A Thesis

entitled

Freshwater Salinization Alters the Biology and Ecology of Zooplankton.

by

Eric David Huber

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in Biology (Ecology and Organismal Biology)

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An Abstract of

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Human-induced environmental change creates novel stressors which populations have not experienced over the course of their evolutionary histories. These stressors are unique selective pressures which may alter species' survival, growth, reproduction, or behaviors. Freshwater salinization is of growing interest in the scientific community because of the substantial increase in salinity concentrations from irrigation, mining runoff, groundwater pumping, and road salt use. Freshwater salinization is particularly harmful to zooplankton. Zooplankton are critically important to the health of freshwater ecosystems and maintaining ecosystem services such as fisheries, recreation, and clean drinking water. Recent research demonstrates that zooplankton may be capable of rapid adaptation to elevated salinity, which has implications for ecological stability. However, many questions remain regarding how changes in zooplankton tolerance to salinization alters survival, growth, reproduction, and behaviors. To address these important questions, I conduced two separate studies. My first chapter addresses how a multigenerational exposure to salt pollution alters the life history traits of zooplankton and if life history tradeoffs can promote ecological stability. I found that freshwater zooplankton are able to make life history tradeoffs to cope with low levels of salt stress.

However, these tradeoffs only occurred in specific salt types and concentrations. Additionally, I found that prolonged, multigenerational exposure to salinity can lead to maladaptation where a long-term exposure history does not confer adaptation, but further reduces lifetime reproduction. My second chapter consists of two experiments to address how the non-consumptive effects of predation may interact with increased freshwater salinity, and a multigenerational exposure to salt pollution to affect 1) the population-level abundance of zooplankton, and 2) the anti-predatory escape behavior of zooplankton. I found in the abundance experiment that salinity and the non-consumptive effects of predation reduced zooplankton abundance 50%. Further, it appears that predation can likely mask the effect of salinity because the effects of salinity are sub-lethal relative to the lethality of predation. In the escape behavior experiment, I found that salinity decreased the vertical escape velocity of zooplankton following a physical disturbance reminiscent of a predation attempt. A prolonged multigenerational exposure to salinity further decreased the swimming velocities of zooplankton. However, when these treatments where combined with predator cues, I found that zooplankton swam at the same speed as control conditions lacking salinization or a predator cue. This finding suggests that predation is a driving force in the behavior of zooplankton and is able to mask the effect of salinity. Changes in zooplankton behavior due to human-induced stress and subsequent masking by natural stressors has implications for community interactions in human-dominated environments. My thesis demonstrates that dramatic effects on zooplankton biology and ecology can occur at environmentally relevant concentrations of salinity, and regulatory thresholds set by the United States Environmental Protection

Agency are insufficient in protecting freshwater organisms from the adverse effects of freshwater salinization.

This thesis is dedicated to my grandfather, Jack Huber, who sparked my passion for the outdoors.

Acknowledgements

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List of Abbreviations

CI.....Confidence Interval

GLM.....Generalized Linear Model

PPT.....Parts Per Thousand

SPP.....Several Species

List of Symbols

Ca^{2+} .....Calcium Ion
$CaCl_2$ .....Calcium Chloride
Cl^- .....Chloride Ion
Na^+ .....Sodium Ion
NaCl .....Sodium Chloride

cm.....Centimeter
mm .....Millimeter
µm .....Micrometer
mg .....Milligram
L .....Liter
mL .....Milliliter
d.....Day
s .....Second

℃.....Degrees Celsius

F_O .....Initial Generation

$\Delta\bar{x}$ .....Difference in means

Chapter 1

Coping with Stress: Changes in Life History in Response to Freshwater Salinization

1.1 Introduction

Organisms must deal with energetic tradeoffs to ensure survival, growth, and reproduction to optimize fitness [1]. Changing environmental conditions can lead to stress that can alter the tradeoffs that affect survival, growth, or reproduction. Plastic responses in life history traits or the evolution of new life history strategies can facilitate successful adaptation to changing environmental conditions. Such successful adaptation can then have implications on population and community dynamics [2, 3] potentially facilitating ecological stability [4]. However, life-history tradeoffs and adaptation to novel, human-induced stressors that might facilitate ecological stability remains to be well understood.

In freshwater ecosystems, a novel stressor of growing concern is human-induced salinization caused by the application of road deicing salts [5]. Road salts are typically inorganic, chloride (Cl^-)-based salts. Since the start of their use in 1938 [6], road salt pollution has increased the salinity of freshwater ecosystems from historical Cl^-

concentrations of 0-10 mg Cl^-/L [7, 8] to concentrations reaching 200 to 5000 mg Cl^-/L [6, 9-11]. Currently, the United States Environmental Protection Agency has published thresholds of 230 mg Cl^-/L and 860 mg Cl^-/L for chronic and acute chloride concentrations, respectively, to protect freshwater organisms from Cl^--based salts [12]. However, these Cl^- thresholds are insufficient to protect many freshwater ecosystems from the harmful effects of salinization caused by sodium chloride (NaCl), one of the most common inorganic salt types leading to freshwater salinization and the most common deicer [13, 14]. Additionally, these Cl^- thresholds do not account for the differences in toxicity between different road deicing salts such as NaCl and calcium chloride ($CaCl_2$) [15], two of the most common deicers used globally [6]. Deicers have led to the stark decline in abundance and population growth rates of several freshwater zooplankton species leading to trophic cascades and altered community structure [13, 16-19]. Yet, few studies have investigated how salinization changes the life history traits of freshwater zooplankton [14, 20, 21], which may provide insight into the changes in population dynamics as a result of road salt use [2].

Changes in the life history of primary consumers such as zooplankton has considerable implications for the functioning of freshwater ecosystems. Large-bodied cladocerans such as *Daphnia pulex* are important species in many freshwater ecosystems and serve as one of the main grazers of phytoplankton. The algal-zooplankton energy pathway is critically important in the stability and functioning of freshwater food webs because it transfers energy from primary producers to higher-level consumers [22]. Thus, understanding the life history trade-offs associated with plastic or evolved responses to

the rising salinity of freshwater ecosystems is fundamental to understanding the contemporary functioning of ecosystems contaminated by salt pollution [2, 23].

Many species may be capable of facilitating resilience via rapid adaptive change to human-induced stressors like salt pollution [24, 25]. Adaptation to higher salinity resulting from road salt use has occurred in freshwater zooplankton populations [26]. Coldsnow et al. (2017) demonstrated that *Daphnia pulex,* a cosmopolitan zooplankton important to aquatic food webs, evolved a tolerance to high salinities in as few as two to three months, or eight to 10 generations. A trade-off to the cost of maintaining evolved tolerance, in this example, was a reduction in population growth rate [27]. We currently do not know how tradeoffs associated with elevated salinity will affect life history traits such as lifespan, maturation rates, number of reproductive events, or brood size. Such changes in organismal life history could drive changes in population dynamics and stability [2].

Life history traits are strongly influenced by resource availability, which can be highly variable in salinized systems of varying trophic state. Resource availability, such as phytoplankton density, can have large impacts on the ability of zooplankton to survive, grow, and reproduce under a range of environmental conditions [28, 29]. Food availability at or near subsistence levels might influence the life history traits such as survival, age at first reproduction, and lifetime reproductive output [30-32]. A greater food supply provides organisms with additional or sufficient energy to respond physiologically or behaviorally to the effects of stressors. Some suggest the tolerance of zooplankton to road salts increases with food concentration [33]. Therefore, zooplankton communities in lakes and wetlands of varying food density (e.g., trophic state) could have

very different responses to freshwater salinization caused by road salts. In general, most natural water bodies have phytoplankton densities at or above the subsistence rate necessary to maintain zooplankton population size [34]. However, more energy is often needed to maintain homeostasis under stressful conditions [35, 36], and it remains unclear how prolonged stress in a population will interact with food availability among freshwater ecosystems. Understanding the role of food availability on stress-induced changes in life-history traits is essential to drawing generalizable conclusions about how zooplankton will respond to road salt pollution, a problem plaguing lakes, wetlands, and streams in cold regions worldwide [37-39].

Here, I tested the effects of freshwater salinization and the prolonged exposure of populations to salinization on zooplankton life history traits. My goal was to better understand life history tradeoffs and the potential for demographic resistance (i.e., no decline in life history traits) and resilience (i.e., recovery in life history traits) to road salt stress over the course of a lifetime. I also asked whether food density interacts with the effects of salt pollution to alter life history traits. I conducted a cohort life history analysis by tracking the reproductive output of individuals from birth to death. I evaluated the effect of the treatments on six life-history response variables: 1) lifespan, 2) maturation rate, 3) brood interval, 4) average brood size, 5) body size, and 6) lifetime reproductive output. I used lifetime reproductive output as an indicator of potential cohort fitness, and I used changes in other life history traits to explain variation in lifetime reproductive output. Due to osmotic stress, I predicted that exposure to elevated concentrations of road salt would reduce lifespan, brood size, and body size and increase time to maturation, and brood interval leading to lower lifetime reproductive output. I also predicted that the

magnitude of these life history changes would be reduced with an increase in food availability and a prior exposure history to elevated salinity over multiple generations.

1.2 Methods

1.2.1 Creating an Exposure History

To determine the effect of prolonged exposure to various salt types and concentrations, I cultured *Daphnia pulex* populations in five treatment combinations: NaCl – 230 mg Cl^-/L, NaCl – 860 mg Cl^-/L, $CaCl_2$ – 230 mg Cl^-/L, $CaCl_2$ – 860 mg Cl^-/L, and a control with no salt additions. I standardized the salts by chloride concentration as it is thought to be the more toxic ion in these salts [40] and the ion for which water quality guidelines were developed [12]. I used two Cl^- concentrations as they are the acute (230 mg Cl^-/L) and chronic (860 mg Cl^-/L) thresholds set by the United States Environmental Protection Agency [12], they are environmentally relevant concentrations often observed in field studies [9, 41], and the low and high concentrations show varying magnitudes of sublethal effects as determined by pilot experiments.

Culturing water consisted of a 1:1 mixture of aged municipal tap water and reverse osmosis water to minimize ambient chloride concentrations without depleting necessary minerals for *Daphnia*. I dosed NaCl and $CaCl_2$ road salts to bring the total Cl^- content to the target concentrations of 230 and 860 mg Cl^-/L for the respective treatments. Specifically, I used the *Safe Step 3300 Rock Salt Ice Melter* for the NaCl

mixture and *Green Gobbler SNOW & ICE Melt Pellets* for the $CaCl_2$ mixture. I used a *YSI ProDSS* multiparameter digital water quality meter with a calibrated Cl^- sensor to determine the Cl^- concentrations of treatment solutions and adjusted as necessary to match the desired nominal concentrations. Actual concentrations were on average within 10% of the nominal concentrations, thus I chose to report nominal target concentrations in the results.

I inoculated each culture combination with five adult *Daphnia* from a stable culture housed in the same culture media for over six months. I initially housed these cultures in beakers with 500mL of their respective treatment solutions on a 16:8-hour light cycle and maintained at 21.5 °C (± 0.03°C). As population size increased, I transferred cultures to 15L containers filled with six liters of their respective treatment solutions. I fed all cultures equal densities of *Raphidocelis subcapitata* throughout the culturing period. I renewed culture media weekly while cultures where in 500mL beakers and biweekly when in 15L containers to prevent excessive algae and metabolite accumulation.

These cultures were maintained in elevated road salt concentrations, along with the control, for 102 days. Based on pilot life history experiments, I estimate that a minimum of 13 to 17 generations were produced during this time. Research demonstrates that eight to 10 generations were sufficient to produce evolved responses in the form of an increased tolerance to salt pollution in other populations of *Daphnia* [26].

To test for changes in life history as a response of a multigenerational exposure to salts, I relied on the assumption that the F_O and several subsequent generations were completely removed from the populations by the time I sampled populations for

individuals for the life history assay. *Daphnia pulex* has an estimated median lifespan of 38 days [42]. Additionally, I observed several population declines over the course of the 102-day culturing period were large, mature *Daphnia* died off and left only neonates in culture. Thus, I was confident that several generations of *Daphnia* were removed from culture prior to sampling efforts.

1.2.2 Experimental Design

I utilized a fractional factorial design to compare the effects of prolonged, multigenerational salt exposure to a naïve, first-time exposure (Figure 1.1). The design resulted in 18 experimental treatments replicated 10 times with individual *Daphnia* for a total of 180 experimental units. For the life history experiment (see below), *Daphnia* originating from the salt-exposed cultures were kept in their respective treatment solutions. *Daphnia* originating from the naïve, no-salt control culture were placed into five treatment combinations: NaCl – 230 mg Cl^-/L, NaCl – 860 mg Cl^-/L, $CaCl_2$ – 230 mg Cl^-/L, $CaCl_2$ – 860 mg Cl^-/L, and control with no salt additions. The *Daphnia* originating from the control population and kept in control conditions for the life history experiment served as the baseline for all treatment comparisons. With this design, I was able to compare the effect of a first-time exposure to salts to that of an organism originating from a salinized population of the same salt type and concentration.

I crossed these treatments with two food concentrations: low – 30,000 cells/mL, and high – 80,000 cells/mL. This was done to determine the effect of food availability on life history traits. Food concentrations were chosen based on published literature of *Daphnia* starvation thresholds [43], salinity-food availability interactions in *Daphnia*

[33], phytoplankton densities observed in field studies [44, 45], and previous food density pilot studies with *Daphnia*.

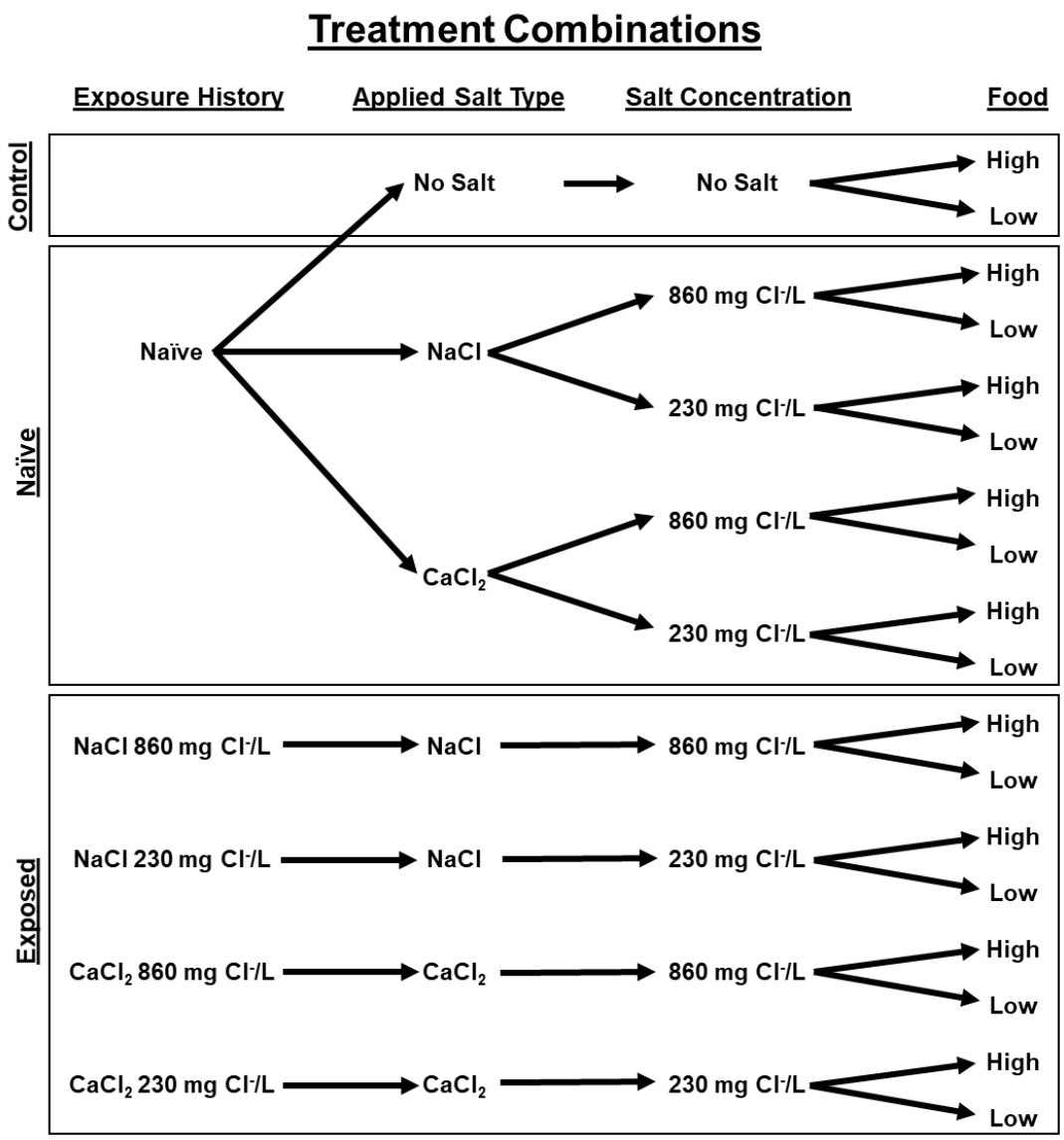

Figure 1–1: A visual of the fractional factorial design utilized in this experiment. Treatments are first organized by 'Exposure History,' where *Daphnia* were either cultured Naïve (no-salt control media), or in a combination of either NaCl or $CaCl_2$ salts in 230 mg Cl^-/L or in 860 mg Cl^-/L for several generations. For the cohort life history analysis, organisms were taken from their source "Exposure History" populations and neonates were placed into 70mL cups with the appropriate treatment combination of salt type and concentration. These treatments were finally crossed with high and low food concentrations. This design resulting in 18 experimental treatments.

1.2.3 Life History Experiment

To evaluate changes to life history over multiple generations in populations of *Daphnia*, I adapted methods from similar studies concerned with individualistic life history assays [14, 20, 46, 47]. I required neonates (i.e., juveniles) of a known age to quantify timing to various life history events. To do this, I sampled individuals from each culture to monitor and produce neonates in a controlled setting. This also provided an additional known generation removed from the initial culturing. Once a sufficient number of neonates were collected, I housed them individually in 70mL of their respective treatments and quantified life history traits.

1.2.3.1 Neonate Production

After 102 days of culturing, I randomly sampled adult *Daphnia* from each of the five cultures. I sampled 24 *Daphnia* from the NaCl – 230 mg Cl^-/L culture, 24 from NaCl – 860 mg Cl^-/L, 24 from $CaCl_2$ – 230 mg Cl^-/L, 26 from $CaCl_2$ – 860 mg Cl^-/L, and 36 from the naïve control. I placed individual *Daphnia* in 70mL of their respective culture solutions and fed to satiation daily with *R. subcapitata*. The enclosure used for both phase one and phase two were Dixie® Plastic Cups. These cups provide adequate space for individual *Daphnia*. I renewed each cup with 70mL of the appropriate treatment solution every 72 hours. I checked the *Daphnia* daily for neonate production until I obtained enough individuals to fully populate all experimental units. After six days, the phase one *Daphnia* produced all the necessary neonates. I then mixed the neonates with others from the same population and randomly sampled individuals to create each experimental unit. I then ended neonate production and began the life history experiment with the new offspring of known age.

1.2.3.2 Life History Experiment

On 15 August 2021, I randomly assigned < 24 hour-old neonates into 70mL of the treatment solutions. I housed the *Daphnia* in a controlled environmental chamber with a 16:8-hour light cycle and held at a temperature of 21.5 °C (± 0.03°C). For the first three days, I fed neonates to satiation to mitigate the effects of stress from transfer into the experiment. On the fourth day, I began feeding at the low and high food concentrations. I renewed treatment solutions every three days for the first 12 days of the experiment to reduce the buildup of algae and metabolites. I found no ammonia accumulation in pilot experiments when *Daphnia* were housed in 70mL of solution and fed higher food densities for five days without solution renewal. Because of this, on day twelve I switched to a solution renewal regime for every three and four days repeating. I monitored the experiment daily to determine: 1) date of reproductive events, 2) number of neonates in each reproductive event, and 3) mortality. I define mortality as inactivity and a lack of flight response within 15 seconds of being gently prodded. All *Daphnia* had died by 19 October 2021, and I concluded the experiment.

To determine if body size co-varied with life history traits, individual *Daphnia* that survived past day 12 were preserved in 4mL of 2.5% Lugol's solution for at least 48 hours before I took body size measurements using a *Wild M5* stereo microscope outfitted with an *INFINITY3-3UR* 2.8-megapixel digital microscopy camera. I measured the standard length of the *Daphnia* by taking the length from the base of the tail spine to the carapace anterior to the compound eye [48].

1.2.4 Statistical Analysis

For each treatment combination, I specifically analyzed 1) lifespan, 2) timing to maturation, 3) average brood size, 4) brood interval (the timing between broods being produced), 5) body size at death, and 6) lifetime reproductive output. I employed generalized linear models (GLMs) for each of these variables to test specifically for treatment interactions with food availability. Lifespan, average brood size, total reproductive output, and body size were analyzed with a gaussian distribution. I analyzed timing to maturation with a Poisson distribution, and brood interval with a Quasi-Poisson distribution to account for the overdispersion of the data. I removed the salt-exposed population in $CaCl_2$ – 860 mg Cl^-/L from the body size analysis because of insufficient replicates (n = 2 for low food individuals, n = 4 for high food individuals) to evaluate salt-by-food interactions in this treatment.

Post-hoc to the GLMs for salt-by-food interactions, I evaluated the effect size of each treatment as described by Cumming (2012). I estimated the standard effect size as the difference between a treatment mean and the control mean for each variable. I derived the 95% confidence intervals (CIs) through nonparametric bootstrap resampling of the means of each treatment 5,000 times which enables the calculation and visualization of CIs as a graded sampling distribution. I chose this method as it provides a transparent way to visualize and describe complex datasets and to highlight the biological importance of treatment responses without the need for extensive null-hypothesis significance testing [49]. Differences in the life history traits were determined by identifying biologically relevant effect sizes (generally $\Delta\bar{x} \geq 10\%$) and the degree of overlap between 95% CIs.

To derive and plot estimation statistics, I used the '*DABESTR'* package [50] in RStudio version 4.1.2 to generate effect sizes and CIs for each response variable. In

addition, *'DABESTR'* generates "Cummings estimation plots" [49] for effect size and CI visualization which I chose to report results.

I performed a Pearson product-moment correlation analysis to measure the strength and direction of associations between 1) lifespan, 2) timing to maturation, 3) average brood size, 4) brood number, 5) brood interval, 6) body size at death, and 7) lifetime reproductive output. This analysis creates a matrix of correlation coefficients with a range of +1 to -1. Values greater than 0 indicate a positive association where both values increase. Values less than 0 indicate a negative association where one value decreases while another increases. I calculated Pearson correlation coefficients and p-values using the *'Hmisc'* package [51], and the Pearson correlation coefficient matrix was plotted using the *'corrplot'* package [52] in RStudio version 4.1.2.

1.3 Results

1.3.1 Salt-by-Food Interaction

Across all response variables, I found no treatment interactions between food availability and the measured life history traits ($F_{1,8} \leq 1.54$, $p \geq 0.111$). For this reason, I combined the data from the low and high food density treatments when I analyzed the responses of the life history traits to the different salt types, salt concentrations, and exposure history. I illustrate the low and high food density data in the Cumming estimation plots.

1.3.2 Food Main Effects

High concentrations of food had no effect on lifespan, brood interval, or body size relative to low food concentrations (Table 1.1). Increased food concentrations decreased

Table 1.1: The main effects of food availability on all response variables. Means of each treatment ($\triangle\bar{x}$) are represented, and 95% CIs calculated for the High Food treatments are displayed. Bolded observations indicate that the 95% CIs of the High Food treatment does not overlap with the mean of the Low Food treatment.

Response	Unit	Low Food $\triangle\bar{x}$	High Food $\triangle\bar{x}$	95% CI
Lifespan	Days	28.3	28.17	23.15, 33.32
Maturation	**Days**	**8.06**	**7.52**	**6.39, 8.04**
Brood Interval	Days	3.8	3.51	2.58, 3.72
Brood Size	**Neonates**	**9.07**	**10.78**	**11.46, 13.56**
Body Size	Neonates	2.37	2.35	2.23, 2.43
Total Output	**Neonates**	**82.34**	**108.72**	**110.72, 159.02**

maturation rates by 7% ($\Delta\bar{x}$: −0.54 d) and increased average brood sizes by 19% ($\Delta\bar{x}$: 1.7 neonates). Finally, high food concentrations increased the total reproductive output of organisms by 32% ($\Delta\bar{x}$: 26 neonates).

1.3.3 Lifespan

1.3.3.1 NaCl

To determine how salt type, concentration, and exposure history affected the lifespan of organisms, I analyzed the number of days from birth to death of individuals in each treatment (Figure 1.2). In the low concentration of NaCl, the average lifespan for the naïve and salt-exposed population had increased by 22-25% ($\Delta\bar{x}$: 6.9 - 7.7 d) relative to

the control. The naïve population in the high concentration of NaCl increased by 19% ($\Delta\bar{x}$: 5.8 d). The salt-exposed population in the NaCl high concentration decreased by 10% ($\Delta\bar{x}$: −3.2 d) from the control. In the high concentration of NaCl, the lifespan of the salt-exposed population was 24% ($\Delta\bar{x}$: −9 d) lower on average than the naïve population.

1.3.3.2 $CaCl_2$

Lifespan was lower than the control population in all elevated concentrations of $CaCl_2$ (Figure 1.2). In the low concentration of $CaCl_2$, the average lifespan for the naïve population was reduced by 10% ($\Delta\bar{x}$: −3.2 d). The lifespan of the salt-exposed population in low $CaCl_2$ decreased by 30% ($\Delta\bar{x}$: −9.2 d) relative to the control. Compared to the control in the high concentration of $CaCl_2$, the lifespan of the naïve population decreased by 63% ($\Delta\bar{x}$: −19.3 d) and the salt-exposed population decreased by 33% ($\Delta\bar{x}$: −10.2 d). When comparing the naïve and salt-exposed populations in the high $CaCl_2$ concentrations to one another, the salt-exposed population lived on average 80% ($\Delta\bar{x}$: 9.2 d) longer than the naïve population.

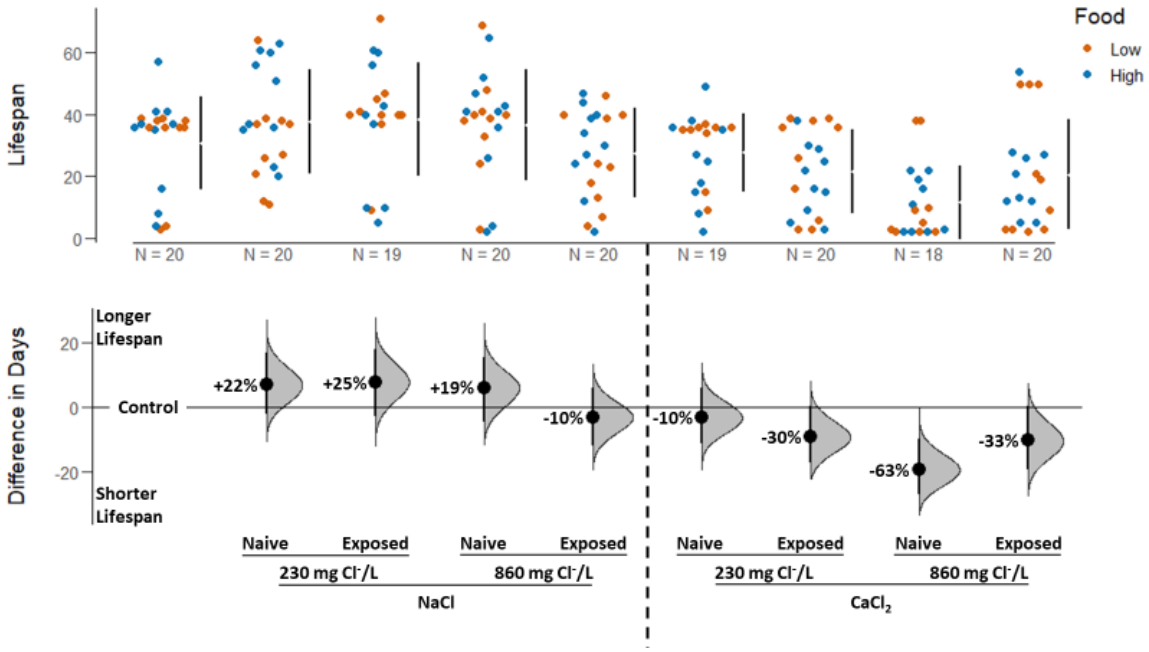

Figure 1–2: Effects of treatments on average lifespan shown in a Cumming estimation plot. Data was analyzed as number of days survived in each replicate. Estimation plots include two sections to show the nature of the raw data (top section) and the magnitude and variability of the effect sizes (bottom section). For the top section, the x-axis displays sample size for each treatment. The points are the raw data presented in a swarm plot. Standard deviation is indicated by the black vertical lines, and the central point of which represents the mean. The bottom section of the plot displays the standard effect size between the treatment and the control group. The central dot represents the difference in means, and the vertical bars show the 95% CIs for each treatment. The greyed, vertical distribution along the confidence interval bar shows the sampling-error curve from the bootstrapping analysis. To the left of the vertical dashed line are NaCl treatments, and to the right are $CaCl_2$ treatments

1.3.4 Maturation

1.3.4.1 NaCl

I analyzed the number of days from birth to first reproduction of individuals in each treatment to determine how maturation was affected. I found no difference in maturation between the control and the naïve and salt-exposed populations in the low NaCl concentration (Figure 1.3). Compared to the control, maturation in the high NaCl concentration in the salt-exposed and naïve populations was delayed by 18% ($\Delta\bar{x}$: 1.3 d) and 30% ($\Delta\bar{x}$: 2.2 d), respectively. There were no differences in maturation between the salt-exposed and naïve populations in the NaCl treatments.

1.3.4.2 $CaCl_2$

In the low concentration of $CaCl_2$, the maturation for both the naïve and salt-exposed populations showed no difference from the control. Similarly, the naïve and salt-exposed populations in the high $CaCl_2$ concentration showed no difference from the control.

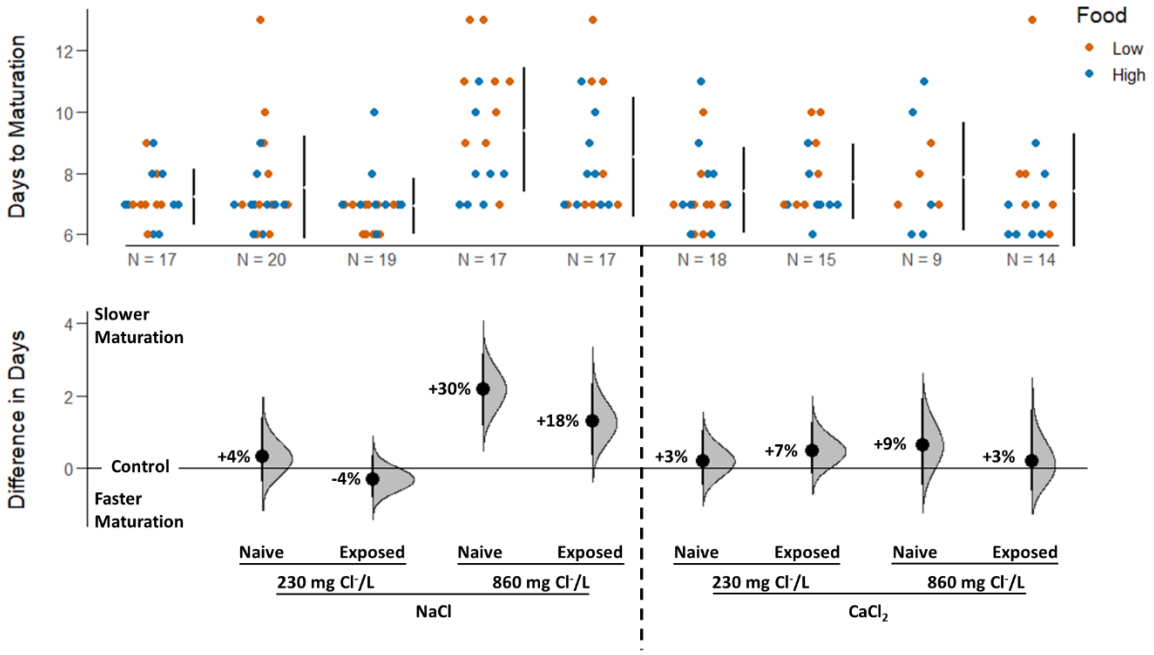

Figure 1–3: Effects of treatments on average days to maturation shown in a Cumming estimation plot.

1.3.5 Brood Interval

1.3.5.1 NaCl

Post-maturation, I analyzed the average number of days between subsequent broods. Brood interval was generally similar (1-3% difference) between the naïve and salt-exposed populations and the control in the low NaCl treatment (Figure 1.4). There was also no difference in brood interval between the naïve and control populations in the high NaCl concentration. However, brood interval increased by 31% ($\Delta\bar{x}$: 1.0 d) compared to the control population in the high NaCl concentration.

1.3.5.2 $CaCl_2$

In the low concentration of $CaCl_2$, the brood intervals for both the naïve and salt-exposed populations were less than 10% higher than the control (Figure 1.4). In the high concentration of $CaCl_2$, both the naïve and salt-exposed populations had brood intervals that were 43% ($\Delta\bar{x}$: 1.4 d) and 64% ($\Delta\bar{x}$: 2.0 d) longer than the control, respectively. There were no differences in brood interval between the salt-exposed and naïve populations in the $CaCl_2$ treatments.

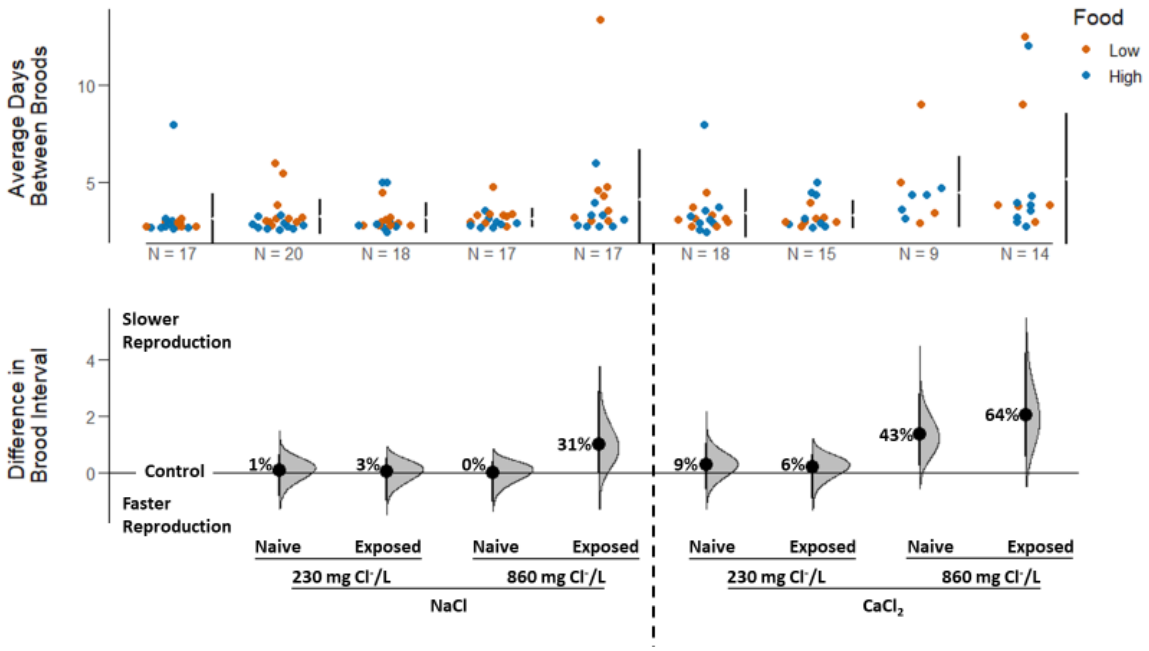

Figure 1–4: Effects of treatments on average days between broods shown in a Cumming estimation plot.

1.3.6 Brood Size

1.3.6.1 NaCl

I analyzed the average number of neonates (offspring) *Daphnia* produced per brood in each treatment. In the low NaCl concentration, the average brood size when compared to the control population decreased by 11% ($\Delta\bar{x}$: −1.5 neonates) in the naïve population and 17% ($\Delta\bar{x}$: −2.3 neonates) in the salt-exposed population (Figure 1.5). In the high NaCl concentration, both the naïve and salt-exposed populations showed clear decreases in brood size. Compared to the control, the brood size of the naïve population declined by 36% ($\Delta\bar{x}$: −4.8 neonates) and by 51% ($\Delta\bar{x}$: −6.7 neonates) in the salt-exposed population. Populations with an exposure history also had 23% ($\Delta\bar{x}$: −2.5 neonates) smaller broods than the naïve populations in high NaCl.

1.3.6.2 $CaCl_2$

In the low concentration of $CaCl_2$, the average brood size of the naïve population decreased by 13% ($\Delta\bar{x}$: −1.7 neonates) compared to the control population (Figure 1.5). The brood size of the salt-exposed population declined by 22% ($\Delta\bar{x}$: −2.95 neonates) compared to the control. In the high concentration of $CaCl_2$, both the naïve and salt-exposed populations showed clear decreases in brood size. Compared to the control, brood size decreased by 40% ($\Delta\bar{x}$: −5.3 neonates) in the naïve population and 46% ($\Delta\bar{x}$: −6.1 neonates) in the salt-exposed population. There was no difference between the brood sizes of naïve and salt-exposed populations in $CaCl_2$ within each salt concentration.

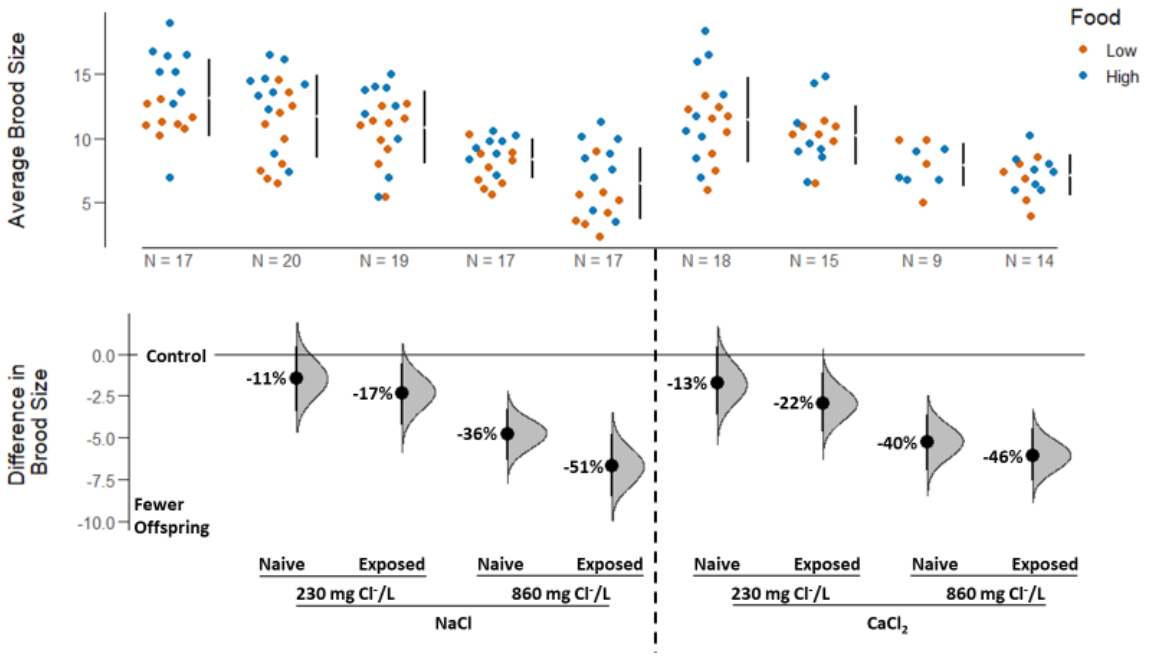

Figure 1–5: Effects of treatments on average brood size shown in a Cumming estimation plot.

1.3.7 Body Size

1.3.7.1 NaCl

In both the low and high concentrations of NaCl, I found little difference in body size compared with the control populations using the 10% effect-size threshold. Worth noting, however, was a decrease in body size of 7-9% ($\Delta\bar{x}$: −0.18-0.23 mm) in the high NaCl concentration of both the naïve and salt-exposed populations when compared to the control (Figure 1.6). This smaller difference was distinct form the control population when examining the 95% CIs and the sampling-error curve from the bootstrapping analysis.

1.3.7.2 $CaCl_2$

In the low concentration of $CaCl_2$, the average body size in both the naïve and salt-exposed populations showed no difference from the control. However, both the salt-exposed and naïve populations in the high $CaCl_2$ concentrations showed decreases in body size of 13% ($\Delta\bar{x}$: −0.31mm) and 14% ($\Delta\bar{x}$: −0.35 mm) compared to the control population.

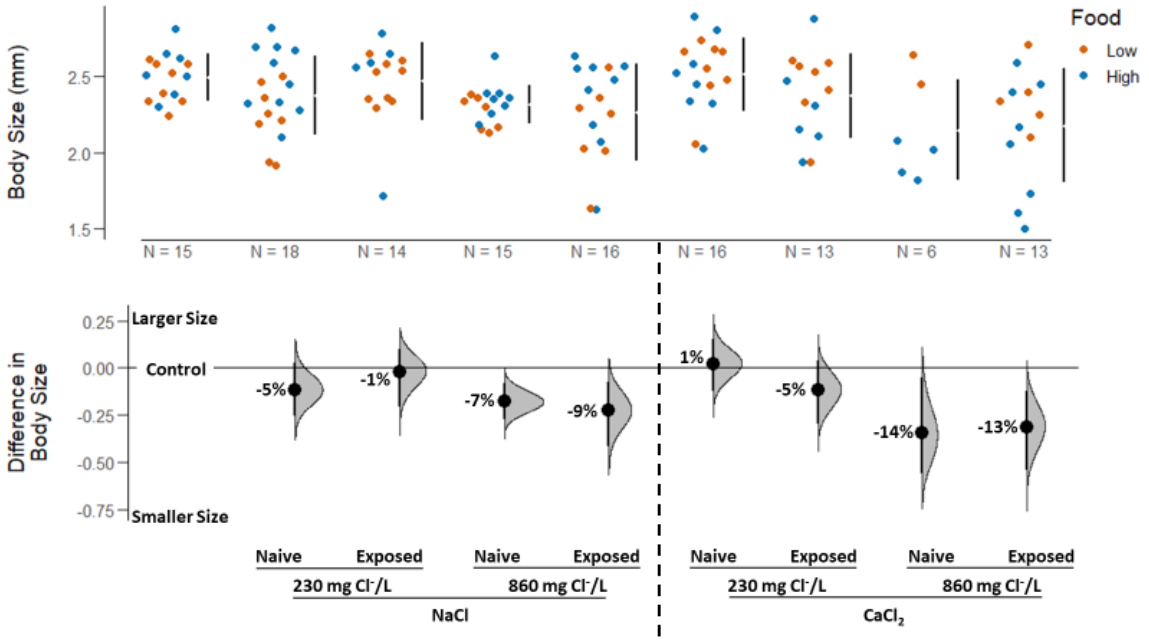

Figure 1–6: Effects of treatments on body size shown in a Cumming estimation plot.

1.3.8 Lifetime Reproductive Output

1.3.8.1 NaCl

I analyzed the total number of neonates produced over an organism's lifespan to determine the total reproductive cost of each treatment (Figure 1.7). In both the naïve and salt-exposed populations in the low concentration of NaCl, I found little difference in total reproductive output using the 10% effect-size threshold. In the high concentration of NaCl, there was a decrease of 31% ($\Delta\bar{x}$: −45 neonates) in the naïve population and a decrease of 58% ($\Delta\bar{x}$: −84 neonates) in the salt-exposed population. When comparing the naïve and salt-exposed populations in the high NaCl concentrations, salt exposure decreased lifetime reproductive output by 40% ($\Delta\bar{x}$: −38 neonates).

1.3.8.2 $CaCl_2$

In the low concentration of $CaCl_2$, the average lifetime reproductive output was decreased by 23% ($\Delta\bar{x}$: −34 neonates) in the naïve population and 50% ($\Delta\bar{x}$: −72 neonates) in the salt-exposed population relative to the control. When comparing the naïve and salt-exposed populations, salt exposure decreased lifetime reproductive output by 34% ($\Delta\bar{x}$: −38 neonates). In the high concentration of $CaCl_2$, the average lifetime reproductive output was decreased by 83% ($\Delta\bar{x}$: −120 neonates) in the naïve population and 75% ($\Delta\bar{x}$: −108 neonates) in the salt-exposed population. When comparing the naïve and salt-exposed populations, salt exposure increased lifetime reproductive output by 50% ($\Delta\bar{x}$: −12 neonates) with considerably high variation and CI overlap within this comparison.

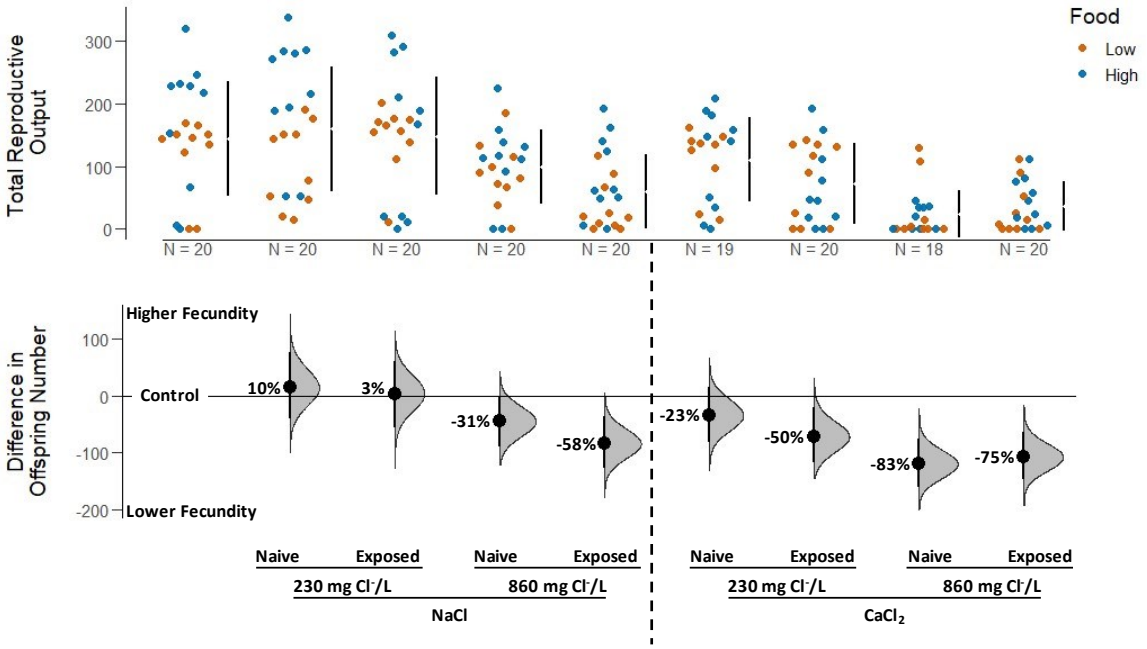

Figure 1–7: Effects of treatments on lifetime reproductive output shown in a Cumming estimation plot.

1.3.9 Pearson Product-Moment Correlation

Pearson product-moment correlation coefficients were computed to assess the relationship between 1) lifespan, 2) timing to maturation, 3) average brood size, 4) brood interval, 5) number of broods produced, 6) body size at death, and 7) lifetime reproductive output across 126 observations. The generated correlation matrix showed that many life history traits are highly correlated with each other (Figure 1.8). Among all response combinations, I found the strongest correlation between lifespan and number of broods produced. Lifetime reproductive output was positively correlated to lifespan, average brood size, number of broods, and body size. Lifetime reproductive output was negatively correlated to brood interval and timing to maturation. Body size was positively correlated to lifespan, average brood size, and brood number. Body size was negatively correlated to brood interval, and there was no correlation between body size and timing to maturation. Brood interval was negatively correlated to all traits except for timing to maturation where there was no correlation. Brood number was positively correlated to lifespan and average brood size with no correlation to maturation rate. Average brood size was positively correlated to lifespan and negatively correlated to maturation rate. Maturation rate was not correlated to lifespan.

Figure 1–8: Pearson's correlation matrix of all life history traits measured. Boxes are colored across a divergent color gradient to indicate positive (green) or negative (pink) correlations between treatments. Pearson's *r* is the value in the center of each box with red asterisks indicating $p \leq 0.001$ (***), $p \leq 0.01$ (**), $p \leq 0.05$ (*), and $p > 0.05$ (no asterisk).

1.4 Discussion

Tracking 180 individuals over the course of their lifetime, I show tradeoffs occur in a freshwater zooplankton that mitigate the ecological impacts of human-induced freshwater salinization. This tradeoff led to similar lifetime reproductive output as the control population, but only occurred in one salt type and concentration suggesting tradeoffs from changes in life history traits have constraints. Further, there were no instances where a multi-generational exposure history conveyed a tradeoff or an advantage in terms of lifetime reproductive output. The one combination of salt type and concentration (two out of eight possible treatments) resulting in life history tradeoffs occurred in the low concentration (230 mg Cl^{-}/L) of NaCl where both naïve and salt-exposed populations had reduced brood sizes. This was coupled with an increase in average lifespan with no changes in the timing between broods relative to the control. Organisms in the low NaCl treatments were able to offset reduced brood sizes and increase lifetime reproductive output by living longer to produce more broods.

Salt type is critically important when considering the impacts and ecological tradeoffs of freshwater salinization. I show that $CaCl_2$ is more harmful to zooplankton than NaCl at similar chloride concentrations. I standardized the treatments by Cl^{-} concentration, which indicates that the cations of Cl^{-}-based salts, not just Cl^{-}, affected the observed changes in life history traits [15]. As others have found, $CaCl_2$ is more toxic than NaCl [33, 40, 53]. Differences in toxicity between these two salts might be due to changes in intracellular homeostatic concentrations. Intracellular concentrations of sodium (Na^{+}) can be 10 to 20 times higher than the concentration of calcium (Ca^{2+}) in

crustaceans like *Daphnia* [54]. Therefore, additional Ca^{2+} from $CaCl_2$ may alter intracellular stoichiometric ratios of Na^+ and Ca^{2+} more than additions of Na^+ from NaCl compared to typical homeostatic ratios. Sodium and Ca^{2+} are both important to cellular processes. For instance, these cations are necessary to the function of sodium-potassium pumps for transepithelial movement of ions across gill membranes [55] and Ca^{2+} is critical to the process of ecdysis [56]. Thus, large changes in intracellular ratios of Na^+ and Ca^{2+} from $CaCl_2$ may alter zooplankton physiology. Changes in physiology could then lead to shifts in life history traits such as lifespan, brood interval, maturation rate, and overall reproductive output. This could account for the differences observed in life history traits between the NaCl and $CaCl_2$ treatments.

The overall rise in salinity from both NaCl and $CaCl_2$ salts imposes osmotic stress on zooplankton and alters life history. Increased salinity requires that organisms actively maintain osmotic pressure as extreme changes in cell volume can denature proteins [57] and breakdown cell volume regulatory capacities leading to apoptosis [58]. Thus, osmotic stress has severe implications for organisms if not properly managed. Osmoregulation through energy-dependent ion transport proteins is a very energy-costly process [59]. To cope with the added stress of road salts, organisms might allocate additional energy to osmoregulation to maintain survival. The increased energetic demand for stressor-mediation would require reallocation of assimilated energy away from growth and reproduction [1, 60]. As with the observations in 230 mg Cl^-/L of NaCl, zooplankton lifespan was increased as brood sizes decreased. It is possible that 230 mg Cl^-/L of NaCl induced a stress response where organisms increased energy allocation to survival and

decreased available energy for reproduction, leading to the reduced brood sizes observed among many of the experimental treatments.

I did not observe a tradeoff in life history in the 860 mg Cl^-/L of NaCl or 230 and 860 mg Cl^-/L of $CaCl_2$ treatments where organisms were able to maintain lifetime reproductive output relative to the control. For 75% (6 of 8) of salinized treatments, zooplankton populations had reduced lifetime reproductive outputs below that of the control. As mentioned above, high salinities increase the energy requirement for metabolic processes and osmoregulation in invertebrates [61, 62] and can reduce reproduction [63]. It is possible that in higher NaCl concentrations and in the more toxic $CaCl_2$ salt type [40], a tradeoff to maintain lifetime reproductive output was not possible due to energetic constraints. For example, in 860 mg Cl^-/L of NaCl, naïve and exposed populations had lifespans that were either longer than or equal to the control. However, the naïve population in the high NaCl concentration showed delayed maturity and much smaller brood sizes compared to the naïve population in the low NaCl concentration. Then, in both concentrations of $CaCl_2$, I observed reductions in brood sizes in similar magnitudes to the NaCl treatments in addition to lifespan reductions and delayed brood intervals. In these treatments, there were changes in life history to varying degrees without any compensatory mechanism to maintain lifetime reproductive output similar to the control population. The osmotic stress from high salinity and the added toxicity of $CaCl_2$ could have physiologically overwhelmed zooplankton, which could be why these populations did not exhibit a tradeoff like what was observed in the low NaCl treatments.

Another possible explanation for changes in zooplankton life history at higher salt concentrations could be changes in foraging efficiency or energy assimilation. If elevated

salinity reduced foraging efficiency or energy assimilation, there would be less energy available for survival, growth and reproduction [64]. This is supported by these results as I observed delayed maturation, delayed brood intervals, smaller brood sizes, and smaller body sizes at higher Cl^- concentrations. Several studies show that foraging and assimilation rates of *Daphnia* are reduced by insecticides [64-67]. This effect is relatively understudied for zooplankton's response to salinity [68, 69]. Li et al. (2008) and Xia et al. (2002) studied the effects of salinity on the foraging rates of estuarine copepods and found reductions in foraging rates outside of typical estuary salinities (<15ppt, >35ppt). It is possible zooplankton in this study suffered from reduced foraging rates or assimilation efficiency due to elevated salinity. If this occurred, these mechanisms could have contributed to the changes in some of the life history traits observed. Osmotic stress altering life history traits directly through energy reallocation and possibly changes in foraging rates or energy assimilation are unlikely to be mutually exclusive. Future research should explore how osmoregulatory costs, foraging rates, and changes in energy assimilation affect tradeoffs and evolving life history traits of freshwater organisms in response to salinization.

In half of the populations exposed to salt for multiple generations, lifetime reproductive output was lower than the naïve populations indicating that prior exposure history did not confer an advantage or tradeoff when re-exposed. This finding might suggest maladaptation because exposed populations exhibited changes in life history such as reduced lifetime reproductive output, reduced lifespan, reduced brood sizes, and increased brood interval compared to the naïve populations. Maladaptation would be surprising as previous research has suggested adaptations can occur in zooplankton in

response to NaCl [26, 27]. Coldsnow et al. (2017) demonstrated evolved tolerance of *Daphnia pulex* to road salts in eight to 10 generations. Others demonstrate that salinity-induced tolerance in zooplankton may be achieved in as short as one generation [70]. In some algal species, an evolved salinity tolerance can occur within three generations [71]. These examples demonstrate adaptation to salinization as either increased survival or reduced salt sensitivity. However, some authors suggest maladaptation may be more common as a result of human-induced environmental change [72, 73]. Rogalski (2017) found that *Daphnia ambigua* showed consistent patterns of heightened toxicity to copper and cadmium following decades of increasing contamination in several lakes. Additionally, Brady (2017) found that anuran tadpoles originating from road-side ponds with more contamination are more sensitive to chloride than populations from ponds more distant from roads with less contamination. Drivers of maladaptation are much less understood than adaptation. Maladaptation is typically attributed to limited gene flow [74] or strong selection in response to another stressor [75]. All of the experimental cultures began from an iso-female line of *Daphnia pulex* which would be limited to one genotype. The specific genetic line used in this experiment could have been poor performing for salinity resistance, and a lack of genetic variation would cause proliferation of this genotype without competition. However, additional work would be needed to determine if the populations evolved in response to chronic salinization as opposed to a mechanism such as transgenerational plasticity [76]. Understanding the prevalence of adaptation and maladaptation to various sources of anthropogenic stressors such as salinization will be essential to understanding the functioning of freshwater ecosystems through the Anthropocene.

One of my goals was to better understand the potential for demographic resistance and resilience [77] to road salt stress by observing changes in life history over the course of several generations [78]. I used naïve populations exposed to salt for the first time to determine the potential for demographic resistance, and salt-exposed populations to determine potential for demographic resilience. In terms of life history, I defined 'resistance' as a lack of change in lifetime reproductive output in the naïve populations when treated with salts [78]. This resistance could manifest as no effect of the salts on life history or life history tradeoffs that maintained lifetime reproductive output similar to the control. I defined 'resilience' as the ability for the salt-exposed counterpart population of each naïve population to recover in lifetime reproductive output when the naïve could not. Based on my results, zooplankton might be able to resist road salts as a stressor through tradeoffs, but this occurred in one concentration and salt type. Zooplankton populations were unable to resist the high NaCl and $CaCl_2$ concentrations as naïve populations had lifetime reproductive outputs below that of the control. Additionally, there were no scenarios where zooplankton populations showed resilience of life history traits following salt exposure for generations as all exposed treatments had lifetime reproductive output either lower than or equal to each naïve counterpart treatment. Many species may be capable of facilitating resilience via rapid adaptive change to human-induced stressors like salt pollution [24, 25], but I demonstrate that this is not always the case. In instances of low abundance, density, or genetic diversity, the potential for evolutionary rescue of stressed populations is limited [4]. Understanding the ability for organisms to demonstrate resistance or resilience to environmental stressors such as salt

pollution will require more research into the multigenerational effects of stressors across a variety of species and populations.

1.5 Conclusions

Freshwater salinization is increasing on a continental scale [37]. Millions of tons of road salts are being applied in the United States alone every winter [79]. In some urban streams, salt concentrations are 25% that of seawater [38]. Thus, it is common for the U.S. Environmental Protection Agency's regulatory guidelines of 230 mg Cl^-/L (chronic) and 860 mg Cl^-/L (acute) to be exceeded among freshwater environments [6, 37, 39]. This study adds to a growing body of evidence that these guidelines are insufficient to protect aquatic biota from the effects of freshwater salinization [13, 14]. Additionally, I have found that persistent salt exposure over multiple generations can increase the negative impacts of salt on zooplankton. The species that I used has relatively high Cl^- resistance compared to other common zooplankton [14, 20, 80, 81]. It is unclear how other more sensitive zooplankton taxa would respond. Current water quality guidelines should be reevaluated to better protect freshwater ecosystems from the adverse effects of Cl^- contamination.

Zooplankton are critically important in nutrient cycling and transferring energy from phytoplankton to higher trophic levels such as fish. Reductions in zooplankton abundance from salinization can induce algal blooms via trophic cascades [13, 82]. Salinity-induced algal blooms could limit light penetration through the euphotic zones of lakes and possibly limit benthic primary productivity, which could reduce fish abundance and recruitment [83]. I demonstrate that salinization can further diminish zooplankton life history traits through time. Future studies should investigate the population-level effects of multigenerational salt exposure in both natural and experimental settings [27].

Freshwater zooplankton are critical to energy transfer in aquatic food webs and ecosystem function. Understanding multigenerational responses to salt pollution and how these responses influence community structure, food webs, and ecosystem function is critical to conserving freshwater resources.

Chapter 2

Hiding Stress in the Face of Predators: The Role of Freshwater Salinization in the Ecology of Fear

2.1 Introduction

Prey express anti-predatory behaviors to maximize the likelihood of survival. In many encounters, the non-consumptive effect of a predator alters prey behavior such as movement patterns and foraging rates [84, 85]. However, naturally occurring interactions between predator and prey are being altered by human-induced environmental change. For instance, some pesticides are > 40 times more lethal when prey perceived a threat of predation [86, 87]. Additionally, pesticides can hinder predator-avoidance behaviors [88], which increases the risk of predation. The ability of contaminants to profoundly alter the natural interactions between predator and prey could alter population dynamics and ecological community structure. Understanding the role of various contaminants in the 'ecology of fear' is paramount to our understanding of species interactions and ecosystem functions in human-dominated landscapes.

The rising salinity of freshwater ecosystems is of ecological concern globally [37], and how interactions between freshwater organisms will change because of increasing

salinity is unclear. Freshwater salinization occurs from a variety of sources such as mining and agricultural operations, climate change (e.g., sea water intrusion), and the application of road deicing salts. Salinization is ranked in the top 15 causes of freshwater impairment in the United States [89] and has negative effects on freshwater organisms across trophic levels [80]. Few studies have investigated how rising freshwater salinity will alter ecological community interactions between predator and prey [19, 90]. Less known is how salt pollution will change prey anti-predatory behaviors and the non-consumptive effects of predators on prey.

The diel vertical migration behavior of freshwater zooplankton is important to the functioning of lake ecosystems [91, 92]. Migrating zooplankton typically spend the day in deeper waters and ascend at night to surface waters. This phototactic behavior is largely a predator-avoidance mechanism where zooplankton trade off food-rich and warm surface waters with decreased predation risk from visual predators [93]. Research shows that sublethal concentrations of various contaminants alter vertical migration and other swimming behaviors in several zooplankton species [94, 95]. It has been well studied that vertical migration and swimming behaviors in zooplankton are altered by predator cues [96-98]. Further, we know that freshwater salinization has substantial direct negative effects on zooplankton abundance [13, 14, 99, 100], which can alter the energy transfer between trophic levels in freshwater ecosystems. However, we do not understand how the predator-induced, daily movements of zooplankton in freshwater ecosystems will change as a result of the global increase in lake salinity.

Recent work has shown that zooplankton are able to rapidly evolve a tolerance to elevated freshwater salinity [26]. One effect of this evolved tolerance was a disruption in

the circadian rhythm of zooplankton where salt-tolerant strains stopped expression of genes responsible for circadian rhythm [101]. Circadian disruption from a prolonged multigenerational exposure to salinization could have dramatic consequences for behaviors such as vertical migration and subsequent species interactions and food web functioning. Thus, understanding the consequences of a prolonged, multigenerational exposure to salinization would be relevant to freshwater lakes that have been salinized for decades and are continuing to increase in chloride concentration [39, 102].

Here, I tested the interactive effects of predatory stress and salinity on the abundance and anti-predatory behaviors of zooplankton to better understand the effects of contaminants on the ecology of fear. I also asked whether a prolonged exposure, or adaptation, of zooplankton to elevated salinity for multiple generations affected the vertical movement of zooplankton. To do this, I performed two experiments. First, I conducted an experiment to determine the effects of predator stress and multiple salt concentrations on the abundance of zooplankton. I predicted that 1) an interaction between salinity and predatory stress would synergistically decrease zooplankton abundance to a greater extent than salt or predatory stress alone, 2) in the absence of an interaction, I would expect salinity and predatory stress alone would both decrease zooplankton abundance. Second, I conducted an experiment to determine the effects of predatory stress, multiple salt concentrations, and a prolonged, multigenerational exposure to salt on the vertical migration rate of zooplankton. Specifically, I invoked the anti-predatory flight response of zooplankton and recorded the average downward movement velocity of individuals. I predicted that 1) an interaction between salt and predatory stress will overwhelm zooplankton and synergistically reduce vertical escape

velocity to a greater extent than salt or predatory stress alone, 2) zooplankton from cultures exposed to elevated salinity for multiple generations will have slower vertical escape velocities than naïve populations (no salt exposure), 3) in the absence of an interaction, I would expect that salinity will decrease vertical escape velocity and predatory stress will increase vertical escape velocity.

2.2 Methods

2.2.1 Zooplankton Abundance Experiment

To determine the effect of various salt concentrations and non-consumptive predator cues on zooplankton abundance, I utilized a 2-by-3 fully factorial design with four replicates comparing the presence and absence of fish kairomone under three salinity regimes – control (no salt added), low (230 mg Cl^- /L of NaCl) and high (860 mg Cl^-/L of NaCl). The full combination of variables resulted in six treatments replicated four times for a total of 24 experimental units. I used NaCl rock salt (Safe Step 3300 Rock Salt) to generate the elevated salinity conditions as NaCl is one of the most common salt types contributing to freshwater salinization. For instance, NaCl is currently the most commonly use road deicing salt [103]. I standardized the salt concentrations by chloride concentration as it is thought to be the more toxic ion in NaCl [40] and the ion for which water quality guidelines are developed around the world for many inorganic salts [12]. The salt concentrations in this study also represent environmentally relevant concentrations often observed in field studies [9, 41].

I used *Daphnia dubia* for the abundance experiment. This species is relatively common in freshwater lakes and the strain used in this study was from the western basin of Lake Erie (North America). I cultured *Daphnia* populations originating from an iso-female line collected from Lake Erie in June of 2021. *Daphnia* were cultured in 5L of culture media consisting of a 1:1 mixture of aged municipal tap water and reverse osmosis water to minimize ambient chloride concentrations. I maintained cultures under a 16:8-hour light cycle with a constant temperature of 21.5 °C and fed *Raphidocelis subcapitata* regularly throughout the culturing period. To prevent excessive algal growth and metabolite accumulation, I renewed culture solutions biweekly. For the fish predator, I use emerald shiners (*Notropis atherinoides*) acquired from a bait shop which were also collected from the western basin of Lake Erie. The emerald shiner is a zooplanktivorous cyprinid native to the Laurentian Great Lakes and co-occurs with and are natural predators of *Daphnia* in many freshwater lakes [104].

To begin the experiment, I filled 10-gallon aquaria (50.8cm x 20.3cm x 30.5cm) with 32L of dechlorinated municipal tap water. The aquaria were kept in an indoor mesocosm facility under a 16:8-hour light cycle with an average temperature of 17.7 °C (± 0.8 °C). Each aquarium was then outfitted with a mesh fish cage (16.5cm x 12cm x 13.3cm) attached to the outer rim of the aquarium. Specifically, I used the brand *Lee's Net Breeder* with a mesh diameter of 30µm. I found this to be sufficient in keeping zooplankton physically isolated from fish. I placed air stones into each fish cage to provide adequate aeration and mixing of treatment media. I then assigned each aquarium a random treatment of presence or absence of fish and one of the three salinity treatments. I used a *YSI ProDSS* multiparameter digital water quality meter with a calibrated Cl^-

sensor to determine the Cl^- concentrations of treatment solutions and adjusted as necessary to match the desired nominal concentrations. Actual concentrations were on average < 10% of the nominal, thus I chose to report nominal target concentrations in my results.

On the first day of the experiment, one emerald shiner was placed into each mesh cage for treatments with fish present. To keep fish satiated and to produce kairomones—a combination of fish odor and conspecific alarm cues common in *Daphnia* spp. [105]—fish were fed 3mL of concentrated *Daphnia* culture every other day for the duration of the experiment. I inoculated each aquarium with a starting population of 30 *Daphnia*, and I added an additional 30 on day seven. I fed *Daphnia* in each treatment equal quantities of *Raphidocelis subcapitata* every other day for an average feeding of $1.46x10^5$ cells/mL day^{-1}. On day 18, I ended the experiment, and *Daphnia* populations from each aquarium were filtered using a 75µm mesh net and preserved in 2.5% Lugol's solution. For analysis, I enumerated preserved *Daphnia* under a stereo microscope using a zooplankton counting wheel to enumerate the entire population size of each tank.

2.2.2 Zooplankton Escape Velocity Experiment

The goal of this experiment was to determine if various salt concentrations and the presence of fish kairomones effects the vertical escape velocity and behavioral responses of zooplankton. Additionally, I wanted to determine if prolonged, multigenerational exposure to salts influences the vertical escape velocity and behavioral responses of zooplankton. To do this, I utilized a 2-by-2-by-3 fractional factorial design to compare the effects of this prolonged, multigenerational salt exposure to a naïve, first-time exposed population of *Daphnia*. This was crossed with the presence and absence of fish

kairomone under the three salinity regimes used in the abundance experiment – control (no salt added), low (230 mg Cl^-/L) and high (860 mg Cl^-/L). The full combination of the fractional factorial design resulted in 10 treatments (Figure 2.1). Each treatment was replicated between 16 and 20 times depending on *Daphnia* survival during acclimation (see below) for a total of 155 experimental trials.

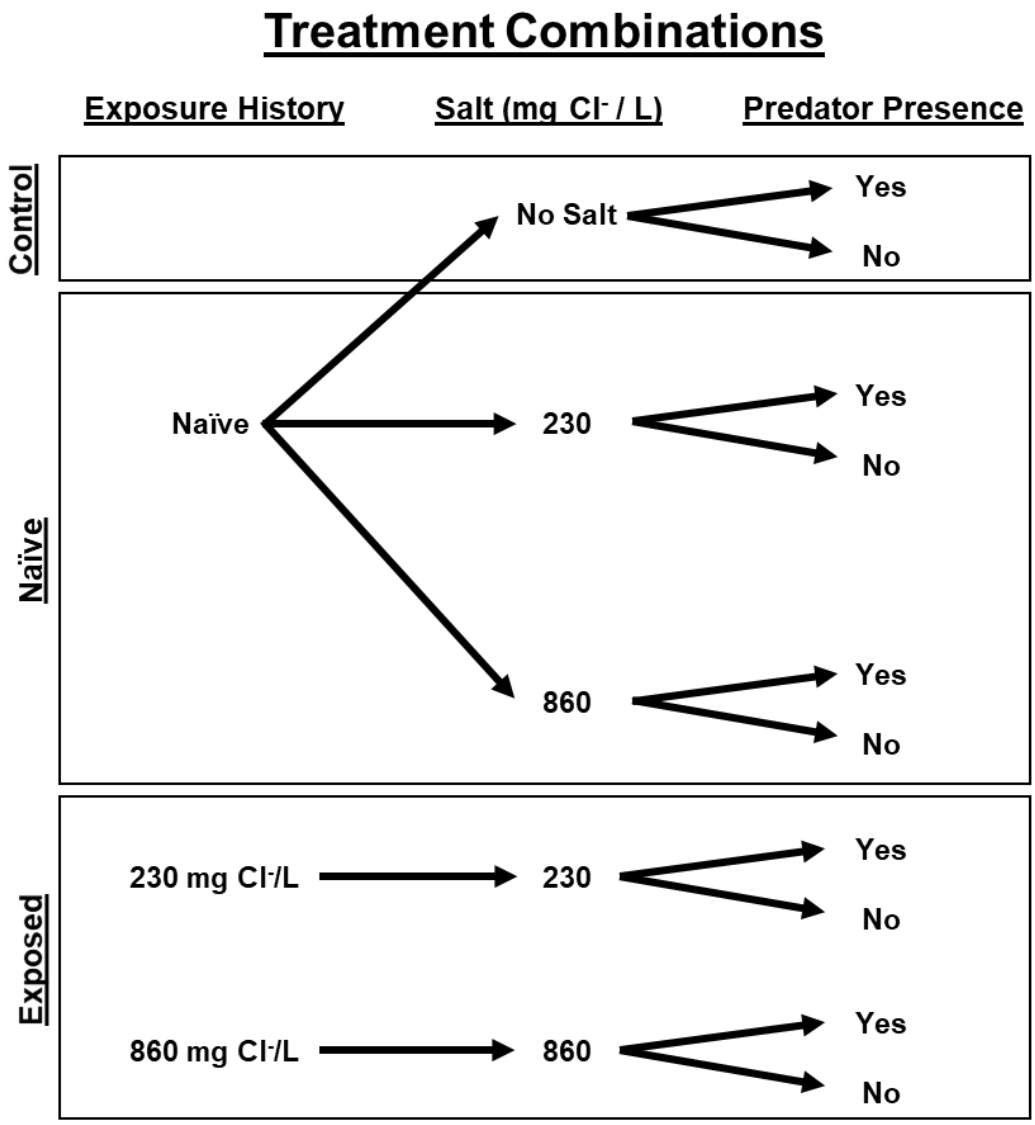

Figure 2–1: A visual of the fractional factorial design of the *Zooplankton escape velocity experiment.* Treatments are first organized by 'Exposure History,' where *Daphnia* were either cultured no-salt control media ("Naïve"), in 230 mg Cl^-/L, or in 860 mg Cl^-/L for several generations generating an exposure history. For acclimation to treatment conditions, organisms were taken from their source 'Exposure History' populations and placed into beakers with either no salt (control), 230 mg Cl^-/L, or 860 mg Cl^-/L. These salt concentrations were additionally crossed with the presence or absence of fish predator cues. This design resulted in a total of 10 experimental treatments, with 10 replicates per treatment.

To determine the effect of prolonged exposure to the salt concentrations, I cultured *Daphnia* populations in three concentrations – control (no salt added), low (230 mg Cl^-/L) and high (860 mg Cl^-/L). Culturing water consisted of a 1:1 mixture of aged municipal tap water and reverse osmosis water. I again used NaCl (*Safe Step 3300 Rock Salt*) to establish Cl^- concentrations of 230 and 860 mg Cl^-/L. I used a *YSI ProDSS* multiparameter digital water quality meter with a calibrated Cl^- sensor to determine the Cl^- concentrations of treatment solutions and adjusted as necessary to match the desired nominal concentrations. Actual concentrations were on average < 10% of the nominal, thus I report nominal target concentrations in the results.

I began each treatment culture for prolonged exposure with 10 adult *Daphnia* originating from the same iso-female line collected in the western basin of Lake Erie. These treatment cultures were housed in 500mL of their respective treatment solutions under a 16:8-hour light cycle and maintained at 21.5 °C (± 0.03°C) and were fed equal densities of *Raphidocelis subcapitata* throughout the culturing period. I renewed culture media bi-weekly to prevent metabolite and algae accumulation.

To establish a population of *Daphnia* with an exposure history to salt, I maintained these treatment cultures in elevated NaCl concentrations along with the no-salt control for 309 days. I estimate a minimum of 40 to 51 generations were produced during this time based on my data from chapter one using *D. pulex* – a closely related species. Research demonstrates that eight to 10 generations of salt exposure in *Daphnia* were sufficient to produce evolved responses in the form of increased salinity tolerance and corresponding changes in circadian rhythms [26, 101]. This is suggestive that 309 days may be sufficient time for behavioral differences to develop.

To create treatment solutions and kairomones for the escape behavior trials, I filled six 2.5-gallon aquaria (30.5cm x 15.2cm x 20.3cm) with 7L of dechlorinated municipal tap water. I had one aquarium for each unique salt concentration by kairomone presence solution. I dosed the NaCl salt mixture to bring the total Cl^- concentrations to the nominal treatment concentrations. I used a *YSI ProDSS* multiparameter digital water quality meter with a calibrated Cl^- sensor to determine the Cl^- concentrations of treatment solutions and adjusted as necessary to match the desired nominal concentrations. I then added 4 mL of concentrated *Daphnia* (average of 142 ± 25 individuals) to each tank followed by two emerald shiners. Emerald shiners had an average length of 76.5mm (± 5.5 mm) in the control solution, 79.5 mm (± 0.5mm) in the 230 mg Cl^-/L solution, and 77.5mm (± 17.5 mm) in the 860 mg Cl^-/L solution. After 21 hours, I removed the emerald shiners and filtered the treatment solutions through 63µm mesh sieves. These solutions were then used to acclimate *Daphnia* to the treatments.

To acclimate the *Daphnia* to new treatment solutions, I prepared 140mL beakers of each treatment solution and randomly selected 20 *Daphnia* from the appropriate population to be put into the beaker. The acclimation beakers with the *Daphnia* were then placed into an environmental chamber on a 16:8-hour light cycle and maintained at 21.5 °C (± 0.03°C) for 19 h prior to the escape velocity trials. Individuals were not fed during this time. In all treatments, mortality during acclimation resulted in treatment sample sizes between 16 and 20 individuals.

The escape behavior trials took place in the same environmental chamber as the acclimation to minimize external disturbances and changes in light or temperature that may have unintentional effects on behavior. A 100mL graduated cylinder was used to

quantify the maximum depth achieved by the *Daphnia* within a 10-second time interval. To minimize unwanted phototactic behavior, I placed a lamp aiming downward over the graduated cylinder and removed all external light sources. The graduated cylinder was filled with 100mL of the respective solution for each treatment, and treatment solution was renewed every 10 replicates. I collected data on the "No Fish" treatments first as to not contaminate the graduated cylinder with fish kairomone.

To begin the experiment, one *Daphnia* was randomly pulled from the acclimation beaker using a 3 mL transfer pipette with an opening greater than 3mm to avoid injuring individuals during transfer. The pipette transfer served a double purpose—to transfer the individuals into the graduated cylinder and to serve as a physical disturbance stimulus to initiate escape behavior. I found from pilot experiments that pipetting and releasing individuals was reliable to induce an escape response. I defined the escape response as the body of an individual angled downward relative to a horizontal plane followed by rapid downward swimming. Once in the pipette, the daphnia were immediately and gently released at the meniscus of the solution in the graduated cylinder. I used a stopwatch set for 10 seconds and recorded the maximum depth reached in the 10-second interval using the milliliter markings on the graduated cylinder. I also recorded whether an escape response occurred. Later, I found the distance in millimeters between the milliliter markings on the graduated cylinder and calculated the average descent velocity of the *Daphnia* over the course of a 10 second trial in millimeters per second.

2.2.3 Analysis

For both the zooplankton abundance and escape behavior experiments, I analyzed the effect size of each treatment as described by Cumming (2012). I estimated the standard

effect size as the difference between a treatment mean and the control mean for each variable. I derived 95% confidence intervals (CIs) through nonparametric bootstrap resampling of the means of each treatment 5,000 times which enables the calculation and visualization of CIs as a graded sampling distribution. I chose this method as it provides a transparent way to visualize and describe datasets simultaneously and to highlight the biological importance of treatment responses without the need for extensive null-hypothesis significance testing [49]. Differences in both abundance and escape velocities were determined by identifying biologically relevant effect sizes (generally $\Delta\bar{x} \geq 10\%$) and the degree of overlap between 95% CIs. To derive and plot estimation statistics, I used the '*DABESTR'* package [50] in RStudio version 4.1.2 to generate effect sizes and CIs for each response variable. In addition, *'DABESTR'* generates estimation plots [49] for effect size and CI visualization.

To test for salt-by-predatory stress interactions, I evaluated the effect sizes of treatment combinations and looked for additive, synergistic, or antagonistic effects. An additive effect (no interaction) would be one where the combined effects of salt and predatory stress equal the sum of each individual treatment alone. A synergism would be one where the combined effects are greater than the sum of the individual effects, and an antagonism would be where the combined effects are less than the sum of the individual effects. I evaluated these effects with consideration to each treatment combination's effect size and CI overlap with comparison means.

To determine if the presence of an escape response varied by treatment, I utilized a generalized linear model (GLM) with the presence of the escape response as a response variable and chloride concentration, salt exposure history, and predator presence as

predictor variables under a binomial distribution. I found no main effects or interactions of the treatments on the probability of a *Daphnia* to display an escape response or not ($F_{1,4} \leq 0.77$ $p \geq 0.392$). Thus, for the vertical escape velocity estimation statistics, I removed all observations where *Daphnia* did not display an escape response. This resulted in sample sizes between nine and 19 individuals for the final analyses.

2.3 Results

2.3.1 Zooplankton Abundance Experiment

To determine how Cl^- concentration and predator presence affected the abundance of *Daphnia*, I enumerated and analyzed the total population of zooplankton in the experimental mesocosms after 18 days. I found that the effect of salt can be similar to the non-consumptive effects of predation, but there was no interaction between salt concentration and predation (Figure 2.2). In the absence of a fish predator, the average population size was reduced by 58% ($\Delta\bar{x} = -248$ individuals) in the 230 mg Cl^-/L treatment and 52% ($\Delta\bar{x} = -222$ individuals) in the 860 mg Cl^-/L treatment compared to the control. Relative to the no-salt control in the absence of a fish predator, the no-salt control in the presence of a fish predator had an average reduction in population size of 56% ($\Delta\bar{x} = -236$ individuals). In the presence of a fish predator, the 230 mg Cl^-/L and 860 mg Cl^-/L treatments had average population reductions of 63% ($\Delta\bar{x} = -267$ individuals) and 72% ($\Delta\bar{x} = -304$ individuals), respectively.

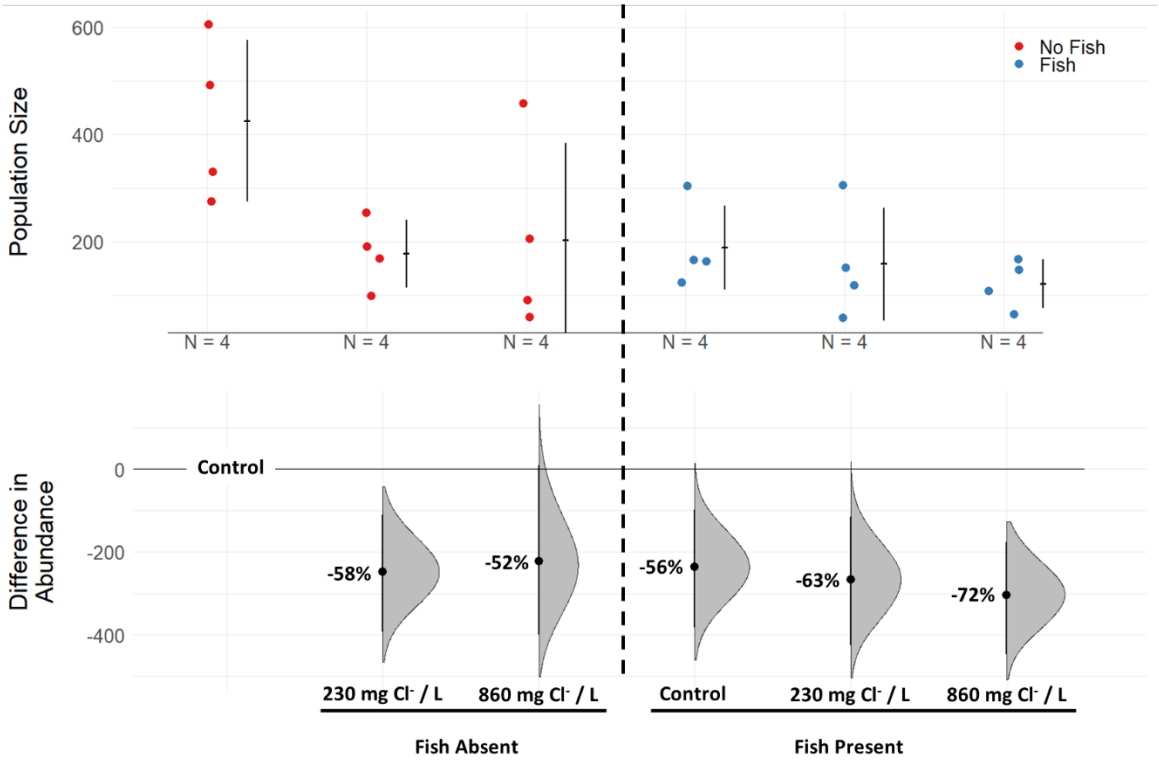

Figure 2–2: Results of the *Zooplankton abundance experiment* shown in a Cumming estimation plot. Data was analyzed as total population size in each replicate. To the left of the vertical dashed line are salt treatments not exposed to a fish predator, and to the right are treatments that were exposed to a fish predator.

2.3.2 Zooplankton Escape Velocity Experiment

2.3.2.1 Predator Absence

To determine how chloride concentration, predator presence, and population exposure history to chloride affected the escape behaviors of zooplankton, I analyzed the average vertical escape velocity of individuals within a 10-second time interval (Figure 2.3). Compared to the control, average escape velocity in the 230 mg Cl^-/L treatment decreased by 22% ($\Delta\bar{x}$: −1.96 mm /s) in the naïve population and by 36% ($\Delta\bar{x}$: −3.24 mm/s) in the salt-exposed population. For the 860 mg Cl^-/L treatment, average escape velocity decreased by 34% ($\Delta\bar{x}$: −3.07 mm/s) in the naïve population and by 47% ($\Delta\bar{x}$: −4.23 mm/s) in the salt-exposed population compared to the control.

2.3.2.2 Predator Presence

Escape velocity was the same in the predator-present, no-salt control as it was in the predator-absent, no-salt control (Figure 2.3). In the presence of the fish predators, the only treatment to vary from the predator-absent control was the naïve population in 230 mg Cl^-/L which had an average decrease in escape velocity of 16% ($\Delta\bar{x}$: −1.42 mm /s).

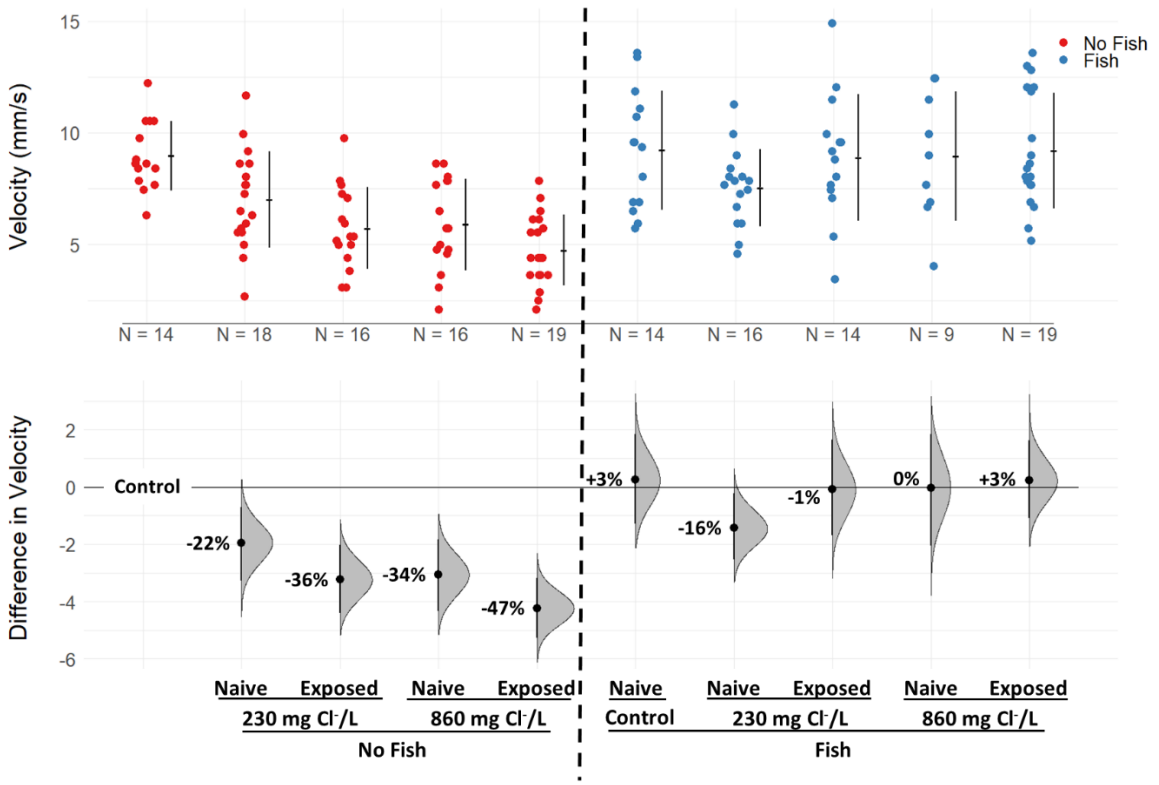

Figure 2–3: Results of the *Zooplankton escape velocity experiment* shown in a Cumming estimation plot. Data was analyzed as millimeters per second. All observations where *Daphnia* did not display an escape response behavior were removed from the analysis.

2.4 Discussion

2.4.1 Zooplankton Abundance

I predicted a salt-by-predatory stress interaction where salinity and predatory stress would synergistically decrease zooplankton abundance to a greater extent than salt or predatory stress alone. However, my results revealed an antagonism where the combination of salinity and predatory stress was comparable to the main effects of salinity or predatory stress. While I predicted a synergistic effect, a growing body of evidence suggests that antagonism and additivity may be the most common stressor interactions [106, 107]. It is possible that the effect of either salinity or predatory stress is physiologically overwhelming and masks the effect of the other [108]. Alternatively, one stressor may impose a stronger selective pressure on the population and eliminate individuals poorly fit to the stronger stressor which could promote co-tolerance [109]. Recent work has identified salinity as the masking stressor in an interaction between salinity and heatwaves on the abundance and biomass of zooplankton communities [110]. However, it remains challenging to identify which stressor may be imposing a masking or co-tolerance response in this study as both stressors produced comparable reductions in abundance.

I found that *Daphnia dubia* reacts to non-consumptive predatory stress with substantial reductions in population size. When exposed to the predatory cues of the native emerald shiner, *D. dubia* in this experiment had an average reduction of over 50% in population size. Recent work with a closely related species (*Daphnia pulex*) showed predator cues alone reduced zooplankton abundance by at least 11% [90]. Another study

evaluating Lake Michigan (North America) zooplankton found a 13-fold reduction in copepod abundance was caused by the non-consumptive effect of the invasive spiny waterflea (*Bythotrephes longimanus*) [111]. Bourdeau et al. (2016) found no effect of the spiny waterflea on the abundance of *D. mendotae*—a closely related and co-occurring daphnid with *D. dubia*. Predator cues alone can also reduce the abundance of estuarine amphipods [112] and offspring viability of fairy shrimp (*Anostraca*) [113]. The response to the non-consumptive effects of predation is also be tied to the evolutionary history between predator and prey [114]. The species used in this study are a natural predator-prey system found in Lake Erie (North America) and these results show that the non-consumptive effect of the emerald shiner can have a strong effect on *D. dubia* population size, perhaps owing to their long evolutionary history. While the mechanism for this reduction is unclear, it is possible reduced reproduction leading to smaller population sizes in *D. dubia* occurred due to a reduction in foraging rate [111], a shift in energy allocation [115], or reduced assimilation efficiency due to predator stress [116]. Interestingly, the impact of salt pollution had the same overall effect on *D. dubia* population size in the absence of emerald shiners.

Supporting my prediction, increased salinity reduced *D. dubia* population size. This reduction was > 50% in both the low (230 mg Cl^-/L) and high (860 mg Cl^-/L) salt concentrations. Similar results have been observed in several other zooplankton taxa [13, 18, 80, 81, 100]. This may be caused by sublethal effects such as increased energy demand for osmoregulation resulting in reduced reproduction from smaller brood sizes or fewer lifetime broods [14, 20, 21, 61]. This suppressed lifetime reproduction could be

one mechanism behind the reduction in *D. dubia* population size due to elevated salt concentration [2].

2.4.2 Zooplankton Escape Velocity

I found an interaction where the threat of predation masks the effect of both salinity and exposure history on zooplankton vertical escape velocity. In the absence of predatory stress, escape velocity was substantially reduced with any elevated salt concentration. An exposure history to elevated salinity further reduced escape velocity by 18%-19% compared to naïve populations. Both findings support my prediction for the individual effects of salt and exposure history on escape velocity. I predicted a synergistic effect would occur where salinity and predatory stress combine and overwhelm zooplankton reducing escape velocity to a greater extent than the individual effects of salinity and predatory stress alone. However, the results indicate an antagonism where the effect of these combined stressors is less than the sum of individual effects. This antagonism occurred in all treatments where predatory cues increased the vertical escape velocity of zooplankton, and all but one treatment (naïve population in 230 mg Cl^-/L) had comparable velocities to that of the control. These findings demonstrate a strong masking effect the ecology of fear can impose in a multi-stressor context.

Zooplankton experienced substantial reductions to vertical escape velocity when exposed to elevated salinity. This is consistent with findings that salinity decreases the swimming velocity of *D. magna* [117]. Heightened salinities requires organism to actively maintain internal osmotic pressure as extreme or abrupt changes can denature proteins [57] and breakdown cellular volume regulatory capacities which can damage cells and induce apoptosis [58]. Because of this, osmotic stress is incredibly important to

manage as deviations from typical homeostatic conditions can lead to adverse physiological consequences. Managing osmotic pressure through energy-dependent ion transport proteins is very energetically taxing to aquatic invertebrates [59]. Thus, I hypothesize that a tradeoff exists where zooplankton exposed to heightened salinities decrease movement speeds to conserve energy, and this energy is then used to maintain homeostatic conditions which are stressed due to changes in osmotic pressure. While this hypothesis remains to be tested, it might be possible that salinization is changing the behavior of freshwater organisms on a much larger scale due to the energetic constraints imposed by higher salinity [118]. Exploration of such sub-lethal tradeoffs and constraints are needed as the salinity of freshwater ecosystems continues to rise globally [37].

Zooplankton originating from populations exposed to higher salt concentrations for multiple generations had reduced swimming velocities relative to the naïve populations. In contrast, Baillieul et al. (1998) found that *D. magna* had reduced swimming velocity in elevated salinity, but *Daphnia* swimming velocity returned to control conditions following acclimation for several days. This finding suggests that zooplankton which have undergone several generations of exposure to salinity vary in behavior from intragenerational acclimation such as what is observed in Baillieul et al. (1998). Differences in behavior between intragenerational and intergenerational exposure could be explained by evolutionary adaptation or transgenerational plasticity in response to salinity [70, 119]. In such cases, changes in gene expression across generations could alter the behavioral response of zooplankton rather than acclimation to conditions. Given these results, organisms that undergo several generations of exposure to contaminants can experience greater behavioral changes than naïve populations with immediate exposure.

This trend may exist in other zooplankton behavioral traits such as foraging or diel vertical migration, however, this is understudied. The extent and magnitude of these behavioral changes in freshwater organisms should be a focus of future research.

Contrary to my predictions, predatory stress masked the salinity-induced behavioral response. I expected the combined effects of predatory stress and salinity to overwhelm zooplankton and result in reduced swimming velocities compared to salinity or predatory stress alone. This finding demonstrates that zooplankton exposed to salinity, even for several generations where there were observed greater reductions in escape velocity, are able to escape at a rate similar to naïve zooplankton in the no-salt control treatment. The landscape of fear is a strong driving force in the physiology and behavior of prey organisms [120] and can interact with multiple stressors in various ways [87, 90, 121]. One explanation for the strong masking effect observed on zooplankton escape velocity is the deterministic nature of predation, which is death. Salinization at the tested concentrations impose sublethal effects [20, 80], and the threat of predation induces behavioral decisions to minimize predation risk in a way that maximizes fitness [120]. I posit that zooplankton in this study became lethargic or inactive when stressed by salinity to minimize energy expenditure and the sublethal effects of osmoregulatory stress. However, detection of predator cues overcame the behavioral response of salinity to minimize the threat of predation. Though predatory cues allowed zooplankton to swim at control speeds, the combination of stressors may impact individuals in other ways that I did not measure such as sensitivity to contaminants [86, 87] or changes in life history strategies such as cyclic parthenogenesis [90]. Evaluating the role of non-consumptive effects from predators on prey exposed to other stressors such as salinity will be essential

to our understanding of multi-stressor interactions in ecological communities in human-dominated environments.

2.5 Conclusion

Though I did not test the effects of salinity and predatory stress on diel vertical migration in this study, vertical escape velocity may serve as a surrogate for understanding diel vertical migration in zooplankton. Vertical migration behavior is an important aspect of lake ecosystems as it contributes to zooplankton population dynamics [91] and affects energy flow through aquatic food webs. These results demonstrate that freshwater salinization can have a large effect on zooplankton vertical movement rate. While we do not know how this reduction in movement might affect DVM in salinized lakes, this is an area in need of further research. It will be important to understand how salinization could alter behavioral responses that might decouple energy pathways in food webs and alter community interactions supporting freshwater biodiversity.

The role of multiple stressors in ecology can be misinterpreted due to the complexity of interactions and needs further study [106, 108]. Here, I demonstrate that the natural threat of predation can mask the behavioral responses of zooplankton to freshwater salinization. Although the non-consumptive effects of predation can mask the impact of salinity on escape velocity, it does not mean the energetic constraints imposed by salinity disappear. Predatory stress at some level will always exist in natural environments. I predict the energetic costs associated with anti-predatory behaviors to be enhanced in salinized conditions compared to an un-salinized environment—this prediction remains

to be tested. Additionally, the effects of salinization on *D. dubia* population size was similar to that of the non-consumptive effects of a natural predator with > 50% reduction in population size, which have implications for current water quality guidelines.

Our salt treatments were the chronic and acute Cl^- thresholds set by the United States Environmental Protection Agency to protect freshwater organisms [12] and are commonly observed in field studies [6, 9, 39, 41]. These results indicate that substantial reductions in *D. dubia* population size will occur at or below regulatory thresholds, and the observed population-level effect was similar to the effect natural predators might have on *D. dubia* population size. Not much is known about the tested organism, *D. dubia*, and this is largely true for most zooplankton taxa outside of the few common model species. *D. dubia* appears to be more susceptible than commonly tested species such as *D. pulex* or *D. magna* [12, 122]. Recent work consistently demonstrates that many zooplankton taxa globally are not protected by government regulatory thresholds [13, 14, 17, 90, 123]. Understanding the complex interactions between multiple stressors, both naturally occurring and human-induced, is essential to understanding the context for changes in freshwater ecosystems. I suggest revised water quality guidelines need to account for natural stressors in the environment in addition to human-induced stressors to better protect functionally important ecosystems.

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