to cope better than if they were in soft water lakes in the wild. The Muskoka region of south-central Ontario is an area of soft water lakes, with the average conductivity only $\sim 38.7 \mu\text{S cm}^{-1}$ (unpublished CAISN data). If daphniids are being tested from this region, using a soft water media such as FLAMES (Celis-Salagado et al. 2008) with a conductivity of $\sim 30.2 \mu\text{S cm}^{-1}$ (Marth Celis-Salagado, unpublished data) would allow for a controlled environment better resembling common conditions found in that region.

Food concentrations have been shown to affect the reproduction, growth, and survival of *Daphnia* in many studies (Hessen et al. 2000, Heugens et al. 2006, Ashforth and Yan 2008, Olijnyk and Nelson 2013, Sarpe et al. 2014, Perez-Fuentetaja and Goodberry 2016). Low food can cause reduced reproduction (Hessen et al. 2000, Heugens et al. 2006, Olijnyk and Nelson 2013, Sarpe et al. 2014, Perez-Fuentetaja and Goodberry 2016), reduced individual growth rates (Hessen et al. 2000, Heugens et al. 2006, Olijnyk and Nelson 2013, Sarpe et al. 2014), and reduced (Ashforth and Yan 2008, Olijnyk and Nelson 2013, Sarpe et al. 2014) or increased survival (Perez-Fuentetaja and Goodberry 2016). Because of these known implications of food on *Daphnia* life-history traits, most studies are conducted at high food concentrations to reduce the likelihood of confounding effects (e.g. Rukke 2002b, Muysen et al. 2008, Tan and Wang 2009). However, the use of high food concentrations may provide unrealistic conditions to assess the response to an environmental stressor in a region with soft water lakes. In the Muskoka region, for example, the average food concentration is $\sim 0.44 \text{mgC L}^{-1}$ (Brown

and Yan 2015), which is a much lower than concentrations used in other *Daphnia* studies (e.g., 0.9mgC L⁻¹ Rukke 2002b, 2.73mgC L⁻¹ Tan and Wang 2009, 4mgC L⁻¹ Prater et al. 2016).

To assess intra-specific variation in *Daphnia* response to calcium decline in soft water, nutrient-poor lakes, we must use media and food concentrations in our experiments that resemble common conditions in oligotrophic lakes. This is especially true in the Muskoka region, an area being exposed to substantial declines in lake water calcium (Palmer et al 2011, Jeziorski et al. 2012a). We are also interested in determining if intra-specific variation in response to calcium decline in *Daphnia* can be explained by source lake (lake calcium concentrations where these populations exist naturally). To achieve these goals, we used multiple iso-female lines (clonal lines started from a single wild individual) from different source lakes and regions.

Using ten iso-female lines that were collected from ten source lakes that differ in calcium concentration across two regions, we are the first to assess the variation in response of *Daphnia* to a large calcium concentration gradient (0.7 – 7mg Ca L⁻¹, which encompasses 96% of 287 sampled lakes in the Muskoka-Haliburton region, CAISN unpublished data) in a low food and soft-water media life-history experiment. We predict that *Daphnia* life history responses will be affected by calcium concentration and that: (1) the response to calcium will be determined by source lake calcium concentration, as *Daphnia* with prior exposure to low calcium conditions are expected to perform better under low calcium conditions, or (2) the response to calcium will be determined solely by iso-female line, as iso-female lines may have different coping or tolerance abilities.

If variation exists, we want to try to understand if differences are related to region/location, source lake calcium or iso-female line differences alone. These results will address the lack in knowledge about intra-specific variation in this important aquatic invertebrate, and help us to better apply and understand the implications of continued calcium decline in the Muskoka region.

Methods

Sampling Iso-Female Lines

To assess variation in calcium sensitivity, we collected life-history responses of ten iso-female lines across five calcium concentrations in a highly replicated, laboratory experiment. We used ten iso-female lines of *Daphnia pulex* (sensu lato; Cristescu et al. 2012) that originated from lakes in either the Muskoka or Frontenac region. The Muskoka region, located in south-central Ontario, Canada, is characterized by lakes that are nutrient-poor and low in ionic strength; including low calcium concentrations due to underlying Precambrian Shield bedrock (median = $2.2 \text{ mg Ca L}^{-1} \pm 1.97 \text{ SD}$; CAISN unpublished data). The Frontenac region is in southern Ontario, Canada, on the edge of the Canadian Shield with heterogeneous bedrock, mostly late Precambrian (Wolff 1982). Lakes are characterized by nutrient-rich, high ionic strength, and high calcium concentrations (median = $20.5 \text{ mg Ca L}^{-1} \pm 8.56 \text{ SD}$; Arnott unpublished 30 lake survey). Daphniids were collected from lakes in the Muskoka region during the summer of 2015, except the Red Chalk iso-female line which was collected in 2008. Daphniids were collected from the Frontenac lakes at Queen's University Biological Station (QUBS) on 20 April 2016 (Table 2.1). The *Daphnia* originated from lakes along a calcium gradient

ranging from 1.5 to 40mg Ca L⁻¹ (Table 2.1). *Daphnia* from southern Ontario have been identified as *D. pulex*, *D. pulicaria*, or a *D. pulex/pulicaria* hybrid using *Ldh* (Lactase dehydrogenase) genotyping (Cristescu et al. 2012). We identified the daphniids based on morphological characteristics alone using a Practical Guide to Identifying Freshwater Crustacean Zooplankton (Whity 2004). As no genetic analysis was performed, our experimental *Daphnia* could be identified as belonging to one of the groups from the *Daphnia pulex* sensu lato (Cristescu et al. 2012) complex, and from here on will be referred to *Daphnia pulex*.

***Daphnia* Cultures**

Daphnia were maintained in stock cultures in the Field Laboratory for the Assessment of Multiple Ecological Stressors (FLAMES) located at the Dorset Environmental Science Centre (DESC) in Dorset, Ontario. *Daphnia* were cultured in FLAMES media (Celis-Salagado et al. 2008); a freshwater media based on water-chemistry of two Muskoka lakes: Red Chalk and Blue Chalk lakes. Stock populations were cultured in 2.5mg Ca L⁻¹ FLAMES media over the course of the experiment at approximate concentrations of 150-200 daphniids per 1L glass jar. Water was changed once or twice a week, with fresh algae and media being added at a ratio of ~40% old and ~60% new media. Stock populations were fed approximately 87µgC *Daphnia*⁻¹ of *Chlamydomonas reinhardtii* three times per week (equivalent to ~43.5µgC *Daphnia*⁻¹ day⁻¹ or 1.3mgC L⁻¹). Stock populations were housed in Conviron chambers (Model E7/2) on a 16:8 light dark cycle at 20°C.

Algae

Chlamydomonas reinhardtii were grown in Bold Basal Medium (BBM; Stein 1973) using Conviron chambers (Model E7/2) at 20°C on an 18:6 light dark cycle with 100 $\mu\text{mol sec}^{-1} \text{cm}^{-2}$ of fluorescence during light hours. Algae were harvested weekly in its logarithmic growth phase and were stored at 4°C. To prevent the addition of nutrients from BBM to stock and experimental *Daphnia* individuals, algae was spun at 3000 rpm in a Thermo IEC Centra CL2 centrifuge for 10 minutes. The supernatant was discarded and algae cells were re-suspended in FLAMES media with calcium concentrations equivalent to calcium treatments. Every second day before feeding daphniids, a subsample of re-suspended algae was fixed using Iodine Tincture (25mg mL⁻¹ Iodine and 25mg mL⁻¹ Potassium Iodine) then counted using a Bright Line hemocytometer on a Lecia Wezlar (Germany) Compound Microscope. This ensured accurate algal cell concentrations for experimental trials.

Experimental Set-up

Prior to initiating the experimental life-history trials, we isolated gravid daphniid mothers (approximately nine) from each iso-female line into 125mL jars containing FLAMES and algae. Jars were then examined daily for neonates that were then used in experimental trials. Life-history trials were initiated using neonates (< 24 hours old). Neonates from individual mothers were randomly assigned to calcium treatments, ensuring that neonates from a single mother were dispersed across treatments. *Daphnia* were exposed to one of five nominal calcium concentrations: 0.7, 1.0, 1.5, 2.5, and 7mg Ca L⁻¹ (Table 2.2), created by adjusting amounts of CaSO₄ in the FLAMES media recipe with a pH = 6.40 (± 0.39 SD). These treatment concentrations were chosen based on past

sensitivity and reproductive thresholds of *Daphnia* in published studies (Hessen et al. 2000, Rukke 2002b, Ashforth and Yan 2008), and is a realistic range of calcium concentrations found in the study area as it encompasses approximately 96% of lakes in the Muskoka-Haliburton region (CAISN, unpublished data). Each individual was grown in a 40mL glass vial containing 30mL of treatment media and algae. Individual daphniids were transferred to clean vials containing 100% fresh media and 14.4µgC of *Chlamydomonas* every second day (equivalent to 7.2µgC *Daphnia*⁻¹ day⁻¹ or 0.24mgC L⁻¹); this food concentration is typical of summer carbon concentrations found in the Muskoka region (Brown and Yan 2015). A small flake of acetyl alcohol (0.8-1.5mg), which has no known toxic effects (Desmarais 1997), was placed in each vial to prevent daphniids from becoming entrapped in the surface tension. Experiments were conducted in three Percival Incubators (Model 130BLL) at 20°C on a 16:8 light dark cycle. Vials were grouped by iso-female line and calcium treatments in racks and rotated among shelves and incubators every other day to prevent individual incubator effects. Vials used in the experiments were machine washed and rinsed three to five times with reverse-osmosis water after every water change. FLAMES media was made weekly for each experimental concentration and an 80mL subsample was analysed for base cations at the DESC Chemistry Laboratory (Ministry of the Environment and Climate Change 2017).

Twelve to 25 (median = 24.5) replicate daphniids were cultured in each calcium treatment for each iso-female line (Table 2.3); the number of replicates varied based on reproductive output of stock populations. Each trial was run for 21 days with survival and reproduction (clutch size and number) being recorded daily. One size measurement, from the top of the head (above the eye) to the base of the tail spine, was taken for each

individual at day six. Because of the high number of experimental individuals involved in the study (1094 individuals), life-history trials could not be conducted simultaneously. Therefore, life-history trials were conducted between June 21, 2016 and August 27, 2016, with ‘start day’ being recorded for each set of trials.

Intrinsic rate of natural increase (r) was calculated for each calcium treatment and iso-female line combination using the equation $\sum e^{-rx} l_x m_x = 1$, where l_x is the number of individuals surviving to time x , m_x the mean number of eggs produced until x , and e the natural logarithm (Ashforth and Yan 2008; Jesus et al. 2014; Perez-Fuentetaja and Goodberry 2016). The equation was solved through an iterative process in R-Studio and an accurate value of r was found (i.e. sum of the equation was equal to 1 ± 0.0001). Because the equation gives a single value of r for an entire population, the sampling error for each calcium treatment and iso-female line combination was obtained through a jackknifing procedure (Meyer et al. 1986, Jesus et al. 2014). See Appendix B for details.

Statistical Analysis

To test the influence that ‘source-lake calcium’ and calcium concentration (labelled here as ‘calcium treatment’) had on daphniid life-history responses, we examined predictor variables as both continuous and categorical to assess the nature of the relationship. Other predictor variables were also tested to control for ‘region’ (categorical) and ‘start day’ (continuous) to control for any variability due to region or time in the summer daphniids were started. Life-history response variables tested were: ‘total reproduction in 21 days’, ‘size at day six’, ‘age at reproductions’, ‘size of clutches’, and ‘intrinsic rate of increase (r)’. All continuous predictor variables were examined including a square term in models to account for non-linear relationships. To account for

variation in clutch size due to time of maturation in daphniids, ‘age at reproduction’ was included in respective ‘size of clutch’ models as a continuous predictor. ‘Size at day six’ was included as a continuous predictor in ‘age at reproduction’ and ‘size of clutch’ models to control for size differences among and within iso-female lines. The response variable ‘size at day six’ was transformed by multiplying the measured value by 1000 to convert values into integers for model selection in GLMs.

To assess the influence that ‘calcium treatment’ and ‘source-lake calcium’ had on response variables, model selection was performed (using the ‘dredge’ function in the ‘MuMIn’ v.1.15.6 package; Barton 2016) to find the minimum adequate model (MAM; chosen through lowest AIC value). If more than one top model existed (models had the same lowest AIC value) the simplest model was chosen as MAM. Full models included all predictor variables and interactions between ‘calcium treatment’ and ‘region’, and ‘calcium treatment’ and ‘source-lake calcium’. The assumptions of normality and homoscedasticity were assessed for the final model (MAM) by viewing residual vs fitted values, normal quantile-quantile, scale-location, and residuals vs leverage plots. Significance and the relationship of predictor variables were tested using an analysis of variance (ANOVA) for categorical predictors (with a ‘TukeyHSD’ test), and linear regression for continuous predictors (with the ‘summary’ command; R Core Team 2016).

For response variables with non-normal distributions, generalized linear models (GLMs) with a poisson, quasi-poisson, or negative binomial distribution were determined as above. For data with negative binomial distributions, we used the ‘glm.nb’ function from the ‘MASS’ v.7.3-45 package (Venables and Ripley 2002). To test for homoscedasticity and influential points, models were assessed by viewing residual vs

fitted, scale-location, and residuals vs leverage plots, as well as determining an estimate of model dispersion (ϕ ; which was found acceptable between 0.8-5). To find significance relationships of predictor variables, categorical predictors were assessed with a multiple comparison of means controlling for false discovery rates ('BH' test with the 'glht' function in the 'multcomp' v.1.4-6 package; Benjamini and Hochlberg 1995, Hothorn et al. 2008) and continuous predictors were assessed using linear regression (with the 'summary' command; R Core Team 2016).

'Age of reproduction' and 'size of clutch' from the second to sixth reproductive events were not assessed as most iso-female lines had only a single reproductive event in all calcium treatments (i.e. we could not run accurate model selection for 'source-lake calcium' against 'calcium treatments'). If the effect of 'calcium treatment' was found to significantly vary among 'source-lake calcium' (i.e. an interaction was present), 'calcium treatment' was retested individually within each iso-female line to determine if the relationship was continuous or categorical (Appendix A); with p-values being adjusted to control for false discovery rates ('BH' method; Benjamini and Hochlberg 1995).

Survival was assessed using two methods. First using the above model selection technique, 'calcium treatment' and 'source-lake calcium' were assessed on 'age at death'. Cox Regression (Cox 1972, Fox and Weisberg 2011) was then used to perform a survival analysis; models were created (using the 'Surv' and 'coxph' functions in the 'survival' v.2.38 package; Therneau 2015) with 'calcium treatment', 'source-lake calcium', and their interaction as both continuous and categorical predictor variables, and were tested for significance using an analysis of variance (ANOVA). Proportional hazards,

influential observations, and nonlinearity were checked through the viewing of plots (using the ‘survminer’ v.0.2.4 package; Kassambara and Kosinski 2016).

All data manipulation and statistical analysis was performed in R v.3.3.1 (R Core Team 2016).

Results

In total, we assessed 1094 individual daphniids from ten lakes across five calcium concentrations. As expected, under low food conditions, reproductive output tended to be low; the average clutch size was 2.75 neonates (± 0.07 SE) and the average total number of eggs produced over 21-day monitoring period was 4.05 eggs (± 0.19 SE). 60% of individuals who survived to day 21 produced at least one clutch, whereas only 34% produced two clutches. This low-reproduction was the result of both delayed reproduction (average day to primiparity was 13.5 days ± 0.14 SE) and high mortality (36% died before reproducing). For all response variables our ‘calcium treatment’ and ‘source-lake calcium’ treatment were found to be categorical predictors; therefore, ‘source-lake calcium’ will be referred to from here as ‘iso-female line’.

Survival

Overall, survival was influenced by both iso-female line and calcium treatment ($X^2=159.1$, $df=13$, $p>0.001$). Survival was best described by iso-female line (Chisq, deviance= 27.62, $df=9$, $p>0.001$) and calcium treatment (Chisq, deviance=143.32, $df=4$, $p>0.001$) separately (i.e. the model with the lowest AIC did not contain an interaction). There was large variation in survival, both across iso-female lines and calcium treatments (Figure 2.1.), with no consistent calcium gradient effect being observed (i.e. survival did

not display any continuous effect with either increasing calcium or linearly across iso-female lines).

Total Reproduction

Reproduction occurred at all calcium concentrations for all iso-females lines (Figure 2.2, Table 2.4). The effect of calcium treatment on total egg production in 21 days varied among iso-female lines (calcium by iso-female line interaction: ANOVA, $n=687$, $F_{36, 637}=2.84$, $p<0.001$). Three trends were apparent across the iso-female lines. The first trend was a positive linear increase of total reproductive output as calcium increased, exhibited by only Red Chalk Lake iso-female line; reproductive output increased 0.64 total eggs per unit calcium increase (or 28% increase from lowest to highest calcium treatment) (Figure 2.2; Appendix A, Table A.1). The second trend was observed in four iso-females lines: Ridout, Mckay, Grandview, and Round (Figure 2.2; Appendix A, Table A.1). These iso-female lines had high reproduction at 1.0mg Ca L⁻¹ with a 43% increase on average over the other four calcium treatments. Calcium response was not significantly different among these four iso-female lines (iso-female line: ANOVA, $n=206$, $F_{3, 201}=1.44$, $p=0.231$). No obvious pattern associated with the effect of calcium was observed in the third group of five iso-female lines; calcium treatment was a significant predictor of total egg production (Figure 2.2; Appendix A, Table A.1) in four iso-female lines (Henshaw, Big Glamour, Glen, and Lindsey); however, the effect of calcium treatment on total egg production in Big Glamour and Lindsey lakes was weak as calcium treatments did not differ significantly within the iso-female lines (Figure 2.2). Henshaw had low reproduction in 1mg Ca L⁻¹ and 1.5mg Ca L⁻¹ treatments, and Glen had increased reproduction in 1mg Ca L⁻¹ and 7mg Ca L⁻¹ (Figure 2.2). Calcium treatment

had no effect on total egg production in 21 days for the iso-female line from Elbow lake (calcium: ANOVA, $n=77$, $F_{4, 72}=0.54$, $p=0.707$) (Figure 2.2; Appendix A, Table A.1). Age at first reproduction and size of first clutch showed no obvious calcium treatment trends; results are located in Appendix C.

Size at Day Six

The effect of calcium treatment on size at day six varied among iso-female lines (calcium by iso-female line interaction: ANOVA, $n=1000$, $F_{9, 980}=3.97$, $p<0.0001$, Table 2.4). Two calcium treatment trends were found across the ten iso-female lines (Figure 2.3). *Daphnia* tended to be larger at low and high calcium concentrations for group one; size at day six exhibited a convex parabolic relationship with calcium treatment (Figure 2.3). This trend was statistically significant in Elbow, Big Glamour, and Lindsey, and was visually present in Ridout, Grandview, Glen, and Round as treatment was a categorical predictor in these four iso-female lines (Figure 2.3; Appendix A, Table A.2). The second trend was found in Red Chalk, McKay, and Henshaw iso-female lines, which showed no obvious pattern of size at day six associated with calcium treatment (Figure 2.3; Appendix A, Table A.2). Size at day 6 was larger in daphniids whose trials were started later in the summer, but effects were minimal with an average increase of only 10% across the 48 separate start days (start day: ANOVA, $n=1000$, $R^2=0.017$, $F_{1, 998}=17.95$, $p<0.001$).

Intrinsic Rate of Population Increase (r)

The influence of calcium treatment on intrinsic rate of population increase (r) varied among iso-female lines (calcium by iso-female line interaction: ANOVA, $n=1137$, $F_{36, 1087}=5.0$, $p<0.0001$, Table 2.4). There were three trends observed. In the first group

(Red Chalk, Henshaw, Elbow, and Big Glamour), r increased with increasing calcium concentration; with Red Chalk and Big Glamour lake iso-female lines exhibiting a 22% or a 40% increase in r , respectively, from the lowest to highest calcium treatment (0.0066 or 0.0024 increase in r per unit calcium increase, respectively) (Figure 2.4; Appendix A, Table A.3). In the second group, consisting of Ridout, Grandview, Glen, and Round lake iso-female lines (Figure 2.4; Appendix A, Table A.3), r was largest at 1.0mg Ca L⁻¹ and, on average, 44% higher than the other four calcium treatments combined. There was no significant difference of calcium response among these four iso-female lines (iso-female line: ANOVA, n=405, F_{6, 403}=0.15, p=0.99). The third group included Mckay and Lindsey lake iso-female lines, which exhibited no obvious change in r across calcium treatments (Figure 2.4; Appendix A, Table A.3).

Discussion

We assessed the variation in calcium sensitivity of *D. pulex*, an important herbivore in freshwater food webs (Leibold 1989, Cyr and Curtis 1999). To our knowledge, our study is the first to explicitly assess whether life-history responses of *Daphnia* to calcium varies across multiple iso-female lines (except Rukke 2002b, who compared two lines of *D. galeata*) in a soft water media and low food conditions. It was important for our experiment to have environmental conditions commonly found in lakes of the Muskoka region as environmental conditions have been shown to have large impacts on the calcium sensitivity of *Daphnia* (Hessen and Rukke 2000, Ashforth and Yan 2008, Perez-Fuentetaja and Goodberry 2016, Prater et al. 2016). Therefore, we used a low food quantity (~0.24mgC L⁻¹ day⁻¹) and a soft water media (FLAMES, Celis-

Salagado et al. 2008), resembling lakes in the Muskoka region, to make experimental conditions as close to natural lake conditions as possible. Many studies also use clones that have been housed in the laboratory for long periods of time (e.g. Hessen and Rukke 2000, Ashforth and Yan 2008, He and Wang 2009, Perez-Fuentetaja and Goodberry 2016) that can be selected over many years as ‘good’ performers. To reduce the risk of laboratory selection influence, nine of our iso-female lines had been in the lab for less than one year. However, we acknowledge that selection still occurred for good performers of laboratory conditions as daphniids that could not survive in laboratory conditions did not make it to the DESC facility for experimental trials.

We found that calcium concentration influenced important life-history attributes of *D. pulex* (reproduction, size at day six, and intrinsic rate of increase), but effects varied across iso-female lines (Figures 2.2-2.4). The calcium trends of the iso-female lines also did not group according to any region or source-lake calcium concentration. Based on other studies that have found evidence of local adaptation to environmental (biotic or abiotic) variables (Weider and Hebert 1987, Boersma et al. 1999, Sarnelle and Wilson 2005, Agra et al. 2011, Geerts et al. 2014, Hochmuth et al. 2015, DeMille et al. 2016) we hypothesized daphniids from low-calcium source lakes would perform better than daphniids from high-calcium source lakes. However, there was no advantage in low calcium conditions for daphniids from low-calcium source lakes compared to those from high-calcium source lakes. One possible explanation for the absence of a source lake calcium effect is that only a single iso-female line or daphniid was collected from each lake; therefore, we may not be best representing the population associated with each source-lake calcium concentration. To gain a more reliable understanding of the

relationship between calcium sensitivity and source-lake calcium concentration, using multiple iso-female lines from each source-lake calcium concentration would allow for a better representation of calcium sensitivity of each lake system. However, at a community level, Azan and Arnott (*in press*) also found no relationship between historical source lake calcium and calcium tolerance on functional growth rates or community richness of zooplankton, suggesting no advantage to calcium decline for individuals/communities who were collected from low calcium lakes.

Daphniids exposed to the same environmental conditions from the same iso-female lines (i.e. identical genotypes) exhibited large amounts variation in their response to a calcium gradient (e.g. Figure 2.2). Daphniids of identical genotypes have shown variation in their responses when exposed to the same environmental treatments (Gliwicz and Guisande 1992, Sakwinska 2004, Olijnyk and Nelson 2013, Cressler et al. 2017). This has sometimes been attributed to maternal effects, which can cause within genotype variation in *Daphnia* (Gliwicz and Guisande 1992, Sakwinska 2004). However, Cressler et al. (2017) found that when testing nine genotypes of *D. pulicaria* across a food gradient, that variance of individuals within a genotype was larger than the variance of individuals among genotypes. They attributed this large amount of variation to developmental noise, as both the genotype and the environmental conditions were identical (Cressler et al. 2017). Despite our large number of replicates (median = 24.5 daphniids treatment⁻¹ iso-female line⁻¹), the large amount of variation in our results could have obscured potential calcium patterns in daphniid responses. We did not detect calcium thresholds, for reproduction and survival, as there was no significant drop in either response in the low calcium treatments from the high calcium treatments. Overall,

the presence of intra-specific variation in calcium sensitivity in our main response variables, suggests that experiments using single iso-female lines may not be the best representation in response of *Daphnia* to this stressor.

Life History Responses

Survival

Survival was influenced by calcium treatment and iso-female lines alone, showing no obvious trends due to the calcium or source lake calcium gradients (Figure 2.1).

Differences in survival among iso-female lines (across all calcium treatments) may be attributed to variation in tolerance to low food, which has been shown to differ among *Daphnia* genotypes (Olijnyk and Nelson 2013). Although calcium has been shown to influence the survival of *Daphnia* (Hessen et al. 2000, Rukke 2002b, Ashforth and Yan 2008, Perez-Fuentetaja et al. 2016, Prater et al. 2016), the absence of a calcium gradient effect (increased survival with increasing calcium) in our experiment may have been driven by differences in food tolerances of the ten iso-female lines. Ashforth and Yan (2008) found that in their low food treatment ($\sim 3\mu\text{g chlorophyll } a \text{ L}^{-1}$) at 20°C , no daphniids survived past day 13 regardless of the calcium concentration. When the food concentration was raised to $\sim 30\mu\text{g chlorophyll } a \text{ L}^{-1}$, survival increased in all calcium treatments, except 0.1mg Ca L^{-1} . However, there was not a linear relationship between calcium treatment and survival; daphniids in the lowest calcium treatments did not have the lowest survival rates (Ashforth and Yan 2008). In contrast, Perez-Fuentetaja and Goodberry (2016) found that survival was higher in their low food (0.16mgC L^{-1}) treatment compared to their high food (1.67mgC L^{-1}) treatment regardless of calcium concentration; their low food treatment also did not show a calcium gradient effect. These

studies match our results as a calcium gradient effect was not exhibited by our *Daphnia*, and survival varied across iso-female lines, and as with both of these studies (Ashforth and Yan 2008, Perez-Fuentetaja and Goodberry 2016), survival varied across calcium concentration.

Reproduction

As expected at low food, total reproduction was low across all iso-female lines and calcium treatments (Figure 2.2). The highest number of neonates produced by one individual was 28 (by Red Chalk in 1.0mg Ca L^{-1}), which is low compared to other high food (0.9mgC L^{-1} – 4mgC L^{-1}) calcium studies (e.g. 10-70 neonates Ashforth and Yan 2008; 20-40 neonates Perez-Fuentetaja and Goodberry 2016). We attribute low reproduction to low food, as it has been shown to reduce reproduction when compared to high food treatments (Ashforth and Yan 2008, Olijnyk and Nelson 2013, Sarpe et al. 2014, Perez-Fuentetaja and Goodberry 2016).

Intra-specific variation was present in total reproduction as the calcium response varied among iso-female lines. We hypothesized total reproduction to increase with increased calcium, but this was only exhibited by a single iso-female line, Red Chalk line (Figure 2.2). This increase in total reproduction is suggested to be due to the fact that Red Chalk line was our longest lived laboratory clone and that the FLAMES media was based off water chemistry variables from its historical source lake, possibly giving it an overall advantage. Other iso-female lines exhibited an increase in reproduction in 1.0mg Ca L^{-1} over all other treatments (trend 2), or no obvious calcium effect (Figure 2.2). The reason why multiple iso-female lines exhibited trend 2 is unknown, as no other studies showed similar results; however, four out of the ten lines repeated this response, suggesting this

may be a general response among some populations. Perez-Fuentetaja and Goodberry (2016) found daphniids had higher reproduction at high calcium (2.5mg Ca L^{-1}) as daphniids in low calcium (0.5mg Ca L^{-1}) were stimulated to delay reproduction to sustain self-maintenance. Although we do not see highest reproduction in our high calcium treatments, this does suggest that *Daphnia* may have coping abilities when under stressful conditions causing increased performance even in low calcium conditions. A few studies have modelled resource allocation in *Daphnia* with the relationship between maintenance, growth, and reproduction being assessed (Kooijman et al. 1989, McCauley et al. 1990, Bradley et al. 1991). When Glazier and Calow (1992) tested two clones of *D. magna* in two food treatments, they found that resource allocation can change among clones and among life-stages. Both clones allocated the most of their energy to self-maintenance; however one clone allocated more energy to reproduction over growth, while another clone allocation equal amounts of energy to reproduction and growth. Although, we do not suspect that our daphniids applied the same coping mechanism as Perez-Fuentetaja and Goodberry (2016) as our daphniids did not display delayed reproduction in the low-calcium treatments compared to the high-calcium treatments (Appendix C, Figure C.1). Daphniids exhibiting trend 2 could be following similar trends as clone 2, in the experiment by Glazier and Calow (1992), by allocating energy to both reproduction and growth.

A calcium effect on average size of first clutch was only found in two iso-female lines that exhibited either a positive increase in clutch size with increasing calcium or a larger clutch in 1.5mg Ca L^{-1} treatment (Appendix C, Figure C.2). Although our average clutch size in our experiment was low ($2.75\text{ neonates} \pm 0.07\text{ SE}$), it was consistent with

recorded clutch sizes of *Daphnia* sp. found in Muskoka lakes (~ 1.97 neonates ± 0.06 SE; Angela Strecker, unpublished data). We do not know the long term effects of low calcium, as daphniids were only exposed for a 21 day period; however, if daphniids cannot cope with further calcium decline, the overall low reproduction of our daphniids suggests that abundance of *Daphnia* may decline in lakes that are experiencing similar conditions of our experiment.

Size at Day Six

Daphnia grew larger at high and low calcium (Figure 2.3) consistently across seven iso-female lines. We hypothesized a positive relationship between calcium concentration and size at day six (Hessen et al. 2000, Jesus et al. 2014, Prater et al. 2016), as *Daphnia* in the high-calcium treatments should be under reduced energetic stress and grow faster. This hypothesis explains the increase in size we see from mid to high calcium concentrations. However, size at day six was also high in the lowest calcium treatment. One hypothesis for this convex relationship is that *Daphnia* allocated energy to growth instead of reproduction in low calcium (Perez-Fuentetaja and Goodberry 2016), allowing them to be larger. In a *Daphnia pulex* X *pulicaria* hybrid it was found that in low food and low calcium conditions, daphniids allocated more energy to lipid storage instead of their ovaries (Perez-Fuentetaja and Goodberry 2016). In *D. magna* this was also observed as daphniids allocated more energy towards growth instead of reproduction in high and low food treatments (Glazier and Calow 1992). Although we did not assess lipids in our daphniids, if they exhibited a similar trend we would have observed reduced reproduction with the increased size in the low-calcium treatments. However, total reproduction was not reduced in iso-females lines who exhibited larger size at low

calcium (Figure 2.2, 2.3). Another hypothesis is that daphniids delayed maturity in low calcium conditions (Jesus et al. 2014, Perez-Fuentetaja and Goodberry 2016), allowing more energy for growth. If this was exhibited, *Daphnia* should have later reproduction in low calcium conditions compared to mid calcium conditions when looking within iso-females lines. However, maturity was not delayed in low-calcium treatments compared to mid-calcium treatments (Appendix C, Figure C.1). Although we cannot be certain what factors were driving larger size at low calcium, this trend was exhibited by multiple replicate daphniids across seven of the ten iso-female lines tested (Figure 2.3). Further research needs to be done to fully understand the affect low calcium has on size of *Daphnia* and address the drivers of variation.

Intrinsic Rate of Population Increase (r)

The intrinsic rate of population increase, r , calcium effect varied with iso-female line. We hypothesized a positive relationship between calcium treatment and intrinsic rate of population increase (Perez-Fuentetaja and Goodberry 2016) as higher calcium treatments should allow for better reproduction and survival. However, this trend was only found in four of the ten iso-female lines, with two iso-female lines exhibiting no overall calcium effect, and four following the same trend 2 found in total reproduction above (Figure 2.2, 2.4, 2.5). Similar trends observed in reproduction and population growth rates are most likely driven by the mechanisms affecting reproduction, as population growth, r , includes reproductive output of individuals. Overall, most iso-female lines had negative population growth rates across all calcium treatments, with only Red Chalk line exhibiting positive growth rates in all calcium treatments. This could be due to the fact that, as mentioned previously, Red Chalk line was our longest lived

laboratory clone and the FLAMES media was based off water chemistry variables from its historical source lake. Ashforth and Yan (2008) found that in their 20°C treatment at low food ($\sim 3\mu\text{g}$ chlorophyll $a\text{ L}^{-1}$), *Daphnia pulex* had variable population growth rates across calcium treatments. They did not find a positive relationship between calcium treatment and population growth rate, as *Daphnia* in their mid-calcium treatments had higher growth rates than the high-calcium treatments (Ashforth and Yan 2008). This variation of population growth rates with calcium treatment was found in our results, as many iso-female lines had the highest population growth rates at 1.0mg Ca L^{-1} (Figure 2.4). As growth was not greatly reduced in calcium treatments of iso-female lines showing this trend, we suggest that daphniids may be allocating resources to both reproduction and growth in low calcium conditions (as also seen by clone 2 in Glazier and Calow 1992, as mentioned above). In contrast, Perez-Funetetaja and Goodberry (2016) found that their clone of a *Daphnia pulex* X *pulicaria* hybrid had relatively high positive growth rates across all calcium treatments even in low food. Although we cannot fully explain the mechanisms by which our daphniids increased their performance at low calcium, we show that responses to calcium decline can vary largely based on iso-female line being tested. Resource allocation techniques have been found to vary among clonal lines (Glazier and Calow 1992), and we suggest that energy allocation in our daphniids for life history traits differ among our iso-female lines.

Overall

As described above, a large amount of variability can be seen among iso-female lines in their response to low calcium (Figure 2.5). Many lakes do not share similar trends across all life-history characteristics, highlighting the presence of intra-specific variation

in their response to calcium. However, even though intra-specific variation was present among iso-female lines, some share common trends in life-history responses to calcium decline (Figure 2.5). Ridout, Grandview, and Round share similar life-history responses to calcium (total reproduction, size at day six, and population growth rate, r). With both total reproduction and population growth rate, r , increasing at mid-low calcium concentrations, and size exhibiting a convex parabolic relationship across calcium treatments. Two other lines, Elbow and Big Glamour, also share common calcium trends, with total reproduction not changing across calcium treatments, size showing the same convex parabolic relationship as those above with calcium, and the population growth rate, r , increasing with increased calcium. Even though intra-specific variation is present in the response of *Daphnia* to calcium decline, some iso-female lines still share similar trends across response variables, suggesting similar tolerances and mechanisms by which they react to the stressor. However, the effect of calcium still widely varies on life-history responses with some lines have high growth and reproduction, and others possibly exhibiting energy to growth over reproduction. The “Principle of Allocation” suggests that life-history response, survival, reproduction, and growth should be negatively correlated as they are energetically expensive. However, in our results we find multiple iso-female lines which exhibit both increased reproduction and increased growth in low calcium conditions (Figures 2.2, 2.3, 2.5). Olijnyk and Nelson (2013) found a similar trend when tested four genotypes of *D. pulicaria* in low food conditions. They suggested an alternative resource mechanism called “differential resource utilization”, which allows for variation in resource utilization efficiency and maintenance costs among individuals. Although we cannot be sure if our study followed this same mechanism, this supports the

validity of our study as *Daphnia*, in this study, had both increased reproduction and growth when exposed to a stressor (Olijnyk and Nelson 2013). With the response to calcium decline varying among iso-female lines and daphniids possibly allocating resources differently, the effects of calcium decline across a landscape could be variable depending on which population of *Daphnia* you sample from.

Contributing Factors

To thoroughly discuss all aspects of our study design, we would like to address the influence that maternal identity and food concentration have on our study.

Maternal Effects

As mentioned above maternal effects are thought to be a contributor to the large amount of within iso-female line variation found in daphniids exposed to the identical treatment types. We recognize that the identity and environment of experimental individuals' mothers and grandmothers is known to affect the performance of daphniids (Sakwinska 2004). A recent study by Mikulski and Pijanowska (2017) found when looking at maternal effect of *D. magna* exposed to a fish kairomone, that maternal effects were only present in some life-history characteristics (e.g. age and size at first reproduction and size of first clutch). To take into account as much variation from maternal effects as possible, experiments were suggested by Sakwinska (2004) to be run for three generations, with experimental individuals all having different mothers and grandmothers exposed to the same conditions. This would reduce inconsistent maternal effects from individuals born to the same mothers, and allows for all levels of genotype variation to be accounted for. To try to control for any maternal effects, we set out to run

a three generational study, with daphniids herein originally being labelled as “grandmothers” of experimental individuals. However, due to low reproduction and survival, a second and third generation could not be obtained in our experimental conditions (i.e. low food and soft-water media). However, stock mothers, used to collect experimental individuals, were grown in 2.5mg Ca L⁻¹ FLAMES media, allowing for consistent maternal advantages or disadvantages to calcium treatments across all iso-female lines. Our methods for randomly selecting mothers from stock populations and separating neonates randomly among treatment types were also uniform; we suggest any maternal effect would be equally distributed across treatments in our study. Another study by Giardini et al. (2015) found that maternal environmental on *Daphnia* embryos, when exposed to three different calcium concentrations, only lasted 48 hours. Even though maternal effects alter daphniid performance, possible only for 48 hours, our methods allowed for consistent affects across all calcium treatments and iso-female lines, and leads us to believe that maternal effects did not play a large role in our results.

Food Concentrations

Low food was a large part of our experimental design, as we wanted to make laboratory conditions resemble lakes in the Muskoka region as best as possible; however, as mentioned above, it is thought to be a contributing factor to the low performance of our *Daphnia*. We know from previous research that low food conditions can hinder the performance of *Daphnia* (Hessen et al. 2000, Heugens et al. 2006, Ashforth and Yan 2008, He and Wang 2009, Sarpe et al. 2014, Perez-Funetetaja and Goodberry 2016), and adequate food levels have shown to increase performance of daphniids facing other environmental stressors, such as increased temperature (Ashforth and Yan 2008) and

cadmium stress (Heugens et al. 2006). Ingestion rates have been found to increase in low calcium conditions (Muysen et al. 2008); this could allow daphniids to compensate for stressful conditions by either using energy to pump in calcium or by trying to ingest small amounts of calcium from dietary sources; excess food could allow daphniids to use extra metabolic energy to cope with another stressor. This hypothesis suggests that the concentration of food provided can alter the response of *Daphnia* to a stressor. As an example, when testing the calcium sensitivity of *D. pulex* in low ($\sim 3\mu\text{g chlorophyll } a \text{ L}^{-1}$) and high food ($\sim 30\mu\text{g chlorophyll } a \text{ L}^{-1}$), Ashforth and Yan (2008) found that daphniid performance was reduced at low food; this was also found in *D. magna*, by Hessen et al. (2000) in low food conditions. Both of these studies also share a common characteristic that was found in our results, the absence of a continuous calcium effect (increase in performance with increasing calcium). In many studies increased calcium has been found to increase the calcium performance of individuals at high food levels (Hessen and Rukke 2000, Rukke 2002b, Ashforth and Yan 2008, Jesus et al. 2014); however, at low food, we see that the continuous calcium effect is reduced or absent altogether (Hessen et al. 2000, Ashforth and Yan 2008, results here within). One hypothesis is that food stress outweighs the calcium stress when it is very low. This causes the response in low food to be more variable and show either no continuous calcium effect, or no obvious calcium trends across treatments. Not only does it make the calcium response more variable, Olijnyk and Nelson (2013) found that at low food there was variation in survival, growth, and reproduction within a genotype. This not only creates variation among the calcium treatments, but is shown through the large amount of variation seen within iso-female lines in the same treatment groups in our results.

As food levels affect the performance of daphniids in other environmental stressors (Hessen et al. 2000, Heugens et al. 2006, Ashforth and Yan 2008, Brown and Yan 2015) it is important to choose the correct concentration. In the Muskoka region, many lakes have low food concentrations (0.44mgC L^{-1} , Brown and Yan 2015) and low conductivity ($\sim 38.7\mu\text{S cm}^{-1}$, unpublished CAISN data). Phosphorus (P) levels are also declining in many lakes (Palmer et al. 2011, Raney and Eimers 2013), and with food quality being important for the performance of *Daphnia* (He and Wang 2009, Sarpe et al. 2014, Prater et al. 2016) it is important to have representative food conditions. Our food concentration (0.48mgC L^{-1} every other day) was chosen to resemble these conditions to make our laboratory experiment as natural as possible. However, we acknowledge that we only used a single species of phytoplankton in our study, and that we did not account for natural variation in edibility of phytoplankton. Although food quality has been found to be an important aspect in the response of *Daphnia* (He and Wang 2009, Prater et al. 2016) and that food quality has been found to be more important than quantity (Sarpe et al. 2014), we believe that food quality was not a driving factor in the performance of our daphniids as algae growth conditions were ideal and algae stocks looked healthy.

Even though we expected low reproduction and performance because of low food, to understand the effects that calcium decline has on intra-specific variation in *Daphnia* it was important to use realistic conditions. Even though laboratory experiments are useful to identify the effects of single stressors, without using conditions commonly found in the area of interest, the results cannot be applied to natural systems across the landscape. Thresholds of *Daphnia* survival and reproduction have been suggested to be higher than that found in the lab (Cairns 2010), as natural conditions are generally harsher

and animals can experience other stressors. Doing experiments with realistic conditions are beneficial to understand what animals are experiencing in the wild.

Conclusions

Our study aimed to determine if variation in sensitivity to calcium was present within a single *Daphnia* species to calcium decline. Using conditions commonly found in lakes of the Muskoka region, we found that large within and among iso-female line variation in life-history response to calcium exists. These results highlight that daphniid response to environmental stressors can vary among iso-female lines, and laboratory experiments conducted on single iso-female lines/genotypes may not reflect responses by populations throughout the region. Using multiple iso-female lines is important when testing the sensitivity of *Daphnia* to environmental stressors to control and account for phenotype/genotype variation. If this is ignored, suggested thresholds and tolerances of *Daphnia* sp. could be inaccurate, and the potential impacts of stressors could be better or worse than expected. Although further research is needed to understand the drivers of variation among iso-female lines, it is also important to understand and disentangle the effects calcium decline has in combination with other stressors such as increased temperature and food levels. As climate change is expected to affect many aspects of aquatic ecosystems (as reviewed by Wrona et al. 2006), it is important to understand how calcium decline will interact and affect zooplankton with other stressors. Understanding the effects that calcium decline has on zooplankton communities is important to understand how aquatic systems will change with calcium continuing to decline in the future.

Table 2.1. Iso-female line lake calcium, region, and collection date.

| Iso-female Line | Source-lake Ca (mg L⁻¹) | Region | Collection Date |
|------------------------|---|---------------|------------------------|
| Ridout | 1.5 | Muskoka | Summer 2015 |
| Red Chalk | 1.8 | Muskoka | 2008 |
| Mckay | 2.7 | Muskoka | Summer 2015 |
| Grandview | 4.5 | Muskoka | Summer 2015 |
| Henshaw | 6.8 | Muskoka | Summer 2015 |
| Elbow | 10.8 | Frontenac | April 2016 |
| Big Glamour | 13.8 | Muskoka | Summer 2015 |
| Glen | 23 | Muskoka | Summer 2015 |
| Round | 24.4 | Frontenac | April 2016 |
| Lindsey | 39.6 | Frontenac | April 2016 |

Table 2.2. Nominal and actual calcium concentrations for treatments (mg Ca L⁻¹) ± standard error (SE).

| Nominal Calcium (mg Ca L⁻¹) | Actual Calcium (mg Ca L⁻¹) |
|---|--|
| 0.7 | 0.77 (± 0.14) |
| 1.0 | 1.11 (± 0.17) |
| 1.5 | 1.65 (± 0.04) |
| 2.5 | 2.70 (± 0.05) |
| 7.0 | 7.69 (± 0.07) |

Table 2.3. Number of replicates within each calcium treatment (mg Ca L⁻¹) for each iso-female line.

| Iso-female Line | 0.7 | 1.0 | 1.5 | 2.5 | 7.0 | Total |
|------------------------|------------|------------|------------|------------|------------|--------------|
| Ridout | 25 | 25 | 25 | 25 | 25 | 125 |
| Red Chalk | 25 | 25 | 25 | 25 | 25 | 125 |
| Mckay | 23 | 24 | 23 | 23 | 23 | 116 |
| Grandview | 25 | 25 | 25 | 25 | 25 | 125 |
| Henshaw | 25 | 25 | 25 | 25 | 25 | 125 |
| Elbow | 20 | 20 | 20 | 20 | 19 | 99 |
| Big Glamour | 23 | 22 | 24 | 24 | 24 | 117 |
| Glen | 12 | 14 | 12 | 12 | 13 | 63 |
| Round | 14 | 15 | 16 | 15 | 13 | 73 |
| Lindsey | 25 | 25 | 25 | 25 | 26 | 126 |

Total number of daphniids: 1094

Table 2.4. Model output using lowest AIC for dependant variables for each response variable. Model terms are abbreviated as: calcium – calcium treatment, source-lake – source-lake calcium, region – region, and start day – start day. Discrete and continuous predictors are denoted with d and c respectively; square terms are denoted with X².

| Dependant Variable | Model | Model Type | AIC | df | Log-Likelihood |
|--|--|-------------------|------------|-----------|-----------------------|
| Total Eggs | calcium ^d * source-lake ^d | linear | 3672.3 | 51 | -1678.422 |
| Size at Day 6 | calcium ^c * source-lake ^d + calcium ² + start day ^c | linear | -468.4 | 23 | 257.765 |
| Age at 1 st Reproduction | calcium ^d * source-lake ^d | linear | 1912.6 | 51 | -905.308 |
| Size of 1 st Clutch | calcium ^d * source-lake ^c + region ^d + start day ^c | linear | 1388.7 | 13 | -680.902 |
| Age at 2 nd | source-lake ^d | linear | 1056.4 | 11 | -516.601 |
| Size of 2 nd | calcium ^d + region ^d + start day ^c | linear | 894.7 | 8 | -439.052 |
| Intrinsic Rate of Increase (r) | calcium ^d * source-lake ^d | linear | -602.75 | 51 | 352.380 |

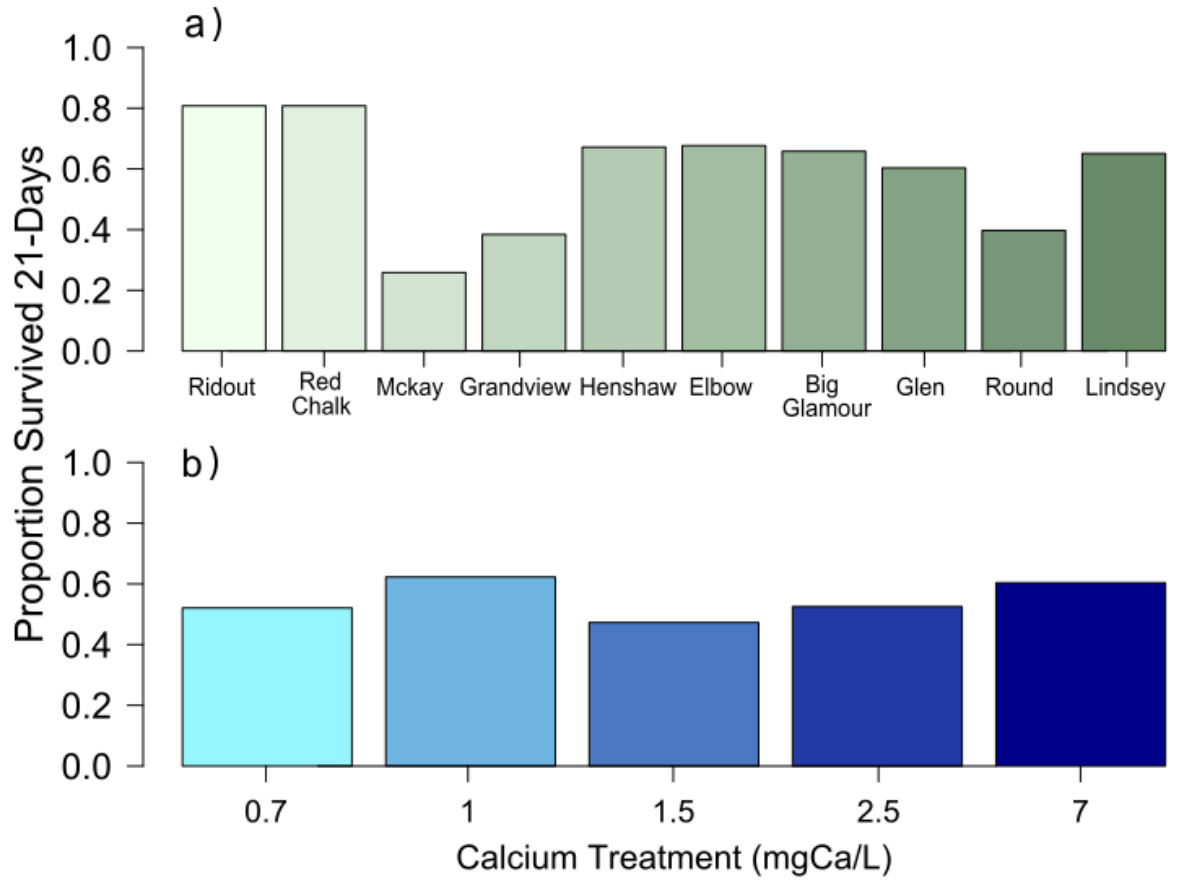


Figure 2.1. Proportion of *Daphnia pulex* surviving until day 21 in (a) each iso-female line and (b) calcium treatments. Iso-female lines are ordered by source-lake calcium concentration from left to right.

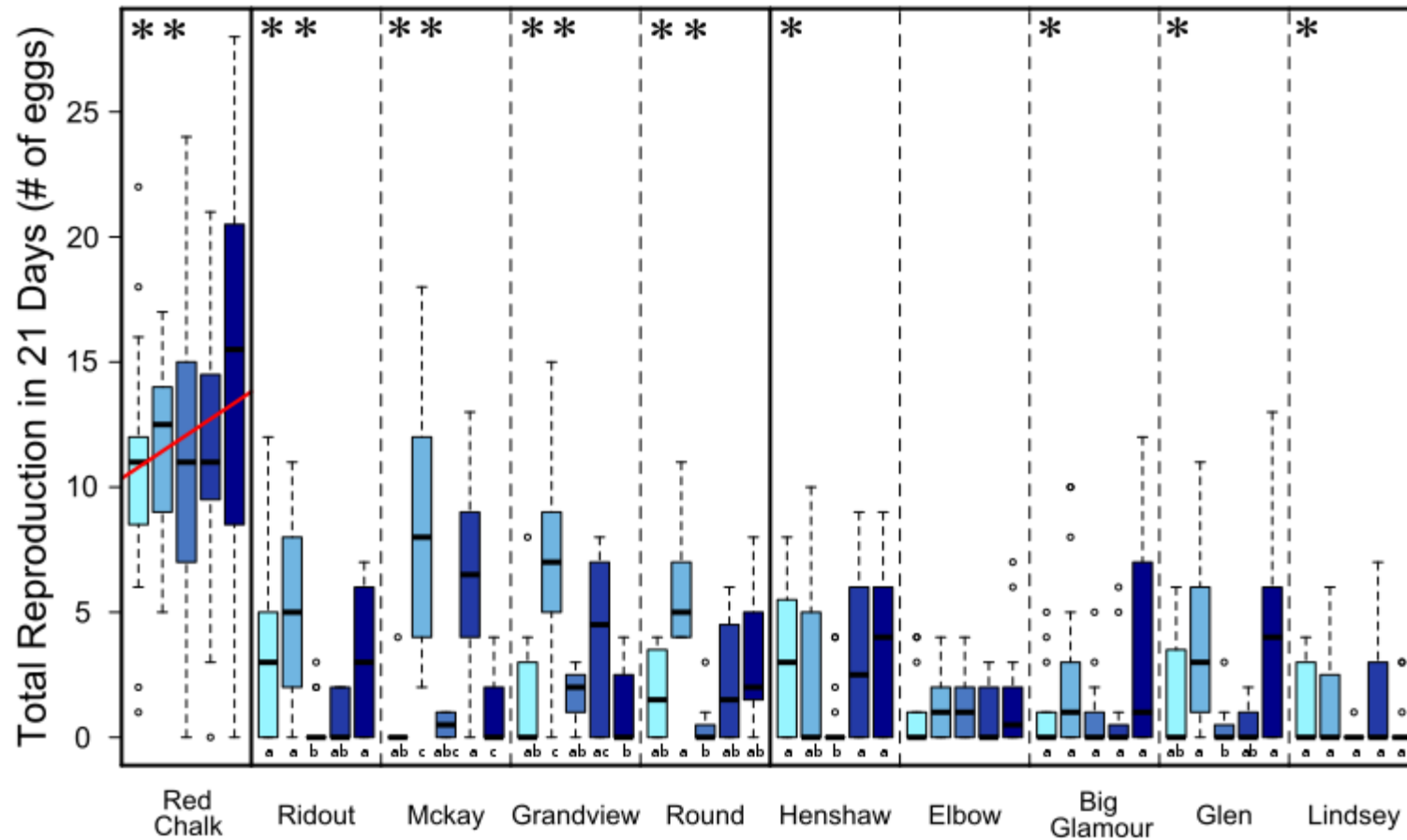


Figure 2.2. Total egg production in 21 days for separate iso-female lines of *Daphnia pulex* in calcium treatments. Calcium treatments are in order from lowest to highest calcium (0.7, 1.0, 1.5, 2.5, and 7.0 mg Ca L⁻¹) from left to right (light to dark colours). Iso-female lines are grouped by trends, then ordered by source-lake calcium concentration within trends; trends are separated by solid lines between graphs, with dotted lines separating iso-female lines graphs within trends. Boxplot horizontal lines indicate the median, with top and bottom edges of the box representing the first and third quartiles respectively. Graphs denoted with a red line shows a significant continuous effect of calcium treatment; graphs are indicated with a star in the top left corner if calcium treatment was a significant predictor of total egg production in 21-days (* p<0.05, ** p<0.01). Stats shown below plots are for within iso-female lines alone for iso-female lines with calcium as a significant categorical predictor.

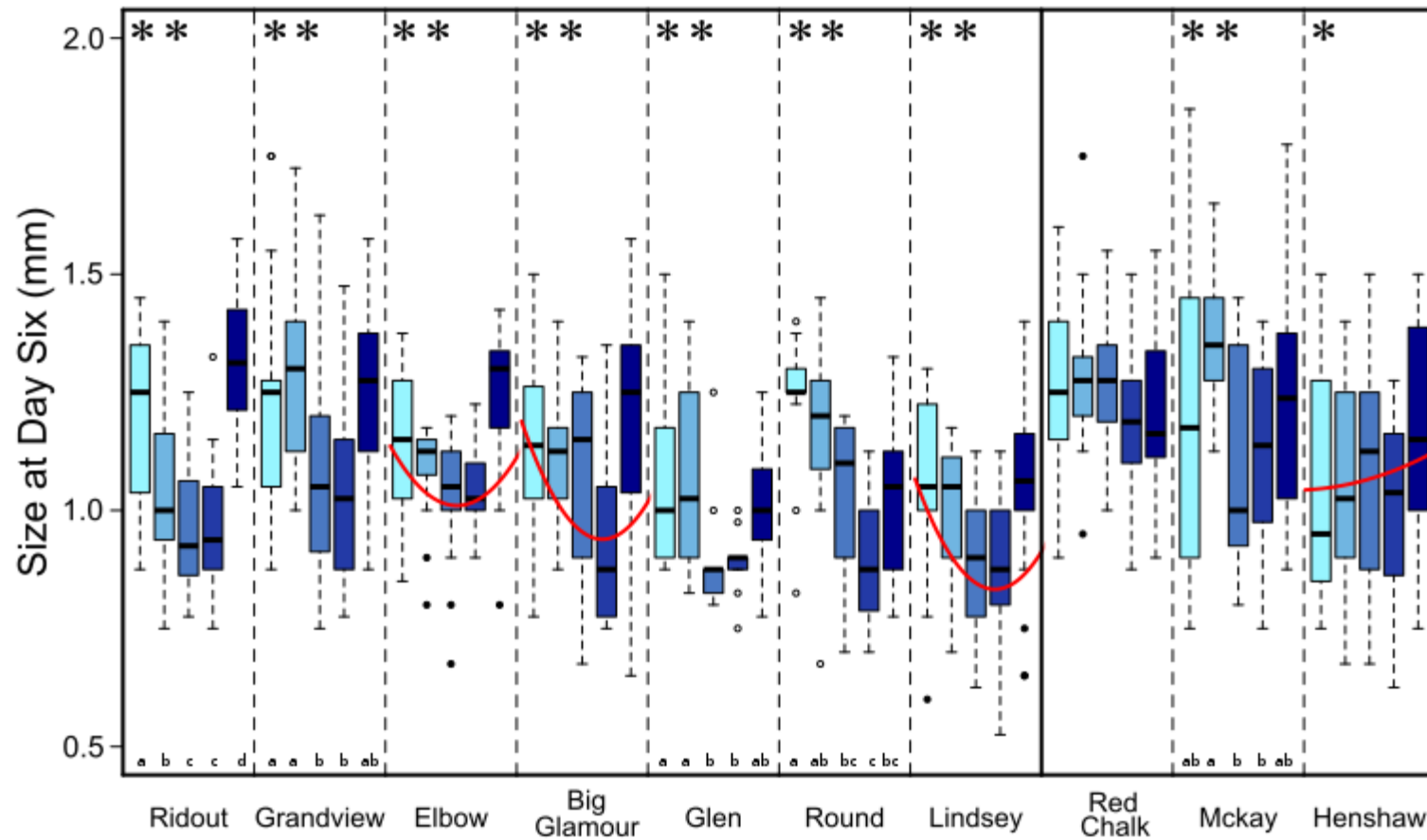


Figure 2.3. Size at day six (mm) for separate iso-female lines of *Daphnia pulex* in calcium treatments. Calcium treatments are in order from lowest to highest calcium (0.7, 1.0, 1.5, 2.5, and 7.0 mg Ca L⁻¹) from left to right (light to dark colours). Iso-female lines are grouped by trends, then ordered by source-lake calcium concentration within trends; trends are separated by solid lines between graphs, with dotted lines separating iso-female lines graphs within trends. Boxplot lines indicate the median, with top and bottom edges of the box representing the first and third quartiles respectively. Graphs denoted with a red line shows a significant continuous effect of calcium treatment; graphs are indicated with a star in the top left corner if calcium treatment was a significant predictor of size at day six (* p<0.05, ** p<0.01). Stats shown below plots are for within iso-female lines alone for iso-female lines with calcium as a significant categorical predictor.

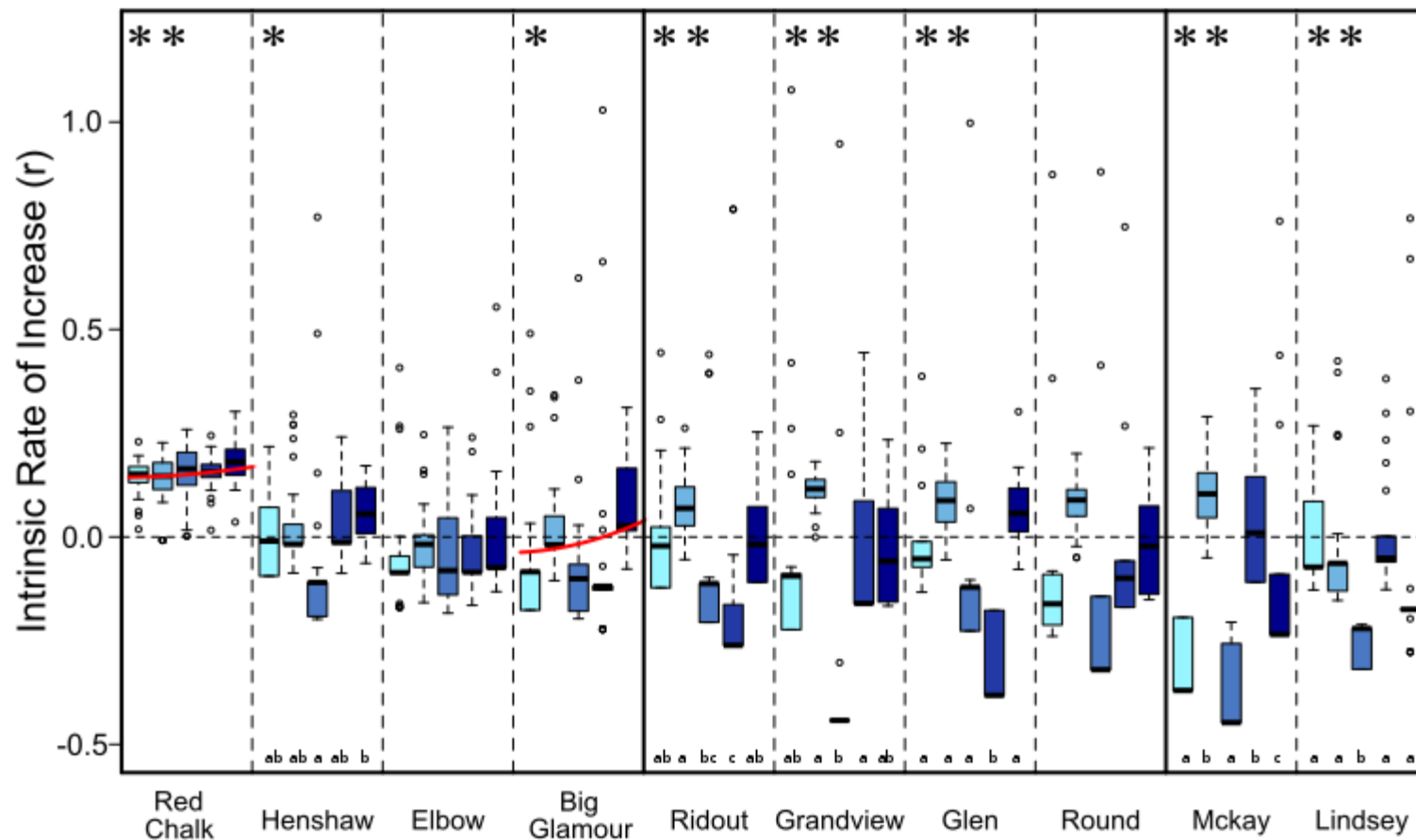


Figure 2.4. Intrinsic rate of increase (r) (day^{-1}) for separate iso-female lines of *Daphnia pulex* in calcium treatments. Calcium treatments are in order from lowest to highest calcium ($0.7, 1.0, 1.5, 2.5,$ and 7.0 mg Ca L^{-1}) from left to right (light to dark colours). Iso-female lines are grouped by trends, then ordered by source-lake calcium concentration within trends; trends are separated by solid lines between graphs, with dotted lines separating iso-female lines graphs within trends. Boxplot lines indicate the median, with top and bottom edges of the box representing the first and third quartiles respectively. Y-axis was truncated for visual presentation; a single point from 1.5 mg Ca/L in Grandview at $r = 2.1$ was excluded from the graph (but not from analyses). Graphs denoted with a red line shows a significant continuous effect of calcium treatment; graphs are indicated with a star in the top left corner if calcium treatment was a significant predictor of intrinsic rate or increase (* $p < 0.05$, ** $p < 0.01$). Stats shown below plots are for within iso-female lines alone for iso-female lines with calcium as a significant categorical predictor.































| | Ridout | Red Chalk | Mckay | Grandview | Henshaw | Elbow | Big Glamour | Glen | Round | Lindsey |
|--|---|---|---|--|---|---|---|---|---|---|
| Total Eggs |  |  |  |  |  |  |  |  |  |  |
| Size at Day Six |  |  |  |  |  |  |  |  |  |  |
| Intrinsic Rate of Population Increase (<i>r</i>) |  |  |  |  |  |  |  |  |  |  |

Figure 2.5. Stylized depiction of summarized calcium treatment trends within each iso-female line for three major responses in *Daphnia pulex*. Iso-female lines are ordered (left to right) based on lowest to highest source-lake calcium concentrations. Dotted trend lines refer to observed trends where calcium treatment was not a significant predictor. The trend with increased performance at 1.0mg Ca L⁻¹ (trend 2) is shown with a wavy line.

Chapter 3

General Discussion

Many lakes in south-central Ontario have recently experienced declines in lake-water calcium (Jeziorski et al. 2008, Palmer et al. 2011), with changes having negative impacts on *Daphnia* communities (Jeziorski et al. 2008, Jeziorski et al. 2012a, Jeziorski et al. 2015). While many studies have assessed the impacts on individual clones of single *Daphnia* sp. (Hessen et al. 2000, Ashforth and Yan 2008, Perez-Fuentetaja and Goodberry 2016), the effect of intra-specific variation has only been looked at in a single study (Rukke 2002b). With both media type (i.e. hard or soft water; Jesus et al. 2014) and food concentrations (Hessen et al. 2000, Ashforth and Yan 2008, Perez-Fuentetaja and Goodberry 2016) affecting the survival and reproduction of daphniids, it was deemed important to assess intra-specific variation in conditions that were common in our study region. To our knowledge, we are the first to test the presence of intra-specific variation in *Daphnia pulex* (sensu lato; Cristescu et al. 2012) to calcium decline in conditions commonly found in lakes of the Muskoka region. We predicted that a calcium effect would be present and that daphniids responses could vary by source-lake calcium concentration or iso-female line. Understanding what drives the variation within a species could allow for better predictions of how calcium decline will affect daphniids in the future.

To test our prediction, we exposed ten iso-female lines of *Daphnia pulex* to a gradient of five calcium concentrations. We found that intra-specific variation to calcium decline was present, and a large amount of within iso-female line variation was also present. Historical lake calcium concentration did not explain any variation in daphniid

life-history response to calcium, as low-calcium source lake individuals did not have higher performance in low calcium conditions, and response patterns were not grouped by source-lake concentration. Our results suggest that daphniids of the same species, and of the same genotypes, can have highly variable responses when tested in the same environmental conditions.

If variation is present in a system, it has been suggested to allow selection to occur and to favour adapted types (Loreau et al. 2003). In the case of our results, the large amount of variation present in the response of *Daphnia* to calcium decline may allow for adaptation to occur. With calcium expected to continue to decline in the future (Watmough and Aherne 2008, Reid and Watmough 2016), the ability of *Daphnia* to possibly adapt to low calcium conditions could help populations to persist. Walsh et al. (2016) suggests that adaptation processes could occur over few generations in *Daphnia*, and that the level of adaptation can vary among locations. Hochmuth et al. (2015) found this to be true, as lines of *D. magna* that had been exposed to either copper or zinc rapidly adapted. Even after daphniid lines were removed from the metal stress, and placed in control conditions, if adapted populations were again exposed to copper or zinc, they had a much higher reproductive rates than populations who had never had a prior exposure (Hochmuth et al. 2015). Populations of *D. longispina* that were exposed long term to copper stress due to pollution, were also found to be more resistant by having faster reproduction, higher survival, and better growth in high copper conditions (Agra et al. 2011). *Daphnia* even have been able to adapt to salinity changes (Weider and Hebert 1987) and mild temperature gradients (Geerts et al. 2014). With *Daphnia* adapting to other environmental stressors, the large amounts of variation in calcium response present

in our results could allow adaptation to exist in some populations of *Daphnia*, and help them to better cope with future decline in aqueous calcium.

As calcium concentrations are expected to continue declining (Watmough and Aherne 2008, Reid and Watmough 2016) the survival of *Daphnia* populations are of concern. *Holopedium*, a calcium-poor cladoceran, has been shown to out-compete *Daphnia* in low calcium conditions (Hessen et al. 1995), and has been increasing in abundance on the Canadian Shield (Jeziorski et al. 2015). This shift in cladoceran community structure could have negative impacts on the freshwater food webs, as *Holopedium* has a lower body content of phosphorus (Andersen and Hessen 1991), experience less predation pressure, and could cause reduced nutrients being transferred up the food chain (Jeziorski et al. 2015). *Bosmina*, which is a less efficient grazer of algae, has been showed to increase in abundance in lakes of Kings Country (Nova Scotia) causing concern for increased frequency of algae blooms (Korosi et al. 2012). If large variability is present within, and possibly among species, *Daphnia* populations may be able to adapt and survive to low calcium conditions and continue to be the primary grazers and food source for many aquatic invertebrates and fish. However, there are still a lot of aspects of the effects of calcium decline on *Daphnia* that we do not know.

Our results highlight the presence of variation in response to calcium decline in *D. pulex*, however we do not know what drives this variation. As we tested ten iso-female lines from ten different source lakes along a calcium concentration gradient, we tested to see if historical exposure to low calcium influenced individual response. As mentioned above, we found that historical source-lake calcium concentration had no detectable effect across our calcium concentration gradient. *Daphnia* from different source lakes did

not share common trends in their responses based on source-lake concentration.

Therefore, as we only tested for one possible method of variation and we do not know what the drivers in variation are to calcium decline.

From previous laboratory studies, we know that when in low calcium and low food conditions the survival and reproduction of *Daphnia* are greatly reduced (Hessen et al. 2000, Ashforth and Yan 2008, Perez-Fuentetaja and Goodberry 2016), however we do not fully understand how calcium declines interacts with other stressors. Freshwater systems face many challenges in lake systems, some examples being: invasive species, acidification (Palmer and Yan 2013), urbanization (Kozlowshi and Bondallaz 2013), and nutrient loading (Palmer et al. 2011). Calcium decline has negative effects on *Daphnia* life-history traits (Hessen et al. 2000, Rukke 2002b, Muysen et al. 2008, results here within), however we do not know how calcium will interact with other natural stressors. For example, the survival of a single clone of *D. magna* and *Daphnia tenebrosa* was greatly reduced in low calcium conditions under UV light stress (Hessen and Rukke 2000). As well in low calcium conditions, high temperatures affected the mobility and behaviour of *Daphnia* (Betini et al. 2016) as well as reducing both survival and reproduction (Ashforth and Yan 2008). Even though we have classified the Muskoka region as an area with low calcium, oligotrophic lakes, landscapes are known to be heterogeneous; with lakes ranging in their pH, dissolved organic carbon (DOC), conductivity, and alkalinity (CAISN, unpublished data). With so many variables changing from lake to lake within a region, the effect that calcium decline has on *Daphnia* populations could vary from lake to lake. Without fully understanding the

impacts that calcium has with other stressors we cannot fully understand the overall effects calcium decline will play on *Daphnia* across the landscape.

Conclusions and Future Directions

We found that intra-specific variation was present in the response of *Daphnia* to calcium decline. With future work assessing the effects of calcium decline on populations (variation in a single species from a common lake) and assessing variation within other species, we can start to better understand the full effects of calcium decline. With calcium decline reducing population growth rates of zooplankton communities (Arnott et al. 2017), understanding the variation present in response to this stressor on a community scale will allow for better predictions as to how communities will shift in the future and how large the impacts will be.

Summary

1. Calcium has been declining over the last few decades in many North American and northern European lakes. This decline has affected many crustacean species as calcium is a vital nutrient for growth and survival.
2. The response of *Daphnia*, a freshwater herbivore, to calcium decline is of concern as they are primary consumers and an important part of aquatic food webs. Studies have shown that low calcium conditions have reduced growth, reproduction, and survival in different *Daphnia* species. However, only one study has assessed whether variation exists to calcium decline within a species.
3. We set out to test whether the response to low calcium from ten iso-female lines collected from lakes with a range of calcium concentrations. To our knowledge we are the first to test the presence of intra-specific variation in *Daphnia pulex* under conditions typical of the Muskoka region (i.e. low food and a soft-water media). We hypothesized that a calcium effect would be present and may differ by (1) source-lake calcium concentration or (2) iso-female line.
4. To test our hypothesis we ran a 21 day life-history experiment on ten iso-female lines of *Daphnia pulex*. Daphniids were exposed to one of five calcium concentrations (0.7, 1.0, 1.5, 2.5, 7.0mg Ca L⁻¹) in a soft-water media (FLAMES) with low food. Survival and reproduction were recorded daily, with one size measurement taken at day six.
5. We found that intra-specific variation was present in calcium sensitivity and that the calcium response differed by iso-female line. We found no evidence of

adaptation to calcium decline, as daphniids from low calcium source lakes did not perform better in low calcium conditions.

6. We also found large amounts of variation present within iso-female lines, as identical individuals exposed to the same environmental conditions had large ranges in their response to low calcium.
7. Overall, we found that intra-specific variation exists to calcium decline, which may allow *Daphnia* to persist if tolerant populations exist across a landscape. Our results also highlight the need for experimental studies to include multiple genotypes/iso-female lines to fully understand the effects a stressor has on *Daphnia* spp.

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APPENDIX A: Top Model Relationship between Calcium Treatment and Iso-Female Lines

Table A.1. Model output and statistics on calcium treatment effect for total eggs of each iso-female line. Test abbreviations are: ANOD = analysis of deviance, ANOVA = analysis of variance and LRT = log-ratio test, model type abbreviations are: q-poisson = quasi-poisson, neg-bin = negative binomial, degrees of freedom abbreviations are: df_B= degrees of freedom between, df_W= degrees of freedom within. Test statistics are reported as deviance for LRT test and F-statistic for ANOD and ANOVA. Adjusted p-values used the Benjamini and Hochlberg (1995) method to control for false discovery rates. Iso-female lines are ordered by trends exhibited, defined by dotted lines, then source-lake calcium concentration within the trends.

| Line | Model Type | Test | n | R ² | φ | df _B | df _W | Statistic | p-adjusted |
|----------------|------------|-------|-----|----------------|------|-----------------|-----------------|-----------|------------|
| Red Chalk | linear | ANOVA | 113 | 0.06 | - | 1 | 111 | 8.72 | 0.008** |
| Ridout | q-poisson | ANOD | 66 | - | 2.95 | 4 | 62 | 6.44 | <0.001** |
| Mckay | q-poisson | ANOD | 44 | - | 2.91 | 4 | 39 | 10.77 | <0.001** |
| Grandview | neg-bin | LRT | 62 | - | 1.11 | 4 | 57 | 44.5 | <0.001** |
| Round | q-poisson | ANOD | 33 | - | 2 | 4 | 28 | 5.89 | 0.004** |
| Henshaw | q-poisson | ANOD | 91 | - | 3.31 | 4 | 86 | 3.65 | 0.013* |
| Elbow | linear | ANOVA | 77 | - | - | 4 | 72 | 0.54 | 0.707 |
| Big Glamour | q-poisson | ANOD | 81 | - | 4.21 | 4 | 76 | 3.38 | 0.017* |
| Glen | neg-bin | LRT | 41 | - | 0.88 | 4 | 36 | 11.62 | 0.022* |
| Lindsey | q-poisson | ANOD | 80 | - | 2.63 | 4 | 75 | 3.59 | 0.013* |

Table A.2. Model output and statistics on calcium treatment effect for size at day six of each iso-female line. Test abbreviations are: ANOVA = analysis of variance and LRT = log-ratio test, model type abbreviations are: q-poisson = quais-poisson, neg-bin = negative binomial, degrees of freedom abbreviations are: dfB= degrees of freedom between, dfW= degrees of freedom within. Test statistics are reported as deviance for LRT test and F-statistic for ANOVA. Iso-female lines with two lines are indicated with which predictor variable each statistic refers too in the case of a non-linear relationship (ca = calcium treatment). Adjusted p-values used the Benjamini and Hochlberg (1995) method to control for false discovery rates. Iso-female lines are ordered by trends exhibited, defined by dotted lines, then source-lake calcium concentration within the trends.

| Line | Model Type | Test | n | R ² | ϕ | df _B | df _W | Statistic | p-adjusted |
|-----------|------------|-------|-----|----------------|--------------------|-----------------|-----------------|-----------|------------|
| Ridout | neg-bin | LRT | 115 | - | 1.05 | 4 | 110 | 81.97 | <0.001** |
| Grandview | neg-bin | LRT | 114 | - | 1.08 | 4 | 109 | 28.81 | <0.001** |
| Elbow | linear | ANOVA | 95 | 0.27 | (ca) | 1 | 92 | 10.10 | 0.003** |
| | | | | | (ca ²) | 1 | 92 | 15.79 | <0.001** |
| Big | linear | ANOVA | 102 | 0.09 | (ca) | 1 | 99 | 10.08 | 0.002** |
| Glamour | | | | | (ca ²) | 1 | 99 | 11.28 | 0.003** |
| Glen | neg-bin | LRT | 56 | - | 1.16 | 4 | 51 | 16.90 | 0.004** |
| Round | linear | ANOVA | 64 | - | - | 4 | 59 | 7.97 | <0.001** |
| Lindsey | linear | ANOVA | 114 | 0.14 | (ca) | 1 | 111 | 16.04 | <0.001** |
| | | | | | (ca ²) | 1 | 111 | 18.36 | <0.001** |
| Red Chalk | linear | ANOVA | 122 | 0.04 | (ca) | 1 | 119 | 3.87 | 0.056 |
| | | | | | (ca ²) | 1 | 119 | 2.76 | 0.098 |
| Mckay | linear | ANOVA | 97 | - | - | 4 | 92 | 3.8 | 0.008** |
| Henshaw | linear | ANOVA | 121 | 0.03 | (ca ²) | 1 | 119 | 4.76 | 0.036* |

Table A.3. Model output and statistics on calcium treatment effect for intrinsic rate of natural increase (r) of each iso-female line. Test abbreviations ANOVA stands for analysis of variance, with degrees of freedom abbreviations being: df_B = degrees of freedom between, df_W = degrees of freedom within. Statistic for ANOVA reported as the F-statistic. Adjusted p-values used the Benjamini and Hochlberg (1995) method to control for false discovery rates. Iso-female lines are ordered by trends exhibited, defined by dotted lines, then source-lake calcium concentration within the trends.

| Line | Model Type | Test | n | R² | ϕ | df_B | df_W | Statistic | p-adjusted |
|-------------|-------------------|-------------|----------|----------------------|--------------------------|--------------------------|--------------------------|------------------|--------------------|
| Red Chalk | linear | ANOVA | 130 | 0.06 | - | 1 | 128 | 8.72 | <i>0.0063**</i> |
| Henshaw | linear | ANOVA | 130 | - | - | 4 | 125 | 3.03 | <i>0.025*</i> |
| Elbow | linear | ANOVA | 103 | 0.003 | - | 1 | 101 | 1.39 | 0.24 |
| Big Glamour | linear | ANOVA | 121 | 0.04 | - | 1 | 119 | 6.38 | <i>0.018*</i> |
| Ridout | linear | ANOVA | 130 | - | - | 4 | 125 | 6.20 | <i><0.001**</i> |
| Grandview | linear | ANOVA | 130 | - | - | 4 | 125 | 4.95 | <i>0.0019**</i> |
| Glen | linear | ANOVA | 67 | - | - | 4 | 62 | 10.33 | <i><0.001**</i> |
| Round | linear | ANOVA | 78 | - | - | 4 | 73 | 1.97 | 0.12 |
| Mckay | linear | ANOVA | 119 | - | - | 4 | 114 | 42.61 | <i><0.001**</i> |
| Lindsey | linear | ANOVA | 130 | - | - | 4 | 124 | 11.70 | <i><0.001**</i> |

APPENDIX B: Annotated R-code for Solving $\sum e^{-rx} l_x m_x = 1$ to find the Intrinsic Rate of Natural Increase (r)

The equation $\sum e^{-rx} l_x m_x = 1$, where l_x is the number of individuals surviving to time x , m_x the mean number of eggs produced until x , and e the natural logarithm, was solved to find the intrinsic rate of natural increase (r) with the following r-code in R-Studio Version 1.0.136 (RStudio team 2016). The ‘SummaryBy’ function was used in the ‘doBy’ v.4.5-15 package (Højsgaard and Halekoh 2016) in the primary loop to jackknife the data. Outputs from the jackknife were then run through a second series of loops until an accurate value of r was found (ie. sum of the equation was equal to 1 ± 0.0001). Jackknifing was done within each lake for each treatment, as well as within each lake alone and treatment alone ignoring any interactions to get accurate outputs for r. Below is an example of how the code works and how to use it with an example dataset.

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Secondary Contributors: James Sinclair and Caleb Yee

References

Højsgaard, S., and U. Halekoh. 2016. doBY: Groupwise Statistics, LSmeans, Linear Contrasts, Utilities. R package version 4.5-15. <https://CRAN.R-project.org/package=doBy>

RStudio Team. 2016. RStudio: Integrated Development for R. RStudio, Inc, Boston, MA.

<http://www.rstudio.com/>

Loading the dataset

```
#setwd(#location of dataset)

#jack.life <- read.csv(#dataset name)
```

Jack-knifing the dataset

When jack-knifing we want to find the sampling error around our value. In the jack-knifing technique we calculate average survivorship (lx) and reproduction (mx) for the population by removing each individual (with replacement). We repeat this until we have dropped every individual and have multiple sets of lx and mx for our population.

```
# make sure that your dataset is the correct length and that at least one factor has levels associated with it (i.e. if there are 5 calcium treatments, it needs levels 1:5)
# your dataset also must have a unique identifier for each individual in the population. We use these identifiers to drop out each individual to recalculate the little r value in the jackknife.
```

```
#example dataset would be. (see Table B.1. for full print out)
```

```
setwd("~/Masters/Experiment Data/Stats/Little R/Lakes Life Table")
jack.life <- read.csv("Example dataset.csv")
jack.life[c(1:5, 11:15),]
```

```
##  day colour  ca trt id lx mx
## 1  1 purple 0.5  1 p1 1 0
## 2  2 purple 0.5  1 p1 1 0
## 3  3 purple 0.5  1 p1 1 1
## 4  4 purple 0.5  1 p1 1 0
## 5  5 purple 0.5  1 p1 0 0
## 11 1  blue 0.5  1 b1 1 0
## 12 2  blue 0.5  1 b1 1 0
## 13 3  blue 0.5  1 b1 1 0
## 14 4  blue 0.5  1 b1 1 3
## 15 5  blue 0.5  1 b1 0 0
```

```
#here the calcium treatments are our levels and under the column 'trt' we see there is a numeric level associated with each treatment. In this created data set (i.e. fake data) we see there are 4 individuals in each treatment (2 treatments), 2 from purple and 2 from blue, and the experiment ran for 5 days.
```

```
library(doBy) #this package gives us the summaryBy command which lets us choose which dependant variables we want to average our response variables by.
```

```
#dependant variables for my example: ca + day
```

```
#response variables: lx + mx
```

```
# *NEED TO CHANGE*: you will need to change the names in the loop depending on your dependant variables.
```

```
# *NOTE*: the first set of lx and mx values are for the full dataset (the real r)
```

```
#e.g. if you have 25 individuals in each trt, you will end up with 26 (# of individuals plus the real r)
```

*# *NOTE*: remember the first set of 5 days (or how long your experiment went on for) for each trt will be the 'real' r values to calculate from (these will not need the pseudo-r equation applied to them at the end)*

```
(jack <- jack.life[1,]) #creating a dataset to add onto
```

```
## day colour ca trt id lx mx  
## 1 1 purple 0.5 1 p1 1 0
```

```
(jack2 <- jack.life[1,]) #again creating a second dataset to add onto
```

```
## day colour ca trt id lx mx  
## 1 1 purple 0.5 1 p1 1 0
```

```
(jack3 <- summaryBy(mx + lx ~ ca + day, data = jack, FUN = mean)) #creating the final template, what we want our dataset to look like in the end
```

```
## ca day mx.mean lx.mean  
## 1 0.5 1 0 1
```

```
for(i in 1:length(unique(jack.life$trt))){ #this first loop subsets the data by your levels (i.e. my calcium treatment, using my numeric identifier column 'trt')
```

```
jack <- rbind(subset(jack.life, jack.life$trt == i)) #going through the first time will subset out data whose 'trt' level is 1, with each loop it will take out each level to do the analysis on.
```

```
print(head(jack)) #will show us the head of the new dataset to make sure it subset it correctly  
print(list.life <- unique(jack$id)) #will print out all the unique individual ids so we can compare them to the jack-knife and confirm it is working. This also allows us to check if we have accidentally mislabelled one.
```

```
jack3 <- rbind(jack3, summaryBy(mx + lx ~ ca + day, data = jack, FUN = mean)) #this is the full set of data, will produce the real little r
```

```
for (j in 1:length(list.life)){ #jack-knifing, to the length of the individuals  
k <- (j-1) #to make it easier to add one every time without missing the first one  
ids <- list.life[k+1] #giving the id a value (first time through will be in position 1, second time through id will be the name of position two, i.e. the second individual)
```

```
jack2 <- rbind(subset(jack, jack$id != ids)) #subsetting the data and taking away the given individual (i.e. in first time through the id in the 1st position will be removed)
```

```
print(unique(jack2$id)) #printing the new set of ids to make sure we dropped one  
jack3 <- rbind(jack3, summaryBy(mx + lx ~ ca + day, data = jack2, FUN = mean)) #calculating the mx and lx with the 1st individual gone.
```

```
} #will repeat until all individuals have been dropped, use the printed ids to check that it is correctly dropping and re-inserting the individuals every time
```

```
} #will repeat for the number of trts I have
```

```
## day colour ca trt id lx mx  
## 1 1 purple 0.5 1 p1 1 0  
## 2 2 purple 0.5 1 p1 1 0  
## 3 3 purple 0.5 1 p1 1 1  
## 4 4 purple 0.5 1 p1 1 0  
## 5 5 purple 0.5 1 p1 0 0  
## 6 1 purple 0.5 1 p2 1 0  
## [1] p1 p2 b1 b2
```

```

## Levels: b1 b2 b3 b4 p1 p2 p3 p4 #here we can check the ids to make sure it is working
## [1] p2 b1 b2
## Levels: b1 b2 b3 b4 p1 p2 p3 p4
## [1] p1 b1 b2
## Levels: b1 b2 b3 b4 p1 p2 p3 p4
## [1] p1 p2 b2
## Levels: b1 b2 b3 b4 p1 p2 p3 p4
## [1] p1 p2 b1
## Levels: b1 b2 b3 b4 p1 p2 p3 p4
##   day colour ca trt id lx mx
## 21  1 purple 5  2 p3  1  0
## 22  2 purple 5  2 p3  1  0
## 23  3 purple 5  2 p3  1  3
## 24  4 purple 5  2 p3  1  0
## 25  5 purple 5  2 p3  1  2
## 26  1 purple 5  2 p4  1  0
## [1] p3 p4 b3 b4
## Levels: b1 b2 b3 b4 p1 p2 p3 p4
## [1] p4 b3 b4
## Levels: b1 b2 b3 b4 p1 p2 p3 p4
## [1] p3 b3 b4
## Levels: b1 b2 b3 b4 p1 p2 p3 p4
## [1] p3 p4 b4
## Levels: b1 b2 b3 b4 p1 p2 p3 p4
## [1] p3 p4 b3
## Levels: b1 b2 b3 b4 p1 p2 p3 p4

head(jack3) #the data set, take note there is an extra line at the top, this is as we made a template
it doesn't override when adding the new data

##   ca day mx.mean lx.mean
## 1 0.5  1  0.00  1.00
## 2 0.5  1  0.00  1.00
## 3 0.5  2  0.00  1.00
## 4 0.5  3  0.50  1.00
## 5 0.5  4  0.75  0.75
## 6 0.5  5  0.75  0.25

# NOTE - it ordered my colour column alphabetically

jack3 <- jack3[-1,] #deleting the template line
#NOTE: again the first set of in each trt is the data for the real r, DO NOT do a pseudo-r equation on this
jack3$lx.mx <- (jack3$mx)*(jack3$lx) #creating a lxm column to make it easier for the little r calculations

head(jack3)

##   ca day mx.mean lx.mean  lx.mx
## 2 0.5  1  0.00  1.00 0.0000
## 3 0.5  2  0.00  1.00 0.0000
## 4 0.5  3  0.50  1.00 0.5000

```

```
## 5 0.5 4 0.75 0.75 0.5625
## 6 0.5 5 0.75 0.25 0.1875
## 7 0.5 1 0.00 1.00 0.0000
```

*#if you want, you can store this dataset in your computer
#write.table(jack3,#location to store this dataset, sep="\t")*

#as an example, if I would also like to separate out the colours from one another, I can subset my data prior to entering the jack-knife loop by other variables and get more defined results. (i.e. here we could have input the purple and blue individuals separately to see changes in not only calcium treatment but changes in calcium treatment and colour), or I could add the variable to my summaryBy equation to separate them out.

Finding little r and the sampling error of little r

```
# Little R calculations
```

```
#setwd(#location of template data)
```

```
# or
```

```
#we will use jack3 made from above
```

#in this loop we need a template to put our results into, this should have the number of columns for each factor you have (i.e. trt is our column), then the number of rows should be the number of r values expected

```
# trt = 2
```

```
# #individuals in each trt = 4
```

```
# length of experiment = 5
```

```
# 5 for each trt (4 + the real r dataset) * 2 = 10 rows in the template
```

make sure you label each row with the proper factor A (trt) this will make it easy to compare when we pull from the dataset

```
#jack 3 = 5*5*2 = 50 rows going into this loop
```

```
# read in your empty template
```

```
setwd("~/Masters/Experiment Data/Stats/Little R/Lakes Life Table")
```

```
life.table <- read.csv("example.template.csv")
```

```
head(life.table) # see Table B.2 for full template
```

```
## trt
```

```
## 1 1
```

```
## 2 1
```

```
## 3 1
```

```
## 4 1
```

```
## 5 1
```

```
## 6 2
```

```
View(life.table) #double check the length is correct
```

Adding the empty columns to fill during the loop, add as many columns of factors that you need for your given dataset. Make sure they are all the same length

```

life.table$trt <- rep(0, times = length(life.table$trt))
# here we repeat out factor columns to make sure that when we output the data it is pulling the correct line. This is a good way to check in the end that you have the correct number of combinations as if the pre-set factor columns don't match ones pulled from the loop something is wrong.

# adding the columns for the results to be put into
life.table$ro <- rep(0, times = length(life.table$trt)) #using the length of an already present column
life.table$sum <- rep(0, times = length(life.table$trt))

#this is what the template should look like
head(life.table)

## trt etrt ro sum
## 1 1 0 0 0
## 2 1 0 0 0
## 3 1 0 0 0
## 4 1 0 0 0
## 5 1 0 0 0
## 6 2 0 0 0

#re-naming the dataset
rcalc <-jack3 #pulled the dataset from above

head(rcalc)

## ca day mx.mean lx.mean lx.mx
## 2 0.5 1 0.00 1.00 0.0000
## 3 0.5 2 0.00 1.00 0.0000
## 4 0.5 3 0.50 1.00 0.5000
## 5 0.5 4 0.75 0.75 0.5625
## 6 0.5 5 0.75 0.25 0.1875
## 7 0.5 1 0.00 1.00 0.0000

for(j in 1:(nrow(rcalc)/length(unique(rcalc$day)))){ #number of sets that I have, see above in template
k <- (j-1)*length(unique(rcalc$day)) #gets me to each set of the length of my experiment, make sure this length is the number of days your experiment went on.

#if your experiment is 31 days make sure the length (unique(rcalc$day)) equals that number

r0 <- 0.05 #random estimate of r, you can vary this depending on if you think you population did well or not
r <- 0 #setting up the variable, we want an empty variable to fill
erxlxmx<-0 #see above

for (i in 1:length(unique(rcalc$day))) { #for length of experiment : **NEED TO CHECK**
#for within the first set of 5, we want to calculate r, we estimate the r, see the sum we get, then estimate it again and compare the sums. We do this until when we minus 1 from each the value is 0 we know the sum is 1 and the r is correct, Follow below

rcalc$erxlxmx[i+k]<-exp(-r0*rcalc$day[i+k])*rcalc$lx.mx[i+k] #using the estimate term we create one side of the equation  $erx * lx * mx$  for each day of the experiment

```

```

}
result0 <- sum(rcalc$erlxxm[(k+1):(k+length(unique(rcalc$day)))] #we then sum all the values
and put the result into result0

ifelse(result0>1,r <- r0+0.0001,r <- r0-0.0001) #if the sum (result0) is greater than 1, add the above
value to r0, if it is less then minus that. (this will allow us to get the sum of the values closer to 1)

repeat{ #getting another r to compare to previous r, using the r values from above
  erlxxm<-0 #setting up an empty vector again
  for (i in 1:length(unique(rcalc$day))) { #same as above
    rcalc$erlxxm[i+k]<-exp(-r*rcalc$day[i+k])*rcalc$lx.mx[i+k]
  }

  result <- sum(rcalc$erlxxm[(k+1):(k+length(unique(rcalc$day)))] #same as above
  #here we have another sum, we are using the new r suggested from the ifelse statement above (addition or subtraction from the r in the first loop, depending on if the sum was greater than or less than 1)

  if(abs(result-1)>=abs(result0-1)){break} #break allows an escape from the loop if previous r provided a better sum (i.e. sum closer to 1)
  #if this isn't the case the loop results will be printed

  print(c("Loop Result=",result )) #see if the program is running
  r0<- r #set my result r to new r0 to guess with for next iteration, now we will restart back at the first r loop and do it again until the sums are equal to 1, gives us another comparison

  result0<- result #set result so it matches with my new r0

  ifelse(result>1,r<- r+0.0001,r<- r-0.0001) #again since it wasn't 1 we add or subtract depending on which way we need the r to go, this will go back to the second loop and allow us to compare the sums.
  #this helps us catch if the sum goes to high or too low, i.e. if we are at sum = 1.0009, then go to sum = 9.998 we went too far, and it will cause the loop to stop. It makes sure that the r values is as accurate as possible without going over
}

print(c(r0,result0,r,result)) #printing the final results if the loop breaks
#for these next steps you will have to correctly label the column names of your dataset
life.table$setrt[j] <- rcalc$scf[k+1] #pulling the factorA allows us to check we have the correct number of rows in our template
life.table$ro[j] <- r0
life.table$sum[j] <- result0

} #will repeat for each combination

example of loop results output while running

## [1] "Loop Result=" "1.00311443330386"
## [1] "Loop Result=" "1.00274119399856"
## [1] "Loop Result=" "1.00236809837352"

```

```
## [1] "Loop Result=" "1.00199514637158"
## [1] "Loop Result=" "1.00162233793557"
## [1] "Loop Result=" "1.00124967300836"
## [1] "Loop Result=" "1.00087715153284"
## [1] "Loop Result=" "1.00050477345192"
## [1] "Loop Result=" "1.00013253870855"
## [1] 0.0597000 1.0001325 0.0598000 0.9997604
```

*#can store the dataset in computer
#write.table(life.table,#location to store dataset, sep="\t")*

head(life.table) *#the final results*

```
## trt etrt      ro      sum
## 1  1 0.5 5.970000e-02 1.0001325
## 2  1 0.5 7.220000e-02 1.0001850
## 3  1 0.5 1.285000e-01 1.0001272
## 4  1 0.5 -3.987425e-16 1.0000000
## 5  1 0.5 8.240000e-02 1.0001306
## 6  2 5.0 4.768000e-01 0.9998256
```

#make sure to check that the pulled factorA from the loop (i.e. columns efactorA), match the pre-s et columns from the template. This is a good way to check that you have to correct number of combinations.

*****!!!NOTE!!!****: this loop does not give an error if there is no reproduction for that set of 5. I t will calculate an incorrect 1. Make sure you check the sum values and if any are equal to 0, th at set of 5 days had no reproduction. You should delete that r as it is incorrect.*

Again as mentioned above, the first r in each trt will be the real r. All following are pseudo r's. You must apply the equation below to those values to get the estimated r's.

#ps.r= pseudo r values

#r.r = real r

#n = number of individuals in that group (i.e. trt, factor A)

*#estimated r = n * r.r - (n - 1) * ps.r*

I did this equation in excel as it was easier.

Table B.1. Example Dataset for R-code

| day | colour | ca | trt | id | lx | mx |
|-----|--------|-----|-----|----|----|----|
| 1 | purple | 0.5 | 1 | p1 | 1 | 0 |
| 2 | purple | 0.5 | 1 | p1 | 1 | 0 |
| 3 | purple | 0.5 | 1 | p1 | 1 | 1 |
| 4 | purple | 0.5 | 1 | p1 | 1 | 0 |
| 5 | purple | 0.5 | 1 | p1 | 0 | 0 |
| 1 | purple | 0.5 | 1 | p2 | 1 | 0 |
| 2 | purple | 0.5 | 1 | p2 | 1 | 0 |
| 3 | purple | 0.5 | 1 | p2 | 1 | 1 |
| 4 | purple | 0.5 | 1 | p2 | 0 | 0 |
| 5 | purple | 0.5 | 1 | p2 | 0 | 0 |
| 1 | blue | 0.5 | 1 | b1 | 1 | 0 |
| 2 | blue | 0.5 | 1 | b1 | 1 | 0 |
| 3 | blue | 0.5 | 1 | b1 | 1 | 0 |
| 4 | blue | 0.5 | 1 | b1 | 1 | 3 |
| 5 | blue | 0.5 | 1 | b1 | 0 | 0 |
| 1 | blue | 0.5 | 1 | b2 | 1 | 0 |
| 2 | blue | 0.5 | 1 | b2 | 1 | 0 |
| 3 | blue | 0.5 | 1 | b2 | 1 | 0 |
| 4 | blue | 0.5 | 1 | b2 | 1 | 0 |
| 5 | blue | 0.5 | 1 | b2 | 1 | 3 |
| 1 | purple | 5 | 2 | p3 | 1 | 0 |
| 2 | purple | 5 | 2 | p3 | 1 | 0 |
| 3 | purple | 5 | 2 | p3 | 1 | 3 |
| 4 | purple | 5 | 2 | p3 | 1 | 0 |
| 5 | purple | 5 | 2 | p3 | 1 | 2 |
| 1 | purple | 5 | 2 | p4 | 1 | 0 |
| 2 | purple | 5 | 2 | p4 | 1 | 0 |
| 3 | purple | 5 | 2 | p4 | 1 | 0 |
| 4 | purple | 5 | 2 | p4 | 1 | 5 |
| 5 | purple | 5 | 2 | p4 | 1 | 0 |
| 1 | blue | 5 | 2 | b3 | 1 | 0 |
| 2 | blue | 5 | 2 | b3 | 1 | 0 |
| 3 | blue | 5 | 2 | b3 | 1 | 0 |
| 4 | blue | 5 | 2 | b3 | 1 | 6 |
| 5 | blue | 5 | 2 | b3 | 1 | 0 |
| 1 | blue | 5 | 2 | b4 | 1 | 0 |
| 2 | blue | 5 | 2 | b4 | 1 | 0 |
| 3 | blue | 5 | 2 | b4 | 1 | 4 |
| 4 | blue | 5 | 2 | b4 | 1 | 0 |
| 5 | blue | 5 | 2 | b4 | 0 | 8 |

Table B.2. Example Template

| trt | blank row |
|-----|--------------|
| 1 | |
| 1 | |
| 1 | |
| 1 | |
| 1 | |
| 2 | |
| 2 | |
| 2 | |
| 2 | |
| 2 | |

APPENDIX C: Additional Reproductive Responses

Age at first reproduction and size of first clutch were analyzed as described in the ‘statistical analysis’ of the ‘Methods’ section. Variables did not show obvious trends across iso-female lines, and could not be easily described. Therefore results were added into the following appendix

Age at Reproduction

The effect of calcium treatment on age at first reproduction was found to vary among iso-female lines (ANOVA, $n=416$, $F_{36, 366}=2.88$, $p<0.001$, Table C.1). Age at first reproduction increased as calcium concentration increased for some iso-female lines, whereas for others age at first reproduction was found to decrease or even show no obvious visual trend (Appendix C, Figure C.1).

Size of Clutch

The size of first clutch was also found to be influenced within each iso-female line by calcium treatment (ANOVA, $n=415$, $F_{4, 405}=2.93$, $p=0.021$, Table C.2). Size of first clutch increased as calcium concentration increased for some iso-female lines, whereas for others size of first clutch was found to decrease, while in others still there was no obvious visual trend (Appendix C, Figure C.2). The mean size of first clutch was found to be 0.86 neonates (± 0.16 SE) higher in the Muskoka region compared to the Frontenac region (ANOVA, $n=415$, $F_{1, 413}=30.63$, $p<0.0001$).

Table C.1. Model output and statistics on calcium treatment effect for age at first reproduction of each iso-female line. Test abbreviations are: LRT = log-ratio test and ANOVA = analysis of variance, degrees of freedom abbreviations are: dfB= degrees of freedom between, dfW= degrees of freedom within. Test statistics are reported as deviance for LRT test and the F-statistic for ANOVA. Iso-female lines with two lines are indicated with which predictor variable each statistic refers too in the case of a non-linear relationship (ca = calcium treatment). Adjusted p-values used the Benjamini and Hochlberg (1995) method to control for false discovery rates. Iso-female lines are ordered by source-lake calcium concentration from lowest to highest (top to bottom).

| Line | Model Type | Test | n | R² | φ | df_B | df_W | Statistic | p-adjusted |
|-------------|-------------------|-------------|----------|----------------------|--------------------|-----------------------|-----------------------|------------------|-------------------|
| Ridout | linear | ANOVA | 38 | - | - | 4 | 33 | 5.94 | 0.004** |
| Red Chalk | linear | ANOVA | 109 | - | - | 4 | 104 | 0.26 | 0.902 |
| Mckay | linear | ANOVA | 31 | 0.22 | (ca) | 2 | 28 | 2.46 | 0.056 |
| | | | | | (ca ²) | 2 | 28 | 1.92 | 0.118 |
| Grandview | linear | ANOVA | 44 | 0.17 | (ca ²) | 1 | 42 | 9.84 | 0.002** |
| Henshaw | linear | ANOVA | 49 | - | - | 4 | 44 | 1.57 | 0.270 |
| Elbow | poisson | LRT | 40 | - | 0.66 | 1 | 38 | 2.23 | 0.057 |
| Big Glamour | linear | ANOVA | 34 | - | - | 4 | 33 | 7.41 | 0.001** |
| Glen | linear | ANOVA | 23 | - | - | 4 | 18 | 1.74 | 0.270 |
| Round | linear | ANOVA | 23 | - | - | 4 | 18 | 1.59 | 0.270 |
| Lindsey | linear | ANOVA | 25 | - | - | 4 | 20 | 1.42 | 0.291 |

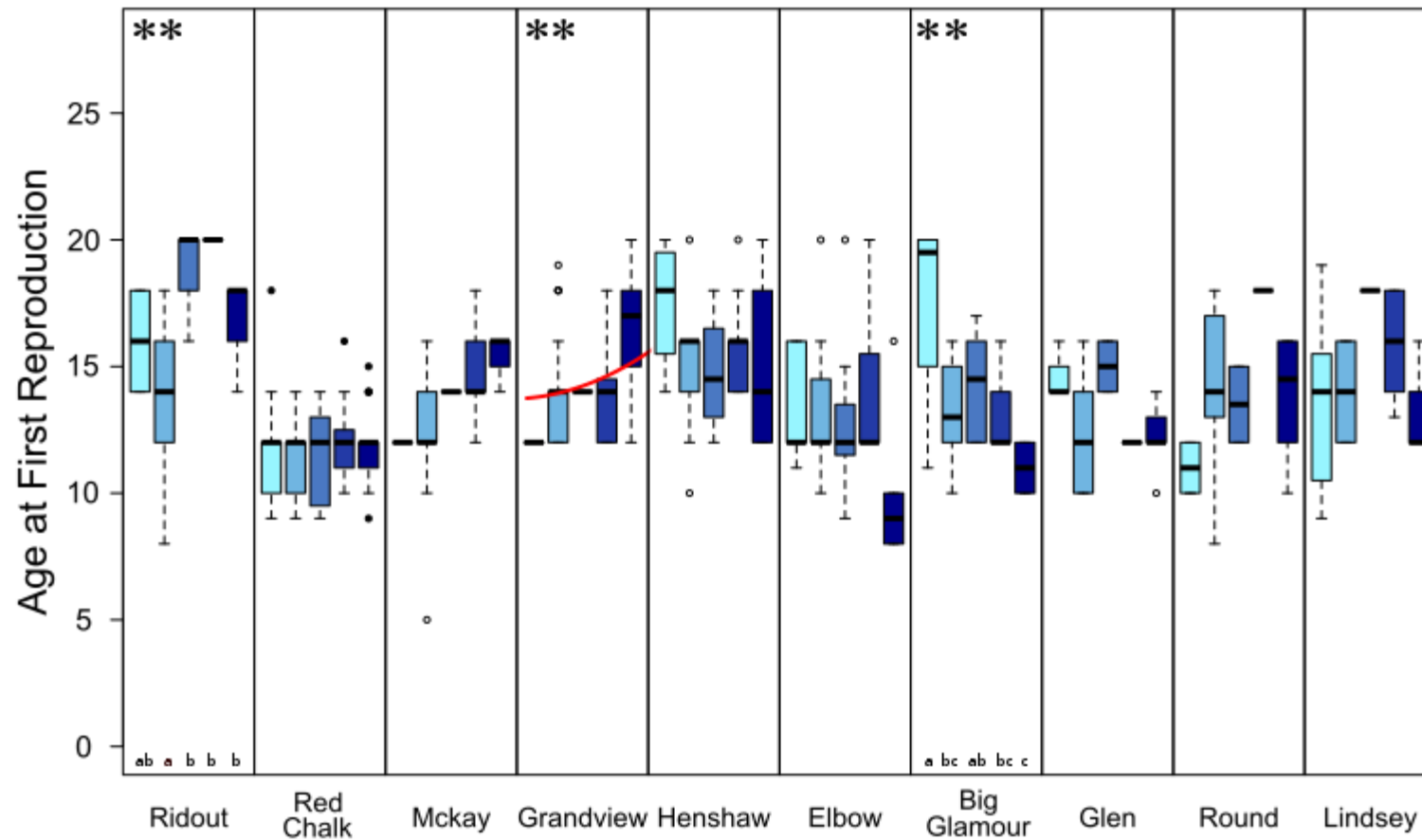


Figure C.1. Age at first reproduction (in days) for separate iso-female lines of *Daphnia pulex* and calcium treatments. Calcium treatments are in order from lowest to highest calcium (0.7, 1.0, 1.5, 2.5, and 7.0 mg Ca/L) from left to right (light to dark colours). Iso-female lines are ordered by source-lake calcium concentration from left to right. Boxplot lines indicate the median, with top and bottom edges of the box representing the first and third quartiles respectively. Graphs denoted with a red line shows a significant continuous effect of calcium treatment; graphs are indicated with a star in the top left corner if calcium treatment was a significant predictor of age at first reproduction (* $p < 0.05$, ** $p < 0.01$). Stats shown below plots are for within iso-female lines alone, for iso-female lines with calcium as a significant categorical predictor.

Table C.2. Model output and statistics on calcium treatment effect for size of first clutch of each iso-female line. ANOVA = analysis of variance, degrees of freedom abbreviations are: dfB= degrees of freedom between, dfW= degrees of freedom within. Test statistics are reported as F-statistic for the ANOVA. Iso-female lines with two lines are indicated with which predictor variable each statistic refers too in the case of a non-linear relationship (ca = calcium treatment). Adjusted p-values used the Benjamini and Hochlberg (1995) method to control for false discovery rates. Iso-female lines are ordered by source-lake calcium concentration from lowest to highest (top to bottom).

| Line | Model Type | Test | n | R² | ϕ | df_B | df_W | Statistic | p-adjusted |
|----------------|-------------------|-------------|----------|----------------------|--------------------|-----------------------|-----------------------|------------------|-------------------|
| Ridout | linear | ANOVA | 38 | 0.29 | (ca ²) | 1 | 36 | 16.37 | 0.002** |
| Red Chalk | linear | ANOVA | 109 | - | - | 4 | 104 | 5.53 | 0.002** |
| Mckay | linear | ANOVA | 31 | - | - | 4 | 26 | 1.66 | 0.475 |
| Grandview | linear | ANOVA | 44 | - | - | 4 | 39 | 3.70 | 0.040* |
| Henshaw | linear | ANOVA | 49 | - | - | 4 | 44 | 0.55 | 0.943 |
| Elbow | linear | ANOVA | 40 | - | - | 4 | 35 | 0.28 | 0.943 |
| Big Glamour | linear | ANOVA | 33 | - | - | 4 | 28 | 0.28 | 0.943 |
| Glen | linear | ANOVA | 23 | - | - | 4 | 18 | 1.17 | 0.629 |
| Round | linear | ANOVA | 23 | - | - | 4 | 18 | 0.19 | 0.943 |
| Lindsey | linear | ANOVA | 25 | - | - | 4 | 20 | 1.11 | 0.629 |

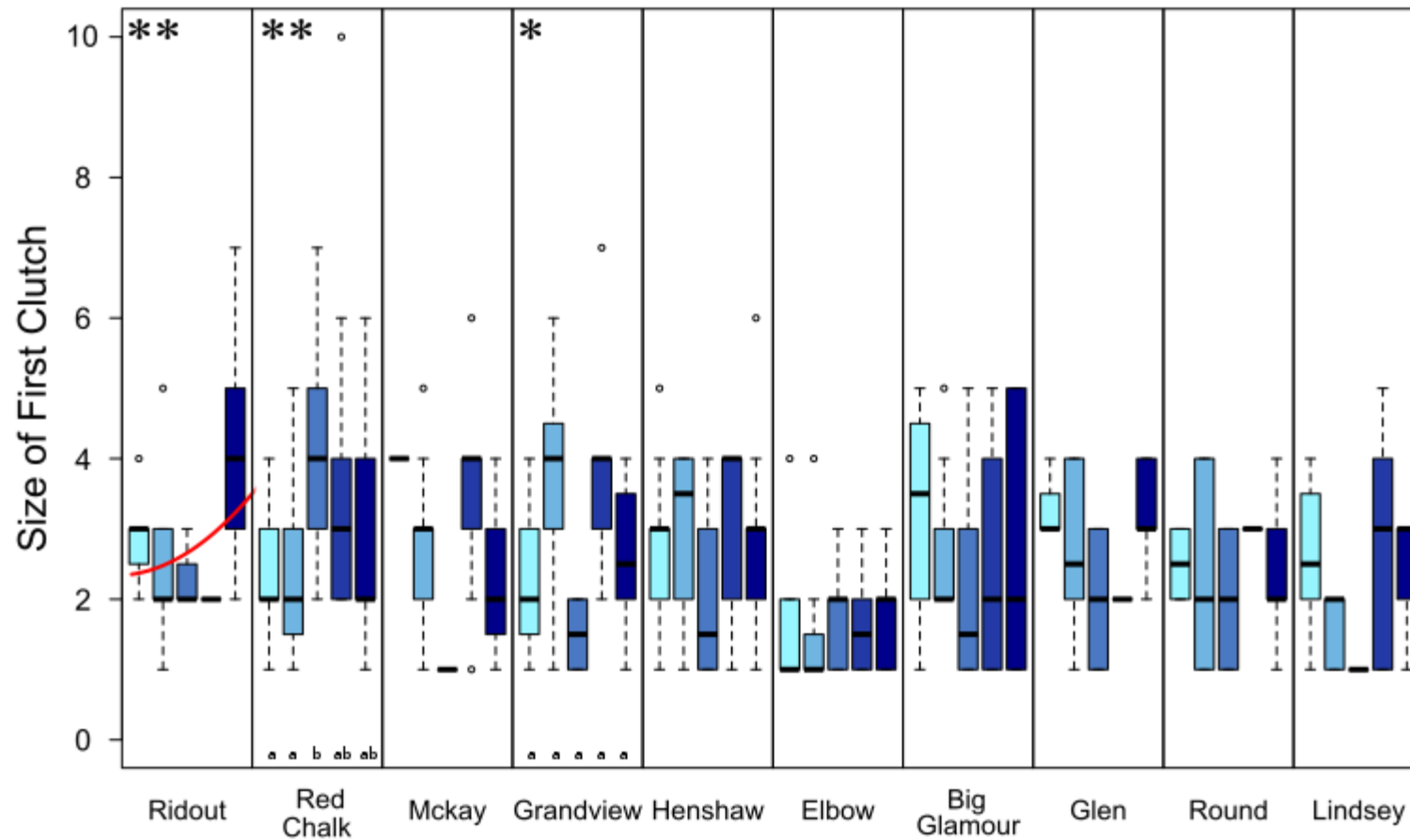


Figure C.2. Size of first clutch (in # of neonates) for separate iso-female lines for *Daphnia pulex* and calcium treatments. Calcium treatments are in order from lowest to highest calcium (0.7, 1.0, 1.5, 2.5, and 7.0 mg Ca/L) from left to right (light to dark colours). Iso-female lines are ordered by source-lake calcium concentration. Boxplot lines indicate the median, with top and bottom edges of the box representing the first and third quartiles respectively. Graphs denoted with a red line shows a significant continuous effect of calcium treatment; graphs are indicated with a star in the top left corner if calcium treatment was a significant predictor of size of first clutch (* $p < 0.05$, ** $p < 0.01$). Stats shown below plots are for within iso-female lines alone, for iso-female lines with calcium as a significant categorical predictor.