Variable Sensitivity of Growth Rate to Calcium Limitation and Sodium Chloride Toxicity Tolerance Among Populations of *Daphnia pulicaria* from Kawartha Highland Lakes

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ABSTRACT

Variable Sensitivity of Growth Rate to Calcium Limitation and Sodium Chloride Toxicity Tolerance Among Populations of *Daphnia pulicaria* from Kawartha Highland Lakes

Sarah Katelyn Cook

Calcium (Ca) limitation and sodium chloride (NaCl) toxicity tolerance are potential controllers of *Daphnia* populations. *D. pulicaria* neonates collected from 14 Kawartha Highland lakes were exposed to 6 Ca concentrations (0.5-5.0 mg Ca L^{-1}) over 6 days. We did not find differences in maximum mass-specific growth rate or half-maximum thresholds between source lakes. Growth rates were reduced <1.5 mg Ca L^{-1}, and there was a significant interaction between Ca and source lake lineage. We also compared log-logistic NaCl dosage models estimating 48-hour lethal concentration $(LC)_{10}$, LC_{25}, and LC_{50} by exposing 9 clones to 10 concentrations, 18-2700 mg Cl L^{-1}. Survivorship decreased >1500 mg NaCl L^{-1}, and there were differences between lake lineage LC_{50} estimations. This suggests our *D. pulicaria* genotypes may not have experienced extreme enough selection pressure from either stressor to develop local adaptation, but allele variability between lakes may explain differences in NaCl toxicity and Ca limitation responses.

Keywords: Zooplankton, *Daphnia pulicaria*, Kawartha Highlands, Ontario, Freshwater, Mass Specific Growth Rate, Lethal Concentration, Calcium, Road Salt, Sodium Chloride, Log-Logistic Modelling, Non-linear Modelling

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DEDICATION

For my family, always my strongest supporters, who may not understand everything in this thesis but see the work and effort all the same.

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LIST OF ABBREVIATIONS AND SYMBOLS

AICc – Akaike Information Criterion with correction for small sample sizes (<40:1; samples: model parameter ratio)

ANS – Anstruther Lake

BIG – Big Cedar Lake

C – Carbon

Ca – Calcium

CaCl – Calcium Chloride

$CaCO_3$ - Calcium Carbonate

CAT – Catchacoma Lake

CCME - Canadian Council of Ministers of the Environment

CI – Confidence Interval

Cl – Chloride

°C – Degrees Celsius

Df – Degrees of freedom

ECCC – Environment and Climate Change Canada

EEL – Eels Lake

FLAMES – Field Laboratory for the Assessment of Multiple Ecological Stressors

H_2 – Dihydrogen

KAS – Kasshabog Lake

KWH – Kawartha Highlands

LC – Lethal Concentration Value (mg L^{-1})

LCI – Lower Confidence Interval (2.5%)

LON – Long Lake

LOO – Loon Call Lake

LOU – Loucks Lake

LSS – Least Sum of Squares

MIS – Mississauga Lake

Mg – Magnesium

MSGR – Mass Specific Growth Rate (d^{-1})

n – Sample Size

N – Nitrogen

NaCl – Sodium Chloride

P – Phosphorus

PEN – Pencil Lake

RAC – Raccoon Lake

RSS – Residual Sum of Squares

SAL – Salmon Lake

SE – Standard Error

SD- Standard Deviation

SO_2 – Sulfur Dioxide

SPC – Specific Conductivity (µS/cm)

SUC – Sucker Lake

UCI – Upper Confidence Interval (97.5%)

WOL – Wolf Lake

WQC – Trent University Water Quality Centre

GLOSSARY

Anthropogenic- originating from human activity or human-made material, usually in the context of pollution or environmental changes.

Holm-Bonferroni Correction for Multiple Comparisons- referred to as Holm-Bonferroni correction, a method used in statistical hypothesis testing to adjust for multiple comparisons. As the probability for Type 1 errors (false positive) increases with number of comparisons, this sequentially adjusts the significance level (alpha) for each individual test. Used when the number of comparative groups are high and is less conservative than standard Bonferroni correction.

Lethal Concentration (LC)- the estimated concentration of a substance (e.g. NaCl) needed to kill a specific percentage of a population. E.g. LC_{50} is the concentration of a substance estimated to kill 50% of the *Daphnia* population.

Mass Specific Growth Rate (MSGR)- the rate of the natural logarithm of an individual *Daphnia*'s mass increase, relative to its initial mass, over a period of time. This allows for a proportional comparison of growth across different initial neonate sizes.

Parthenogenesis/Parthenogenic- a form of asexual reproduction where offspring develop from unfertilized eggs and are female clones of the mother.

Phenotypic Plasticity- when an organism can display a range of different trait presentations, either physical or metabolic, in response to environmental conditions. Particularly, the ability for different *Daphnia* individuals to express different phenotypes in response to environmental stimuli despite having the same underlying genotype (e.g. are clones of each other).

Salinization- the accumulation of salts in soil or water, increasing overall salt concentration.

Chapter 1. General Introduction

Human activity has left a lasting impact on both terrestrial and aquatic ecosystems across North America. This impact has been created by a myriad of environmental stressors including invasive species, alterations to nutrient cycling through anthropogenic point sourcing (Carpenter et al., 1992; Carpenter et al., 2011) and changes to global climate systems. The onset of lake chemistry changes and forest damage (Likens et al., 1996), associated in part with widespread lake acidification (Schindler, 1997), led to public concern and regulatory responses based on the findings of several federal, university, and industry led research and monitoring programs. These regulatory responses were driven by legislation that included the *Clean Air Act* of 1970 and *Eastern Canada Acid Rain Program* of 1985. The primary aim was to reduce acid rain correlated emissions, sulphur dioxide (SO_2) and nitrogen oxides (Likens et al., 1996; Stoddard et al., 1999; Jeffries et al., 2003). Since the introduction of these regulations, SO_2 emissions have been significantly reduced at most Ontario research sites (Jeffries et al., 2003). Recovery of lakes has been found to be site-specific with some significant time lags (Stoddard et al., 1999; Jeffries et al., 2003).

Researchers have proposed that watershed acidification and recovery has strongly affected ion cycling and has led to declines of critical aqueous cations such as calcium (Ca) in recent decades (Stoddard et al., 1999; Keller et al., 2001; Jeffries et al., 2003; Weyhenmeyer et al., 2019). Many interacting biotic and abiotic factors may have contributed to recent trends of declining Ca. For example, acid rain increased aqueous cation release and caused ionic balance shifts before 1970 (Likens et al., 1996), slow-release sources like watershed soil and rockbound Ca were depleted by acid rain erosion

(Tomlinson, 2003; Jeziorski and Smol, 2017), and logging removed sequestered Ca stores from forests preventing its re-entry to the local nutrient cycle (Likens et al., 1996; Watmough et al., 2003).

A consequence of Ca decline in soft water lakes where concentrations are already low, such as in the Canadian Shield (Jeziorski and Smol, 2017), are changes to zooplankton community assemblages (Desellas et al., 2008; Shapeira et al., 2012; Jeziorski et al., 2015; Azan and Arnott, 2018). Recent studies of zooplankton communities indicated a risk to biodiversity, productivity, population stability, and growth rates (Shapiera et al., 2012; Azan & Arnott, 2018; Huang et al., 2022) as Ca availability declines. This may have far reaching impacts on higher trophic levels, nutrient cycling rates, and algal bloom control. In Ontario, Sudbury lakes have experienced a Ca decline of >2 mg L^{-1} over 20 years and are 46-67% lower than pre-industrial concentrations (Keller et al., 2001; Keller et al., 2019). Similar patterns have been observed in the nearby Muskoka River Watershed (Reid & Watmough, 2016).

During the same period, shifts in Cladoceran relative abundance away from *Daphnia* and copepods toward smaller, less nutritious species have been seen in Ca-declining European soft water lakes (Bergström et al., 2024). *Daphnia* play a vital role as model organisms for environmental monitoring and assessment, particularly for large scale collaborations due to the world-wide distribution of various species and species complexes within the genus (Ebert 2022). In Ontario, there have been reports of increasing *Holopedium glacialis* abundance and decreasing *Daphnia longispina* since the 1970's (Redmond et al., 2016). *Daphnia* have been the focus of many previous Ca decline studies due to their Ca-rich body composition and relatively high Ca needs (Cairns & Yan, 2009).

Ca decline is a critical stressor to both zooplankton assemblages and individual species populations that may leave them more vulnerable to environmental co-stressors such as sodium chloride (NaCl) toxicity. Road salt, usually in the form of NaCl, is integral to road winter management strategies in the province of Ontario. Since road salt use has increased post-1950's, there have been significant consequences to many freshwater ecosystems exhibiting increased salinization (Dugan et al., 2017). One comprehensive study examined North American lakes with 10+ years of ecological data and found 44% of study lakes had undergone salinization. High chloride acted as a significant indicator and warning signal of recent salinization (Dugan et al., 2017). Canada uses more than 5 million tonnes of road salt annually, broadly regulated by environmental assessments. These regulations were based on recognition of NaCl road salts as a toxic substance under the *Canadian Environmental Protection Act* in 1999 (Environment Canada, 2022). Current Canadian Water Quality Guidelines recommend keeping long-term exposure below 120 mg Cl L^{-1} and acute exposure below 640 mg Cl L^{-1} for the protection of aquatic life (Canadian Council of Ministers of the Environment, 2011).

In Ontario, chloride trends are variable and correlate with proximity to human presence and urban development. Undisturbed watersheds exhibit decreasing Cl concentrations since the 1970s and lakes near cities or areas of high human activity having increasing Cl (Yao et al., 2020). While south-central Ontarian lakes are generally low Cl, recent studies indicate that the current guidelines may be too high for aquatic life to be safe under long-term exposure (Brown & Yan, 2015; Arnott et al., 2020). Concern for the protection of freshwater ecosystems deepens as population growth coupled with climate change is projected to increase total use of road salts into potentially harmful local concentrations (Yao et al., 2020).

Salinization greater than 1000 mg Cl L^{-1} has been shown to reduce primary producers like filamentous algae, have sublethal effects on Cladocerans, and to shift zooplankton assemblages due to variable toxicity tolerances (Hintz et al., 2017; Valleau et al., 2020). *Daphnia* sourced from soft water Canadian Shield lakes have high sensitivity to Cl with decreased reproductive potential and increased mortality in as low as 5-40 mg Cl L^{-1} (Arnott et al., 2020), far below federal guidelines. Despite this, there is some evidence that evolved tolerance to salinization may emerge quickly (e.g., after 20-30 generations) in *Daphnia* populations (Coldsnow et al., 2017; Hintz et al., 2018). This indicates that the past conditions of *Daphnia* source lakes could have an impact on their sensitivity to NaCl stress and performance in lab-based experiments. Co-stressors like food limitation have been shown to decrease the tolerance of *Daphnia* to salinity (LC_{50} = 55–248 mg Cl L^{-1}) (Brown & Yan, 2015). It is possible that calcium limitation may similarly predispose *Daphnia* populations to greater sub-lethal NaCl toxic effects.

The Kawartha Highlands Provincial Park and surrounding area (herein referred to as KWH or Kawartha Highlands region) encompasses an area >400 km² containing over 300 freshwater lakes and rivers in south-central Ontario, north of Peterborough. Environmental monitoring of these lakes has been ongoing (unpublished Frost, Trent University survey data, 2015-2025), however extensive research into locally sourced *Daphnia* stress responses has been limited. The goal of this research was investigate the potential impact of Ca and NaCl interactions on locally souced *Daphnia* in the KWH region by developing a baseline understanding of both calcium limitation response and sodium chloride tolerance. This may offer greater applicability to lake management than lab-sourced *Daphnia* experimentation, and assist in predicting population changes if KWH conditions decline.

Chapter 2. Variable Sensitivity of Growth Rate to Calcium Limitation among Populations of *Daphnia pulicaria* from Kawartha Highland Lakes

Introduction

Calcium (Ca) is the backbone mineral of aquatic invertebrates, used to create and strengthen exoskeletons through biomineralization which is essential for growth, survival, and protection. The effects of low Ca concentrations in freshwater ecosystems on zooplankton communities, with particular interest in the response of herbivorous *Daphnia* species, have been extensively studied (Ashforth and Yan, 2008; Tan and Wang, 2009; Shapeira et al., 2012; Prater et al., 2016; Huang et al., 2022). Following reports of declining Ca, these studies have found shifts in relative and absolute abundances of zooplankton taxa (Redmond et al., 2016), changes to population dynamics of Ca-sensitive species (Riessen et al., 2012), and shifts in zooplankton species composition toward *Holopedium* and smaller Cladocerans (Bergström et al., 2024).

Zooplankton community responses to declining Ca concentrations are consistent with expectations based on their presumed Ca requirements. Among zooplankton, *Daphnia* have high-Ca body content, 2-8% dry weight. (Muyssen et al., 2009; Tan and Wang, 2009). In comparison, other zooplankton taxa have lower Ca requirements. For example, the gelatinous Cladoceran *Holopedium* has a body Ca content of 0.2-0.4% dry weight (Tan and Wang, 2009; Jeziorski et al., 2015). Elevated body Ca in *Daphnia* translates into an elevated vulnerability to Ca malnutrition in lake water. For example, *D. magna* and *D. pulex* have a reported minimum requirement of 1.5 mg Ca L^{-1} when food and temperature conditions are optimal. Survival is usually reduced below 0.5 mg Ca L^{-1} after 15 days (Ashforth and Yan, 2008). Sub-lethal effects of calcium limitation include delayed growth, maturation, reproduction (Ashforth and Yan, 2008; Cairns and Yan, 2009; Akbar et al., 2017) and diminished phenotypic plasticity to form protective structures in response to predator cues (Riessen et al., 2012). Low water Ca concentrations are especially problematic for *Daphnia* because their primary uptake pathway is through aqueous transport (Tan and Wang, 2009). Ca that has been acquired by *Daphnia* is primarily used for body structure with 72-92% of body Ca locked in the carapace (Cairns and Yan, 2009). Frequent moulting and a lack of Ca storage structures causes a regular loss of ~90% of body Ca during the moulting process (Cairns and Yan, 2009). All together these factors lead *Daphnia* to rely heavily on readily available environmental Ca.

It has been suggested that minimum Ca requirements of *Daphnia* vary within and among species in the genus and their lake of origin (Tan and Wang, 2009; Jeziorski and Smol, 2016). Significant intra-specific differences have been reported in the survival thresholds between *D. galeata* sourced from soft water (2-3 mg Ca L^{-1}) vs hardwater (>10 mg Ca L^{-1}) lakes (Tan and Wang, 2009; Rukke, 2002). This variable sensitivity may not be universal, as conflicting results were reported by Azan and Arnott (2018), who found no differences in *Daphnia* low calcium response from different lakes in a community-level experiment.

In this chapter, we examine whether there is variable sensitivity to low Ca supply among *Daphnia* clones originating from different lakes. To do so, we tested the effect of Ca limitation on mass specific growth rates (MSGR) of *D. pulicaria* clone populations sourced from 14 lakes within the Kawartha Highlands, Ontario region. We predicted that the tolerance of *D. pulicaria* to low Ca concentrations would differ among lakes. Specifically, we expected *Daphnia* from low average Ca lakes, measured from 2015-2023, to grow faster at lower Ca concentrations than those from high Ca lakes. These differences would imply genotypic differences exist among these clones, presumably from varying degrees of selection pressure on Ca-use efficiency. We also investigated if different environmental Ca concentrations among lakes in the recent past explain differences between growth curves. An additional goal of investigating the Ca sensitivity of local KWH *Daphnia* populations is to see if their Ca-limited responses are comparable to other generalized Ontario populations reported in literature.

Methods

Study Sites

This study was conducted on iso-female parthenogenic *D. pulicaria* clone populations collected April-June 2023 from 14 lakes (Table 1) in the KWH region located in south-central Ontario, Canada.

Daphnia Collection and Establishing Source Lake Iso-Mother Populations

Daphnia were collected at each lake's deepest point using 3 draws of a plankton tow net lowered 5 m into the water column. Once returned to the lab, zooplankton assemblages were sorted. Visually identified *Daphnia spp.* were acclimated to lab conditions for 48 hrs in a 50:50 dilution of filtered lake water and COMBO (Kilham et al. 1998). After this acclimation, all animals were transferred and housed in COMBO media. *Daphnia* cultures were monitored for approximately 7 days to assess their ability to survive and reproduce under lab conditions. From surviving lake-specific populations, single females were randomly selected to parthenogenetically source an iso-female test population representative of each source lake. This resulted in 14 lake-specific iso-female populations, which were raised for up to 8 months before experimental trials began (culturing details below).

Table 1. Collection and experimental conditions for each of the iso-mother *D. pulicaria* clone culture lines collected in lakes of the Kawartha Highlands in south-central Ontario, Canada. Shown are lake name, experimental ID, date of iso-female clone line collection, coordinates, mean depth, surface area, average lake calcium concentration with standard deviation (measured years 2015; 2018-2023), and *n* representing day 6 surviving neonate number per experiment trial (max 3 trials; 36 per trial). † Sucker lake was performed in triplicate during its first trial as a procedural test run, resulting in greater *n* than other lakes.

Lake	ID Code	Date (in 2023)	Coordinates	Mean Depth (m)	Surface area (ha)	Average Lake Ca (mg/L) ± SD	*n*
Anstruther	ANS	20 June	44°44” N, 78°13” W	12.6	639.5	5.08 ± 1.33	36, 35, 34
Big Cedar	BIG	28 April	44°36” N, 78°10” W	5.8	222.6	28.89 ± 3.46	36, 35, 32
Catchacoma	CAT	21 June	44°44” N, 78°19” W	19.5	707.4	6.04 ± 0.54	35, 35, 33
Eels	EEL	22 June	44°53” N, 78°09” W	6.0	936.0	7.64 ± 0.50	33, NA, NA
Kasshabog	KAS	22 June	44°38” N, 77°57” W	3.51	997.9	7.75 ± 0.58	36, 35, 30
Long	LON	20 June	44°41” N, 78°09” W	9.4	96.7	4.36 ± 0.33	33, NA, NA
Loon Call	LOO	20 June	44°44” N, 78°08” W	5.6	84.3	7.56 ± 1.31	34, 35, 36

Loucks	LOU	20 June	44°40” N, 78°13” W	7.5	34.0	4.12 ± 1.44	35, 32, NA
Mississauga	MIS	21 June	44°42” N, 78°19” W	15.9	672.4	6.33 ± 0.46	34, 36, 35
Pencil	PEN	21 June	44°48” N, 78°20” W	7.2	90.8	15.34 ± 1.16	32, 36, 34
Raccoon	RAC	22 June	44°36” N, 78°11” W	3.3	50.4	17.99 ± 2.16	35, 36, 32
Salmon	SAL	21 June	44°49” N, 78°26” W	12.5	174.6	27.79 ± 2.05	36, 36, 35
Sucker	SUC	23 June	44°45” N, 78°15” W	8.0	155.0	2.72 ± 0.45	98 †, 34, 34
Wolf	WOL	20 June	44°44” N, 78°10” W	4.8	138.4	5.66 ± 0.64	34, 29, NA

D. pulicaria was verified as the target species as opposed to its visually identical sister species *D. pulex* using DNA extraction, PCR, digestion, and agarose gel electrophoresis with an adapted and modified cleaved amplified polymorphic sequence (CAPS) protocol (S. Arnott, personal communication, 2023). Details can be found in Appendix B and C.

Algae and Daphnia Culturing

Algal 'food' source *Tetradesmus obliquus* (formerly *Scenedesmus obliquus*; Canadian Physiological Culture Centre strain 10) was grown in-lab in multiple semicontinuous culture flasks diluted daily to maintain phosphorus-rich media and support continuous growth. Harvested cells were centrifuged (5000 x *g*) for 10 min and resuspended in N, P, and Ca-free COMBO media (Kilham et al. 1998). Concentrated algal 'food' was then stored for up to 2 weeks at 4°C. Elemental carbon content of algae was estimated by first filtering algae onto pre-weighed GF/C glass fiber filters and dried for a minimum of 2 hrs at 60 °C. Carbon content was assumed to be 50% of total algal cell mass and used to calculate a mg C mL^{-1} of 'food' used to feed *Daphnia* brood mothers in excess of 6 mg C $L^{-1}day^{-1}$.

To produce experimental animals, on the day before each experiment, trial neonates were removed from culture jars. The next morning, we collected <24 h old neonates that were born in the mother's third to sixth brood. We placed them within Ca-free COMBO media until transfer into experimental media, rinsing with Ca-free media to remove Ca carry-over from brood mother COMBO.

Growth Experiment

Methodology generally followed protocols previously used to study calcium and phosphorus limitation (Prater et al., 2016). We manipulated aqueous Ca availability during a 6-d growth experiment. On day 0, neonates were provided dietary carbon from algal food totalling 3 mg C L^{-1}. This was increased to 6 mg C L^{-1} on day 3 along with refreshed experimental media to maintain relatively constant Ca exposure concentrations. Media Ca concentrations (0.5 mg L^{-1}, 1 mg L^{-1}, 1.5 mg L^{-1}, 2 mg L^{-1}, 3 mg L^{-1}, 5 mg L^{-1}) were manipulated by adjusting the amount of CaCl added to culture COMBO. Ca concentrations were chosen to encompass the range we expected calcium limitation to be present based on previously reported negative effects below 1.5 mg Ca L^{-1} and the biologically relevant concentrations expected in Canadian Shield lakes in south-central Ontario (Huang et al., 2022, Weyhenmeyer et al., 2019).

For individual growth experiments, neonates were rinsed with Ca-free COMBO media and 36 individuals were randomly assigned to 40 mL falcon tubes, 6 individuals per Ca availability level. Samples of experimental growth media were saved in falcon tubes and later analyzed to quantify true calcium concentration using an Agilent 8900 ICP-QQQ-MS, measured in MS/MS mode with a H_2 cell gas. The NRC SLRS-6 (River Water Certified Reference Material for Trace Metals) was used for quality assurance.

Initial neonate mass (*n* = 3 of 20 pooled individuals) was measured from dried samples using a microbalance and was included in MSGR calculations along with 6 d final juvenile dried masses. After 6 d of growth, all animals from individual falcon tubes were placed into pre-weighed aluminum cups, dried at 60°C for at least 24 h, and weighed on a microbalance to determine individual post-treatment body mass. MSGR values were calculated assuming nonlinear growth within this age period:

$$\mu = \frac{(\ln \mathrm{m6} - \ln \mathrm{m0})}{t}$$

where μ is the mass-specific growth rate, m_6 is a daphnid's day 6 dry mass, m_0 is the average day 0 dry mass of pooled neonates (n = 20), and *t* is the experiment duration in days. Experiments were performed in triplicate for a final pooled sample 36 individuals per target treatment concentration.

Statistical Analysis

For all statistical tests the significant result threshold was $\alpha = 0.05$. P values were adjusted using Holm-Bonferroni methods where multiple comparisons were appropriate, which controls for family-wise error rate while retaining more statistical power than standard Bonferroni correction (Holm, 1979; Armstrong, 2014; Jafari & Ansari-Pour, 2018). Correction was deemed appropriate for analyzing bootstrap parameter results where t 14 pairwise comparisons were repeated 400 times using data subset with replacement. Further, we did not encounter the problem of further reduced power when analyzing multiple response variables (Nakagawa, 2004) as we focused only on one, MSGR. This increased the chance of false-positive significance (Cabin & Mitchell, 2000) compared to the general linear model post-hoc testing, which only compared all 14 lakes pairwise once. Though there is no formal consensus for when p-value correction should be used, Bonferroni corrections are used often in *Daphnia* research and ecology as a broader field for comparing multiple treatment groups or unique source groups (Flaherty & Dodson, 2005; Giardini et al., 2015; Pérez-Fuentetaja & Goodberry, 2016). All analyses were performed using R Statistical Software (v4.3.1; R Core Team 2023).

Effect of Calcium and Source Lake

The overall effect of treatment Ca concentration, source lake, and their interaction as predictor variables were tested using a general linear model. Raw MSGR data were transformed by squaring values to ensure homoscedasticity and improve normality of residuals. The model with the lowest AICc and a relevant ΔAICc > 2 score (Appendix F, Table F1) included both Ca concentration (continuous) and source lake (categorical) as predictor variables ($\alpha = 0.05$) (Table 2), as well as an interaction term. A Levene's test was used to meet the assumption of approximately equal variance on the dependant variable ($\alpha = 0.05$; F value = 0.749; p value = 0.71). The model explains ~87.4% of data distribution. A Two-way ANOVA was used to determine if at least one group MSGR^2 mean was statistically different as Ca increased and between source lakes (Table 3). Differences in MSGR^2 values between lake groups (Table 4) were evaluated *post-hoc* using Tukey's Honestly Significant Difference (HSD) using the pairs function of the emmeans package (Lenth, 2023).

Calcium Limitation Response Curves

MSGRs were fit with treatment Ca concentration following Monod-like (1949) growth kinetics:

$$\mu = \mu_{max} \frac{Ca}{(Ca + K_s)}$$

where μ is the predicted mass-specific growth rate, μ_{max} is the maximum mass-specific growth rate, Ca is the calcium treatment concentration, and K_s is the calcium concentration threshold at which growth rate is one half maximum (half-saturation constant). A precedence for using an asymptotic non-linear model approach for *Daphnia* growth can be found in other studies (Sperfeld & Wacker, 2011; Martin-Creuzburg et al., 2014; Sikora et al., 2016). Estimates of μ_{max} and K_s were determined using a nonlinear Least Sum of Squares (LSS) function from the nlstools R package (v2.1; Baty et al. 2015). For all parameters the lower bound was fit to 0, as negative Ca concentrations, growth rates, and K_s values would be biologically impossible for this experimental design and neonate age range. Estimates were reported with their corresponding 95% confidence intervals.

Table 2. Output from a general linear model testing the effects of overall calcium concentration, source lake, and an interaction between both variables on MSGR. Calcium concentration has been included as a continuous numeric variable and source lake as a categorical variable. Significant predictor coefficients do not exceed $\alpha = 0.05$.

General Linear Formula: $MSGR^2$ ~ Ca. Concentration * Source Lake

Residual standard error: 0.054 on 1263 degrees of freedom

Adjusted R-squared: 0.874

F statistic: 319.4 on 28 and 1263 degrees of freedom

Model p value: < 0.001

Coefficient	Estimate	Standard Error	t value	p value
Ca Concentration	0.008	0.004	2.050	0.041
Lake - ANS	0.142	0.010	14.277	0.000
Lake - BIG	0.043	0.010	4.277	0.000
Lake - CAT	0.104	0.010	10.205	0.000
Lake - EEL	0.117	0.019	6.252	0.000
Lake - KAS	0.048	0.010	4.692	0.000
Lake - LON	0.081	0.019	4.343	0.000
Lake - LOO	0.122	0.010	12.217	0.000
Lake - LOU	0.090	0.013	7.137	0.000
Lake - MIS	0.135	0.010	13.331	0.000
Lake - PEN	0.092	0.011	8.650	0.000
Lake - RAC	0.111	0.010	11.023	0.000
Lake - SAL	0.090	0.010	8.708	0.000

Lake - SUC	0.138	0.008	17.641	0.000
Lake - WOL	0.094	0.014	6.752	0.000
Ca: BIG	0.021	0.006	3.494	0.000
Ca: CAT	0.011	0.005	2.020	0.044
Ca: EEL	-0.002	0.008	-0.295	0.768
Ca: KAS	0.019	0.006	3.267	0.001
Ca: LON	0.015	0.009	1.690	0.091
Ca: LOO	-0.001	0.006	-0.247	0.805
Ca: LOU	0.018	0.007	2.586	0.010
Ca: MIS	0.001	0.006	0.268	0.789
Ca: PEN	0.013	0.006	2.276	0.023
Ca: RAC	0.006	0.006	1.130	0.259
Ca: SAL	0.016	0.006	2.842	0.005
Ca: SUC	0.005	0.005	1.100	0.271
Ca: WOL	0.010	0.007	1.406	0.160

Nonlinear model fits were tested by calculating AICc scores and comparing 3 parameter (*Ca,* K_s*,* μ_{max}) candidate models, referred herein as 'A' models, and 4 parameter (*Ca,* K_s*,* μ_{max}, μ_{zero}) candidate models, referred herein as 'B' models. Some studies have reported more accurate models by implementing a μ_{zero} parameter to represent neonate growth at time 0 of the experiment, which could be at any age >24 hours old (Sperfeld & Wacker, 2011; Martin-Creuzburg et al., 2014; Sikora et al., 2016). To test if this parameter would improve K_s and μ_{max} estimations for our dataset, ΔAICc values and their weights were calculated between 'A' and 'B' models. If the ΔAICc values were within 2 AICc units of each other, neither model was considered a relevantly better fit to the data and the preferred model defaulted to the less complex 3 parameter 'A' model. If the ΔAICc value was greater than 2 AICc units, the model with the lowest AICc score was preferentially selected as it represented the best balance between accuracy and parameter simplicity. If the 'B' model had the preferred AICc score, but its parameter estimates were biologically questionable (e.g. exceeding the Ca testing gradient), this was considered an 'overfit' model. For these exceptions analysis defaulted to the 'A' model (See Appendix F, Table F3 for AICc and ΔAICc scores. See Results: *MSGR Growth Curve Model* for description of exceptions).

To statistically compare μ_{max} and K_s threshold estimations respective to each source-lake clone, we used bootstrap methods to randomly draw the same number of replicates as in the experimental design (n = 3) from 1000 simulated distributions. The nonlinear model was then fit to the subset distribution. This was done for each source lake group and compared using Wilcoxon Rank-Sum testing (Mann & Whitney, 1947) with Holm-Bonferroni correction for multiple comparisons (Holm, 1979; Jafari & Ansari-

Pour, 2018). Following similar protocol used in Sperfeld & Wacker (2011) and Martin-Creuzburg et al. (2014), this procedure was repeated 400 times, and the proportion of 'successful' tests ($p < 0.05$) was reported. Correlation between 2023 collection year Ca concentration, mean 2015; 2018-2023 Ca concentration, and each source-lake's respective K_s was evaluated using Spearman's correlation between paired samples in the stats package of base R (v4.3.1; R Core Team 2023).

Results

Species Verification

D. pulicaria populations originated from 14 lakes that varied in morphology and chemistry and were chosen to represent a gradient of soft water calcium concentrations present within the Kawartha Highlands, ON region. All lake-specific populations of *D. pulicaria* were genetically confirmed via diagnostic Cleaved Amplified Polymorphic Sequence (LdhA-F 5'-CCACGGCTGGATTATCGGAG-3'; LdhA-R 5'-ATTGCCCATGAGGTGTAACCC-3') gel electrophoresis. We confirmed the test populations were neither the visually identical *D. pulex* or a species-complex hybrid. See Appendix B and C for supplementary information.

Effect of Treatment Calcium Concentration

Measured calcium concentration and source lake were significant predictors of MSGR values, where greater calcium concentrations resulted in higher MSGR values until the maximum growth rate had been reached (Figure 1, Table 2). After this point, growth rates plateaued. Ca concentration predicted a change of 0.089 MSGR day $^{-1}$ for every 1 mg Ca L^{-1} increase. An interaction between Ca and Source Lake was found (ANOVA; Table 3. Df = 13, F value = 3.023, p value < 0.001) with each lake having a unique interaction with Ca resulting in different lake-specific slopes. This contributed to significant differences in MSGR between source lake groups (ANOVA; Table 3. Df = 13, F value = 103.16; p value < 0.001), and as Ca concentration increased (ANOVA; Table 3. Df = 1, F value = 7458.77; p value < 0.001).

Differences in MSGR values were found between several lakes analyzed using Tukey's HSD (Table 4; where p values > 0.05 Tukey's HSD statistics reported in Appendix F, Table F2). Big Cedar Lake (9 of 13; coefficient estimate = 0.043 MSGR day $^{-1}$, t value = 4.28, p value = < 0.001), Kassabog (9 of 13; coefficient estimate = 0.09 MSGR day $^{-1}$, t value = 9.32, p value = < 0.001), and Sucker (9 of 13; coefficient estimate = 0.138 MSGR day $^{-1}$, t value = 17.64, p value = < 0.001) were the most frequently different. Big Cedar Lake generally had lower MSGR values than most lakes, while Kasshabog Lake and Sucker Lake had both greater and lesser MSGR values than lakes that were different.

Effect of Natural Lake Calcium Concentration

Across 8 years of monitoring lake water chemistry parameters, aqueous Ca concentrations in all 14 lakes consistently exceeded the generally accepted limitation 'threshold' concentration 1.5 mg Ca L^{-1} (Ashforth and Yan, 2008). Concentrations ranged from 2.72 ± 0.45 mg Ca L^{-1} (Sucker) to 28.89 ± 3.46 mg Ca L^{-1} (Big Cedar) with no obvious changes in Ca concentrations within individual lakes over this period. When added to the general linear model (Table 2), average lake Ca concentration was not a significant predictor of MSGR (estimate = 0.031, SE = 0.04, t value = 0.737, adjusted p value = 0.46). It also did not improve the AICc score compared to the Ca concentration and source lake interactive model (ΔAICc = 1.503). When added as a random effect, the effect was very small and its inclusion did not result in a relevantly different model fit, therefore it was left out to maintain the least complex model. A Spearman correlation test indicated that average source lake calcium concentration did not correlate with faster or slower MSGR response patterns, maximum growth rate estimates (p = 0.5; rho = -0.011), or half-saturation constant estimates (p = 0.22; rho = 0.226).

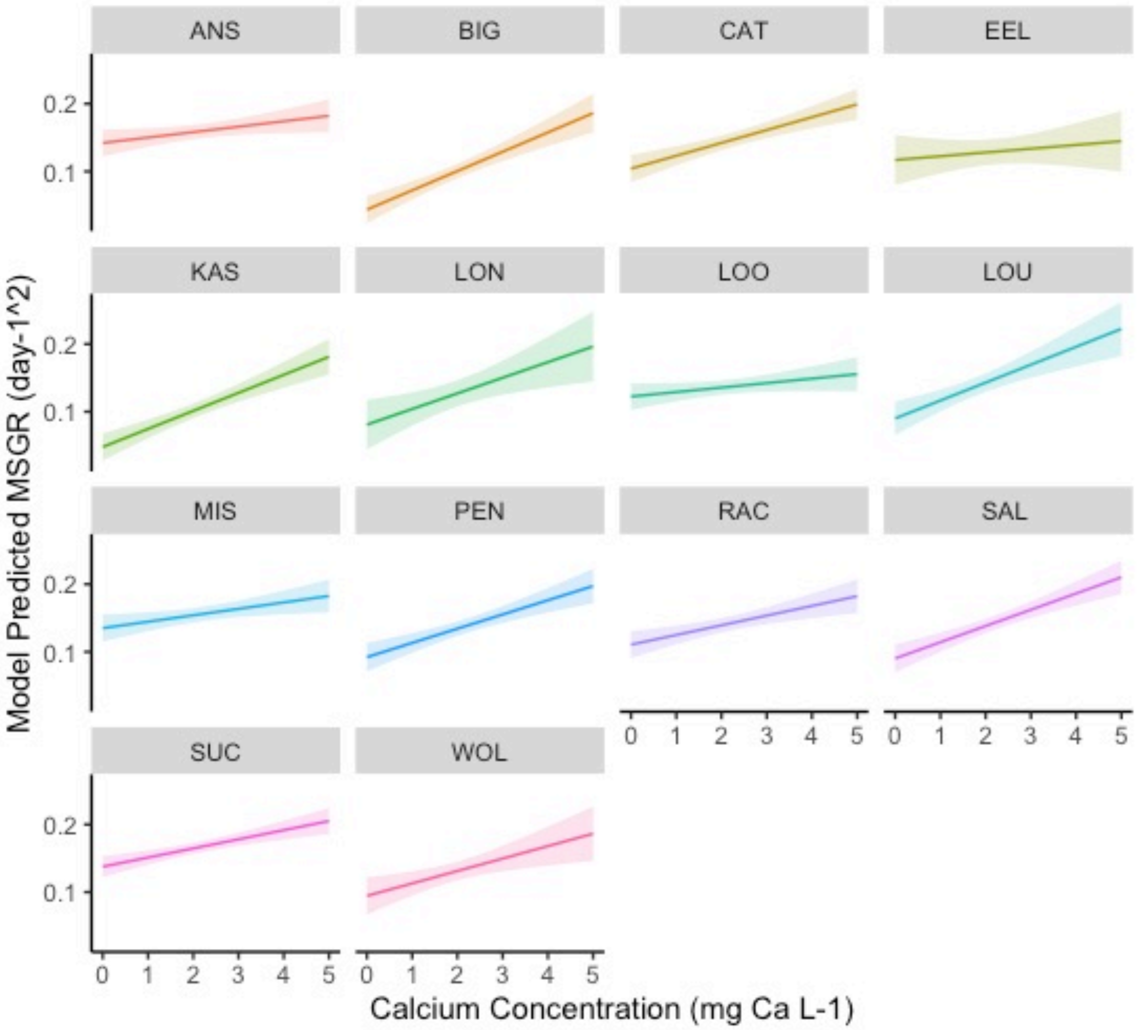

Figure 1. General linear model calcium concentration interaction with source lake on MSGR. Represented regression slopes are the result of MSGR^2 ~ Treatment Ca Concentration (mg Ca L$^{-1)}$ * Source Lake Group. Colour denotes source lake. Sample size ranged, see Methods for a detailed breakdown.

Table 3. Two-Way ANOVA table output of the general linear model of overall calcium concentration, source lake, and their interactive effect on $MSGR^2$. Calcium concentration had been included as a continuous numeric variable and source lake as a categorical variable. Significant differences between at least one group or across the calcium continuous gradient ($p>0.05$) are noted with an asterisk '*'

General Linear Model: $MSGR^2$ ~ Treatment.Ca.Concentration*Source.Lake

Variable	Df	Sum of Squares	Mean Squares	F value	p value
Calcium Concentration	1	22.041	22.041	7458.769	< 0.001*
Source Lake	13	4.268	0.305	103.158	< 0.001*
Calcium : Source Lake	14	0.116	0.009	3.023	0.0002*
Residuals	1263	3.732	0.003		

Table 4: Significant Tukey's HSD pairwise comparisons between source lake group MSGR values. The test estimate, standard error (SE), t value, and p value (α = 0.05) are listed. A negative estimate indicates that the first lake has a lower mean MSGR than the second lake. A positive estimate indicates the first lake has a greater mean MSGR than the second lake.

Comparative Groups	Estimate	SE	t value	p value
ANS-BIG	0.055	0.008	7.287	0.000
ANS-KAS	0.055	0.008	7.206	0.000
BIG-CAT	-0.041	0.008	-5.290	0.000
BIG-LOO	-0.033	0.008	-4.298	0.002
BIG-LOU	-0.042	0.009	-4.775	0.000
BIG-MIS	-0.051	0.008	-6.778	0.000
BIG-PEN	-0.033	0.008	-4.302	0.002
BIG-RAC	-0.037	0.008	-4.883	0.000
BIG-SAL	-0.037	0.008	-4.903	0.000
BIG-SUC	-0.062	0.007	-9.131	0.000
CAT-KAS	0.040	0.008	5.218	0.000
EEL-SUC	-0.038	0.010	-3.621	0.021
KAS-LOO	-0.032	0.008	-4.230	0.002
KAS-LOU	-0.042	0.009	-4.716	0.000
KAS-MIS	-0.051	0.008	-6.699	0.000
KAS-PEN	-0.033	0.008	-4.234	0.002

KAS-RAC	-0.037	0.008	-4.812	0.000
KAS-SAL	-0.037	0.008	-4.831	0.000
KAS-SUC	-0.062	0.007	-9.027	0.000
LON-SUC	-0.037	0.011	-3.426	0.041
LOO-SUC	-0.030	0.007	-4.418	0.001
PEN-SUC	-0.030	0.007	-4.327	0.001
RAC-SUC	-0.025	0.007	-3.713	0.015
SAL-SUC	-0.025	0.007	-3.728	0.015
SUC-WOL	0.033	0.008	4.041	0.004

MSGR Growth Curve Model

While designing the model, an additive parameter (μ_{zero}) to represent a basal neonate growth rate at time zero was tested to determine if greater complexity would improve the model's fit to the MSGR data and account for a non-zero initial neonate growth rate. For Anstruther, Kasshabog, and Sucker, no significant differences existed between candidate models. Analysis defaulted to the 'A' model due to its reduced complexity. The ΔAICc value was relevant and favored the 'A' model, which did not include the μ_{zero} parameter for the following lakes: Big Cedar, Catchacoma, Eels, Long, Loon Call, Loucks, Mississauga, Raccoon, and Wolf. AICc scores favored 'B' model, which did include the μ_{zero} parameter, for Pencil and Salmon lakes (Appendix F, Table F3).

Estimates of K_s and μ_{max} from the 'B' models defied biological expectations for both Pencil and Salmon lakes and when compared to the spread of the data were possibly 'overfit' models (PEN; K_s = 5.87 mg Ca L^{-1} d^{-1}, μ_{max} = 0.66 d^{-1}, μ_{zero} = 0.25. SAL d^{-1}; K_s = 6.81 mg Ca L^{-1} d^{-1}, μ_{max} = 0.67 d^{-1}, μ_{zero} = 0.27 d^{-1}). The K_s and μ_{max} 'B' estimates were notably high compared to other lakes' 'A' estimates, outside of the experiment's treatment gradient, and did not match the spread of data well. The asymptote representing μ_{max} appeared to have been approached at much lower concentrations (Figure 2). Given K_s is the concentration at which half μ_{max} has been reached, it was decided that the 'B' models possibly over-estimated this parameter by overfitting the model. μ_{zero} may be capturing 'noise' rather than describing a true relationship (Sutherland et al., 2023). Despite the lower AICc score, the 'A' model produced more biologically realistic parameter estimations, thus all analysis defaulted to using the 'A' model (Appendix F,

Table F3). By doing so, we prioritized biological plausibility.

All lake MSGR values followed a logarithmic curve with horizontal asymptotes representative of the estimated maximum growth rate (μ_{max}) by ‘A’ models. This value represents the biological maximum rate at which neonates/early juveniles grow within the 6-day testing period, assuming that their treatment level Ca concentrations were not nutritionally limiting. Individual MSGR values varied widely depending on both lake and treatment group. Ca concentration was reported as measured concentration values rather than nominal to mitigate overfitting or misleading representation.

Table 5. Model ‘A’ estimations of maximum growth rate (μ_{max}) and half-saturation constant (K_s) parameters with 95% confidence intervals, and model residual sum of squares.

Lake	μ_{max} (d^{-1})	CI	K_s (mg Ca $L^{-1}d^{-1}$)	CI	RSS
ANS	0.417	0.394-0.440	0.083	0.010 - 0.156	0.415
BIG	0.474	0.423-0.524	0.853	0.574 - 1.132	0.389
CAT	0.447	0.414-0.480	0.300	0.164 - 0.436	0.528
EEL	0.389	0.366-0.411	0.136	0.044 - 0.227	0.027
KAS	0.449	0.380-0.518	0.764	0.372 - 1.157	0.818
LON	0.444	0.341-0.547	0.447	-0.011 - 0.905	0.259
LOO	0.387	0.359-0.416	0.101	0.000 - 0.203	0.518
LOU	0.444	0.410-0.478	0.269	0.153 - 0.385	0.206
MIS	0.413	0.381-0.445	0.103	-0.004 - 0.210	0.700
PEN	0.419	0.375-0.463	0.258	0.072 - 0.445	0.959
RAC	0.431	0.409-0.454	0.240	0.154 - 0.326	0.250
SAL	0.410	0.363-0456	0.213	0.028 - 0.397	1.351
SUC	0.428	0.402-0.455	0.111	0.028 - 0.195	1.484
WOL	0.414	0.364-0.463	0.255	0.060 - 0.449	0.397

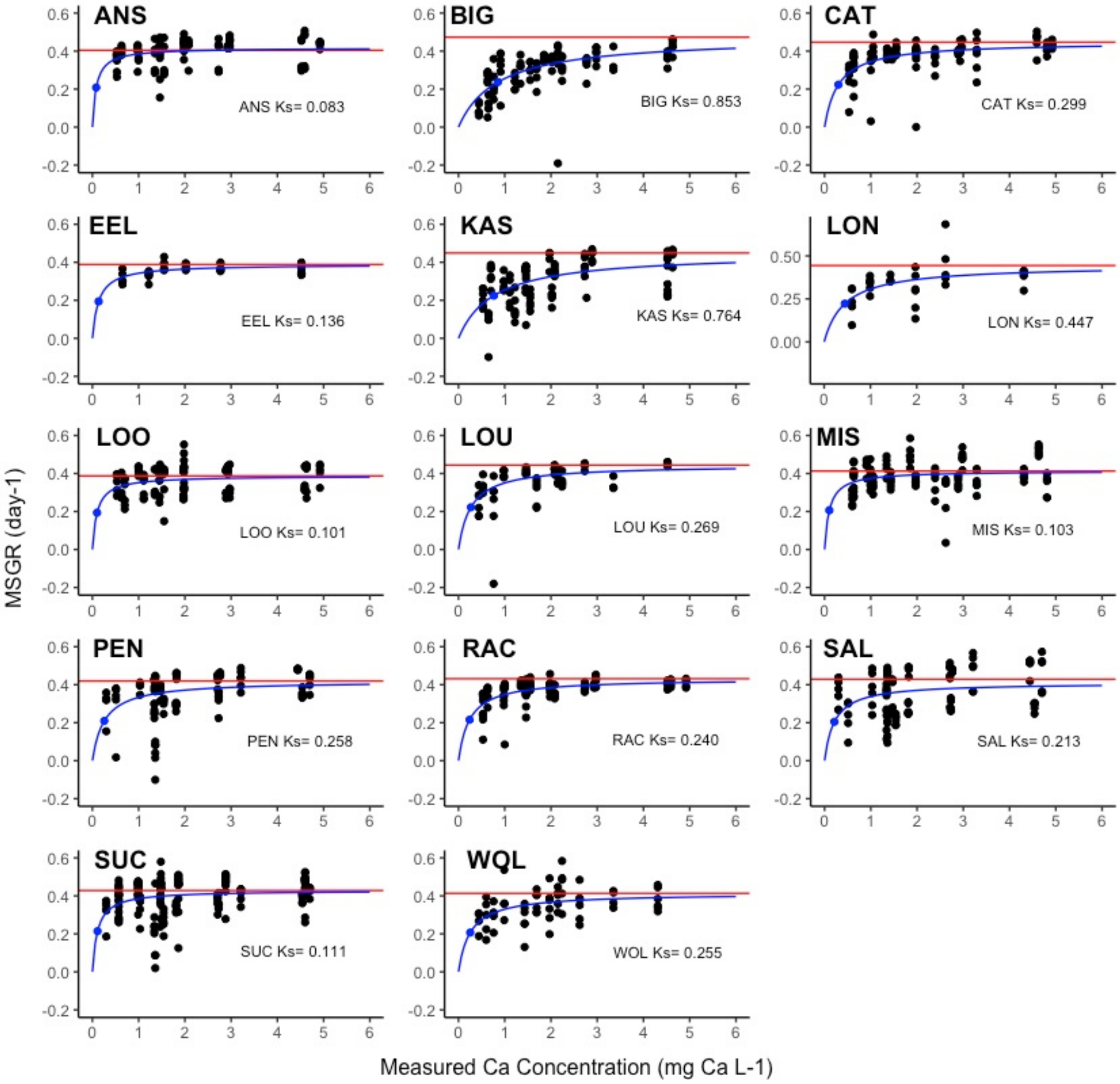

Figure 2. Mass-specific growth rate ‘A’ model curves by source lake and measured calcium concentration (mg Ca L^{-1}). Individual MSGR values are presented in black with model estimations of the half-μ_{max} saturation point (K_s) in blue along the model’s predicted growth curve. Estimated maximum growth rate (μ_{max}) is presented as a red asymptote. Sample size ranged, see Methods for a detailed breakdown.

Estimated Half-Saturation Constant

Parameter estimations of K_s coincided with the MSGR value of half μ_{max} for each source lake and acted as a representation of the lake populations' potential response capacity under Ca limitation. This allowed for a comparable threshold where limitation was reasonably occurring, and growth would be inhibited by at least 50%. Four hundred *post-hoc* Wilcoxon Rank-Sum tests estimating K_s used 3 estimates drawn from 1000 distribution iterations. Despite some lakes such as Big Cedar overlapping its confidence interval with only 2 of 13 other lakes (Figure 3), no lake had a statistically different K_s estimation from the others after multiple comparison adjustment (all p values > 0.05, adjusted following Holm-Bonferroni methods).

Estimated Maximum Mass Specific Growth Rate

All lake populations approached μ_{max} within the testing Ca concentration gradient. Parameter estimations of μ_{max} remained relatively consistent between lakes, ranging from Loon Call (0.387 d^{-1}) to Big Cedar (0.474 d^{-1}). μ_{max} estimation confidence intervals exhibited extensive confidence interval overlap (Figure 4). Notably, both Eels Lake and Loon Call Lake estimation confidence intervals overlap with each other and other lakes in the set. Of 400 *post-hoc* Wilcoxon Rank-Sum tests estimating μ_{max} using 3 estimates drawn from 1000 distribution iterations, no lake had a significantly different μ_{max} estimation from the others (all p values > 0.05, adjusted following Holm-Bonferroni methods).

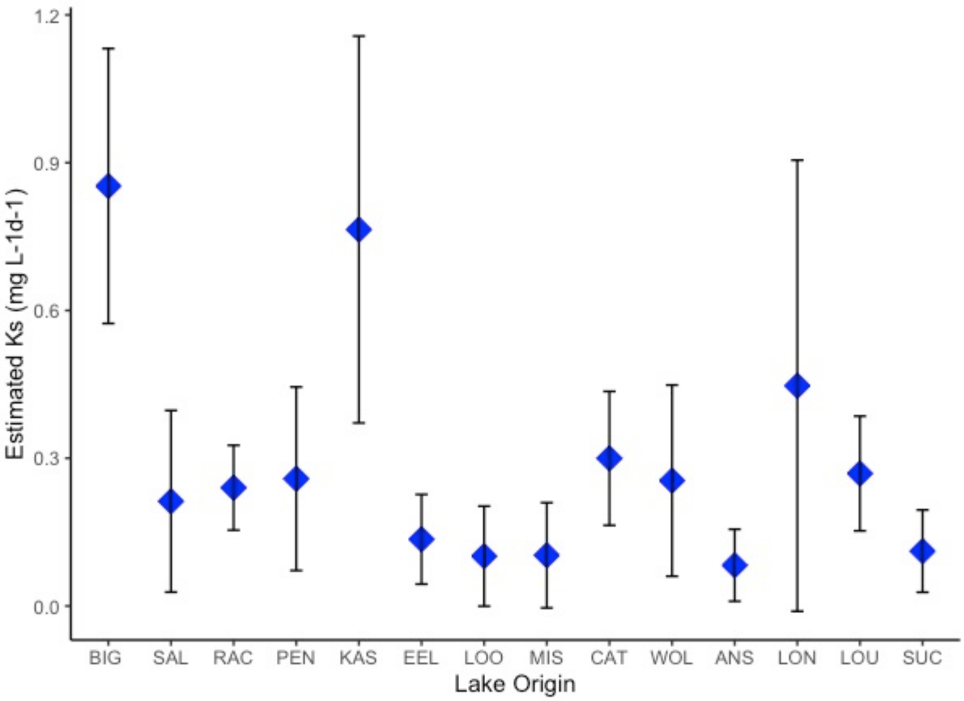

Figure 3. Estimated half-saturation constant (K_s; mg $L^{-1}d^{-1}$) values by source lake ordered by decreasing calcium concentration average (2015; 2018-2023). Error bars are representative of 95% confidence intervals where overlapping intervals indicated no significant difference between value estimations.

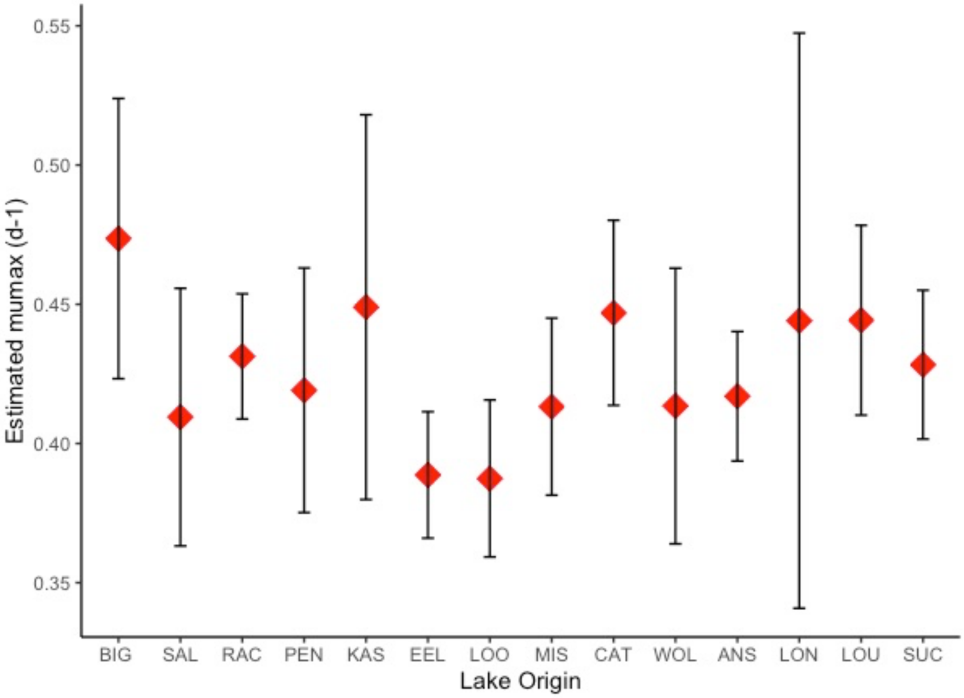

Figure 4. Estimated maximum mass specific growth rate (μ_{max}; d^{-1}) values by source lake ordered by decreasing calcium concentration average (2015; 2018-2023). Error bars are representative of 95% confidence intervals where overlapping intervals indicated no significant difference between value estimations.

Discussion

Calcium Effect

Aqueous Ca concentration is a well-studied predictor variable for *D. pulex* (Riessen at al., 2012; Prater et al., 2016; Overhill, 2017; Huang et al., 2022) and *D. magna* neonate growth (Muyssen et al., 2009; Tan & Wang, 2009; reviewed in Ebert, 2022). However, its influence over genetically confirmed *D. pulicaria* has been infrequently explored because *D. pulicaria* is most often included with *D. pulex* as part of a species complex. We expected that there would be significant differences in MSGR values within lakes as Ca increased, where neonates exposed to the highest concentration of Ca would have consistently grown the fastest. Individuals from high Ca availability treatments were also expected to have a larger final mass on day 6 than low availability treatment groups.

We found that Ca treatment concentration and source lake were significant predictors of MSGR, and there were differences between source lake MSGR means as Ca concentration increased. MSGR values were predicted to increase 0.089 day $^{-1}$ for every 1 mg Ca L^{-1} increase. Differences in slope specific to each lake group caused diverging response patterns of MSGR to Ca limitation, and were the result of differential interactions between Ca concentration and source lake. These differences indicated that source lake lineage influenced MSGR and supports that underlying genotypic or phenotypic plasticity mechanisms may exist between our lake populations, driving variable response patterns.

Our findings are contrary to a general linear model approach taken by Overhill (2017), who found either no pattern in day 6 body size or a convex parabolic relationship as Ca concentration increased. While body size is not an equivalent measure of mass, the two variables are often correlated and vary by body shape (Geller & Müller, 1985). Big Cedar appears to be the most distinct population of the set, with lower MSGR values that are different from most other source lakes. Notably, Big Cedar Lake has the greatest ambient Ca average concentration of the sample sites (28.88 mg Ca L^{-1}). However, two Kasshabog and Sucker lakes were also different from 9 others and did not follow the same pattern of high ambient lake Ca. Sucker had the lowest ambient lake Ca of the set, but higher average MSGR than Big Cedar. As there is no clear grouping factor for these three lakes versus the other eleven, out model may be missing unaddressed genotypic or ecological factors. While average lake Ca concentration in the recent past was not a predictor of MSGR, there may be other lake-specific conditions or factors not explored in this experiment that may influence iso-female populations. These unconsidered factors, such as characteristics of the lake's chemical components or Ca related allele profiles of its *Daphnia* populations, could contribute to why source lake was a predictor variable and interactive with Ca in our general linear model.

Low Ca treatment levels, particularly below a lake's K_s concentration, could have been inadequate for providing enough Ca to build a neonate's calcified cuticle. This could lead to slower growth rates and nutritional stress (Ashforth & Yan 2008). Over time, *D. pulicaria* neonates in lakes with declining Ca availability may experience higher stress during moulting, even when provided with ample energy from an algal food source. During high temperature events like summer heat waves (Ashforth & Yan 2008), or periods of low algal availability or quality (Muyssen et al., 2009), low Ca has a strong effect on maturation, reproduction, and survival (Giardini et al., 2015). Neonates in declining Ca lakes could take longer to mature and reproduce in the future, potentially decreasing a population's intrinsic rate of natural increase between 1.0-1.5 mg Ca L^{-1} (Ashforth & Yan 2008).

Our results agree with Azan & Arnott (2018) and Overhill (2017) whose studies indicated that, against our initial hypothesis, the average Ca availability in source lakes did not influence our populations' responses to low Ca treatments. This was shown when average source lake Ca concentration was not a significant predictor of MSGR in our general linear model (estimate = 0.031, SE = 0.04, t value = 0.737, adjusted p value = 0.46). These findings are contrary to differences in *D. galeata* Ca limitation responses between soft and hardwater sources (Rukke 2002), and local adaptation of *Daphnia* sister-species to other environmental stressors such as heavy metal pollution (Agra et al., 2011) and regional temperature (Yampolsky et al., 2014; reviewed in Ebert, 2022). It is possible that the lake sites chosen for this study did not have average Ca concentrations low enough to be a significant selection pressure, as all are above the accepted reproductive effectiveness threshold 1.5 mg Ca L^{-1} (Ashforth & Yan 2008). Overall, we do not believe that KWH populations have faced selection pressures strong enough or for enough time to drive the formation of lake-specific ecotypes, and differences in MSGR may be the result of genotypic differences in Ca related gene families or different expression responses (Omilian and Lynch, 2009).

Model Parameter Predictions

While using a general linear approach is effective for visualizing the overall effect of increasing Ca concentration on MSGR among source lakes and their interactive effects, it can generalize the relationship as infinitely linear. We expected that *Daphnia* growth would approach a natural maximum asymptote when Ca availability was adequate, and the rate would not infinitely increase after Ca availability had overcome the neonate's Ca requirements. An asymptotic growth rate pattern was consistent as Ca concentration increased above the estimated K_s limiting threshold in all lake populations.

The half saturation constant (K_s) is the estimated Ca concentration a neonate needs to achieve half the maximum estimated growth rate (half μ_{max}). In this study it acts as a comparative threshold between lakes across the continuous Ca concentration gradient (Figure 3). This analytical approach was taken because measured treatment concentrations were not within an acceptable margin of nominal target concentrations. Generally, greater K_s values would mean greater Ca concentrations are required to reach half μ_{max} for a given source lake population. Therefore, more limitation-related stress is expected to impact neonate growth from populations with high K_s values than neonates from lakes with lower K_s values. After computing the successful proportion ($p> 0.05$) of 400 Wilcoxon Rank-Sum comparisons with Holm-Bonferroni correction (Sperfeld & Wacker, 2011; Martin-Creuzburg et al., 2014), we found that source lake K_s values were not statistically different (0% comparisons successful). However, all values were notably lower (Table 5) than the generally accepted Ca-limitation threshold 1.5 mg L^{-1} (Ashforth & Yan 2008).

This implies our lake populations may achieve reasonable growth with minimally impacted survival at lower concentrations than recognized in most literature. With a mean K_s of 0.295 ± 0.231 SD mg Ca $L^{-1}d^{-1}$, KWH *Daphnia* may be less sensitive overall to Ca limitation than some other study populations (Ashforth & Yan 2008; Overhill, 2017). To increase the confidence in this conclusion, this experiment could be replicated over a longer period until neonates have entered the adult stage and are reproducing from 15-21 days old. Neonates greater than 6 days old would require more calcium to supply frequent moulting until they have reached full sexual maturity, and it is possible that we have underestimated the true limiting threshold of our populations.

K_s may be misrepresentative of how our population would respond to Ca-limitation in its native lake system. Source lake as a significant predictor of MSGR in the general linear model seems to contradict K_s not being statistically different between lakes in our nonlinear model. It is possible that the 50% μ_{max} threshold that K_s represents is missing important growth kinetic patterns that are occurring under extreme limitation, which could influence the nonlinear response curve's overall shape. Differences between source lake MSGR values may be more apparent at lower Ca concentrations than we tested in our gradient, however we would then question the biological relevance of said differences. Neonates at low Ca concentrations < 1.5 mg Ca L^{-1} would be unlikely to survive past 15 days (Ashforth & Yan, 2008; Overhill, 2017). Therefore, we would not encourage that K_s values be used as a comparative 'threshold' value applied to a lake's natural population. Interactive effects between Ca treatment levels and multiple life history events such as survival, reproduction, and stoichiometric body content should be considered alongside growth rate to have a more complete picture of both resource and energy allocation.

As expected, maximum growth rate (μ_{max}) did not significantly vary between source lakes (Figure 4) after computing the successful proportion ($p > 0.05$; 0%) of 400 Wilcoxon Rank-Sum comparisons with Holm-Bonferroni correction (Sperfeld & Wacker, 2011; Martin-Creuzburg et al., 2014). We estimated that KWH populations had an average μ_{max} value of 0.426 ± 0.023 SD d^{-1}. Similar μ_{max} estimations among lakes within a small geographic range are unsurprising within the same species, suggesting there is a biological limit for the speed of growth when all other conditions and food quality are optimal (reviewed in Dmitriew, 2011). Theoretically, a 0.1 d^{-1} greater increase in MSGR could equate a reproductive advantage, as faster growth coincides with faster sexual maturation in some *Daphnia pulicaria* populations (Dudycha & Tessier, 1999). Conversely, pairing with a relatively high K_s value could suggest that a *D. pulicaria* population may have a lesser capacity to respond to limited Ca availability. They may be impacted to a greater degree at lower Ca concentrations than lake counterparts with lower K_s estimations. Overall, there may be a trade-off between faster growth and Ca limitation tolerance that requires further investigation.

Environmental and Biological Relevance

Ca decline is a concern in many North American freshwater ecosystems (Azan & Arnott, 2018; Weyhenmeyer et al., 2019). However, this trend has been minimal in KWH over the course of active monitoring. Data suggests that KWH Ca has remained relatively stable over the last decade (unpublished Frost survey data, 2015-2024). Changes in ambient Ca concentration could partially explain differences in zooplankton assemblages collected from the surface sediment of oligotrophic lakes, particularly between competing Cladocerans with lower calcium requirements like *Holopedium* (Jeziorski et al., 2015). We did not find any correlation between average source lake Ca concentration in the recent past and greater MSGR values, however all lakes in this study set have had ambient concentrations above the limiting threshold over the last decade. If this study were to broaden its scope to include lakes < 1.5 mg ambient Ca L^{-1} there may be difference response curve patterns not captured in our dataset. Though, finding *Daphnia* present in such lakes is not guaranteed.

Daphnia multi-generational adaptation has been shown in toxicity studies involving sodium (Hintz et al., 2018), implying that annual adaptation can occur after as few as 30 generations in *D. pulex*, with relatively few founders, and persist for at least 1 year (Chaturvedi et al., 2021). On average, 10-15 asexual and 1 sexual generation of *Daphnia* are produced per year (Chaturvedi et al., 2021) in Ontario populations. If Ca limitation has a similar capacity for adaptation, many small changes to Ca limitation responses may occur over time and eventually result in a population different from what is currently found in KWH, especially if Ca declines significantly.

Maternal Effects

Experimental neonates were raised from mothers provided with a Ca concentration above their nutritional requirements where we would not expect epigenetic modifications to Ca related gene regulation (Giardini et al., 2015). The sudden exposure of neonates to a Ca limited environment could introduce unrealistic responses and result in growth rate changes more drastic than second or third generation low Ca exposure would indicate (Giardini et al., 2015; reviewed in Agrelius & Dudycha, 2025). Ca declines reported in North America have been in terms of years (Weyhenmeyer et al., 2019) and hundreds of *Daphnia* generations. Their environmental maternal effects would reflect gradual changes in Ca availability rather than this experimental design's sudden exposure.

Multi-generational exposure of brood mothers to low Ca culture media may have resulted in different parameter estimations or overall responses than seen in our results.

Some evidence shows that second and third generation Ca limited *Daphnia* may impact Ca provisioning to embryos and faster embryonic development, resulting in smaller size-at-hatch neonates (Giardini et al., 2015). Conversely, across generations the number of offspring produced was not significantly different between high and low calcium environments, with effects to body size still present but slightly diminished (Giardini et al., 2015). Since some populations face survival and reproductive limitations below 0.1-1.5 mg Ca L^{-1} (Ashforth & Yan 2008), adaptation to low Ca may only go so far and there may be a lower limit that adaptation cannot compensate for.

Environmental maternal effects can be similarly observed when food quantity or quality are compromised, causing resource and energy allocation to alter the methylation of genomic DNA that can be passed down to offspring (Glazier 1992; Frost et al., 2010) and vary between clones (reviewed in Agrelius & Dudycha, 2025). Over the 13-month period between iso-female collection and the conclusion of testing, there were periods of time where the quantity and quality of lab-grown algal food was compromised. While quickly rectified, there was a period of 2-3 weeks where brood mothers may not have been receiving optimal carbon or phosphorus from their diet. Through maternal effect inheritance, this may have contributed further to the response variability seen in this experiment. In sister species *D. magna,* under caloric restriction clones sampled from the same environment differentially methylated (reviewed in Agrelius & Dudycha, 2025). This led to phenotypic variability sourced outside of genotypic differences (reviewed in Agrelius & Dudycha, 2025). This is one possible source of clonal variation, but the topic has not been comprehensively studied in *D. pulicaria.* Differences in growth rate from underlying factors other than Ca treatment may be poorly captured or explained in the nonlinear model and could be explored through a multi-generational exposure study.

Conclusion

Daphnia species are found in most standing freshwater on Earth (reviewed in Ebert, 2022), proving their adaptability and effectiveness in their ecological niche and lending significance to their decades long use as a model system for ecotoxicology and environmental monitoring. Based on the findings of this study, source lake average Ca availability was not a significant predictor for the Ca limitation response capacity of KWH *D. pulicaria*. Rather, it is likely a piece of a larger puzzle incorporating generational effects, the timeline of water chemistry changes, genotypic diversity, and the high level of phenotypic plasticity the genus exhibits. For the purposes of environmental monitoring, our findings do not indicate that any KWH lake population should be given special attention or is at elevated risk of decline because of changing Ca availability compared to other lakes in Southern Ontario. This does not undermine the value of investigating growth responses, as it uncovers that significantly different responses within a relatively small geographic area do exist.

Our results have shown that Ca and source lake both contribute to predicting MSGR values, and that there are differences between KWH lake populations due to differential interactions specific to each source lake. More investigation into the effect of low Ca concentration treatments 0.5-2 mg Ca L^{-1}, including additional life history endpoints such as reproduction and body Ca content, could provide further insight into how these populations differ. This may help explain why K_s values were not different between lakes despite source lake being a significant MSGR predictor. As expected, μ_{max} was not different among KWH lakes, which suggests they all share a similar maximum growth rate capacity.

In some management scenarios, generalizations should not be made about the responses of *Daphnia* and zooplankton as a greater whole. We advise against using single iso-female clonal lineages per source lake in future studies, as our results reflect one possible genotype's response per lake collected in 2023. We recommend researchers collect multiple iso-female lines to observe a more comprehensive and natural range of responses. This should allow for more accurate and practicably applicable predictive models in future work, however our findings are a critical first step to understanding local *Daphnia* response patterns.

Chapter 3. Sodium Chloride Toxicity Tolerance among Populations of *Daphnia pulicaria* from Kawartha Highland Lakes

Introduction

Of the approximately 5 million tonnes of road salt used annually in Canada, most is applied in Quebec and Ontario (Environment and Climate Change Canada, 2022) and is projected to increase as urban development expands in major metropolitan areas like Toronto (Howard & Maier, 2007; Ontario Ministry of Finance, 2024). Reporting on the amount, type, and storage of road salts in Canada has improved over the last decade, but less than 60% of reporting municipalities review salt management plans annually (Environment and Climate Change Canada, 2020). Finite quantities of used road salt remain poorly tracked or documented. Peterborough and the city of Kawartha Lakes are the closest metropolitan areas to the KWH lake region, and while Peterborough participated in reporting road salt use and management protocols to Environment and Climate Change Canada (ECCC), Kawartha Lakes City is not included in a publicly available list of participants (Environment and Climate Change Canada, 2020).

Tolerance of KWH *Daphnia* to NaCl toxicity has not been thoroughly explored, as Na and Cl measurements are infrequently included in annual monitoring projects. When they are sampled, Cl concentrations have been stable remaining under 1 mg Cl L^{-1} (unpublished Frost survey data, 2015-2024). In contrast, lakes with more thoroughly documented road salt impacts in the nearby Muskoka River Watershed (MRW) had 10-year average Na and Cl concentrations ranging 32.8-90.9 mg Cl L^{-1} in 2016 (Valleau et al., 2020). Still, zooplankton assemblages changed following the onset of road salt application in Jevins and Tooke lakes, increasing *D. pulex* abundance and decreasing more Cl-sensitive *Bosmina spp*. (Valleau et al., 2020).

While Brown and Yan (2015) found limited tolerance of *Daphnia* to salinity ranging Lethal Concentration $(LC)_{50}$ = 55–248 mg Cl L^{-1}, many other studies have shown a wider range of inter and intra-specific tolerances (Liao at al., 2015) for the *D. pulex* group (including *D. pulex, D. schødleri, and D. pulicaria)* than found in other zooplankton taxa (Bos et al., 1996). *D. pulicaria* specifically have reported LC_{50} values above 600 mg Cl L^{-1} (Latta et al, 2012; Buren & Arnott, 2025). Rapid adaptability to increasing salinity environments has been shown to occur in *D. pulex* in as few as 15-30 generations (Coldsnow et al., 2017; Hintz et al., 2018). Many studies have shown correlations between higher lake salinity conditions and greater tolerance to road salt toxicity (Latta et al., 2012; Liao et al., 2015). NaCl toxicity is usually considered in terms of lethality, however sub-lethal toxic effects to reproduction may occur (Arnott et al., 2020; Buren & Arnott, 2025) below current Canadian guidelines 640 mg Cl L^{-1} (Canadian Council of Ministers of the Environment, 2011), particularly if other co-stressors are present. There is some evidence that water hardness and calcium availability may shift tolerance thresholds in *Daphnia* (Buren & Arnott, 2025), therefore differences in ambient calcium in source lakes may predispose iso-female populations to different responses.

In this chapter, we examine whether there is variable tolerance to sodium chloride (NaCl) toxicity among *Daphnia* clones from KWH lakes with low measured chloride (Cl) in the recent past. To do so, we examined the effect of increasing NaCl concentrations on *D. pulicaria* clone survivorship in populations sourced from 9 lakes within the Kawartha Highlands, Ontario region. These iso-female clone populations were also used in the experiment presented in Chapter 2. We predicted that the tolerance of *D. pulicaria* to acute short-term NaCl exposure would differ among lakes with different average Ca availability in the recent past. This may have resulted in different historical selection pressure to NaCl sensitivity. Specifically, we expected that *Daphnia* from softer low Ca lakes would be less sensitive to acute NaCl toxicity than harder high Ca lakes. High Ca lakes could have had a tolerance buffer provided by water hardness. These differences justify an investigation of interactive effects between NaCl toxicity and water hardness to better predict how increasing road salt use may impact local KWH *Daphnia* populations. As *Daphnia* were provided with ample Ca in this experiment to simulate Ca recovery in declined lakes, we explore if differences in NaCl tolerance may be genetically predisposed within our lineages.

Methods

Initial *Daphnia* population collection, culturing, algal food source culturing, and species verification all followed the protocol outlined in Chapter 2 Methods without deviation. See Appendix B and C for species verification procedures, and Appendix D for media components.

Study Sites

This study was conducted on iso-female parthenogenic *D. pulicaria* clone populations referred to previously in Chapter 2. All lakes in the study set have had stable Cl concentrations < 1 mg Cl L^{-1} over the recent past (unpublished Frost survey data, 2015-2024). The following 9 lakes were included in this experimental group: Anstruther (ANS), Big Cedar (BIG), Eels (EEL), Kasshabog (KAS), Loon Call (LOO), Mississauga (MIS), Pencil (PEN), Salmon (SAL), and Sucker (SUC). The KWH region had low Cl concentrations relative to other lakes being considered in the Arnott Salt Tolerance Collaboration Project. As the focus of the project was *Daphnia* tolerance in road salt impacted and naturally high Cl environments, the 9 lakes in this subset were selected to encompass the range of Cl typically measured in the KWH region (unpublished Frost survey data, 2015-2024). To reduce the cost and work of genome sequencing that will be included in the project's publication, lakes of similar Cl concentration to those listed in this subset were considered redundant and not included.

Standardizing Maternal Effects

Lab conditions in the prior 2 generations before this experiment were standardized to keep maternal effects consistent across all *Daphnia* individuals. F1 (first generation) females were raised in isolation and fed 4 mg C L^{-1} every other day with media renewed every second day. The first F1 brood was discarded, as is typical of *Daphnia* toxicology experiments as the first brood neonates are typically smaller in size and number (Frost et al., 2024). Using the second brood onward ensured more consistency in neonate size and better representation of the clone line (Frost et al., 2024). The second brood was moved to new culture tubes (1 per tube) to form the F2 (second generation). Feeding and culture renewal was continued, and the first brood of F2 discarded. F2's second brood onward were used in this experiment until F2 mothers were 5 weeks old, when they were discarded. When the testing period exceeded this 5-week age guideline, F2 neonates were taken to form an F3 generation and the process repeated, using F3 neonates as experimental individuals.

LC50 Toxicity Experiment

This experimental protocol was conceived and designed by Dr. Shelley Arnott (Queen's University) as part of a multi-lab collaborative project. We manipulated aqueous NaCl to conduct an LC exposure test across a concentration gradient for 48 hrs. At time 0, individual *D. pulicaria* neonates <24 hrs old were placed within falcon tubes with 10 mL of COMBO medium spiked with various concentrations of NaCl, quantified by concentration of Cl^- anions. Ten treatment levels were used: 18, 300, 600, 900, 1200, 1500, 1800, 2100, 2400, 2700 mg Cl L^{-1}. A single trial consisted of 10 individual neonates per treatment level and trials were triplicated for a total of 300 neonates per source lake, 30 neonates per source lake per treatment level. Initial test conditions were measured including specific conductivity (SPC) to approximate Cl^- concentration. This was based on a Cl^- and conductivity calibration curve model relationship used by all collaborating labs to ensure standardization of conversion (see Appendix E, Figure E1). Oxygen saturation and pH were also measured for quality assurance. After 48 hrs, the number of surviving neonates based on movement of appendages was recorded. A test was considered valid if survival in the control group (18 mg Cl L^{-1}) was >90% over the 3 trial replicates.

Statistical Analysis

Actual Cl concentration was estimated based on SPC measurements using the relationship (S. Arnott, personal communication, 2023) between ionic concentration and conductivity specific to COMBO ($R^2 = 0.999$):

$$Cl = (0.3138 * SPC) - 93.9267$$

where Cl is the concentration of chloride ions which translates to the same approximate concentration of Na^+ present in solution (NaCl stoichiometry Na:Cl is 1:1). SPC is specific conductivity standardized to 20 °C ambient lab temperature (see Appendix E). Actual Cl estimates were used in place of nominal concentrations to estimate LC_{10}, LC_{25}, and LC_{50} dosage points in R (v4.3.1; R Core Team 2023), using the dlm function of the drc R package (Ritz & Strebig, 2016). Models were constructed as 4-parameter log-

logistic curves with the lower limit fixed at 0, upper limit fixed at 1, and a binomial distribution. For each source lake we tested the "lack-of-fit" using Neill's test grouped for n=3 experimental replicates (neill.test in the R package drc). Confidence intervals (95%) were examined for overlap. LC50 values were compared using the EDcomp function in the drc package (Ritz & Strebig, 2016), which reported if significant differences existed between source lake values based on an LC ratio test (Wheeler., 2006; Ritz et al., 2015). To evaluate differences in overall dose responses, each lake's unique model curve was fit to the other lakes' datasets and tested for significant deviations in fit accuracy. Slopes were compared using the compParm function of the drc package (Ritz & Strebig, 2016).

Results

Control performance requirements (ECCC, 2014) were met in all NaCl toxicity control (nominal 18 mg Cl L^{-1}) replicate tests and cumulative survival across all 3 replicate tests (≥ 90%; ≥ 95% survival, respectively). ANS, PEN, and LOO were exceptions, although each had an acceptable cumulative survival of 93.33%. Converting SPC to estimated Cl concentration resulted in negative estimations for the control nominal group (18 mg Cl L^{-1}). The conversion relationship model's negative intercept ($Cl = (0.3138 * SPC) - 93.9267$) resulted in a negative Cl concentration when SPC is below 299.32 µS/cm. This led to an overcorrection for Cl concentrations that were near 0 mg Cl L^{-1}. Negative Cl concentration estimates could not be included in standard log-logistic analyses as it would result in computational error. These points were removed from the model dataset. Mortality at the next lowest concentration (300 mg Cl L^{-1}) also exceeded ≥ 90% survival in all replicates except one trial of SAL (70%; cumulative survival 86%) and one of EEL (80%, cumulative survival 93%). Removal of the 18 mg Cl

L^{-1} was not expected to significantly alter log-logistic model interpretation. Dose-response curves showed declines in survival as Cl concentration increased (Figure 4) with some variation in predicted response slopes.

Estimated dosages predicted to kill 10, 25, and 50% of the population varied between some lakes. In all cases, survival probability decreased as estimated Cl concentration, and consequently estimated NaCl, increased. Model statistics, parameters, and LC ratio tests can be found in Appendix F. All Neill's test results were non-significant ($p > 0.05$, Table F5) indicating all lake specific models fit their respective datasets well. A lake with a significantly steeper slope may have a greater overall LC_{50} tolerance value but had an increased mortality probability over a narrower Cl dosage gradient. Eel lake was the most often different from other lakes, with a shallower slope than Sucker, Kasshabog, Pencil, and Loon Call (Figure 5; Appendix F Table F8). Sucker lake had the steepest slope, different from Anstruther, Eel, Mississauga, and Salmon (Figure 5).

Three groups can be formed based on LC_{50} value confidence interval overlap and LC ratio results (Appendix F, Table F6 and F7): least tolerant Group A (ANS, BIG, EEL, MIS, SAL, SUC), intermediate tolerance Group B (KAS), and most tolerance Group C (PEN, LOO). Group A was less clearly defined than Groups B and C, with some lakes having significantly different LC_{50} values than others (See Appendix F) but a high degree of 95% confidence interval overlap. The average LC_{50} toxicity threshold for Group A was 1097 mg Cl L^{-1}. Group B's Kasshabog Lake had intermediate NaCl tolerance and was significantly different from all other lakes. Its LC50 value 1246 mg Cl L^{-1} was significantly greater than all lakes in Group A, and lower than Group C. Group C PEN and LOO LC_{50} values were greater than all other lakes averaging to 1364 mg Cl L^{-1} and had a trend of greater overall values than other lakes. This was approximately 24.3% greater tolerance than Group A, and 9.5% greater than Group B.

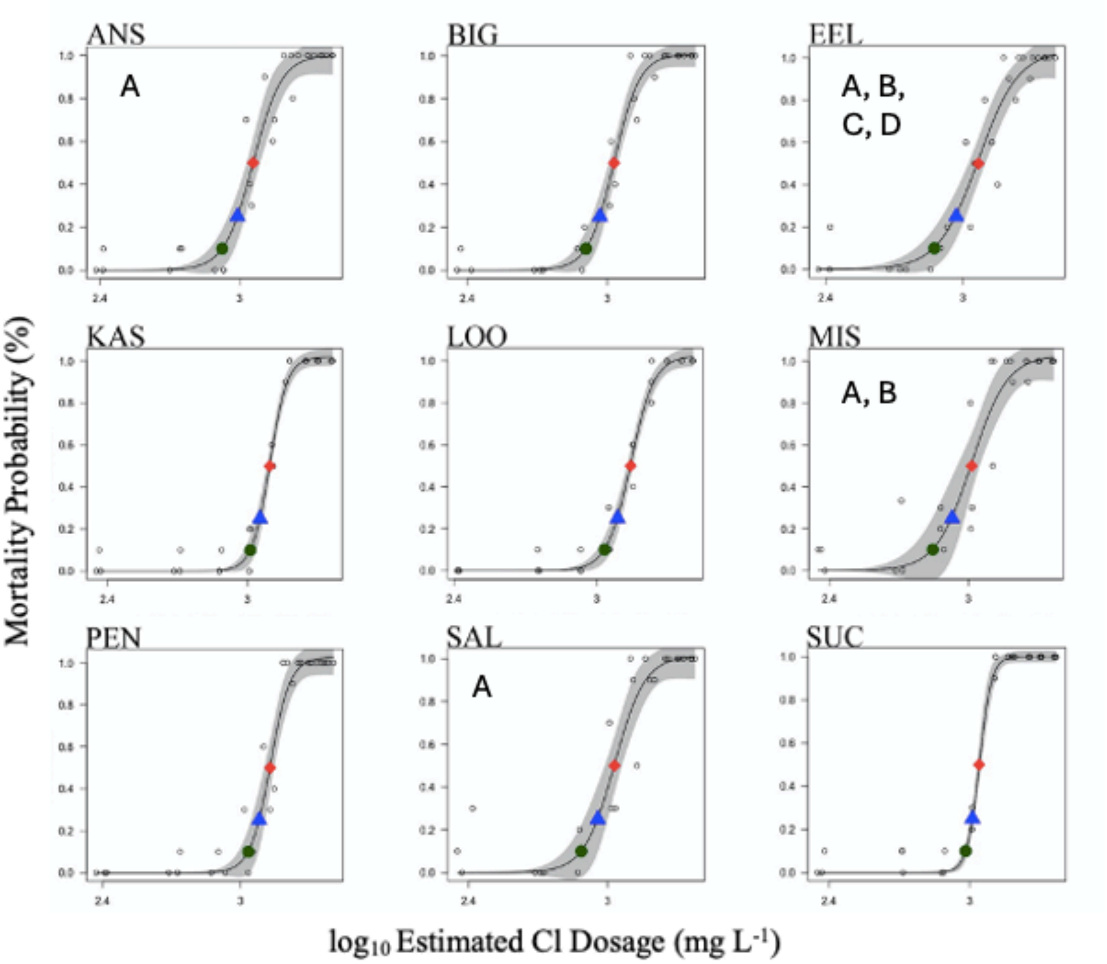

Figure 5. Estimated 4-parameter $\log_{10}$ logistic NaCl mortality dose-response curves with 95% confidence range (based on estimated $\log_{10}$ Cl^- concentration mg L^{-1} via SPC conversion) by source lake. LC_{10}, LC_{25}, and LC_{50} points are marked in green, blue, and red, respectively. Slope significant differences are labelled: Sucker (A), Kasshabog (B), Pencil (C), Loon Call (D).

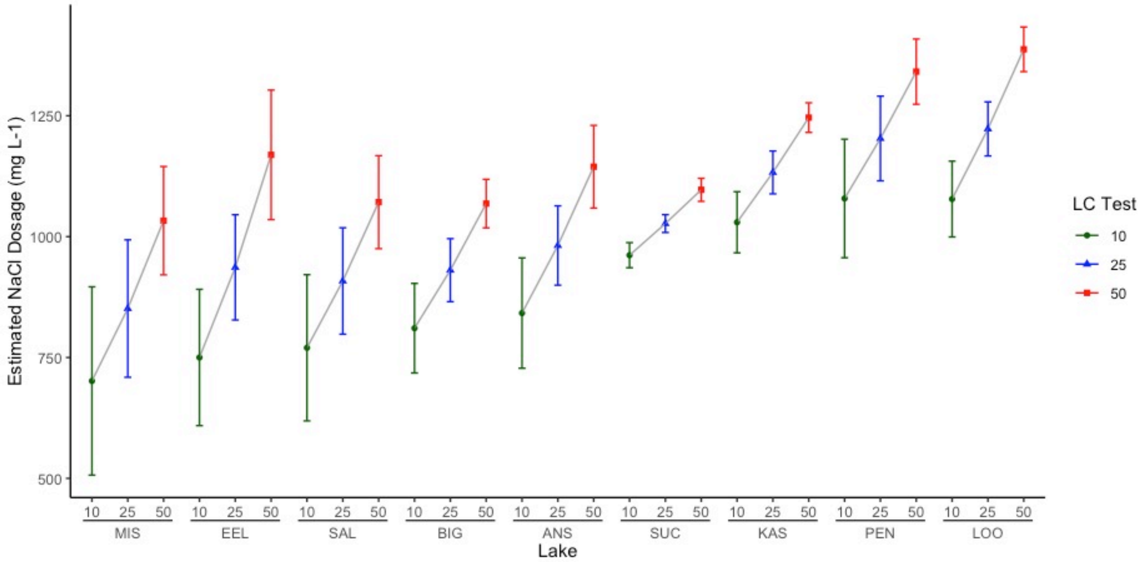

Figure 6. Estimated LC_{10}, LC_{25}, and $LC5_0$ mortality NaCl dosage (mg L^{-1}) from a 4-parameter log-logistic model analysis. Lakes are ordered left to right by increasing LC_{10} estimation. Data is grouped by source lake and nested LC test level. Error bars indicate 95% confidence intervals, colour and symbol corresponds to LC test level.

Table 6. Estimated *D. pulicaria* chloride toxicity values in mg $Cl^- L^{-1}$ by source lake and lethal concentration test level. The chloride concentrations from road-salt equivalent (NaCl) that resulted in 10% (LC_{10}), 25% (LC_{25}), and 50% (LC_{50}) *Daphnia* experimental mortality. 95% confidence intervals are shown in brackets. Results were obtained by fitting a four-parameter $\log_{10}$ logistic model where the lower limit was fixed at 0 and upper limit fixed at 1.

Source Lake	LC_{10}	LC_{25}	LC_{50}
Anstruther	842	982	1145
	(728-956)	(900-1064)	(1059-1230)
Big Cedar	811	931	1068
	(718-903)	(865-996)	(1018-1118)
Eels	750	936	1169
	(609-891)	(828-1045)	(1035-1303)
Kasshabog	1030	1133	1246
	(966-1093)	(1088-1177)	(1215-1277)
Loon Call	1078	1223	1387
	(999-1156)	(1167-1279)	(1341-1433)
Mississauga	701	851	1033
	(507-896)	(709-993)	(921-1145)
Pencil	1079	1203	1341
	(956-1202)	(1115-1290)	(1274-1409)
Salmon	770	908	1071
	(619-921)	(798-1018)	(975-1167)
Sucker	962	1027	1097
	(936-988)	(1009-1045)	(1073-1121)

Discussion

NaCl Toxicity

As expected, survival in all iso-female clones decreased as acute NaCl concentration increased. Contrary to our expectations, there was no correlation between lower source lake average Ca concentrations and greater tolerance at any of the LC testing levels. This corroborates Cicchetti (2023) who found no evidence of evolved tolerance in road salt polluted Ontario lakes, but did see similar variation in results between their *D. pulicaria* clone lines.

Current CWQGs (Canadian Water Quality Guidelines) referenced the genus representative *Daphnia pulex*'s chloride tolerance as 1248 mg Cl L^{-1} (CCME, 2011). Group A study populations had greater sensitivity (1097 mg Cl L^{-1}) than those used in determining CWQ guidelines, Kasshabog in Group B roughly equivalent (1246 mg Cl L^{-1}), and Group C was more tolerant (1364 mg Cl L^{-1}). All lakes were slightly less tolerant than Buren & Arnott's (2025) Muskoka-sourced *D. pulicaria* populations at roughly equivalent water hardness (LC_{50} = 1525 mg Cl L^{-1}, 95% CI 1444-1607). Their study identified significant differences between two *D. pulicaria* population's LC_{50} points which aligns with the findings of our study. This may be due to higher exposure concentrations in MRW than KWH, indicating some local adaptation to NaCl in their systems could be present giving them an advantage over our KWH lineages.

Kasshabog, Pencil, and Loon Call generally had greater LC values than other lakes at the same Cl dosage concentration with some degree of confidence interval overlap. Any unifying traits that resulted in these greater values are unclear. For example, these lakes are not grouped by North or South geographic placement within the region, do not have significantly different flow or chemistry patterns, and do not experience extreme fluctuations in conditions year-to-year. Perhaps these lakes are in closer proximity to frequently used roads and road salt runoff than others, which may have exposed ancestors to small amounts of NaCl leading to increased inheritable tolerance. During environmental monitoring over the recent past chloride concentrations have remained below 1 mg Cl L^{-1} (unpublished Frost survey data, 2015-2023), therefore it is unlikely that the KWH study site populations have been exposed to concentrations great enough for selection pressure to result in local adaptation.

Though significantly different, the quantitative difference between the greatest and lowest LC_{50} value could be as small as 38 mg Cl L^{-1} (LOO LCI =1341 mg Cl L^{-1}; EEL UCI =1303 mg Cl L^{-1}) or as great as 512 mg Cl L^{-1} (MIS LCI = 921 mg Cl $L^{-1;}$ LOO UCI =1433 mg Cl L^{-1}) given respective confidence intervals. It is possible but unlikely that increases in NaCl concentration from road salt application would land within this narrow window for our observed tolerance variation to have a differential impact between *Daphnia* lineages on survival in the natural ecosystem. There is not a robust system in place to track total municipal road salt use and expected runoff into local lakes. For KWH management it is practical to default to the lowest LC_{50} threshold value which protects the widest range of lakes, Group A's 1097 mg Cl L^{-1}.

Acknowledging the possibility of significant differences between source lake clones is important for understanding *D. pulicaria*'s capacity for adaptation but it is more realistic to recognize that none of our lakes appear to have an ecologically relevant higher tolerance to acute NaCl toxicity than others. Chloride concentrations, and in turn NaCl, have remained below 1 mg Ca L^{-1} in KWH over an extended period (unpublished Frost survey data, 2015-2024), providing little support that previous exposure has driven our observed variation in NaCl toxicity responses between lineages. It is possible that some lineages have had acute, point source exposure to NaCl at some point outside of annual monitoring activity, which would be undocumented in survey data. Epigenetic modifications in response to acute exposure could be inherited by offspring (Coldsnow et al., 2017), leading to enhanced tolerance in some of our lineages without supporting lake chemistry monitoring data even months after an exposure event (Hintz et al., 2018). More work could be done to investigate if these differences are evident over long-term exposure or differ in *Daphnia* collected at different times of the year.

Daphnia's metabolic response to salt stress includes upregulation of Na-K-ATPase (Sun et al., 2024), an active ion transporter in nuchal glands and epipodites (Aladin & Potts, 1995). This helps maintain internal fluid osmoregulation (Bal et al., 2021) in freshwater species, but is energetically costly by increasing metabolic rates, oxygen consumption, and contributing to the damaging accumulation of reactive oxygen species. The more energy that is sequestered into regulatory responses, the less is available for maintaining growth rates, avoiding development complications, and survival. Long term, this could potentially decrease the population after stress events and has implications for genetic diversity if some genotypes within the population are more susceptible than others.

Though survivorship of our experimental clones consistently exceeded 85% at 300 mg Cl L^{-1}, neonate life history parameters during critical developmental stages could be impacted by osmotic stress and a buildup of cell damage at this concentration level. Sublethal toxic effects of NaCl include decreased predation escape ability, longer maturation times below 600 mg Cl L^{-1} (Liu & Steiner, 2017), and smaller first brood neonate sizes and number below 120 mg Cl L^{-1} (Liu & Steiner, 2017; Arnott et al., 2020, Sun et al., 2024). More investigation into sub-lethal effects of NaCl on KWH lakes may reveal other response differences. There may be more value in determining if there is a genetic basis for these observed differences, or if the hardness of an iso-female clone's source lake could act as a buffer to NaCl toxicity selection pressure.

Influence of Calcium

The role of water hardness, determined by dissolved Ca and Mg, in decreasing NaCl toxicity is well supported in a range of freshwater species (Gillis, 2011; Arnott et al., 2020; Rogalski et al., 2024; Buren & Arnott, 2025). It's been proposed that calcium operates as a mutual competitor for sodium during ion transport (Griffith, 2017) and helps mitigate uptake and osmotic stress. Soft water lakes are categorized by a low concentration range (0-60 mg $CaCO_3$ L^{-1}) (Health Canada, 2025). High Ca test media may artificially increase NaCl toxicity thresholds (Rogalski et al., 2024; Buren & Arnott, 2025) and could mask the strong influence of small variations of Ca on NaCl toxicity tolerance. Therefore, a standardized Ca test concentration used in 'common garden' experiments may not be reflective of tolerance ranges in a *Daphnia* population's natural ecosystem. As KWH lakes vary from 2.7 - 28.9 mg Ca L^{-1}, and COMBO contains ~36.4 mg Ca L^{-1}, the responses of our iso-female clone populations in nature may not be accurately reflected in this study. Future investigations may benefit from using COMBO with differential Ca concentrations or using filtered lake water.

We found no correlation between source lakes' average calcium concentration and NaCl toxicity tolerance. This was based on the idea that lakes with lower Ca from 2015-2023 could have experienced greater NaCl stress than higher Ca lakes, forcing regulation to be more active and placing greater selection pressure on *Daphnia* populations that did not have a protective ionic 'buffer'. Generally, studies have focused on long-term NaCl exposure and only investigated Ca influence in experimental media (Arnott et al., 2020;

Buren & Arnott, 2025). Some studies have begun placing emphasis on the value of testing populations in filtered source lake water to create more environmentally relevant predictive models (Rogalski et al., 2024). However, few have considered aquatic systems with both high Ca and high Cl, requiring future investigation.

Genetic Considerations

Genetic differences between KWH lake iso-female clones are currently unknown. Genome sequencing to gain an understanding of whole-genome similarities between the iso-types is currently underway in collaboration with Dr. Michael Pfrender (University of Notre Dame, IN, USA) for a global multi-lab project headed by Dr. Shelley Arnott (Queen's University, ON, CA) investigating *Daphnia* salt tolerance. Several studies have established *Daphnia*'s adaptability and rapid tolerance evolution in response to increased salinity (Latta et al., 2012; Coldsnow et al., 2017), with differences found between clone sources (Liao et al., 2015; Liu & Steiner, 2017; Hintz et al., 2018, Buren & Arnott, 2025). *Daphnia* have been shown to evolve increased tolerance when reared under high salt conditions to improve survivorship in 5-10 generations (2.5 months) (Coldsnow et al., 2017) and this gained tolerance could be conserved for at least 30 generations (~1 year) (Hintz et al., 2018). Similar to our findings, all *Daphnia* had reduced survival above 1500 mg Cl L^{-1} (Coldsnow et al., 2017), suggesting there is a limit to how short-term evolution can accommodate salinity pressure (Hintz et al., 2018).

A study by Latta et al. (2012) compared a 'specialist' *D. pulex* clone found in ponds with the salinity range 0.11 to 0.58 g L^{-1} and 'generalist' clone line found in 0.60 to 9.95 g L^{-1} ponds. When exposed to increasing NaCl concentrations, they found that the generalist clone had greater survivorship than the specialist (Latta et al., 2012). Saline environments changed gene expression (393 cases), with greater regulatory plasticity in generalists than specialists (Latta et al., 2012). Genes that regulated changes in salinity response were also involved in predator escape responses. This suggested there could be a trade-off cost to generalizing Cl survival range with other environmental factors (Latta et al., 2012). Salt is unlikely to be an isolated selection pressure nor the most influential in a natural system. Selection acts on overall fitness rather than one specific trait, therefore other factors could influence evolution direction like food quality and quantity (Brown & Yan, 2015), water hardness (Buren & Arnott, 2025), co-stressors (Ashforth & Yan, 2008; Azan & Arnott 2018) and specific nutrient availability like phosphorus (Makino et al., 2002). Additionally, evolved tolerance to one form of salt does not necessarily equate to cross-tolerance of other salt types at similar concentrations, as there is some evidence for NaCl cross-tolerance to $CaCl_2$ but not $MgCl_2$ (Hintz et al., 2018).

Comparison to Regulatory Guidelines

The CWQG for short term Cl exposure was created after extensive study evaluating toxic effects to invertebrate, amphibian, and fish species as a guideline to protect most freshwater species against salt mortality (CCME, 2011). The guidelines recognized acute lethal toxicity (24-96 hr exposure) at 640 mg Cl L^{-1}. In statistical analyses to determine this point, *D. pulex* was reported to have a 48-hour geomean LC_{50} of 1248 mg Cl L^{-1} based on the work of Birge et al. (1985) and Palmer et al. (2004). These studies were both performed in hard water, and the CCME concedes in their publication that data was insufficient to include a chloride-hardness relationship when developing their guideline value. While we did not measure hardness for our experimental COMBO media, it is generally considered 'low hardness' (Kilham et al.,

1998) and more comparable to softness seen in Canadian Shield lakes (Celis et al., 2008) than what was used to form the CWQG. LC_{50} values tested in COMBO media are likely a more realistic representation of KWH *D. pulicaria* NaCl toxicity tolerance but could be improved further by using FLAMES media in future studies. FLAMES has lower, more locally relevant concentrations of Ca and is softer overall (Celis et al., 2008), which may reveal a more statistically significant relationship with lake Ca concentrations over the recent past. Based on review by Arnott et al. (2020), 76% of 822 Ontario inland lakes in 2016-2017 had Cl concentrations <5 mg Cl L^{-1}, 23% 5-40 mg Cl L^{-1}, and 1.1% >40 mg Cl L^{-1}. The current acute guidelines appear to be sufficient for the protection of KWH *D. pulicaria* based on mortality, but it is possible that there are sub-lethal toxic effects occurring below 640 mg Cl L^{-1}. Source lake water chemistry and Ca decline may play a more significant role in survival thresholds than CWQG currently addresses.

Conclusion

Our study supports the assertion that *D. pulicaria* acute NaCl toxicity tolerance varies between iso-female populations sourced from different lakes, even within a small geographic range. KWH sensitivity appears vary between lower, approximately equivalent, and greater NaCl tolerance than populations used to inform CWQ guidelines. While there is a general agreement among literature that CWQG should be revised to acknowledge the role of water hardness and Ca in salt toxicity thresholds, our *Daphnia* populations appear to be adequately protected by current CWQG regulations based on survivorship. Further studies should be conducted on how declining Ca availability in the Canadian Shield could result in shifting NaCl tolerances toward greater sensitivity, and chronic NaCl toxicity may cause sublethal reproductive and growth effects to *Daphnia* and other freshwater species.

Chapter 4. General Discussion

Despite the relatively stable calcium availability in Kawartha Highland lakes in the recent past (unpublished Frost survey data, 2015-2025), calcium decline trends across North America and Ontario continue to be a concern for *Daphnia* populations and their place in zooplankton communities (Giardini et al., 2015; Jeziorski et al., 2015; Bergström et al., 2024; Azan & Arnott, 2025). Cascading effects of Ca decline on *Daphnia* life history could exacerbate other stressors such as road salt toxicity (Arnott et al., 2020; Buren & Arnott, 2025). This can be difficult to predict as road salt use fluctuates annually with generally poor documentation. Road salt use could increase as urban sprawl spreads to new areas (Howard & Maier, 2007; Ontario Ministry of Finance, 2024). Despite warming winter temperature projections from climate change (Hori et al., 2018), it is difficult to predict changes to local road salt use and how it will runoff into KWH lakes. Both Ca limitation and road salt toxicity have been shown to affect *Daphnia* survival (Arnott et al., 2020; Buren & Arnott, 2025), growth (Muyssen et al., 2009), and reproduction (Ashforth & Yan, 2008; Giardini et al., 2015; Arnott et al., 2020), therefore is important to investigate how intra-specific responses could vary between local lake-specific *Daphnia* populations. This would increase our understanding of how changing conditions could shape the future of freshwater lake communities.

Some lakes in Ontario are already experiencing changes in zooplankton communities due in part to Ca decline (Jeziorski et al., 2015). Mesocosm experiments suggest that further Ca decline could lead to lower zooplankton productivity, reduced biodiversity, and slower growth across a wide variety of zooplankton taxa (Azan & Arnott, 2017). If *Daphnia* are unable to adapt to decreasing Ca concentrations, they will be at increased risk of being outcompeted by *Holopedium*. *Holopedium* have lower Ca requirements than *Daphnia*, and while Ca has declined by a median of 25% (Palmer et al., 2011), *Holopedium* abundances have increased in Ontario 3-37% depending on sampled lake over the last 30 years (Jeziorski et al., 2015). Both *Daphnia* and *Holopedium* are easy prey for planktivorous fish, however *Holopedium* have much lower Ca (Jeziorski & Yan, 2006) and P (Andersen & Hessen, 1991) body content, making them less nutritious and an unequal replacement in the diet of many fish. Beyond ecological consequences, *Holopedium* causes 'jellification', the unpleasant formation of jelly-like clumps in lakes that are unsightly to cottagers, could impact local ecotourism, and could block water access infrastructure.

To my knowledge, this is the first study to test *Daphnia pulicaria* intra-specific response variation to Ca availability and road salt toxicity using lake-specific genotypes from the Kawartha Highlands region. We predicted that the variability in Ca availability in the recent past may have predisposed some iso-female lines to greater tolerance to Ca limitation. We expected *Daphnia* growth from low average Ca concentration lake to be impacted less than lakes with higher Ca availability. More limitation-tolerant iso-female lines may have an advantage in the future as Ca concentrations decline across Ontario (Keller et al., 2001). We also predicted that *Daphnia* from lakes that had low Ca concentrations in the recent past may not have had a 'buffer' effect as strong as high Ca harder-water lakes. This could have placed more selection pressure on low Ca lake *Daphnia* to adapt a greater tolerance to NaCl toxicity, and lead to variability in road salt survival thresholds between KWH lakes.

Clonal Variability

In our Chapter 2 experiment, the effect of Ca treatment level and parameter estimates could have been influenced by the high level of response variability seen within iso-female lake lines. There is some evidence that *Daphnia* multi-lake iso-female lines across the same low-Ca gradient could follow non-asymptotic growth patterns, unlike the analytical approach taken in our study. For example, a convex parabolic relationship for body size was observed in Overhill's (2017) Muskoka, ON study where the largest individuals grew at low and high Ca concentrations. This study reported similar high-variability among their own lake iso-female lines, suggesting that even within the same genotype and conditions there is an unpredictable source of 'developmental noise' (Overhill, 2017). The relationship between Ca availability and MSGR in a 'common garden' experiment may not be comparable to natural iso-female limitation responses. This degree of clonal variation was not seen in Chapter 3's NaCl toxicity experiment, which may be due to the inherently different nature of each experiment's endpoints. Mortality is a finite endpoint for NaCl toxicity, while MSGR is a continuous and gradient scale response. It is possible that if we included survival as an endpoint for a longer timescale MSGR experiment, or explored sublethal NaCl toxicity effects on *Daphnia pulicaria*, we would see similar clonal variation.

Experimental Design Considerations

We assumed that using a parthenogenically reproducing organism would mitigate the risk of genetic drift or mutation (reviewed in Ebert, 2022) so that Ca related genes and alleles would remain a reflection of selection pressures placed on a lake's *Daphnia* population over the recent past. In *Daphnia* research, perpetually cultured iso-female populations are often used for decades without routine genome sequence monitoring (reviewed in Ebert, 2022). All populations used in this experiment were sampled in 2023 and reflect one lake-specific genotype's possible capacity to respond to sudden changes in Ca concentration or NaCl toxicity. Mutations in a selection-free laboratory population can spontaneously occur (Flynn et al., 2017; Ho et al., 2020), however the cost and labour of monitoring genotypic conservation by sequencing iso-female lake lines on a regular basis would be financially impractical. We cannot guarantee *Daphnia* iso-females used in this study are genetically identical to the original collected brood mother, but this is an assumption that other *Daphnia* research studies generally accept (reviewed in Ebert, 2022).

We also acknowledge that the populations presented in this study may not have been the dominant genotype in their respective lake or are representative of the genotypes present in their lakes post-2023. Patterns in reproduction can vary both seasonally and between populations (Hebert et al., 1989; Černý & Hebert, 1993; Hebert et al., 1993). For example, sister-species *D. pulex* found in central Canadian ponds were previously reported as obligate asexual reproducers, which did not undergo cyclical parthenogenesis at a detectable frequency. Conversely, western Canadian pond populations most often underwent cyclical parthenogenesis, which has a sexual period (Hebert et al., 1993). In a wide-scale survey of 64 Ontario lakes, the vast majority of *D. pulex* populations contained 6 or fewer genotype clones and overall had low clonal diversity. To our knowledge, similar surveys of *D. pulicaria* have not been done in the KWH. If *D. pulicaria* follows similar patterns to *D. pulex,* our lakes may heavily favor asexual reproduction and have a low number of genotypes present.

It is unlikely our lakes only contain a single genotype at any given time. First generation hybrids with *D. pulex* have been routinely found alongside unhybridized *D. pulicaria* in Ontario ponds (Černý & Hebert, 1993), potentially introducing another source of genetic variation when sexual reproduction does occur. More research into the breeding dynamics of lake-based KWH *D. pulicaria* populations could give insight into the risk of generalizing our results to all genotypes present in a lake. Presently, we may be underestimating the effect of intra-lake differential genotypes on its overall resilience to Ca limitation or NaCl tolerance.

Additionally, laboratory selection influence may have introduced bias to the study, as the iso-female lines that were kept in culture were those who survived acclimation to lab conditions and culture media. If replicated, this study may be improved by using culture media with Ca availability similar to source lake conditions, such as FLAMES (Celis et al., 2008) with softer water (9.41 mg CaCO3 L^{-1}). This would be more comparable to conditions in the Canadian Shield than COMBO (40 mg CaCO3 L^{-1}) (Kilham et al., 1998).

Overall Conclusions

The results of this study suggested that there is extensive clonal and intra-specific response variability to Ca limitation (Chapter 2) and road salt toxicity tolerance (Chapter 3) among *Daphnia pulicaria* populations from Kawartha Highland lakes. When tested under the same experimental conditions, neonates of the same species, and even within the same genotype, displayed highly variable responses. Therefore, generalizing *D. pulicaria* response patterns to low Ca or moderate NaCl as a whole or by their specific lake is inadvisable without testing multiple genotypes from each source lake.

Daphnia pulicaria is studied far less often than sister species *D. pulex,* therefore this assessment is novel for the region and contributes to our understanding of inter-species differences that may exist, even within the same species complex. In general, KWH *Daphnia* performed well under low Ca conditions, with survival not significantly impacted at 0.5 mg Ca L^{-1} and mass specific growth rates achieving greater than half maximum at concentrations lower than the generally accepted limitation threshold 1.5 mg Ca L^{-1} (Ashforth and Yan, 2008). Both Ca treatment concentration and source lake were significant predictors of MSGR, and their interaction resulted in unique slopes for each source lake. This showed that there are underlying differences between responses attributed to their specific lineages. There was no correlation or common trends between the Ca concentration of 14 source lakes in the recent past and MSGR, and its potential influence over Ca response variability was unestablished. Response variability might allow adaptation to occur if Ca declined in the region, however there are many facets to the response mechanism and what drives clonal variability that we still do not know.

Kawartha Highland *D. pulicaria* were less tolerant than nearby Muskoka region *D. pulicaria* (Buren & Arnott, 2025) to NaCl toxicity, with some differences found between 9 tested lakes. In all lakes survivorship was significantly reduced below 50% at NaCl concentrations greater than 1500 mg NaCl L^{-1}. While sensitivity varied between less and more tolerant than the *D. pulex* that was used to inform current CWQ guidelines for acceptable Cl concentration in freshwater (Birge et al., 1985; Palmer et al., 2004; CCME, 2011), this study suggests that current guidelines are sufficient to protect KWH *Daphnia* from road salt lethality. This does not mean that current guidelines are sufficient to protect KWH *Daphnia* populations against sublethal toxic effects to growth or reproduction. Further investigation into these community-impacting sublethal effects at low Cl concentrations over a chronic timescale are required to assess more nuanced variability between lake-specific tolerances.

Future Directions

With Ca decline potentially leading to decreased *Daphnia* growth rates on a community level, it is important to expand research to include multiple genotypes sourced from lakes to better capture the variability present in local populations. *D. pulicaria'*s prevalence in the open waters of Ontarian lakes and distinct genetic differences from sister-species *D. pulex* lends to an increasing need to understand their specific responses. A movement away from imposing *D. pulex* research onto *D. pulicaria* responses without adequate proof that their responses do not significantly differ will allow for more accurate predictions of community shifts in response to change. Nine of the 14 lakes included in this study are currently undergoing genomic sequencing at the time of writing, which should provide insight into potential allele variability in Ca related gene families (Ashforth & Yan 2008; Riessen et al., 2012; Prater et al. 2016; Overhill 2017), individual clonal variation (Overhill, 2017), and environmental maternal effects (Glazier, 1992; Frost et al., 2010; Giardini et al., 2015; reviewed in Agrelius & Dudycha, 2025).

While small geographic regions often have their lakes treated homogenously, we have seen heterogenous conditions within the Kawartha Lakes that could influence their individual *Daphnia* populations (e.g. Ca availability, Appendix A; Figure A1). We have explored the potential risk of generalizing *Daphnia* responses to Ca availability (Chapter 2) and road salt toxicity (Chapter 3), and it is possible that *Daphnia* genotypes sourced from different lakes within the region may differ in other stress responses. In natural ecosystems no variable is acting independently, and many will be present at once to affect the survival, growth, reproduction, health, and genetic diversity of a *Daphnia* population.

This study established the necessary groundwork for understanding Ca limitation and road salt toxicity for KWH *D. pulicaria* populations but could be taken further for more practical applications. An expansion of the work presented in Chapters 2 and 3 would see a co-stressor study similar to Buren & Arnott (2025), where both Ca and NaCl concentrations were manipulated. They explored how water hardness, influenced by Ca and Mg, may have an interactive effect with road salt toxicity. They found that as water hardness increased, so did the concentration of NaCl required to observe lethal and sublethal toxic effects (Buren & Arnott, 2025). Ca decline in North America would in turn decrease the water hardness of Canadian lakes. This could inadvertently lead to a greater sensitivity in the future to road salts than current conditions would imply as the water hardness 'buffer' is slowly removed. Revising current water quality guidelines to consider how multiple factors interact and affect toxicity thresholds of aquatic species would allow for more stringent protection of Canadian freshwater ecosystem health and stability.

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APPENDIX A. Study Lake Selection

We sampled 17 lakes within the Kawartha Highlands region with the goal of finding *D. pulicaria* in as many lakes as possible. Sites were selected to optimize the captured range of calcium concentrations available in the region based on monitoring data collected by the Frost lab since 2015. Emphasis was placed on collecting a viable *D. pulicaria* female from at least one lake below 5 mg Ca L^{-1}. Beaver Lake, Bottle Lake, Crystal Lake, and Gold Lake yielded *Daphnia*, however upon morphological inspection were identified as possibly *D. catawba* or *D. longispina* and discarded from the study set.

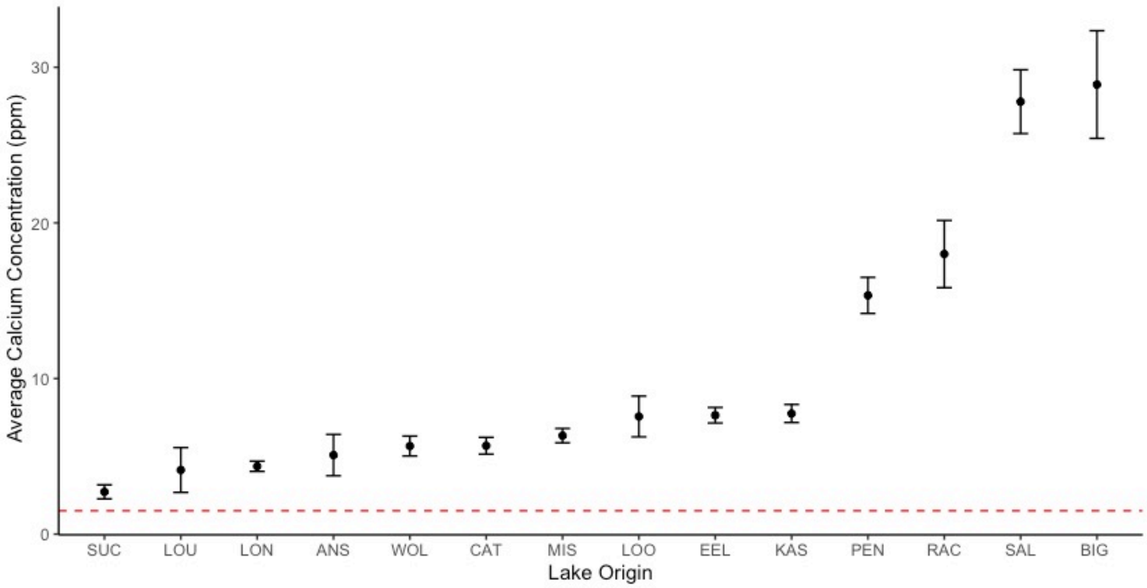

Figure A1. Average measured Calcium concentration of sampled lakes in the recent past. Ordered by increasing concentration with standard deviation, the red line represents the generally accepted threshold of sublethal effects of calcium limitation on *D. pulicaria* (1.5 mg Ca L^{-1}). Source lake ID codes are on the x axis and average concentration of measured calcium in spring 2015, 2018:2023 from Dr. Paul Frost & lab's monitoring activity.

APPENDIX B. Morphological ID

Visual identification was done using the UNH Zooplankton Key and individuals were retained if they displayed 4-6 large middle pecten on their post-abdominal claw, significant abdominal process pubescence, and elongated polygon rostrum cells. These features are more specific to *D. pulex, D. pulicaria*, and their hybrids than other features such as ocellus presence or helmet shape.

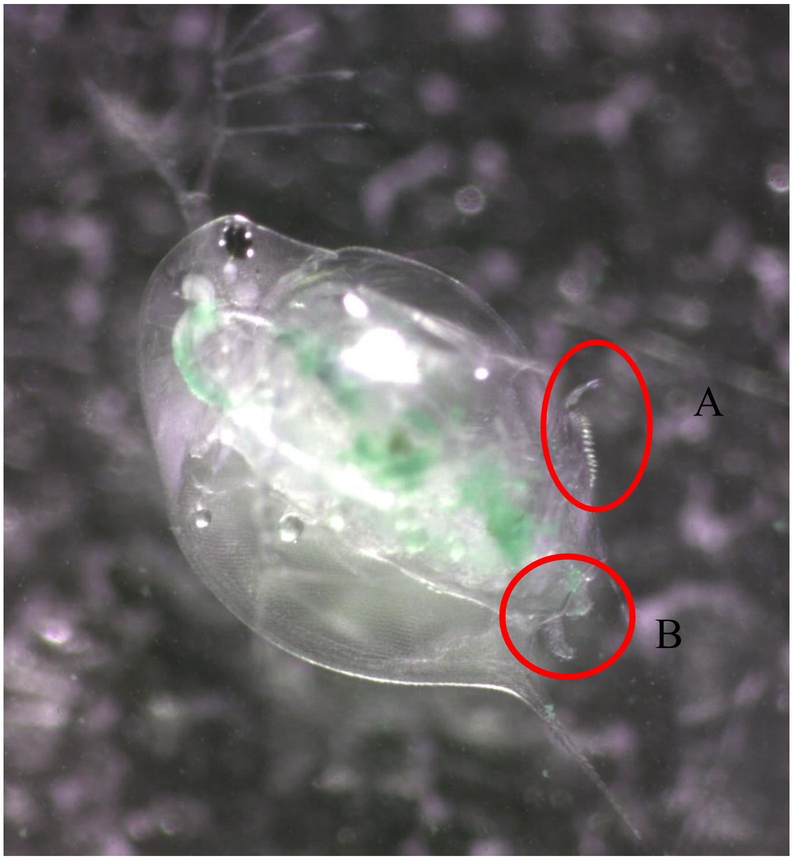

Figure B1. Example image of *Daphnia pulicaria* from Mississauga Lake. Daphniid displays a rounded helmet with elongated rostrum, ocellus present, empty brood chamber, exposed post-abdominal claw (A) with large middle pecten, and pubescent abdominal processes (B).

APPENDIX C. Identifying *D. pulex* vs. *D. pulicaria* via CAPS

(B.E. Miner and S.J. Cargill, unpublished data. Modified by Dr. Zhengxin Sun)

The protocol by Dr. Brooks Miner (Ithica College, NY, USA) was modified by Dr. Zhengxin Sun (Queen's University, ON, CA) in June 2022 for use in the multi-lab salt tolerance project headed by Dr. Shelley Arnott (Queen's Universty, ON, CA). I selected the *Daphnia* clone lines to send for species identification to Dr. Shelley Arnott's lab based on which lines for each lake had the strongest *D. pulicaria* features and had survived acclimation to the Frost lab (Trent University, ON, CA). This protocol was carried out by members of the Arnott lab and results forwarded to Sarah Cook (Frost Lab).

<u>Primers:</u>

LdhA-F 5'-CCACGGCTGGATTATCGGAG-3'

LdhA-R 5'-ATTGCCCATGAGGTGTAACCC-3'

<u>PCR conditions:</u>

Initial denature: 95° 10 min

40 cycles of:

Denature: 95° 30 sec

Anneal: 50.6° 1 min

Extension: 72° 1 min

Final extension: 72° 10 min

PCR products are then digested with HphI enzyme, which must first be diluted. (If enzyme is not diluted, it will devour the PCR products with nonspecific cutting.)

Source: ThermoFisher Scientific

- HphI (#<u>ER1101</u>) – includes Buffer B (or #<u>R0158</u> from NEB)

- B19 Dilution Buffer for Restriction Enzymes (#B19)

HphI comes at a stock concentration of 10U/uL, which we dilute down to 0.5U/uL. To make **diluted** HphI: 10uL HphI + 190uL B19 Dilution Buffer.

Digest LdhA PCR products:

1uL **diluted** HphI (0.5 U/uL)

2uL Buffer B

12uL Water

5uL PCR product

Digestion:

16 hours at 37° C, followed by 20 min at 65° C to stop reaction.

Load complete digestion reaction (20 uL) into agarose gel and visualize.

- Undigested (uncut) PCR products will have one band at ≈400bp and represent homozygotes for the *D. pulex* allele
- Digested (cut) PCR products will have two bands: one at 200-300bp and a second at 100-200bp. Fully cut PCR products with only these two bands represent homozygotes for the *D. pulicaria* allele
- Products with all three bands represent heterozygotes (hybrids) that have one *D. pulex* allele and one *D. pulicaria* allele.

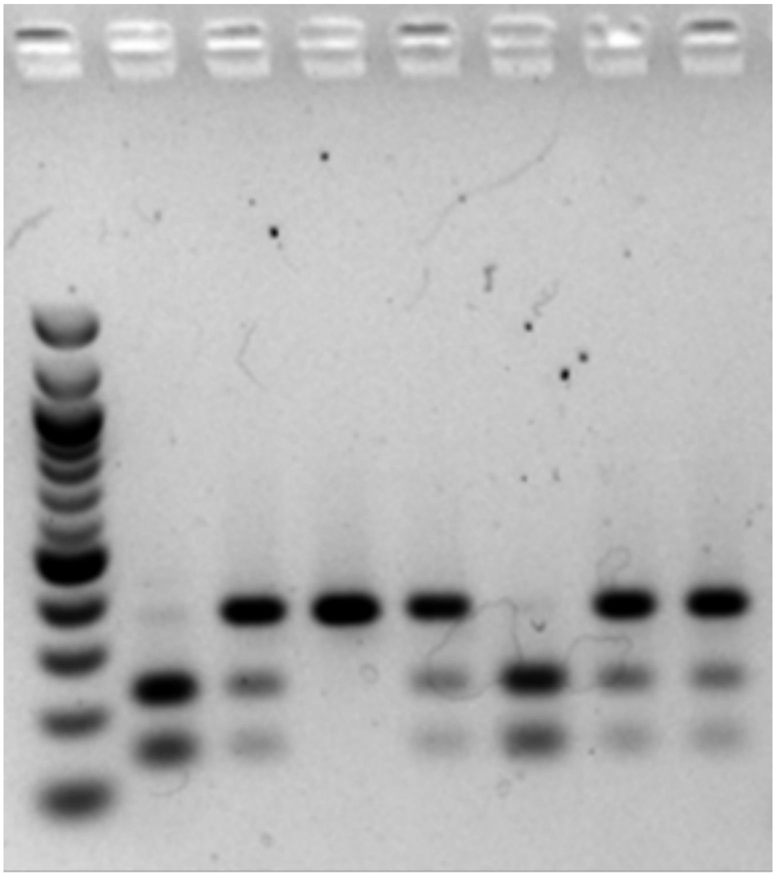

Figure C1. Example of the gel electrophoresis with genetic identification results for *D. pulex, D. pulicaria,* and hybrids. From left to right: 100 bp ladder (lane 1), *D. pulicaria* homozygotes (lanes 2, 6), heterozygotes (hybrids) (lanes 3, 5, 7, 8), and *D. pulex* homozygote (lane 4).

Procedure Modifications

A master PCR reaction mix was created for 18 samples (one extra for error). It contained 18μL of LdhA-F, 18μL of LdhA-R, 180μL of Froggabio mix, and 108μL of ddH2O. 18μL of the master mix was aliquoted to PCR tubes and added 2μL of each extracted DNA sample.

A digestion master mix was created for the 18 samples (one extra for error). It contained 18μL of diluted HphI (0.5U/μL), 36μL of 10x Buffer B and 216μL of ddH2O. 15μL of this master mix was aliquoted to new PCR tubes and 5μL of PCR product thereafter. Digestion proceeded for 16 hours at 37°C, followed by 20 minutes at 65°C to stop the reaction.

The samples were then loaded on a 1.2% (w/v) agarose gel, electrophoresis to separate the bands and visualized using a Gel Doc system.

Daphnia Identification for Study Lakes

Table C1. Species identification results based on morphological identification with the UNH Zooplankton Key and DNA identification following CAPS protocol.

Source Lake	Gel Number	Well	Morphologically IDed?	Species
Anstruther Lake	1	8	Y	*D. pulicaria*
Big Cedar Lake	2	7	Y	*D. pulicaria*
Catchacoma Lake	2	2	Y	*D. pulicaria*
Eels Lake	2	4	Y	*D. pulicaria*
Kasshabog Lake	1	4	Y	*D. pulicaria*
Long Lake	2	6	Y	*D. pulicaria*
Loon Call Lake	2	3	Y	*D. pulicaria*
Loucks Lake	2	5	Y	*D. pulicaria*
Mississauga Lake	1	6	Y	*D. pulicaria*
Pencil Lake	1	5	Y	*D. pulicaria*
Raccoon Lake	1	7	Y	*D. pulicaria*
Salmon Lake	2	9	Y	*D. pulicaria*
Sucker Lake	2	8	Y	*D. pulicaria*
Wolf Lake	1	3	Y	*D. pulicaria*

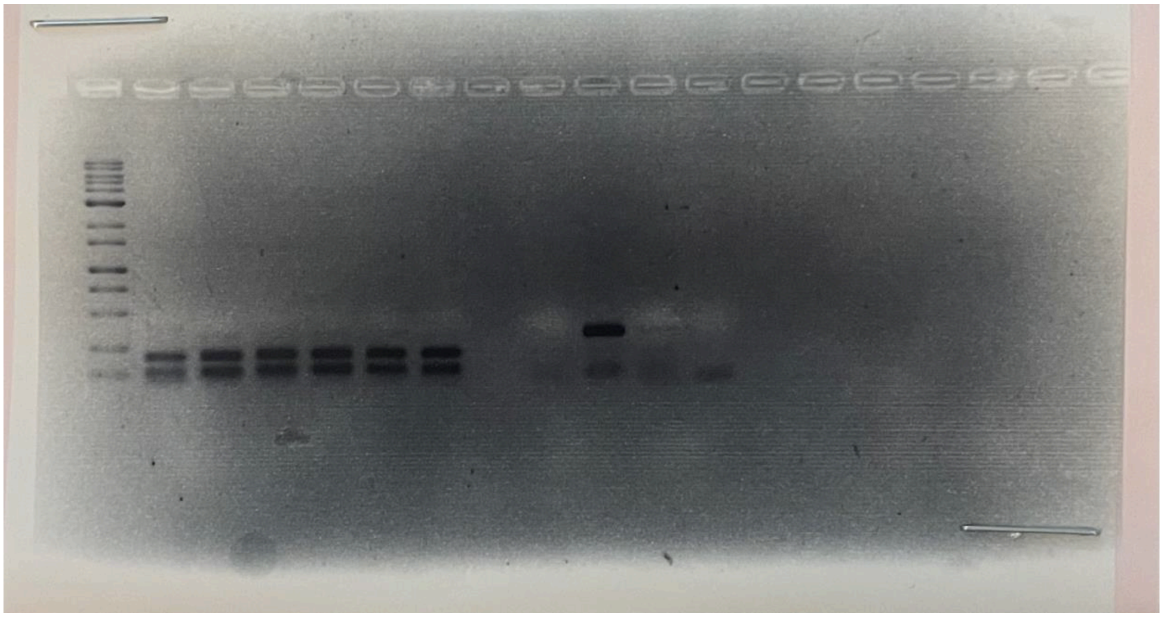

Figure C2: Gel 1 *Daphnia* DNA extraction, PCR, and digest on a 1.2% (w/v) Agarose gel with 6uL of Redsafe for fluorescence. The gel ran on 100 volts for ~40 minutes and a 1x TAE buffer was used. From left to right: Well 1: Empty (partially cropped), Well 2: 1Kb+ Molecular Weight Marker, Well 3: Sample 1, Well 4: Sample 2, Well 5: Sample 3, Well 6: Sample 4, Well 7: Sample 5, Well 8: Sample 6, Well 9: Empty, Well 10: Positive 1, Well 11: Positive 3, Well 12: Positive 5, Well 13: Negative Control (-). (Arnott, 2023).

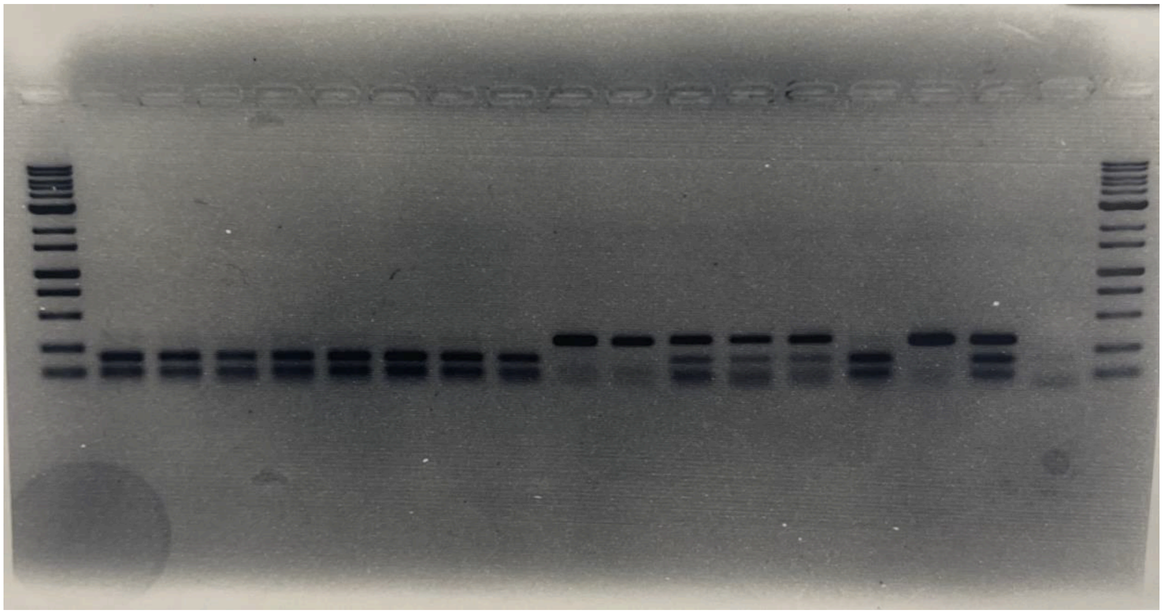

Figure C3. Gel 2 *Daphnia* DNA extraction, PCR, and digest on a 1.2% (w/v) Agarose gel with 6uL of Redsafe for fluorescence. The gel ran on 100 volts for ~40 minutes and a 1x TAE buffer was used. From left to right: Well 1: 1Kb+ Molecular Weight Marker, Well 2: Sample 1, Well 3: Sample 2, Well 4: Sample 3, Well 5: Sample 4, Well 6: Sample 5, Well 7: Sample 6, Well 8: Sample 7, Well 9: Sample 8, Well 10: Sample 9, Well 11: Sample 10, Well 12: Sample 11, Well 13: Sample 12, Well 14: Sample 13, Well 15: Positive 1, Well 16: Positive 3, Well 17: Positive 5, Well 18: Negative Control (-), Well 19: 1Kb+ Molecular Weight Marker. (Arnott, 2023).

APPENDIX D. Media Preparation and Contents

Table D1. COMBO Media Contents. These compounds are added to deionized RO water to a total volume of 21 L. Adapted from Kilham et al., 1998.

Compound	Stock ($g\ L^{-1}$)	Final Medium			Elements	
		($mg\ L^{-1}$)	($\mu mol\ L^{-1}$)		(mgL^{-1})	($\mu mol\ L^{-1}$)
Seven Major Compound Stocks						
$CaCl_2 2H_2O$	36.76	36.76	250	Ca	10.0	250
				Cl	17.7	500
$MgSO_4$ 7H_2O	36.97	36.97	150	Mg	3.65	150
				S	4.81	150
$NaHCO_3$	12.60	1.08	12.9	Na	0.30	12.9
				C	0.15	12.9
Na_2SiO_3 5 H_2O	21.21	21.21	100	Na	4.598	200
				Si	2.808	100
H_3BO_3	24.00	24.00	388.16	B	4.20	388.16
$NaNO_3$	85.01	85.01	1000	Na	23.0	1000
				N	14.0	1000
K_2PO_4	8.71	8.741	50	K	3.91	100
				P	1.55	50
Vitamin stock solution (VIM)						
B_{12}		5.5×10^{-4}	4.0×10^{-4}			
Biotin		5.0×10^{-4}	2.0×10^{-3}			
Thiamine		0.09996	0.03			
Algal Trace Elements Solution (ATE)						
EDTA		3.42	11.7	EDTA	3.42	11.7
$FeCl_3 6H_2O$		1.00	3.7	Fe	0.207	3.7
				Cl	0.394	11.1
$CuSO_4 5H_2O$		0.001	0.00401	Cu	0.000254	0.0040
				S	0.000128	0.0040
$ZnSO_4$ 7H_2O		0.022	0.0765	Zn	0.005	0.0765
				S	0.0024	0.0765
$CoCl_2$ 6H_2O		0.010	0.042	Co	0.00248	0.042
				Cl	0.00298	0.084
$MnCl_2$ 4H_2O		0.180	0.91	Mn	0.05	0.91
				Cl	0.0645	1.82
Na_2MoO4 2H_2O		0.006	0.0248	Na	0.00114	0.0496
				Mo	0.00238	0.0248
H_2SeO_3		0.0016	0.0124	Se	0.000979	0.0124
Na_3VO_4		0.0018	0.00979	Na	0.000676	0.0294
				V	0.0005	0.0098
Animal Trace Elements Solution (ANIMATE)						
LiCl	31	31	731	Li	5.07	731
				Cl	25.92	731
RbCl	7	7	57.89	Rb	4.95	57.89
				Cl	2.05	57.89

$SrCl_2$	15	15	94.63	Sr	8.29	94.63
				Cl	6.71	189.26
NaBr	1.6	1.6	15.55	Na	0.36	15.55
				Br	1.24	15.55
KI	0.33	0.33	2.0	K	0.0778	2.0
				I	0.253	2.0

APPENDIX E. Sodium Chloride Toxicity Tests

Table E1. Specific conductance of media for all control trials with average specific conductance for corresponding lakes.

Source Lake	Specific Conductivity of Control (uS/cm)
Anstruther Lake	269.33
Big Cedar Lake	251.27
Eels Lake	257.00
Kasshabog Lake	239.60
Loon Call Lake	282.14
Mississauga Lake	238.07
Pencil Lake	260.9
Salmon Lake	250.07
Sucker Lake	237.47
Mean	**253.98**
Standard Deviation	**15.17**

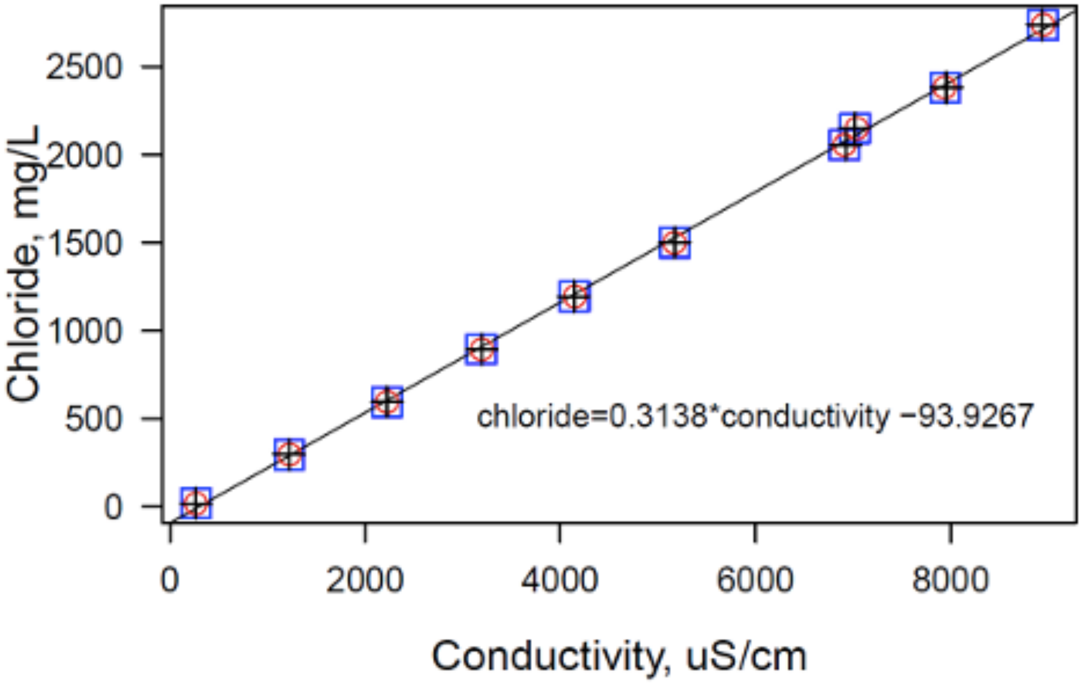

Figure E1. Chloride-specific conductance relationship (Arnott, 2023). This relationship (y = 0.3138x – 93.9267) was used to calculate actual chloride values with the measured specific conductance and is adapted from project protocol distributed by Dr. Shelley Arnott (Queen's University, 2023).

Appendix F: Supplementary Results

Chapter 2 Supplemental Results- Calcium Limitation Sensitivity

General Linear Model

Table F1: Candidate models for general linear model of overall calcium concentration, source lake, and their interactive effects on MSGR. ΔAICc scores greater than 2 or less than -2 are considered significantly different. Where ΔAICc < -2, the model with the lower AICc score is preferred. The favored model is marked with '*'. AICc wt represents AICc weights.

Model	Added Variable	Df	Sum of Squares	RSS	AICc	ΔAICc	AICc wt
Linear 4*	Ca Concentration + Source Lake + Ca Concentration : Source Lake	27	0.12	2.73	-3824.3	0.0	0.9982
Linear 3	Ca Concentration + Source Lake	14	0.47	3.85	-3811.7	12.6	0.0018
Linear 1	+ Ca Concentration	1	0.67	4.32	-3689.6	134.7	<0.001
Linear 2	+ Source Lake	13	0.51	4.47	-3620.0	204.3	<0.001
Null	~1	/	/	/	-3506.1	318.2	<0.001

Table F2. Tukey's HSD pairwise comparisons between source lake group MSGR values. The test estimate, standard error (SE), t value, and p value ($\alpha = 0.05$) are listed. A negative estimate indicates that the first lake has a lower MSGR mean than the second lake. A positive estimate indicates the first lake has a greater MSGR mean than the second lake.

Lake 1	Lake 2	Estimate	SE	t value	p value
ANS	BIG	0.055	0.008	7.287	0.000
ANS	CAT	0.015	0.008	1.942	0.803
ANS	EEL	0.030	0.011	2.797	0.227
ANS	KAS	0.055	0.008	7.206	0.000
ANS	LON	0.030	0.011	2.637	0.318
ANS	LOO	0.023	0.008	3.023	0.131
ANS	LOU	0.013	0.009	1.502	0.967
ANS	MIS	0.004	0.008	0.512	1.000
ANS	PEN	0.022	0.008	2.957	0.156
ANS	RAC	0.018	0.008	2.397	0.483
ANS	SAL	0.018	0.008	2.405	0.477
ANS	SUC	-0.007	0.007	-1.052	0.999
ANS	WOL	0.026	0.009	2.936	0.164
BIG	CAT	-0.041	0.008	-5.290	0.000
BIG	EEL	-0.025	0.011	-2.281	0.570
BIG	KAS	0.000	0.008	-0.050	1.000
BIG	LON	-0.026	0.011	-2.301	0.554
BIG	LOO	-0.033	0.008	-4.298	0.002
BIG	LOU	-0.042	0.009	-4.775	0.000
BIG	MIS	-0.051	0.008	-6.778	0.000
BIG	PEN	-0.033	0.008	-4.302	0.002
BIG	RAC	-0.037	0.008	-4.883	0.000
BIG	SAL	-0.037	0.008	-4.903	0.000
BIG	SUC	-0.062	0.007	-9.131	0.000
BIG	WOL	-0.030	0.009	-3.339	0.053
CAT	EEL	0.016	0.011	1.433	0.978
CAT	KAS	0.040	0.008	5.218	0.000
CAT	LON	0.015	0.011	1.312	0.990
CAT	LOO	0.008	0.008	1.049	0.999
CAT	LOU	-0.002	0.009	-0.179	1.000

CAT	MIS	-0.011	0.008	-1.435	0.978
CAT	PEN	0.008	0.008	1.000	0.999
CAT	RAC	0.003	0.008	0.439	1.000
CAT	SAL	0.003	0.008	0.439	1.000
CAT	SUC	-0.022	0.007	-3.195	0.082
CAT	WOL	0.011	0.009	1.247	0.994
EEL	KAS	0.025	0.011	2.241	0.599
EEL	LON	-0.001	0.014	-0.068	1.000
EEL	LOO	-0.008	0.011	-0.709	1.000
EEL	LOU	-0.017	0.012	-1.465	0.973
EEL	MIS	-0.027	0.011	-2.442	0.450
EEL	PEN	-0.008	0.011	-0.734	1.000
EEL	RAC	-0.012	0.011	-1.130	0.998
EEL	SAL	-0.012	0.011	-1.133	0.998
EEL	SUC	-0.038	0.010	-3.621	0.021
EEL	WOL	-0.005	0.012	-0.392	1.000
KAS	LON	-0.025	0.011	-2.263	0.583
KAS	LOO	-0.032	0.008	-4.230	0.002
KAS	LOU	-0.042	0.009	-4.716	0.000
KAS	MIS	-0.051	0.008	-6.699	0.000
KAS	PEN	-0.033	0.008	-4.234	0.002
KAS	RAC	-0.037	0.008	-4.812	0.000
KAS	SAL	-0.037	0.008	-4.831	0.000
KAS	SUC	-0.062	0.007	-9.027	0.000
KAS	WOL	-0.029	0.009	-3.285	0.063
LON	LOO	-0.007	0.011	-0.606	1.000
LON	LOU	-0.016	0.012	-1.354	0.987
LON	MIS	-0.026	0.011	-2.293	0.561
LON	PEN	-0.007	0.011	-0.631	1.000
LON	RAC	-0.011	0.011	-1.017	0.999
LON	SAL	-0.011	0.011	-1.020	0.999
LON	SUC	-0.037	0.011	-3.426	0.041
LON	WOL	-0.004	0.012	-0.307	1.000
LOO	LOU	-0.010	0.009	-1.089	0.998
LOO	MIS	-0.019	0.008	-2.510	0.402
LOO	PEN	0.000	0.008	-0.040	1.000
LOO	RAC	-0.005	0.008	-0.611	1.000
LOO	SAL	-0.005	0.008	-0.615	1.000
LOO	SUC	-0.030	0.007	-4.418	0.001
LOO	WOL	0.003	0.009	0.350	1.000

LOU	MIS	-0.009	0.009	-1.063	0.999
LOU	PEN	0.009	0.009	1.048	0.999
LOU	RAC	0.005	0.009	0.561	1.000
LOU	SAL	0.005	0.009	0.561	1.000
LOU	SUC	-0.020	0.008	-2.496	0.411
LOU	WOL	0.013	0.010	1.278	0.992
MIS	PEN	0.019	0.008	2.448	0.446
MIS	RAC	0.014	0.008	1.887	0.834
MIS	SAL	0.014	0.008	1.892	0.831
MIS	SUC	-0.011	0.007	-1.621	0.941
MIS	WOL	0.022	0.009	2.497	0.411
PEN	RAC	-0.004	0.008	-0.566	1.000
PEN	SAL	-0.004	0.008	-0.570	1.000
PEN	SUC	-0.030	0.007	-4.327	0.001
PEN	WOL	0.003	0.009	0.383	1.000
RAC	SAL	0.000	0.008	-0.002	1.000
RAC	SUC	-0.025	0.007	-3.713	0.015
RAC	WOL	0.008	0.009	0.872	1.000
SAL	SUC	-0.025	0.007	-3.728	0.015
SAL	WOL	0.008	0.009	0.877	1.000
SUC	WOL	0.033	0.008	4.041	0.004

Non-Linear Model Selection

While many studies have acceptable degrees of deviation from nominal treatment concentrations, we found that between source lake experimental replicates clonal variation and inaccurate media preparation led to us analyzing the data on a continuous concentration scale rather than in discrete nominal concentration groups. A precedence for using an asymptotic non-linear model approach we took can be found in other studies such as Sikora et al. (2016).

Table F3. Candidate models for asymptotic mass-specific growth rate parameters *Ca* (treatment calcium concentration), μ_{max} (maximum MSGR value), K_s (growth rate half-saturation constant), and μ_{zero} (initial neonate growth rate). ΔAICc scores greater than 2 or less than -2 are considered significantly different. Where ΔAICc < -2, the model with the lower AICc score is preferred. Favored models are marked with '*' where applicable, no '*' on either model indicates negligible difference. AIC wt represents AICc weights. Values have been rounded to the nearest hundredth, AICc wt values may not equal 1.

Lake Origin	Model	Model Parameters	Df	AICc	ΔAICc	AICc wt
ANS	A	Ca, umax, Ks	3	-276.78	0.00	0.71
	B	Ca, umax,Ks, uzero	4	-275.01	1.77	0.29
BIG	A*	Ca, umax, Ks	3	-217.50	0.00	0.75
	B	Ca, umax,Ks, uzero	4	-215.33	2.17	0.24
CAT	A*	Ca, umax, Ks	3	-241.17	0.00	0.75
	B	Ca, umax,Ks, uzero	4	-239.00	2.17	0.24
EEL	A*	Ca, umax, Ks	3	-134.41	0.00	0.79
	B	Ca, umax,Ks, uzero	4	-131.81	2.60	0.21
KAS	A	Ca, umax, Ks	3	-177.25	0.00	0.68
	B	Ca, umax,Ks, uzero	4	-175.76	1.49	0.32
LON	A*	Ca, umax, Ks	3	-53.50	0.00	0.79
	B	Ca, umax,Ks, uzero	4	-50.85	2.65	0.21
LOO	A*	Ca, umax, Ks	3	-253.43	0.00	0.75
	B	Ca, umax,Ks, uzero	4	-251.28	2.15	0.25
LOU	A*	Ca, umax, Ks	3	-134.56	0.00	0.73
	B	Ca, umax,Ks, uzero	4	-132.53	2.03	0.27
MIS	A*	Ca, umax, Ks	3	-221.93	0.00	0.75
	B	Ca, umax,Ks, uzero	4	-219.77	2.16	0.25
PEN	A	Ca, umax, Ks	3	-160.80	5.02	0.08
	B*	Ca, umax,Ks, uzero	4	-165.82	0.00	0.92
RAC	A*	Ca, umax, Ks	3	-321.60	0.00	0.75
	B	Ca, umax,Ks, uzero	4	-319.44	2.16	0.25
SAL	A	Ca, umax, Ks	3	-152.87	5.81	0.05
	B*	Ca, umax,Ks, uzero	4	-158.68	0.00	0.95
SUC	A	Ca, umax, Ks	3	-320.35	1.42	0.33
	B	Ca, umax,Ks, uzero	4	-321.77	0.00	0.67
WOL	A*	Ca, umax, Ks	3	-130.77	0.00	0.25
	B	Ca, umax,Ks, uzero	4	-128.52	2.25	0.75

Chapter 3 Supplemental Results- Salt Toxicity Tolerance

Table F4. NaCl Toxicity Tolerance Lethal Concentration Models. Log-logistic 4 parameter (Slope, Upper Limit fixed at 1, Lower Limit fixed at 0, and Effective Dose 50 Intercept) models with parameter estimates, standard error, significance, and overall model residual standard error. All models have 24 degrees of freedom.

Anstruther Lake

Parameter	**Estimate**	**Std. Error**	**t-value**	**p value**
Slope	-7.15	1.60	-4.48	0.00
Upper Limit	1.00	0.05	21.89	< 0.001
ED50	1144.54	41.43	27.63	< 0.001
RSE = 1.16				

Big Cedar Lake

Parameter	**Estimate**	**Std. Error**	**t-value**	**p value**
Slope	-7.96	1.46	-5.46	1.317e-05
Upper Limit	1.01	0.03	33.89	< 0.001
ED50	1068.30	24.30	43.96	< 0.001
RSE = 0.84				

Eels Lake

Parameter	**Estimate**	**Std. Error**	**t-value**	**p value**
Slope	-4.95	1.12	-4.42	0.00
Upper Limit	1.04	0.07	14.33	2.927e-13
ED50	1169.07	64.91	18.01	1.839e-15
RSE = 1.27				

Kasshabog Lake

Parameter	**Estimate**	**Std. Error**	**t-value**	**p value**
Slope	-11.52	1.50	-7.68	6.509e-08
Upper Limit	1.02	0.02	50.27	< 0.001

ED50	1246.08	14.85	83.88	< 0.001
RSE = 0.59				
Loon Call Lake				
Parameter	**Estimate**	**Std. Error**	**t-value**	**p value**
Slope	-8.70	1.11	-7.83	4.604e-08
Upper Limit	1.03	0.03	39.20	< 0.001
ED50	1387.35	22.35	62.06	< 0.001
RSE = 0.65				
Mississauga Lake				
Parameter	**Estimate**	**Std. Error**	**t-value**	**p value**
Slope	-5.68	1.83	-3.10	0.00
Upper Limit	1.03	0.07	5.73	3.862e-14
ED50	1032.96	54.30	19.02	4.999e-16
RSE = 1.53				
Pencil Lake				
Parameter	**Estimate**	**Std. Error**	**t-value**	**p value**
Slope	-10.10	2.21	-4.56	0.00
Upper Limit	1.03	0.04	24.79	< 0.001
ED50	1341.23	32.68	41.04	< 0.001
RSE = 1.11				
Salmon Lake				
Parameter	**Estimate**	**Std. Error**	**t-value**	**p value**
Slope	-6.66	1.82	-3.67	0.00
Upper Limit	1.01	0.05	18.41	1.136e-15
ED50	1071.23	46.60	22.99	< 0.001
RSE = 1.39				

Sucker Lake				
Parameter	**Estimate**	**Std. Error**	**t-value**	**p value**
Slope	-16.70	2.06	-8.12	2.433e-08
Upper Limit	1.00	0.01	73.81	< 0.001
ED50	1096.80	11.56	94.89	< 0.001
RSE = 0.46				

Table F5. Neill's Lack-of-Fit Test Values. Insignificant p values indicate that the data is well explained by the model parameters with replicates n = 3, and there is no 'lack of fit'.

Source Lake	**F Value**	**p Value (α= 0.05)**
Anstruther Lake	0.51	0.79
Big Cedar Lake	0.15	0.99
Eels Lake	0.33	0.91
Kasshabog Lake	1.04	0.43
Loon Call Lake	0.37	0.89
Mississauga Lake	0.27	0.94
Pencil Lake	0.55	0.76
Salmon Lake	0.38	0.88
Sucker Lake	1.10	0.40

Table F6. LC Ratio Test and 95% confidence intervals. Estimates greater than 1 indicated lake 1 has greater LC estimations than Lake 2, equal to1 indicated no difference in estimates, and less than 1 indicated lake 1 had lower LC estimations than lake 2.

Lake 1	Lake 2	LC Level	Estimate	Lower CI	Upper CI
ANS	BIG	10	1.03	0.86	1.21
ANS	EEL	10	1.11	0.91	1.30
ANS	KAS	10	0.81	0.69	0.94
ANS	LOO	10	0.78	0.66	0.90
ANS	MIS	10	1.18	0.94	1.41
ANS	PEN	10	0.78	0.66	0.89
ANS	SAL	10	1.09	0.90	1.27
ANS	SUC	10	0.88	0.77	0.98
BIG	EEL	10	1.07	0.87	1.27
BIG	KAS	10	0.79	0.66	0.92
BIG	LOO	10	0.75	0.62	0.88
BIG	MIS	10	1.14	0.90	1.37
BIG	PEN	10	0.75	0.63	0.87
BIG	SAL	10	1.05	0.85	1.25
BIG	SUC	10	0.85	0.73	0.97
EEL	KAS	10	0.74	0.61	0.86
EEL	LOO	10	0.70	0.58	0.82
EEL	MIS	10	1.06	0.84	1.29
EEL	PEN	10	0.70	0.58	0.82
EEL	SAL	10	0.98	0.79	1.17
EEL	SUC	10	0.79	0.67	0.91
KAS	LOO	10	0.95	0.81	1.09
KAS	MIS	10	1.44	1.16	1.72
KAS	PEN	10	0.95	0.82	1.09
KAS	SAL	10	1.33	1.11	1.56
KAS	SUC	10	1.07	0.95	1.20
LOO	MIS	10	1.52	1.22	1.81
LOO	PEN	10	1.00	0.85	1.15
LOO	SAL	10	1.40	1.16	1.64
LOO	SUC	10	1.13	0.99	1.27
MIS	PEN	10	0.66	0.53	0.79
MIS	SAL	10	0.92	0.73	1.12
MIS	SUC	10	0.74	0.61	0.87
PEN	SAL	10	1.40	1.17	1.63

PEN	SUC	10	1.13	1.00	1.26
SAL	SUC	10	0.81	0.69	0.92
ANS	BIG	25	1.05	0.94	1.17
ANS	EEL	25	1.05	0.93	1.17
ANS	KAS	25	0.87	0.78	0.95
ANS	LOO	25	0.80	0.72	0.88
ANS	MIS	25	1.15	1.00	1.30
ANS	PEN	25	0.82	0.74	0.90
ANS	SAL	25	1.08	0.96	1.20
ANS	SUC	25	0.96	0.88	1.03
BIG	EEL	25	1.00	0.87	1.12
BIG	KAS	25	0.82	0.73	0.91
BIG	LOO	25	0.76	0.68	0.85
BIG	MIS	25	1.09	0.94	1.24
BIG	PEN	25	0.77	0.69	0.86
BIG	SAL	25	1.02	0.90	1.15
BIG	SUC	25	0.91	0.82	0.99
EEL	KAS	25	0.82	0.73	0.92
EEL	LOO	25	0.76	0.68	0.85
EEL	MIS	25	1.09	0.94	1.24
EEL	PEN	25	0.78	0.69	0.86
EEL	SAL	25	1.03	0.90	1.16
EEL	SUC	25	0.91	0.82	1.00
KAS	LOO	25	0.93	0.84	1.02
KAS	MIS	25	1.32	1.16	1.49
KAS	PEN	25	0.94	0.85	1.03
KAS	SAL	25	1.25	1.11	1.38
KAS	SUC	25	1.10	1.02	1.19
LOO	MIS	25	1.43	1.24	1.61
LOO	PEN	25	1.02	0.92	1.12
LOO	SAL	25	1.35	1.19	1.50
LOO	SUC	25	1.19	1.09	1.29
MIS	PEN	25	0.71	0.62	0.80
MIS	SAL	25	0.94	0.81	1.07
MIS	SUC	25	0.83	0.74	0.93
PEN	SAL	25	1.32	1.18	1.47
PEN	SUC	25	1.17	1.08	1.26
SAL	SUC	25	0.89	0.80	0.97
ANS	BIG	50	1.07	1.00	1.15
ANS	EEL	50	1.00	0.92	1.08

ANS	KAS	50	0.92	0.86	0.98
ANS	LOO	50	0.83	0.77	0.89
ANS	MIS	50	1.12	1.03	1.21
ANS	PEN	50	0.86	0.80	0.92
ANS	SAL	50	1.07	0.99	1.16
ANS	SUC	50	1.04	0.97	1.11
BIG	EEL	50	0.93	0.86	1.00
BIG	KAS	50	0.86	0.80	0.91
BIG	LOO	50	0.77	0.72	0.83
BIG	MIS	50	1.04	0.96	1.13
BIG	PEN	50	0.80	0.75	0.85
BIG	SAL	50	1.00	0.92	1.08
BIG	SUC	50	0.97	0.91	1.04
EEL	KAS	50	0.92	0.85	0.99
EEL	LOO	50	0.83	0.77	0.90
EEL	MIS	50	1.12	1.02	1.22
EEL	PEN	50	0.86	0.80	0.92
EEL	SAL	50	1.07	0.99	1.16
EEL	SUC	50	1.05	0.97	1.12
KAS	LOO	50	0.90	0.85	0.96
KAS	MIS	50	1.21	1.12	1.31
KAS	PEN	50	0.93	0.88	0.99
KAS	SAL	50	1.16	1.08	1.25
KAS	SUC	50	1.13	1.07	1.20
LOO	MIS	50	1.35	1.24	1.45
LOO	PEN	50	1.03	0.97	1.10
LOO	SAL	50	1.29	1.20	1.39
LOO	SUC	50	1.26	1.18	1.34
MIS	PEN	50	0.77	0.71	0.83
MIS	SAL	50	0.96	0.88	1.04
MIS	SUC	50	0.93	0.86	1.01
PEN	SAL	50	1.25	1.16	1.34
PEN	SUC	50	1.22	1.14	1.29
SAL	SUC	50	0.97	0.90	1.04

Table F7. Comparison of LC_{50} values using EDcomp (drc package). Significant p values ($p<0.05$) and the lake with the greater LC_{50} estimate are marked with '*'.

Lake 1	Lake 2	Estimate	Std Error	t value	p value
ANS	EEL	-1.62	46.82	-0.03	0.97
ANS	LOO*	-233.58	43.98	-5.31	0.00*
ANS	PEN*	-188.97	41.53	-4.55	0.00*
BIG	ANS	-78.21	40.90	-1.91	0.06
BIG	EEL	-79.83	45.24	-1.76	0.08
BIG	LOO*	-311.79	42.30	-7.37	0.00*
BIG	PEN*	-267.17	39.74	-6.72	0.00*
EEL	LOO*	-231.96	48.04	-4.83	0.00*
EEL	PEN*	-187.34	45.81	-4.09	0.00*
KAS*	ANS	97.74	39.46	2.48	0.01*
KAS*	BIG	175.95	37.57	4.68	0.00*
KAS*	EEL	96.12	43.94	2.19	0.03*
KAS	LOO*	-135.84	40.90	-3.32	0.00*
KAS	PEN*	-91.22	38.25	-2.38	0.02*
KAS*	SAL	175.79	39.55	4.45	0.00*
MIS	ANS*	-121.69	44.84	-2.71	0.01*
MIS	BIG	-43.48	43.19	-1.01	0.32
MIS	EEL*	-123.31	48.83	-2.53	0.01*
MIS	KAS*	-219.43	41.83	-5.25	0.00*
MIS	LOO*	-355.27	46.12	-7.70	0.00*
MIS	PEN*	-310.65	43.79	-7.09	0.00*
MIS	SAL	-43.64	44.92	-0.97	0.33
PEN	LOO	-44.62	42.91	-1.04	0.30
SAL	ANS	-78.05	42.73	-1.83	0.07
SAL	BIG	0.16	40.99	0.00	1.00
SAL	EEL	-79.67	46.89	-1.70	0.09
SAL	LOO*	-311.63	44.06	-7.07	0.00*
SAL	PEN*	-267.01	41.62	-6.42	0.00*
SUC	ANS	-48.12	38.67	-1.24	0.21
SUC	BIG	30.08	36.74	0.82	0.41
SUC	EEL	-49.74	43.23	-1.15	0.25
SUC	KAS*	-145.86	35.12	-4.15	0.00*
SUC	LOO*	-281.70	40.14	-7.02	0.00*
SUC	MIS	73.56	41.08	1.79	0.07
SUC	PEN*	-237.09	37.44	-6.33	0.00*
SUC	SAL	29.93	38.76	0.77	0.44

Table F8. Comparison of slope parameter using compParm (drc package). Significant p values ($p<0.05$) and the lake with the steeper slope estimate are marked with '*'.

Lake 1	Lake 2	Estimate	Std Err	t Value	p value
SUC *	MIS	-10.75	4.63	-2.32	0.02*
SUC	KAS	-4.93	5.23	-0.94	0.35
SUC*	SAL	-10.03	4.65	-2.16	0.03*
SUC	BIG	-8.77	4.78	-1.83	0.07
SUC*	ANS	-9.76	4.64	-2.10	0.04*
SUC*	EEL	-11.56	4.55	-2.54	0.01
SUC	PEN	-6.25	4.92	-1.27	0.21
SUC	LOO	-7.69	4.80	-1.60	0.11
MIS	KAS*	5.82	2.89	2.02	0.04*
MIS	SAL	0.72	1.63	0.44	0.66
MIS	BIG	1.98	1.98	1.00	0.32
MIS	ANS	0.99	1.61	0.62	0.54
MIS	EEL	-0.81	1.33	-0.61	0.54
MIS	PEN	4.50	2.30	1.96	0.05
MIS	LOO	3.06	2.02	1.52	0.13
KAS	SAL	-5.10	2.91	-1.75	0.08
KAS	BIG	-3.84	3.12	-1.23	0.22
KAS	ANS	-4.83	2.90	-1.66	0.10
KAS*	EEL	-6.63	2.76	-2.40	0.02*
KAS	PEN	-1.33	3.34	-0.40	0.69
KAS	LOO	-2.76	3.15	-0.88	0.38
SAL	BIG	1.26	2.01	0.63	0.53
SAL	ANS	0.27	1.65	0.17	0.87
SAL	EEL	-1.53	1.39	-1.10	0.27
SAL	PEN	3.78	2.33	1.62	0.11
SAL	LOO	2.34	2.05	1.14	0.25
BIG	ANS	-0.99	2.00	-0.50	0.62
BIG	EEL	-2.79	1.78	-1.57	0.12
BIG	PEN	2.52	2.59	0.97	0.33
BIG	LOO	1.08	2.34	0.46	0.64
ANS	EEL	-1.80	1.36	-1.32	0.19
ANS	PEN	3.50	2.32	1.51	0.13
ANS	LOO	2.07	2.04	1.02	0.31
EEL	PEN*	5.31	2.14	2.48	0.01*
EEL	LOO*	3.87	1.83	2.12	0.04*
PEN	LOO	-1.43	2.62	-0.55	0.58