THE EFFECTS OF SALINITY ON CANADIAN TOAD (*ANAXYRUS HEMIOPHRYS*) LARVAE AND POST-METAMORPHIC JUVENILES

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MASTER OF SCIENCE

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ABSTRACT

Sodium Chloride (NaCl) production in the United States is at an all-time high. As weather patterns become harsher through climate change and demands on energy and agriculture increase, evidence of long-term NaCl exposure is emerging throughout environments. With a focus on Canadian toads, this research seeks to fill existent gaps of NaCl effects on tadpoles and post-metamorphic juveniles for this species. Chapter 1 of this thesis examined the effects of NaCl on larvae exposed at multiple developmental time periods. Weight, survival, and hatch success were not affected by NaCl, however time taken to complete metamorphosis was extended in tadpoles exposed at an older age. Chapter 2 of this thesis examined the effects of NaCl infused substrates on post-metamorphic juvenile choice, weight, and consumption. Weight gain and decreased consumption rates were observed in high saline treatments, while salinity did not affect the toad's choice between saline and freshwater substrates.

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DEDICATION

I would like to dedicate my thesis to my mom, Dee Audet. She has pushed me and supported me through all the challenges and obstacles that have happened over the past 2 and a half years. Even across the country, she has been willing to stop what she was doing at that moment to help me work through whatever it was that I was going through. She is the real MVP and this simple dedication does not do her justice.

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1.1. Introduction

Salt pollution is increasing globally through road deicing methods, intensive agricultural practices, and energy-related extraction operations (Cañedo-Argüelles et al., 2013; Gleason et al., 2011; Kaushal et al., 2018; Williams, 2001). These methods are continually perpetuated through urban development and accelerated weathering (Cañedo-Argüelles et al., 2013; Gleason et al., 2011; Kaushal et al., 2018; Williams, 2001). Throughout the past 50 years, the northern United States has experienced the largest upsurges of salinization contamination in freshwater waterways (Kaushal et al., 2018). While northern United States (US) freshwater waterways are increasing in unnatural salinity, naturally saline-rich soils exist in select areas across the US. For example, North Dakota (ND) contains salinity-rich soils from natural geological deposits. These naturally occurring salts are primarily sulfate-based and contain a higher solubility threshold. Naturally occurring salts typically lead to saline soil conditions in large quantities (Doll et al., 1989; Seelig, 2000). Salts that are deemed unnatural often contain a soluble component, such as chloride. As the chloride interacts with the soil, it is negatively repelled from the soil, leaving the saline component to bind with the soil. This saline binding ultimately destabilizes the soil, altering its structure, restricting root growth, and disrupting nutrient dispersal (Doll et al., 1989; De Jong, 1982; Seelig, 2000). A combination of a shallow water table and recurrent anthropogenic salt loading throughout the state of ND allows for the continual resurfacing of these salts (Cañedo-Argüelles et al., 2013; Franzen, 2013; Gleason et al., 2011; Williams, 2001).

One of the large contributing factors to the shallow water table existent in North Dakota is the Prairie Pothole Region. Approximately, 90% of the wetlands in ND are a part of the PPR

(Berkas, 1993). The PPR is characterized by millions of shallow, freshwater depressional wetlands ("potholes"), spanning across southern Canada, Montana, North Dakota, South Dakota, Minnesota, and Iowa (Berkas, 1993). The area is considered to be one of the most valuable and largest wetland regions on earth, sustaining habitat for a diverse array of migratory bird, invertebrate, and vertebrate populations (Gleason et al., 2008; Mushet et al., 2014). These wetlands play a vital role in protecting and improving water quality, balancing carbon cycles, absorbing flood surges, nutrient cycling, and maintaining water flow during dry periods (Mushet et al., 2014). Yet, wetlands and (inter)nationally protected areas, account for 86% of salt-affected areas (approximately 971 Mha) throughout the world (Wicke et al., 2011). Unfortunately, many of these habitats have been lost to agricultural practices in ND. The runoff from agriculture, energy production, deicing operations, and urbanization is the second largest threat to wetlands in the PPR Inventory (US Environmental Protection Agency, 2005). These practices affect sensitive inhabitants, such as amphibians, that are dependent on valuable freshwater resources.

1.1.1. Salinity Impacts

1.1.1.1. Agricultural Impacts

Although ND is home to a portion of the PPR, it is an agriculturally dependent state, with 90% of the land dedicated to agriculture (NDDA, 2011). Agriculture is the leading non-point source (NPS) pollution of rivers and lakes, in addition to the second largest NPS pollution to wetlands, according to the 2000 National Water Quality Inventory (US Environmental Protection Agency, 2005). Overgrazing, high-frequency plowing, and improper/excessive/poorly timed application of pesticides, irrigation water, or fertilizers are the driving sources of agriculture pollution (US Environmental Protection Agency, 2005). The main pollutants from agricultural runoff include heavy metals, sediment, pathogens, nutrients, and salt all of which aggregate in

remaining depressional wetlands (US Environmental Protection Agency, 2005). The aggregation of these pollutants, has revealed negative impacts on amphibian communities and have been observed to cause an increase in malformations (Piha et al., 2006). As ND's freshwater environments are at risk and severely exposed to agricultural runoff, amphibians are also susceptible to the state's energy-related operations.

1.1.1.2. Energy-Related Impacts

Western ND, is home to the Bakken Formation in the Williston Basin, which is the second-largest crude oil reserve in the nation (Benko & Drewes, 2008). This portion of the state is susceptible to energy-related salinity contamination through wastewater discharge, accidental spills, and contaminant transport through induced and natural ruptures of storage tanks or pipelines (Lefebvre, 2017). Produced water naturally occurs within formations and is brought to the surface when oil is extracted. With the Bakken Formation yielding the most produced water, compared to any other shale formation in the US, over 17,000 spills have been reported between 2001-2020 (North Dakota Department of Environmental Quality, n.d.). Most of the spills reported inorganic contamination five or more years after the initial spill (Cote & Vengosh, 2017). This is especially important, as produced water from the Bakken Formation can be composed of approximately 90% sodium chloride, often exceeding 450,000 ppm, as it is pulled from a hypersaline formation (Benko & Drewes, 2008; Cote & Vengosh, 2017; Doll et al., 1989). Disposal is a continual challenge for operators and resource managers as produced water from the Williston Basin is untreatable, due to its high salinity content (Benko & Drewes, 2008).

Prior to knowing the toxicity of produced waters, disposal methods dating back to the 1950s consisted of pumping the water in un-lined reserve pits, drainage trenches, or receiving waters (Farag & Harper, 2014). While disposal methods of produced waters have been updated in recent

years, the solubility effects are still persistent throughout the landscape. Continual migration of these soluble chlorides in the groundwater indicates that this source of salinity contamination is a long-lived environmental issue (Farag & Harper, 2014). In recent years, research on the effects of ND's produced waters on aquatic inhabitants has gained attention. The effects of the produced water in ND have been observed to destabilize soil and its nutrients, decrease macroinvertebrate diversity, eradicate native plant growth, decrease amphibian abundance, while additionally decreasing the survival of Boreal Chorus Frog (*Pseudacris maculata*) larvae (Farag & Harper, 2014; Hossack et al., 2017, 2018; Preston et al., 2018).

1.1.1.3. Urbanization Impacts

It is estimated that roads and highways influence ecological decisions for at least one-fifth of the land in the US (Forman, 2000). As of 2018, North Dakota's Department of Transportation adopted adding pure salt to icy roads to cut costs, prevent ice build-up, and clear roads faster for safer travel (NDDOT Get Answers Page: Snow and Ice Control Information, n.d.). As some counties add pure salt during the winter, other counties around the state have adopted policies to spray produced water on dirt roads during the warmer months to control dust and melt ice during the colder months (North Dakota Department of Environmental Quality, 2018). These road salts, being highly soluble, easily migrate from pavement to groundwater and surface water habitats, instigating damaging effects to the environment and its inhabitants (Marsalek, 2003). These incessant accumulations of salinity throughout the state of ND easily allows solutes to be transported over a broad range of aquatic environments and their adjacent habitats.

The urbanization development that is increasing with human populations has been listed as a contributing factor to amphibian declines, while also significantly altering the hydrology of the natural landscape (Azous & Horner, 2001). Urban populations continue to grow globally, often

settling in agriculturally productive, low-lying areas that formerly contained wetlands, disproportionately displacing amphibians that depend on the aquatic habitats (Holzer, 2014). Urban development perpetuates run-off contaminants and habitat fragmentation, potentially entrapping and isolating specimens to a cocktail of chemicals within urban habitats (Azous & Horner, 2001; Holzer, 2014). Urban amphibian populations are often characterized by altered breeding, increased parasite rates, and pathogens within amphibian populations (Collinge & Ray, 2007; Longcore & Rich, 2004; Sun & Narins, 2005).

Freshwater salinization is hastily becoming a chronic toxicity to aquatic habitats, posing an extreme threat to biodiversity (Cañedo-Argüelles et al., 2013; Christy & Dickman, 2002; Denoël et al., 2010; Hintz & Relyea, 2017). This creates an essential need to fundamentally understand how wildlife dependent on freshwater is affected by salinity contaminates. Amphibians are often considered to be a prominent environmental freshwater bioindicator species, due to their complex life cycle, semi-permeable skin, and inability to consolidate and excrete excess salts (Albecker & McCoy, 2017; Waddle, 2006). An estimated 70% of amphibian populations are currently in decline, while at least one-third of the populations are in jeopardy of extinction (Blaustein et al., 1994; Hayes et al., 2010). As a species that solely relies on freshwater for successful breeding/development, long-term salinity contamination has a strong potential to affect survival and development, consequently sustaining population declines (Hayes et al., 2010). Although the toxicity of salts in aquatic environments is not a new concept, research on the toxicity effects of various salinity concentrations on amphibians is still lacking for many species, such as the Canadian toad (*Anaxyrus hemiophrys*).

1.1.2. Amphibians and Salinity

Previously published research outlining the acute effects of NaCl on amphibians has largely shown negative effects, such as decreased survival, delayed development, and increased malformations for a multitude of species (Table 1.1). Spotted salamander (*Ambystoma maculatum*) eggs exposed to various NaCl concentrations displayed a decrease in mass throughout all NaCl concentrations, despite reintroduction to freshwater (Karraker & Gibbs, 2011). While these results suggest a disruption of egg osmoregulation, the research also infers that spring snowmelt/rains do not alleviate the effects that NaCl has on amphibian eggs (Karraker & Gibbs, 2011). In addition, NaCl has been shown to alter behavior in Common frog (*Rana temporaia*) tadpoles exposed to various NaCl concentrations (Denoël et al., 2010). NaCl exposure resulted in decreased movement and speed, suggesting that tadpoles could have delayed responses when trying to escape predators (Denoël et al., 2010). Additionally, Wood frog (*Lithobates sylvaticus*) tadpoles in roadside wetlands with high salinity readings, experienced a delay in development, while also weighing more and consuming less (Hall et al., 2017). Overall, these results suggest that a reduction in growth can reduce fitness, survival, and mating success if sexual maturity is delayed (Hall et al., 2017).

As most previously published impacts of NaCl have largely concluded in negative consequences, a study conducted in 2012 revealed that exposure to NaCl decreased the spread of chytrid fungus (*Batrachochytrium dendrobatidis*) and prolonged the host's lifespan (Stockwell et al., 2012). Higher survival rates were observed in the higher saline treatments, suggesting that NaCl could potentially be an anti-fungal agent. Additionally, other species have shown high tolerances to salinity, while local adaptations have sporadically been observed in coastal regions (Gordon & Tucker, 1965; Karraker, 2007; Gomez-Mestre & Tejedo, 2003).

Table 1.1: Previous research of saline solutions on amphibians. Saline Source abbreviations: (NaCl) various brands of ionized salt sources; (RD) source of road deicing agents; (SW) seawater from a natural source; (BW) brackish water from a natural source: (IO) instant ocean salt solution; (RS) ringer's solutions; (MS) mixed aquarium sea salt; (unk) unknown.

Species	Common Name	Low/High (uS/cm)	Duration	Results	Source	Author
Acris crepitans	Blanchard's Cricket frog	430 - 31,250	72 Hrs	The highest salinities had significant differences in survival time.	NaCl	Hua & Pierce 2013
Ambyostoma maculatum	Spotted salamander	460 - 9500	96 Hrs	Median lethal Cl- concentration of 1840	NaCl	Collins & Russel 2009
Buergeria japonica	Japanese Coastal frog	1100 - 4900	96 Hrs	Survival rate of eggs was low at 2, 3, and 4%, and no eggs hatched at 5% salinity	NaCl	Haramura 2007
Bufo americanus	American toad	460 - 9500	96 Hrs	Median lethal Cl- concentration of 6100	NaCl	Collins & Russel 2009
Bufo marinus	Cane toad	1500 – 18,750	Metamorph	Percentage of larvae surviving to metamorphosis was greatly reduced in 22-25% seawater (12,500 uS/cm)	IO	Rios-Lopez 2008
Eurycea bislineata	Northern Two-Lined salamander	1900 – 14,400	96 hrs	All salamanders survived at concentrations approx. below 6250. All salamanders died concentrations above 9300.	NaCl	Snodgrass & Ownby 2015
Eurycea bislineata	Northern Two-Lined salamander	1900 – 14,400	96 hrs	All salamanders survived at concentrations approx. below 6250. All salamanders died concentrations above 9300.	RD	Snodgrass & Ownby 2015
Exerodonta zera	Puebla Treefrog	400 - 1250	Metamorph	Higher salinities delayed metamorphosis by up to 9 wks; reduced survivorship by approximately 20% at 680 and 800	IO	Woolrich-Pina 2015

Table 1.1: Previous research of saline solutions on amphibians (continued). Saline Source abbreviations: (NaCl) various brands of ionized salt sources; (RD) source of road deicing agents; (SW) seawater from a natural source; (BW) brackish water from a natural source: (IO) instant ocean salt solution; (RS) ringer's solutions; (MS) mixed aquarium sea salt; (unk) unknown.

Species	Common Name	Low/High (uS/cm)	Duration	Results	Source	Author
Hyla cinerea	American Green Tree frog	6250 – 18,750	Egg Hatching	Inland populations had a 3% hatch rate vs. Coastal populations had a 10% hatch rate at 9300	SW	Albecker 2007
Incilius nebulifer	Gulf Coast toad	3125 – 15,600	Metamorph	Embryos exhibited 95.5-99.5% hatching success at 0, 3125, and 6250 uS/cm; 74.4% success at 9300; no hatching at 1250 or 15,600.	IO	Alexander et al. 2012
Incilius nebulifer	Gulf Coast toad	430 - 31,250 (96 Hrs.) 680 - 7800 (7d)	72 Hrs (7d)	The highest salinities had significant differences in survival time.	unk	Hua & Pierce 2013
Incilius occidentalis	Pine toad	625 - 1250	Metamorph	Metamorphosis was delayed by 3 or 4 d; increased salinity did not affect survivorship	IO	Woolrich-Pina 2015
Leptodactylus albilabris	Gunther's white-lipped frog	1500 – 18,750	Metamorph	Percentage of larvae surviving to metamorphosis was greatly reduced in 22-25% seawater (18,750)	IO	Rios-Lopez 2008
Lithobates berlandieri	Rio Grande Leopard frog	430 - 31,250	72 Hrs	The highest salinities had significant differences in survival time.	unk	Hua & Pierce 2013
Litoria ewingii	Brown Tree frog	2180 - 8750	Metamorph	Larva in the highest salinity had decreased survival, slower growth, & delayed growth.	NaCl	Chinathamby et al. 2006

Table 1.1: Previous research of saline solutions on amphibians (continued). Saline Source abbreviations: (NaCl) various brands of ionized salt sources; (RD) source of road deicing agents; (SW) seawater from a natural source; (BW) brackish water from a natural source: (IO) instant ocean salt solution; (RS) ringer's solutions; (MS) mixed aquarium sea salt; (unk) unknown.

Species	Common Name	Low/High (uS/cm)	Duration	Results	Source	Author
Litoria aurea	Green and Golden Bell frog	1100 – 13,600	72 days	Metamorphosis only occurred in <5.5% SW; Survival threshold was between 5.5%-10% SW.	IO	Christy & Dickman 2002
Pseudacris crucifer	Spring Peeper	460 - 9500	96 Hrs	Median lethal Cl- concentration of 4430	NaCl	Collins & Russel 2009
Pelophylax perezi	Perez's frog	1500 - 11,500	96 hrs	Lethal toxicity to embryos (100%; 10,700) & to tadpoles (50%; 11,500)	unk	Santos et al. 2012
Lithobates clamitans	Green frog	51 - 540	58 days	Larval growth unaffected by road salt, but 15% of larvae were malformed at the highest treatment	RD	Karraker 2007
Lithobates slyvaticus	Wood frog	460 - 9500	96 Hrs	Median lethal concentrations were 4250	NaCl	Collins & Russel 2009
Lithobates clamitans	Green frog	460 - 9500	96 Hrs	Median lethal concentrations were 7500	NaCl	Collins & Russel 2009
Rana temporia	Common frog	1500 & 2300	Metamorph	Highest salt concentrations reduced the speed and movement of tadpoles in comparison with control treatment.	unk	Denoel 2010
Lithobates clamitans	Green frog	1500 - 5400	96 hrs	LC50 exposure 3780	NaCl	Snodgrass & Ownby 2015
Lithobates clamitans	Green frog	1500 - 5400	96 hrs	LC50 exposure 3725	RD	Snodgrass & Ownby 2015
Epidalea calamita	Natterjack toad	3100 - 18,750	Metamorph	Local adaptations observed; Salinity levels at 10,000 caused lower rates of survival, delayed development, and growth.	BW	Gomez-Mestre & Tejedo 2003

Table 1.1: Previous research of saline solutions on amphibians (continued). Saline Source abbreviations: (NaCl) various brands of ionized salt sources; (RD) source of road deicing agents; (SW) seawater from a natural source; (BW) brackish water from a natural source: (IO) instant ocean salt solution; (RS) ringer's solutions; (MS) mixed aquarium sea salt; (unk) unknown.

Species	Common Name	Low/High (uS/cm)	Duration	Results	Source	Author
Rhinella marina	Cane toad	200 - 1900	Metamorph	High-salinity treatments increased tadpole body sizes, accelerated metamorphosis, and improved locomotor ability of metamorphs (did not affect metamorph morphology).	SW	Wijethunga et al. 2015
Duttaphryrynus melanostictus	Asian Common toad	0 - 5400	96 Hrs.	Size and survival not effected	MS	Karraker 2010
Fejervarya limnocharis	Paddy frog	0 - 5400	96 Hrs.	Size and survival not effected	MS	Karraker 2010
Kaloula pulchra	Asiatic Painted frog	0 - 5400	96 Hrs.	Salinity decreased size and survival	MS	Karraker 2010
Polypedates megacephalus	Brown Tree frog	0 - 5400	96 Hrs.	Size and survival not effected	MS	Karraker 2010
Microhyla ornata	Pigmy frog	0 - 5400	96 Hrs.	Salinity decreased size and survival	MS	Karraker 2010
Lithobates slyvaticus	Wood frog	930	14 days	930 uS/cm salinity reduced larval activity and foraging behaviors	RD	Hall et al. 2017
Lithobates pipiens	Northern Leopard frog	2700 - 14,000	Metamorph	Salinity > 7800 was lethal to embryos, 5900-7100 was semi-lethal, & < 5900 allowed development, increased abnormalities >3900	RS	Ruibal 1959
Lithobates slyvaticus	Wood frog	0 - 15,200	Metamorph	Acute and chronic exposure caused reduced activity, weight, and increased abnormalities. Higher salinities delayed development and reduced survivorship.	NaCl	Sanzo & Hecnar 2005

While work on various salinity thresholds has been reported for a handful of species in the United States, North Dakota's native amphibian species have received minimal attention regarding salinity research. An overwhelming amount of published salinity research begins exposure as larvae. Few research projects focus on exposing eggs and continuing exposure through to metamorphosis. This research provides insight into longer durations of exposure throughout various life stages. Extensive gaps of information on Canadian toads persist, and a full understanding of salinity effects on hatch success, mortality, larval development period, and time of exposure is a necessity for this species. This research examined the effects of salinity on two different age groups of larvae through the assessment of egg mortality, time taken to complete metamorphosis, weight at metamorphosis, and overall survival. Effects of increased salinity between treatments on hatch success, weight at metamorphosis, time to metamorphosis, and survival are expected as exposure began as eggs and larvae.

1.2. Materials & Methods

1.2.1. Field Measurements

Monthly, starting at the end of April 2020, conductivity and chloride levels were monitored at 20 wetland locations around the urban landscape of Fargo, ND. All locations were considered within the Fargo city limits. Three sites were considered rural, located on private property, and in low trafficked areas. The 17 remaining sites were located off high-trafficked roadways, located in high-density areas of the city. Using a YSI (model 85) meter, conductivity and temperature readings were collected at the edge of the wetland until the end of August 2020. A small sample (approx. 20-30 mL) of water was collected at each location to obtain a reading from the Chloride (Cl-) QuanTab® 30-600 mg/L Test Strip. The strip would require approximately 2-3 minutes of resting in the water to display a color change on the stripe. In between sites, the YSI probe and

water collection cup were rinsed 3 times using deionized water, to ensure an uncontaminated reading from the previous site.

The North Dakota Department of Environmental Quality (NDDEQ) website was used to obtain data for 6 surrounding locations within Cass co. as a comparison outside of the urban landscape of Fargo. The 6 additional sites are from various rivers and drainages located within Cass Co. Data from 3 of the sites were from the spring/summer of 2020 and 3 of the sites were from spring/summer of 2018, due to the unavailability of 2019 or 2020 data collected. Chloride and conductivity levels were the only readings used to obtain averages, as the sodium levels were inconsistently collected and therefore not used. Additionally, the first month of April only includes 4 of the 6 sites as data was unavailable for 2 of the sites during that time.

1.2.2. Species

Canadian toads (*Anaxyrus hemiophrys*), are a medium-sized anuran with a widespread distribution throughout the prairies of the northern mid-west United States and southern Canada. Active only for a small portion of the year, Canadian toads spend a majority of the year underground in hibernation but utilize shallow water bodies to breed (ditches, ponds, wetlands, etc.) in early May. Throughout the summer, Canadian toads disperse into nearby environments but prefer wetter habitats than other species of toads (Constible et al., 2010). Due to their early seasonal breeding, natural disposition for wetter habitats, the harsh winter conditions, and the agriculturally dominant nature of the north, this species has the increased potential to regularly encounter saline conditions. Additionally, the effects of salinity on Canadian toads have not been researched.

1.2.2.1. Ethics Statement

All procedures adhered to the protocols approved by the North Dakota State University Animal Care and Use Committee (Protocol # A20002). No animals were euthanized during the duration of this study.

1.2.3. Collection Site

Collection occurred, approximately two miles south of Hawley, Minnesota (46.8225923, 96.3455016) on private property. The site is bordered on the east, west, and south by agricultural fields. Situated on the northern border, is a neighboring homestead. The habitat composition of the collection site is a mixture of deciduous woodlands to the west, with a small non-native prairie grassland to the east. At the time of the collection in early May, the property had 3 shallow seasonal depressions and 1 semi-permanent wetland. Egg masses were collected from a man-made, seasonal depression. The property is in the process of being developed, containing a single shed that was constructed in the fall of 2019. Conductivity measured at 306 uS/cm, with a 0 reading for Chloride, at the time of oviposition.

1.2.4. Collection

Canadian toad egg masses were hand-collected on May 2, 2020. Copulating events were observed the previous night, marked, and then egg masses were collected the following day. Due to toads laying their eggs in long single-egg strands, anchored to vegetation, it is estimated that approximately five egg masses were collected. Egg masses were then transported to North Dakota State University (NDSU) in a 5-gallon bucket, using a portable air bubbler to ensure proper oxygen levels during transport. Egg masses were aged at stage 2-5, using the Gosner staging system for anurans (Gosner 1960). The Gosner staging system is specific to aging anurans as stages 1-19 are embryos, stages 20-25 are embryos, 26-41 are larva, and 41-46 are considered metamorphs (1960).

1.2.5. Treatments

Treatments were made using tap water, treated with Seachem Prime Freshwater & Saltwater conditioner and API Ammo Lock Ammonia Remover Aquarium Water Conditioner, before adding salt and specimens. Electrical conductivity was measured using an HI 9811-5 Portable EC/TDS/°C Meter. Treatments were individually created in 2000 mL Erlenmeyer plastic sterilized flasks. Based on the electrical conductivity of the tap water, appropriate amounts of non-iodized Morton Canning & Pickling Salt (no additives) were used to create the various salinity treatments.

Treatment 1 (hereafter referred to as the control throughout) contained no additional salt and ranged from 430 uS/cm to 1030 uS/cm with an average electrical conductivity of 625 uS/cm. Treatment 2 (hereafter referred to as the low treatment throughout) had an average electrical conductivity reading of 2268 uS/cm (min/max range: 1600/2867 uS/cm), with an average of 1.75g of salt added. Treatment 3 (hereafter referred to as the moderate treatment throughout) had an average electrical conductivity of 4001 uS/cm (min/max range: 3126/4546 uS/cm), with an average of 3.73g of salt added. Treatment 4 (hereafter referred to as the moderately high treatment throughout) had an average conductivity reading of 5631 uS/cm (min/max range: 4587/6129 uS/cm), with an average of 5.78g of salt added. Treatment 5 (hereafter referred to as the high throughout) had an average conductivity reading of 7957 uS/cm (min/max range: 7592/8260 uS/cm), with an average of 9.50g of salt added. These salinity levels were based on previously published literature on multiple species and wetland soil salinity samples collected by the United States Geological Survey (USGS) throughout ND (USGS, 2019). Additionally, anurans were trapped and observed at the higher salinity levels in ND, where tissue samples were collected (Robinove et al., 1958; USGS, 2019).

For optimal growth and minimal stress, the water parameter guidelines in *Amphibians: Guidelines for the breeding, care, and management of laboratory animals*, were followed (Nace et al., 1974). To monitor salinity fluctuations due to evaporation and to ensure salinity ranges were being met, conductivity measurements were taken weekly. Every other week, a subset of containers was randomly selected to sample and monitor standard water parameters (nitrate, nitrite, chloride, pH, ammonia, hardness, alkalinity), using Tetra® 6 N 1 Aquarium Water and Ammonia Test Strips. Throughout the project duration, water parameters remained within normal, non-harmful levels. To reduce handling, fluctuation of water characteristics, and unnecessary stress, water changes were conducted as needed (approximately every other week). To mitigate evaporation, containers were topped off as needed.

1.2.6. Husbandry

Specimens were housed in a window-less, aquatic-based room, in the basement of Steven's Hall on a 4-tiered metal shelf. Each flask (container) was supplied with an airline and air stone to provide circulation and oxygen. Containers were split between 4 shelves (18/shelf) using a randomized block design. Throughout the project duration, boiled lettuce and spinach were fed at ab libitum. Uneaten lettuce was removed after 24 hours, to prevent spoilage. The lighting was on a 12h light: 12h dark schedule and temperature ranged between 22-24° C.

1.2.7. Early Salinity Exposure

Early salinity exposure is defined as eggs exposed to saline conditions. Throughout the entirety of the document, eggs submitted to salinity will be referred to as early salinity exposure, or as early exposure. Egg exposure mirrors the environmental conditions of spring melt, in which eggs could be exposed to saline conditions upon being laid/oviposited. On May 3, 2020, approximately 5 egg masses were separated, using forceps and scalpels, into chains of 10 eggs.

Fifteen chains of 10 eggs were added to each flask (n = 24) for a total of 150 eggs per container, with 6 replications per treatment (control, low, moderate, moderately high, and high salinity levels).

Age progression, malformations, and mortality checks were conducted and recorded daily. Eggs were determined to be deceased if mold or degradation was observed. After 2 weeks of initial salinity exposure, undeveloped eggs were determined to be deceased. Approximately 6 weeks after initial exposure as individuals developed, water levels were decreased and artificial floating plants were added to each container. This allowed individuals developing lungs, to transition more readily to a terrestrial lifestyle and to prevent drowning. To prevent and reduce the effects of stunted growth and competition for food in containers, containers were split within the respective salinity range when needed. Completion of metamorphosis was defined as Gosner stage 44-46. Upon completing metamorphosis, individuals were removed from their container, weighed, and then released to the original collection site.

1.2.8. Late Salinity Exposure

Late salinity exposure is defined as salinity exposure occurring at Gosner stage 25. Older tadpole exposure mirrors the environmental conditions of spring melt evaporation, where tadpoles could be subjected to increased salinity levels at a later developmental stage. On May 3, 2020, approximately 5 egg masses were collected and separated into various 5-gallon buckets filled with treated tap water. Starting on May 11, 2020, tiered 24-hour acclimating periods began. A subset of individuals was exposed to the low treatment levels of salinity. After 24 hours, a subset of the individuals exposed to the low treatment levels was exposed to the moderate treatment salinity levels. This continued until individuals reached the highest level of high treatment. After

acclimating exposures, each 2000 mL flask (n = 35) contained 25 tadpoles, with 7 replications per treatment (the control, low, moderate, moderately high, and high salinity).

Age progression, malformations, and mortality checks were conducted and recorded daily. Individuals were determined to be deceased if no longer responsive to stimuli or degradation was observed. Approximately 4 weeks after initial exposure as individuals developed, water levels were decreased and artificial floating plants were added to each container. This allowed individuals developing lungs, to transition more readily to a terrestrial lifestyle and avoid drowning. To prevent and reduce the effects of stunted growth and competition for food in containers, containers were split within the respective salinity range when needed. Completion of metamorphosis was defined as Gosner stage 44-46. Upon completing metamorphosis, individuals were removed from their container, weighed, and then released to the original collection site.

1.2.9. Analysis

Egg mortality (eggs unhatched), time to metamorphosis (days), mass at metamorphosis (g), and survival to metamorphosis were all recorded for early salinity exposure. Time to metamorphosis (days), mass at metamorphosis (g), and survival to metamorphosis were all recorded for late salinity exposure. Egg mortality was measured per container by counting individuals that hatched, in combination with unhatched and/or moldy eggs that were removed daily. Mass and time to metamorphosis was analyzed using an individual's weight (g) and time taken to complete metamorphosis (days) for early (n = 109) and late (n = 312) salinity exposure. Both the early and late exposure time to metamorphosis was set at their hatch date, not their exposure date. As individuals within the same egg clutch can develop at various rates, the hatch day was determined when approximately 80% of individuals throughout all containers were in between Gosner Stage 20-25. Survival to metamorphosis was recorded by counting the survivors

per container while recording deceased individuals that were removed from each container. Before splitting containers, survival was analyzed based on original container replication numbers for early (n = 19) and late (n = 32) salinity exposure.

Due to the data breaking normality assumptions of an ANOVA, regardless of transformations, non-parametric permutational analysis of variance (PERMANOVA) was used. A PERMANOVA (10,000 permutations) was used to determine the effects of salinity on mass, time to metamorphosis, survival to metamorphosis, and egg mortality. To assess further differences of NaCl effects between all treatment pairs, pairwise post-hoc permutational t-tests (10,000) were used. To maintain an experiment-wise error rate at 0.05 a Benjamini-Hochberg correction was applied throughout the pairwise comparisons. The Benjamini-Hochberg correction was used to avoid type 1 and type 2 errors while a large number of comparisons were being analyzed. Additionally, a PERMANOVA was used to analyze the difference between early and late salinity experiments, with an additional variable of exposure time added to the analysis. The analysis was performed in RStudio Desktop 1.3.1093 using the plyr, RVAideMemoire, coin, ggplot2, tidyverse, devtools, vegan, pairwise Adonis, and stargazer packages.

1.3. Results

1.3.1. Field Salinity

Conductivity, Cl-, and NaCl were averaged between 20 wetland sites across the urban landscape of Fargo (Figure 1.1). For the month of April, conductivity averaged 1409 uS/cm (s = 994, min/max: 269/3720 uS/cm), with Cl- at 85.9 mg/L (s = 115, min/max: 25/523 mg/L), and NaCl at 140 mg/L (s = 190, min/max: 10/860). In May conductivity measured at 1773 uS/cm (s = 1181, min/max: 457/4773 uS/cm), Cl- at 71.4 mg/L (s = 52, min/max: 29/236 mg/L), and NaCl at 120 mg/L (s = 90, min/max: 30/420). During the month of June conductivity dipped to 1461 uS/cm

(*s* = 1222, min/max: 453/5317 uS/cm), with a spike in Cl- at 127 mg/L (*s* = 253, min/max: 29/1122 mg/L), and NaCl at 210 mg/L (*s* = 420, min/max: 30/1860). In July conductivity measured at 1247 uS/cm (*s* = 1169, min/max: 261/4276 uS/cm), with Cl- dipping to 51.7 mg/L (*s* = 39, min/max: 27/154 mg/L), and NaCl at 80 mg/L (*s* = 70, min/max: 20/250). For the month of August, conductivity continued to drop to 699 uS/cm (*s* = 447, min/max: 162/1581 uS/cm), as Cl- continued to drop to 32.8 mg/L (*s* = 14, min/max: 25/84 mg/L), and NaCl at 40 mg/L (*s* = 30, min/max: 10/140 mg/L).

Conductivity spiked in May and June, with the lowest readings recorded in August. Chloride levels were the highest at the beginning of the warm season, peaking in June, with the lowest levels recorded towards the end of the season in August. Sodium chloride levels followed a similar pattern to Cl-, as levels increased during June and decreased at the end of August. However, when conductivity peaked in May, Cl- and NaCl dipped and subsequently peaked in June, as conductivity continued to decline. These levels coincide with Canadian toad breeding as they tend to lay in early May. Eggs continue to hatch throughout May and larval development continues into June and usually, metamorphosis occurs at the end of June into July when the levels of conductivity and NaCl are at their highest.

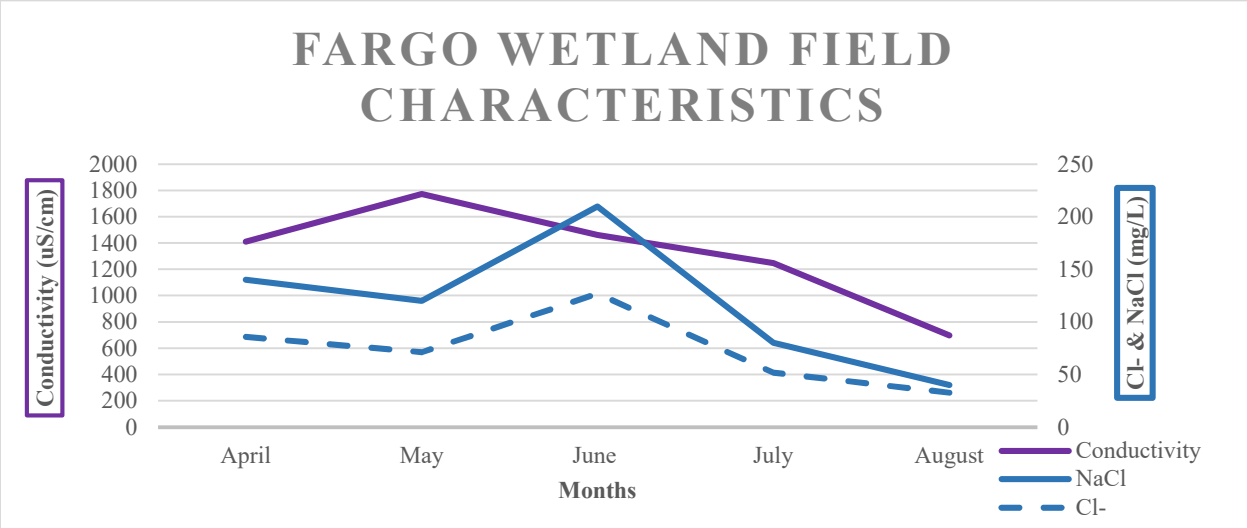

Figure 1.1: Field collected wetland conductivity, chloride (Cl-), and salinity (NaCl) levels around Fargo, ND.

Conductivity, and Cl- were averaged between the 6 sites located within Cass Co. (Figure 1.2). For the month of April (only included 4 of the 6 sites), conductivity averaged 1076 uS/cm (*s* = 192, min/max: 977/1300 uS/cm), with Cl- at 32 mg/L (*s* = 6, min/max: 32/37 mg/L). In May conductivity measured at 1540 uS/cm (*s* = 230, min/max: 1210/1780 uS/cm), Cl- at 70 mg/L (*s* = 14, min/max: 32/70 mg/L). During the month of June conductivity peaked at 1685 uS/cm (*s* = 289, min/max: 1290/2110 uS/cm), with a spike in Cl- at 49 mg/L (*s* = 24, min/max: 26/93 mg/L). In July conductivity averaged at 1344 uS/cm (*s* = 383, min/max: 902/1850 uS/cm), with Cl- dipping to 46 mg/L (*s* = 21, min/max: 17/77 mg/L). For the month of August, conductivity continued to decrease to 1212 uS/cm (*s* = 326, min/max: 964/1460 uS/cm), as Cl- continued to drop to 42 mg/L (*s* = 17, min/max: 23/72 mg/L).

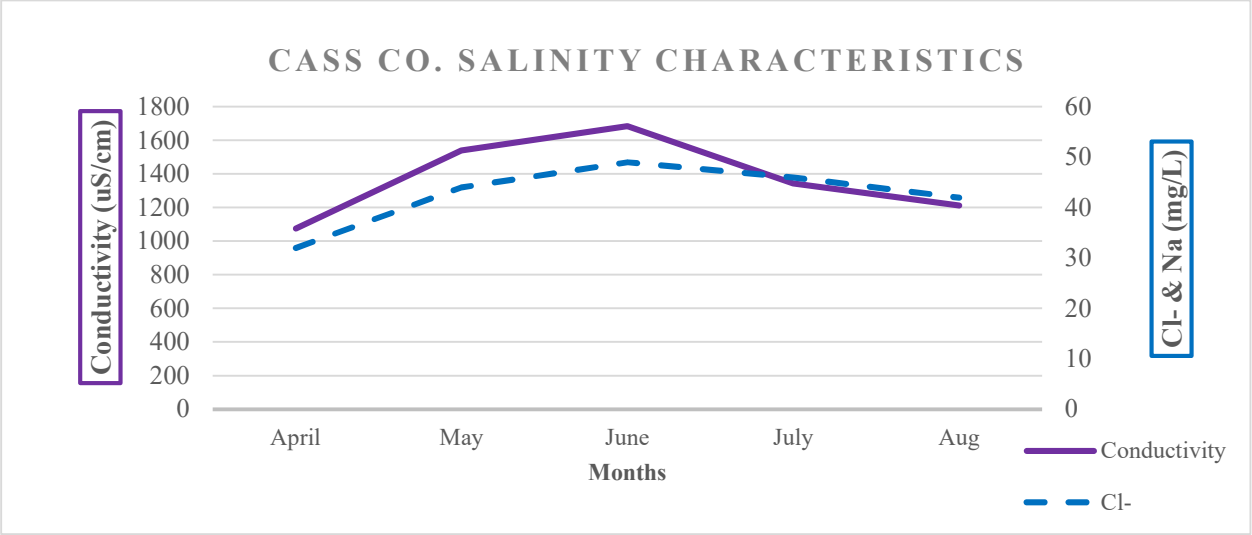

Figure 1.2: NDDEQ collected conductivity and chloride readings for river and drainage sites within Cass Co.

Similar to the Fargo samples, the surrounding Cass co. site's conductivity levels were the highest in May and June with the lowest levels recorded in April and August. Initially, chloride started low at the beginning of spring, peaked in June, and decreased as the weather started to cool. Overall, the wetland sites experienced larger fluctuations of levels than the Cass Co. sites. This is likely due to the seasonal fluctuations that wetlands experience with minimal water input throughout the season.

1.3.2. Early Salinity Exposure

Egg mortality (eggs unhatched), time to metamorphosis (days), mass at metamorphosis (g), and survival to metamorphosis were all recorded for early salinity exposure (Table 1.2). The average of unhatched eggs per container for the control was 82% (± 21, 66.6/100%; +/- standard deviation, min/max). For the low treatment, the average mortality was 76% (± 23.7, 89/98.6%) eggs per container. The moderate treatment averaged 84% (± 16.2, 66.6/92.6%) eggs per container. The moderately high treatment had a mortality of eggs at 90% (± 11.8, 76/98%) per container. The

high treatment averaged egg mortality of 99.3% (± .816, 98.6/100%) eggs per container (Figure 1.3).

Through a PERMANOVA analysis, it was found that salinity did have a significant effect on egg mortality ($F_{4,25}$ = 3.67, p = 0.021). The high treatment was the only treatment that was significantly different from the low treatment (p = 0.020), the moderate treatment (p = 0.012), and the moderately high treatment (p = 0.017). The relationship between the control and the high treatment was borderline significant with a p-value of 0.055. Approximately, 99.3% of eggs in the high treatment did not hatch. Initially, the high treatment was included in the analysis but was removed to view the relationship between other treatments. With the high treatment removed, it was found that salinity did not influence hatch success, with an F-statistic of 1.23, degrees of freedom at 3 and 20, and a p-value at 0.319 (Figure 1.5).

The time taken to complete metamorphosis was significant between treatments ($F_{3,105}$ = 5.18; p = 0.02; Table 1.3 & Figure 1.4). Time to complete metamorphosis did not differ among the two lower salinity treatments; the control (48.9 ± 11, 40/94; average, +/- standard deviation, min/max), the low treatment (47.7 ± 7.14, 40/63), but both had significantly shorter time intervals to metamorphosis when compared to the moderate treatment (55.4 ± 8.44, 40/76; p = 0.014 and p = 0.001, respectively). The moderately high treatment (51.7 ± 8.41, 40/60) did not differ from the control (p = 0.548), the low treatment (p = 0.311) or the moderate treatment (p = 0.311; Table 1.3 & Figure 1.4).

Table 1.2: Descriptive statistics of mass at metamorphosis ($F_{3, 105} = 1.86$, $p = 0.135$), time to metamorphosis ($F_{3,105} = 5.18$, $p = 0.002$), hatch mortality ($F_{3,20} = 1.23$, $p = 0.319$), and survival to metamorphosis ($F_{3,15} = 0.559$, $p = 0.649$) for early exposure between treatments.

	Mass (g)			Days		Egg Mortality			Survival to Meta		
Treatments	*n*	μ	*s*	μ	*s*	*n*	μ (%)	*s*	*n*	μ	*s*
Control	28	0.12	0.04	48.9	11.0	6	82	21.0	6	4.67	3.56
Low	26	0.12	0.03	47.7	7.14	6	76	23.7	4	6.50	5.20
Moderate	44	0.13	0.04	55.4	8.44	6	84	16.2	6	7.33	5.40
Moderately high	11	0.15	0.06	51.7	8.41	6	90	11.8	3	3.67	4.73
High	-	-	-	-	-	6	99.3	.816	-	-	-

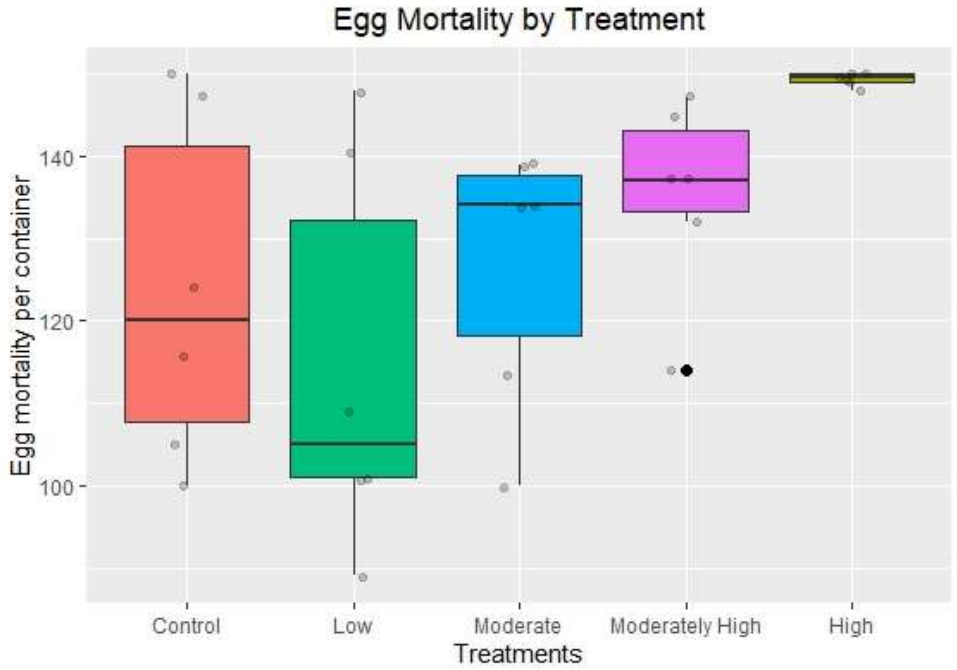

Figure 1.3: Median and IQR values displayed for egg count mortality per container between treatments for early exposure ($F_{3,20} = 1.23$, $p = 0.319$).

Table 1.3: Pairwise comparison of time to metamorphosis displaying the p-values between treatments in early exposure ($F_{3,105} = 5.18$, $p = 0.002$).

	Control	Low	Moderate
Low	0.690		
Moderate	**0.014**	**0.001**	
Moderately High	0.548	0.311	0.311

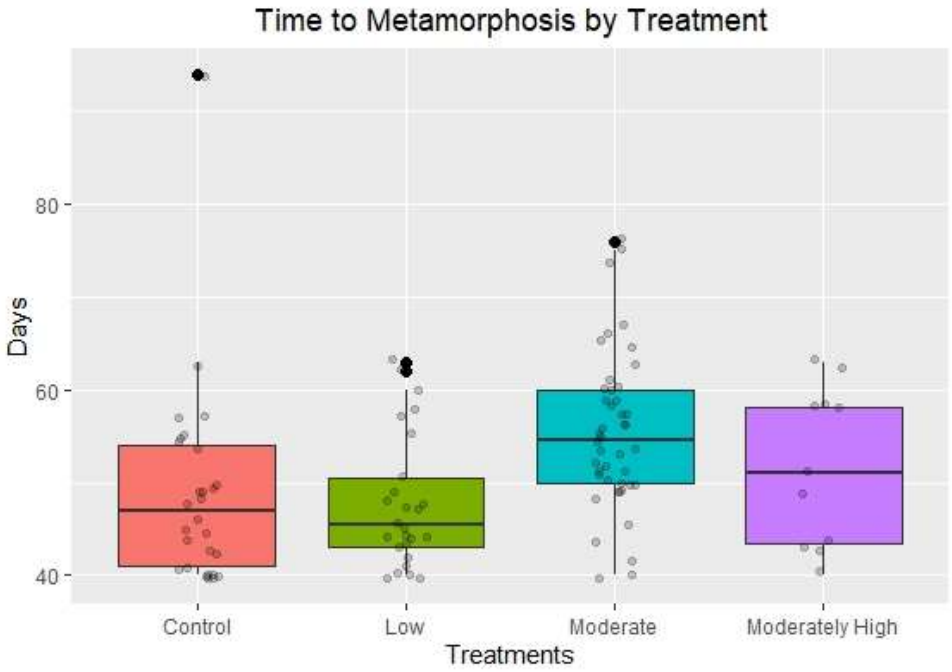

Figure 1.4: *Median and IQR values for time to metamorphosis by treatments for early salinity exposure ($F_{3,105} = 5.18$, $p = 0.002$).*

Masses at metamorphosis were not significantly different among treatments ($F_{3,105} = 1.86$; P = 0.135; Table 1.3 & Figure 1.5). The average mass at metamorphosis for the control (0.12 $\pm$ 0.04) and the low treatment (0.12 $\pm$ 0.03) were the same between treatments. The moderate treatment experienced a 0.01g increase at 0.13g ($\pm$ 0.04), while the moderately high experienced 0.03g increase at 0.15g ($\pm$ 0.06). The high treatment did not have any individuals complete.

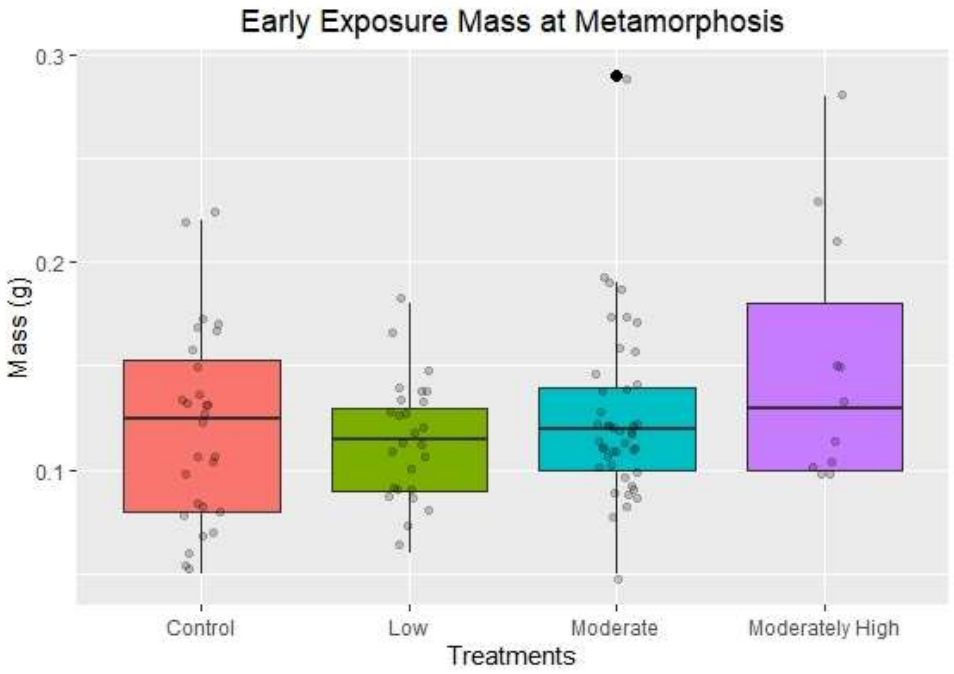

Figure 1.5: Median and IQR values displayed for weight at metamorphosis by treatments for early salinity exposure ($F_{3,105} = 1.86$, $p = 0.135$).

Survival did not differ among the four salinity treatments with salinity below 5600 uS/cm ($F_{3,150} = 0.559$, P = 0.649; Figure 1.6). The average survival per container in the control was 4.67 individuals (± 3.56). The low treatment had an average of 6.50 individuals (± 5.20) survive per container. The moderate treatment contained an average of 7.33 (± 5.40) per container, while the moderately high had an average of 3.67 (± 4.73) survival per container. The high salinity treatment did not have any survivors that completed metamorphosis and were removed from the survival analysis to avoid creating a false effect. In the PERMANOVA analysis, it was found that salinity below 5600 uS/cm did not cause a significant difference in survival to metamorphosis (Figure 1.6: $F_{3,15} = .559$, p = 0.649). Due to a mortality event associated with potential water change issues, 5

replicates were removed from the low treatment during data analysis resulting in a sample size of 5.

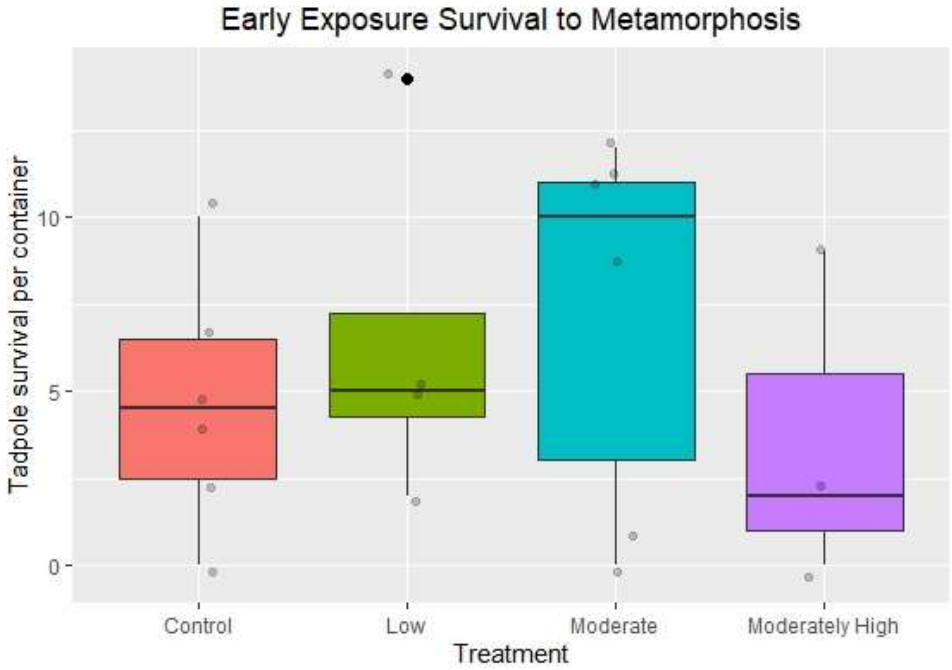

Figure 1.6: Median and IQR values displayed over-all survival to metamorphosis between treatments for early exposure ($F_{3,15} = 0.559$, $p = 0.649$).

1.3.3. Late Salinity Exposure

Time taken to complete metamorphosis (days), mass at metamorphosis (g), and survival to metamorphosis were all recorded for late salinity exposure (Table 1.4). For late salinity exposure the time taken to complete metamorphosis was significant between treatments (PERMANOVA: $F_{3,308} = 3.30$; $p = 0.018$; Table 1.5 & Figure 1.7). The control took an average of 58.2 days (± 9.14, 41/93), while the low treatment took an average of 59 days (± 9.34, 40/85). With a decrease in time taken to complete metamorphosis, the moderate treatment completed metamorphosis in 54.7 days (± 7.44, 40/74), while the moderately high completed metamorphosis in 56.8 days (± 9.58, 41/79).

The relationship between the low treatment (p = 0.579) and the moderately high (p = 0.456) was not significantly different from the control. However, the moderate treatment significantly differed from the control (p = 0.028), and the low treatment (p = 0.017), but did not statistically from the moderately high (p = 0.284). The salinity range of 3100-4500 uS/cm for the moderate treatment, decreased time to metamorphosis within late exposure by an average of 3.5 days and 4.3 days compared to the control and the low treatment (Table 1.4).

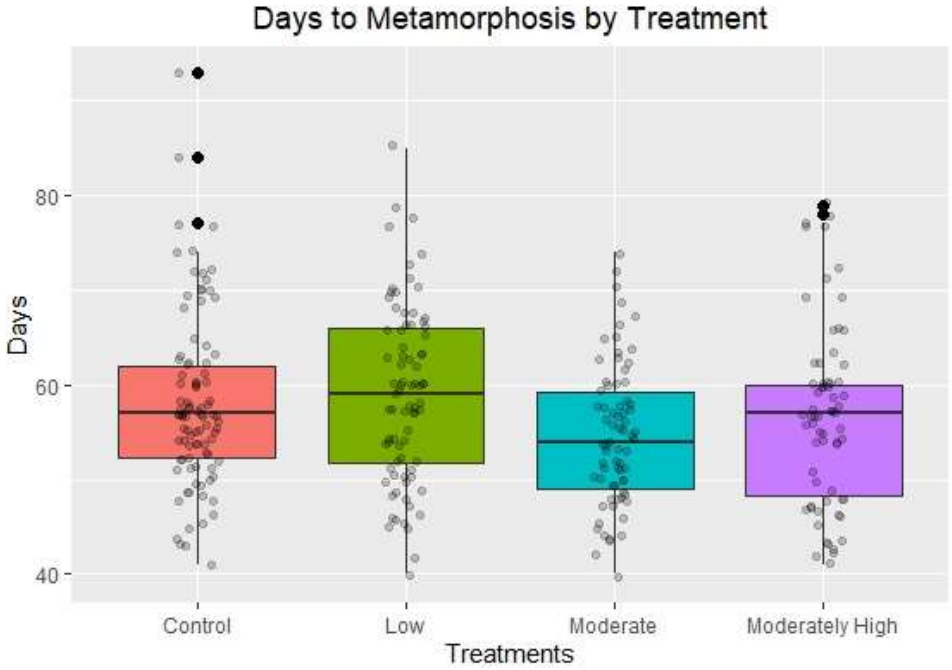

Figure 1.7: Median and IQR values for time to metamorphosis between treatments in late exposure ($F_{3,308}$ = 3.30, p = 0.018).

Table 1.4: Descriptive statistics of mass at metamorphosis ($F_{3,308}$ = 1.23, p = 0.307), time to metamorphosis ($F_{3,308}$ = 3.30, p = 0.018), and survival to metamorphosis ($F_{3,21}$ =1.84, p = 0.180) for late exposure between treatments.

	Mass (g)			Days			Survival to Meta.	
Treatment	*n*	μ	*s*	μ	*s*	*n*	μ	*s*
Control	94	0.12	0.04	58.2	9.14	7	13.4	6.11
Low	80	0.11	0.04	59.0	9.34	5	16.0	1.90
Moderate	72	0.12	0.04	54.7	7.44	7	10.3	2.93
Moderately High	66	0.12	0.03	56.8	9.60	6	11.0	5.50

Table 1.5: Pairwise comparison of time to metamorphosis displaying the p-values between treatments in late exposure ($F_{3,308} = 3.30$, $p = 0.018$).

	Control	Low	Moderate
Low	0.579		
Moderate	**0.028**	**0.017**	
Moderately High	0.456	0.284	0.284

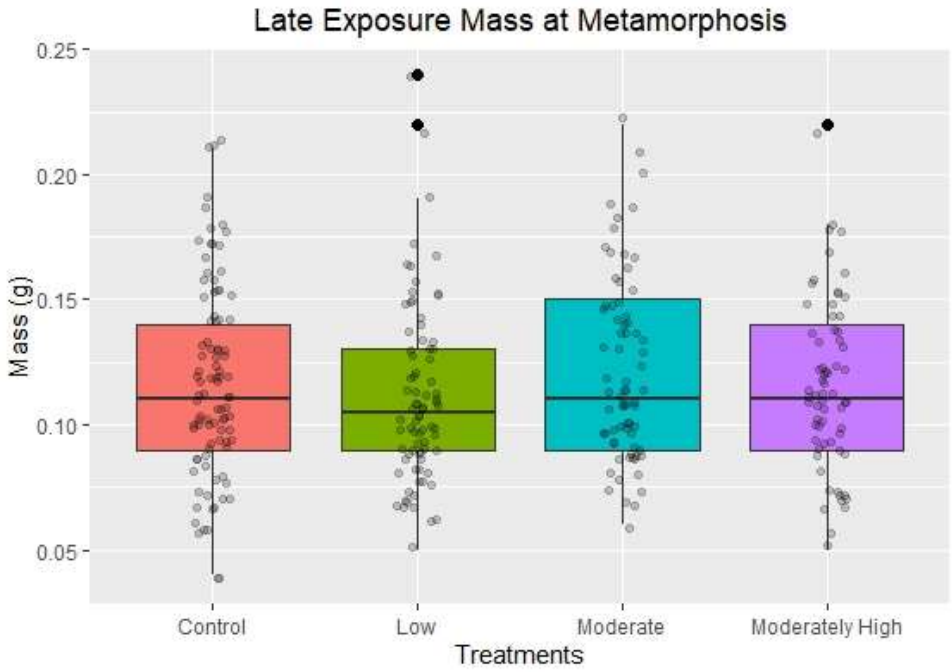

Figure 1.8: Median and IQR values for mass at metamorphosis between treatments for late exposure ($F_{3,308} = 1.23$, $p = 0.307$).

Mass at metamorphosis was not significantly different between treatments with an F-statistic at 1.23, degrees of freedom at 3 and 308, and a p-value at 0.307 (Figure 1.8). The average mass for the control was 0.12g (± 0.04; standard deviation), while the low treatment had a decrease of 0.01g at an average of 0.11g (± 0.04). The moderate treatment maintained the same mass at the control at 0.12g (± 0.04), along with the moderately high at 0.12g (± 0.03). The high treatment did not have any individuals complete metamorphosis. Increased salinity between treatments did not influence mass at the time of completing metamorphosis for tadpoles exposed to saline treatments at Gosner stage 25 (Table 1.4).

To determine survival, all treatments started with 25 individuals per replicate, with 7 replicates per treatment for late salinity exposure. In the PERMANOVA analysis, it was found that salinity below 5600 uS/cm did not cause a significant difference in survival to metamorphosis ($F_{3,21} = 1.84$, $p = 0.180$; Figure 1.9). The average survival per container in the control was 13.4 individuals ($\pm$ 6.11). The low treatment had an average of 16 individuals ($\pm$ 1.90) survive per container. The moderate treatment contained an average of 10.3 ($\pm$ 2.93) per container, while the moderately high had an average of 11 ($\pm$ 5.50) survival per container. The high treatment, the highest salinity, did not have any survivors that completed metamorphosis and were removed from the survival analysis to avoid creating a false effect. Due to a mortality event, likely related to water changes, 2 replicates were removed from the low treatment and 1 replicate was removed from the moderately high. With the high treatment included in the analysis ($F_{4,27} = 14.82$, $p = .001$), significant relationships existed with the control ($p = 0.001$), the low treatment ($p = 0.006$), the moderate treatment ($p = 0.006$), and the moderately high ($p = 0.006$).

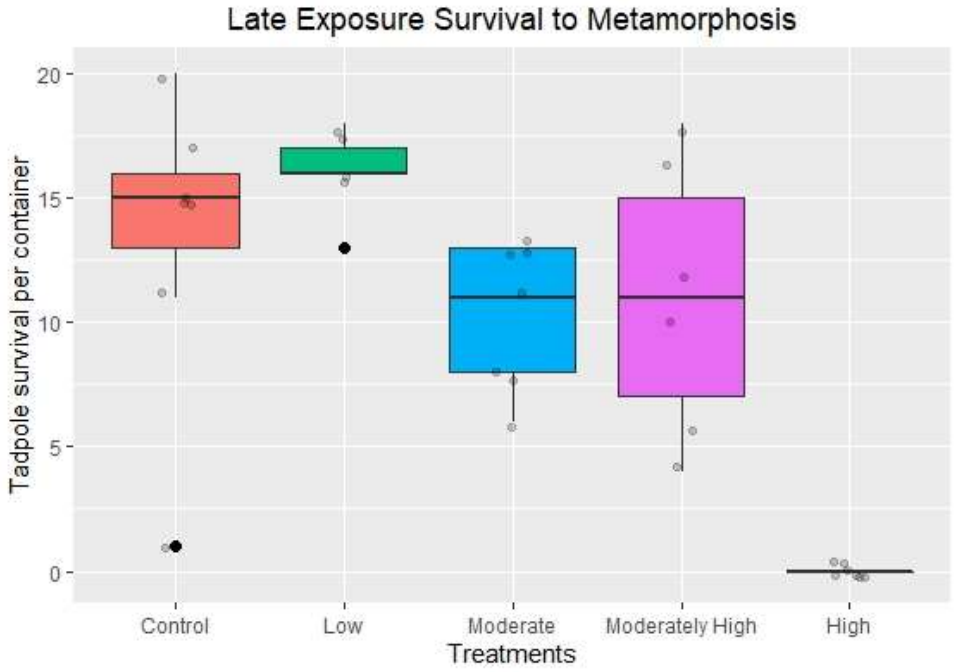

Figure 1.9: Median and IQR values for late salinity exposure survival between treatments ($F_{3,21}$ = 1.84, p = 0.18) measured by survivors per container. The high treatment had a 100% mortality.

1.3.4. Late vs. Early Exposure

The late and early salinity exposures were compared together in order to determine if the timing of salinity exposure affected the time taken to reach metamorphosis. For early salinity exposure, the control took an average of 48.9 days (±11.0, 47; standard deviation, median), while the low treatment only took an average of 47.7 days (±7.14, 45.5). With an increase of time taken to complete metamorphosis observed, the moderate treatment completed metamorphosis in 55.4 days (±8.44, 54.5), while the moderately high completed metamorphosis in 51.7 days (± 8.41, 51). The time taken to complete metamorphosis in the late salinity exposure for the control took an average of 58.2 days (± 9.14, 57), while the low treatment took an average of 59 days (± 9.34, 59). With a decrease in time taken to complete metamorphosis, the moderate treatment completed

metamorphosis in 54.7 days (± 7.44, 54), while the moderately high completed metamorphosis in 56.8 days (± 9.58, 57).

When comparing the two data sets, the controls differ by 9.3 days, with the late salinity control taking an average of 9.3 days longer to complete metamorphosis. This pattern continues into the low treatment as the late salinity exposure the low treatment took an average of 11.3 days longer to complete metamorphosis, the early salinity exposure. Comparing the moderate treatment, the early exposure took approximately half a day longer to complete metamorphosis than the late salinity exposure. The moderately high treatment late salinity exposure average 5.1 days longer to complete metamorphosis than the early salinity exposure.

The timing of salinity exposure affects the time taken to reach metamorphosis (Figure 1.10: $F_{3,415} = 10.44$, p = 0.000009; table 1.6). When treatments (the control, the low, the moderate, & the moderately high treatment) and time to complete metamorphosis were analyzed, the F-statistic was 0.069, with the degrees of freedom of 3 and 415, and a p-value of 0.978. The time to complete metamorphosis and age of exposure were analyzed as the F-statistic was 23.30, degrees of freedom of 1 and 415, and the resulting p-value of <0.001. As the time to complete metamorphosis was grouped by treatments and compared to the age of exposure, the F-statistic was 10.44, degrees of freedom of 3 and 415, with a p-value of < 0.001.

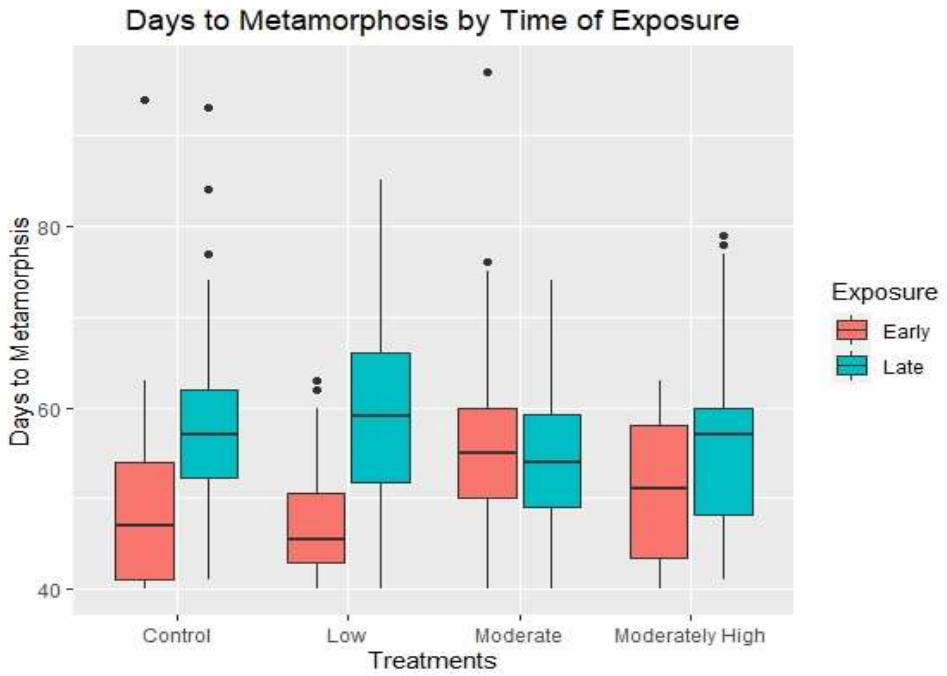

Figure 1.10: Median and IQR values for time to metamorphosis for early and late salinity ($F_{3,415}$ = 10.44, p = <.001).

Table 1.6: PERMANOVA (10,000) table displaying the significance between the early and late data sets with the added variable of exposure.

	Df	*F*	P
Treatment	3	0.069	.975
Exposure time	1	23.31	<.001
Treatment X Exposure time	3	10.44	<.001

1.4. Discussion

The results indicate that Canadian toad larvae survival to metamorphosis, egg mortality, and mass at metamorphosis are largely unaffected by salinity levels below 5600 uS/cm, despite the timing of exposure. Survival was significantly impacted in salinity over 5600 uS/cm. Late salinity exposure experienced a slight decrease in survival with an increase of salinity between treatments, however, this was not statistically significant. Early salinity exposure contained only 11 individuals that survived to metamorphosis in the moderately high, while survival in the moderate

treatment was the highest, with 44 individuals completing metamorphosis. This illustrates that early exposure to saline conditions below 5600 uS/cm is non-lethal, and still allows for metamorphosis to occur. However, it was discovered that for approximately 10 days, all the moderately high containers (early and late exposure) were exposed to lower salinity levels due to a miscalculation. This could account for the non-significant discrepancies observed in the moderately high compared to the moderate treatment, despite a higher salinity exposure on average. A decrease in salinity for a 10-day period could have provided an acclimation opportunity while development was concurrent.

Due to the potential influence of water change mishaps, comparing survival to metamorphosis between early and late salinity exposure would not provide accurate insight into the effects of salinity on survival and the age at which exposure occurred. Throughout all salinity treatments, bent tail malformations, underdeveloped tails, and abdominal edemas were observed in early Gosner stages, but most of the malformed died off early within the first 3-4 weeks. Changes in behavior were not quantified, but irregular behaviors were observed in higher salinities (ie. swimming in tight circles, erratic swimming, etc).

Elevated salinity did not affect tadpole survival, hatch success, or mass at metamorphosis below 5600 uS/cm, suggesting that Canadian toad salinity tolerances are moderate. As the high treatment experienced a 100% mortality in both experiments, this would infer that the lethal salinity threshold for developing tadpoles is between 5600 to 8100 uS/cm. Compared to other established thresholds for amphibians, Canadian toads can tolerate a moderate amount of salinity (Table 1.1) (Hopkins et al., 2013). This result mirrors the closely related American toad (*Anaxyrus americanus*) salinity threshold ranges of 5826 - 6456 uS/cm (Collins & Russell, 2009).

Further research would be advantageous to establish an exact salinity threshold and determine a salinity level where increases in mortality occur for Canadian toad tadpoles. While hatch success proved not to be statistically significant, further research is recommended to determine whether exposure as eggs to saline conditions could affect survival if prolonged exposure is above 4600 uS/cm. Additionally, if research were to continue, an environment with weather-dependent salinity fluctuations, would be recommended and more environmentally relevant. The salinity fluctuation that occurred in the moderately high, provided an opportunity to further research the effects that fluctuating salinity levels could have on survival and developmental periods in Canadian toads. Additionally, the field-collected salinity characteristics confirmed these fluctuations. This would further display a more environmentally relevant effect on survival and development while providing insight into the resiliency of tadpoles when encountering salinity fluctuations.

Our research sought to answer whether the age of exposure affected the time to metamorphosis. While previous research on other species has sought to answer this question, our research largely differed by starting salinity exposure at younger Gosner stages and continued exposure throughout Gosner stage milestones. Our "early salinity exposure" started exposure shortly after ovulation and our "late salinity exposure" started between Gosner stage 20-25, shortly after hatching. Continued exposure throughout the entirety of the larval period mimics the environmental constraints tadpoles experience in their natural habitats. In addition, exposure at ovulation and shortly after hatching mimic the spring runoff salinity spikes experienced in April through June. These high levels and spikes experienced around the Fargo area occur during fundamental time periods when amphibians are breeding and hatching.

As survival, egg mortality, and mass were unaffected despite the age of exposure, the time taken to complete metamorphosis was significant and differed between early and late exposure. Early salinity exposure increased the time to metamorphosis by 3-7 days. The opposite effect was observed in late salinity exposure, with a 1-4 day decrease to metamorphosis in higher salinities. While our comparison between late vs. early exposure does present interesting differences, referencing the previous pairwise comparisons, Figure 1.10, and the controls being dissimilar, an unaccounted-for variable could be driving the significant relationship observed in the analysis. Published salinity research highlights the effects of “early” exposed larvae developing delays to metamorphosis when exposure begins between Gosner stages 25-38 (Welch et al., 2019; Newman, 1989).

Early salinity exposure as eggs, delays their development to metamorphosis. This observed difference may result from the timing of exposure compared to osmoregulatory capabilities. With increased tadpole maturation, osmoregulatory abilities increase, due to gill development (Gordon & Tucker, 1965). In the Crab-Eating Frog, early developing stages, such as egg capsules, have very little capabilities of osmoregulating, making them more susceptible to salinity than larvae (Gordon & Tucker, 1965). Elevated salinity levels that are demonstrated with snowmelt in early spring, increase their time as a tadpole, leaving them exposed to other environmental factors in a vulnerable state. While the results regarding "early" salinity exposure are in accordance with published literature, our "late" salinity exposure results are conflicting.

Our late salinity exposure results suggest that as salinity levels peak during the time that metamorphosis occurs (June-July), tadpoles could still develop with minimal associated mortality events. These results are not in agreement with current literature, as the majority of amphibian species experienced a significant increase in mortality with similarly increased salinity levels

(Karraker & Gibbs, 2011; Collins & Russell, 2009; Welch et al., 2019). Previous research suggests that more developed tadpoles display a greater tolerance to salinity, due to increased gill development, starting around Gosner stage 38 (Welch et al., 2019). However, with survival unaffected by salinity under 5600 uS/cm, our results suggest that tolerances start as early as Gosner stage 20-25, while also decreasing the time taken to complete metamorphosis. This decrease in time taken to complete metamorphosis could possibly be a plastic response due to the environmental stressor of salinity. Previous research has highlighted the abilities that tadpoles can display through plasticity when encountering stressors within their environments (Burraco et al. 2017).

Tadpoles often display growth and developmental shifts in response to environmental changes and stressors (Burraco et al., 2017). A species' plasticity plays an integral role in overcoming unpredictable environmental conditions, often ensuring the survival of the individual. These responses can entail acceleration to metamorphosis under stressful conditions, such as osmotic stress, to ensure survival (Newman, 1989). Seemingly, an acceleration to metamorphosis would prove to be an initial benefit, increasing the chances of short-term survival (Burraco et al., 2017). As short-term survival is achieved, this type of plasticity can concurrently impact long-term survival, through shortened telomeres (Burraco et al., 2017). While the short-term success is beneficial to survival, the long-term implications could influence subsequent life stages, impacting overall fitness.

Salinity alters developmental timing, thus potentially eliciting a developmental plasticity response to ensure survival when encountering unpredictable environmental stressors. Although alterations to developmental timelines have the potential to cause detrimental consequences at the community level, the relatively high tolerances observed are intriguing given that amphibians are

often viewed as vulnerable species. While amphibians are relatively vulnerable, they are often a by-product of their environment. The high salinity tolerances observed, paired with the potential for plastic responses, and the increased levels of salinity throughout the environment could be indicative of adaptations occurring throughout the population. These adaptations to higher salinities have been observed in coastal communities throughout the world (Karraker, 2007; Hopkins & Brodie, 2015).

Salinity research has primarily fixated on species-level impacts in understanding the effects of salinity. Although this is fundamental, efforts for the future should be directed towards the population and community levels as salinity is becoming more prevalent throughout the world. This would provide insight into whether developmental alterations are leaving populations vulnerable or are contributing to the process of adaptations. Mitigation efforts to decrease salt use are recommended to minimize potential negative impacts on populations and habitats.

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2. THE EFFECTS OF SALINE SURFACES ON POST-METAMORPHIC JUVENILE CANADIAN TOAD (*ANAXYRUS HEMIOPHRYS*) SELECTION, WEIGHT, AND CONSUMPTION RATES

2.1. Introduction

2.1.1. Soil, Salts, and Solubility

If lost, soil is a non-renewable resource that cannot be recovered within a human's lifespan (Zaman et al., 2018). Second, to erosion, soil salinity is the leading cause of land degradation across the globe (Zaman et al., 2018). Salinization of soil occurs when water-soluble salts accumulate throughout soil under various climatic conditions (Salama et al., 1999). Naturally occurring salts are usually less soluble compound sulfates, typically only leading to saline soil conditions in large concentrated quantities (Doll et al., 1989; Seelig, 2000). A majority of unnatural salts are enriched with a chloride component, therefore more soluble, allowing the sodium to easily bind with clay particles (Doll et al., 1989; De Jong, 1982; Seelig, 2000). This binding can arise through natural or human-induced processes, consequently undermining the soil's resource base, while decreasing soil quality, altering its structure, restricting root growth, disrupting nutrient dispersal and the self-regulatory capacity of the soil (Doll et al., 1989; De Jong, 1982; Seelig, 2000; Zaman et al., 2018).

A soil is deemed saline when the electrical conductivity (EC) exceeds 4000 µS/cm (Shrivastava & Kumar, 2015). As soluble salts migrate through the soil, many climatic variables, such as increased temperatures, and minimal rain-fall, contribute to a high salinity content accumulating near the surface (Food and Agriculture Organization of the United Nations, 2014; Qadir et al., 2014). Due to climate change, rising sea levels, improper irrigation methods, and anthropogenic salt loading, salt-affected soils account for more than 20% of the soils around the

globe (Food and Agriculture Organization of the United Nations, 2014; Qadir et al., 2014). The presence of salt in the soil is quickly becoming a global issue affecting agriculture yields, environmental health, and economic welfare (Rengasamy, 2006). As saline soils increase globally, the effects on vulnerable species, like amphibians, are inadequately researched.

2.1.2. North Dakota (ND) Soil Salinity Causations

The extent of soil salinization is hard to estimate and quantify throughout the world. However, as of 2006, an estimated 932.2 Mha (approx. 2.3 billion acres) of saline/sodic soils existed throughout the world with a yearly increase of 10% of impacted soils (Rengasamy, 2006; Shrivastava & Kumar, 2015). North Dakota straddles the moist eastern United States and semi-arid western United States, while also central in the Prairie Pothole Region (PPR) (Frankson et al., 2017; Malanu, 1986). As an agriculturally dominant state experiencing rising temperatures through climate change, ND's soil moisture depletion is concurrently increasing (Frankson et al., 2017). Soil moisture depletion contributes to the accumulation of salts in the root zone, causing a reduction in crop yields and destabilizing soil (Food and Agriculture Organization of the United Nations, 2014; Qadir et al., 2014; Vengosh, 2013; Zaman et al., 2018). With ND's high-water table and historical glacial saline deposits, the resurfacing of salts is a common occurrence throughout the state (Franzen, 2013). In 2000, there was a reported 2.6 million acres across 34 of 53 reporting counties that were considered salt-affected in the state (Seelig).

North Dakota's landscape history is the primary source for naturally salt-affected soils found in the state. Before the glacial age, western ND was completely submerged under the sea, as central and eastern ND were glacially covered (Malanu, 1986). Salt-affected soils in central and eastern ND are associated with the shallow saline water table (Malanu, 1986). The hydrology within the state is controlled by the glacially formed closed depressions, better known as

"potholes," thus allowing for salts to travel, leach, and settle near the surface (Seelig, 2000). The majority of the salts naturally found in ND are insolubly sulfate-based, but in concentrated amounts can have detrimental effects on plant growth (Keller et al., 1986; Seelig, 2000). The northern portion of the Red River Valley is known to have extensive, naturally occurring, soluble deposits of chloride-based salts (Seelig, 2000). Other large quantities of chloride-affected soils present in the state are indicative of anthropogenic alterations (Seelig, 2000). The threat of salinity contamination from natural processes, agriculture, and urban causes is present in ND.

2.1.2.1. Agriculture

Salt-affected soils cost approx. $27.3 US billion dollars annually across the world due to production losses (Qadir et al., 2014; Seelig, 2000; Vengosh, 2013). Agriculture dominates the state of ND as approximately 90% of the landscape is dedicated to agricultural use (NDDA, 2011). However, agriculture is often responsible for creating saline soils through overgrazing, improper irrigation practices, overuse of fertilizers, and nutrient depletion (Qadir et al., 2014; Seelig, 2000; Vengosh, 2013). The main pollutants from agricultural runoff include heavy metals, sediment, pathogens, nutrients, and salt; all of which aggregate in depressional wetlands and leech into surrounding areas (US Environmental Protection Agency, 2005). The aggregation of salts in aquatic environments has resulted in negative impacts on larval amphibian diversity, growth, consumption, predatory responses, hatch success, and survival (Karraker & Ruthig, 2009; Hossack et al., 2017; Sanzo & Hecnar, 2006). Even though agriculture is one of the leading causes of pollution, biodiversity loss, and habitat destruction for the environment, it is a necessity for food production, as human populations continue to increase in urban areas.

2.1.2.2. Urbanization

Urbanization is the trend of populations migrating from rural to urban areas. Urban settings are a relatively new concept in human history, as more than half the world's population now live in urban settings (Ritchie & Roser, 2018). This trend is increasing as two-thirds of the world's population is expected to live in an urban setting by 2050 (Ritchie & Roser, 2018). Urbanization is known to displace wildlife, prolong habitat fragmentation, and generate large quantities of run-off contaminates (Azous & Horner, 2001; Holzer, 2014; Ritchie & Roser, 2018). Urbanization perpetuating run-off contaminants, salt loading, and habitat fragmentation frequently entraps and isolates wildlife to a cocktail of chemicals within urban habitats, especially amphibians (Azous & Horner, 2001; Holzer, 2014). Heavily altered urban habitats have correlated with altered breeding behaviors, increased parasite loads, increased pathogens, and diversity declines within amphibian populations (Azous & Horner, 2001; Collinge & Ray, 2007; Longcore & Rich, 2004; Sun & Narins, 2005).

2.1.2.3. Road Salts

It is estimated that roads and highways influence ecological decisions for at least one-fifth of the land in the US (Forman, 2000). As of 2018, North Dakota's Department of Transportation adopted adding pure salt to icy roads to cut costs, prevent ice build-up, and clear roads faster for safer travel (*NDDOT Get Answers Page: Snow and Ice Control Information*, n.d.). In addition to adding pure salt during the winter, some counties around the state have adopted policies to spray energy created produced water on dirt roads during the warmer months (*Guidelines for the Use of Oilfield Salt Brines for Dust and Ice Control*, 2018). This is done to help mitigate dust, and melt ice during colder months (*Guidelines for the Use of Oilfield Salt Brines for Dust and Ice Control*, 2018). Highly soluble road salts, easily migrate from pavement to soil, instigating damaging effects

to the environment and its inhabitants (Marsalek, 2003). These chloride-based salts have been associated with soil, groundwater, and watershed salinization (Vengosh, 2013). Accumulations of salinity throughout the state of ND easily allows for solutes to be transported over a broad range of environments and habitats.

2.1.2.4. Energy Demands

Western ND is home to the Bakken Formation in the Williston Basin. The Bakken formation is the second-largest crude oil reserve in the nation (Benko & Drewes, 2008). Primarily composed of sodium, bicarbonate, and chlorides, produced waters are created during the oil and gas extraction process in large volumes. The Bakken Formation yields the most produced water of any other shale formation in the US (Cote & Vengosh, 2017). Western ND is susceptible to energy-related salinity contamination through produced water discharge, accidental spills, and contaminant transport through induced and natural ruptures of storage tanks or pipelines (Lefebvre, 2017). Disposal methods dating back to the 1950s consisted of pumping the water in un-lined reserve pits, drainage trenches, or receiving waters (Farag & Harper, 2014). Produced water disposal is a continual challenge for operators and resource managers as the produced water from the Williston Basin are considered to be untreatable, due to their high salinity content (Benko & Drewes, 2008).

Continual dispersal and resurfacing of produced water through soil indicate that this source of salinity contamination is a long-lived environmental issue (Farag & Harper, 2014). In recent years, research on the effects of ND's produced water on aquatic inhabitants has gained attention. The effects of the produced water in ND have been observed to, destabilize soil and its nutrients, decrease macroinvertebrate diversity, eradicate native plant growth, decrease amphibian

abundance, while additionally decreasing the survival of Boreal Chorus Frog (*Pseudacris maculata*) larvae (Farag & Harper, 2014; Hossack et al., 2017, 2018; Preston et al., 2018).

2.1.3. Previous Research

North Dakota is susceptible to societal strains with amplified agricultural, urban, and energy demands. The consequences of these demands are currently unfolding as the northern Midwest freshwater environments are experiencing the largest salinity upsurges comparatively to the rest of the United States (Kaushal et al., 2018). Continual salt loading, has negative effects on various environments, thus affecting inhabitants such as amphibians.

Amphibians possess physiological traits that directly contribute to their potential of contaminate exposure. Amphibian skin functions as a respirating organ, while also controlling water uptake to maintain hydration (Smith et al., 2007). Amphibians, particularly toads, contain a highly vascularized pelvic patch on their posterior ventral surface. The pelvic patch functions to regulate water movement through lymph channels into venous circulation when in contact with moist surfaces (Smith et al., 2007). Studies have shown the importance of this dermal uptake when exposed to various contaminants and recognize the effects that the exposure has through this specific dermal absorption (Smith et al., 2007; Van Meter et al., 2016; Hatch et al., 2001; Smith et al., 2007). Previous research in materials such as metals, organochlorines, and nitroaromatics has suggested that amphibians have the capabilities to recognize these materials through their pelvic patch absorption, and actively avoid these surfaces (Hatch et al., 2001; Johnson et al., 2004).

Previous salinity research conducted on eggs and larval life stages has suggested that sodium chloride (NaCl) reduces survival, delays developmental rates, and increases malformations in various larval amphibian species (chapter 1). Spotted salamander (*Ambystoma maculatum*) eggs exposed to various NaCl concentrations displayed a decrease in mass throughout all

concentrations, despite reintroduction to freshwater (Karraker & Gibbs, 2011). While these results suggest a disruption of egg osmoregulation, the research also infers that spring snowmelt/rains do not alleviate the effects that NaCl has on amphibian eggs (Karraker & Gibbs, 2011). Additionally, NaCl has been shown to alter behavior in Common frog (*Rana temporaia*) tadpoles exposed to various NaCl concentrations (Denoël et al., 2010). NaCl exposure resulted in decreased movement and speed, suggesting that tadpoles could have delayed responses when trying to escape predators (Denoël et al., 2010). Wood frog (*Lithobates sylvaticus*) tadpoles transplanted in saline roadside wetlands displayed a delayed growth rate (Hall et al., 2017). Despite a delay in development, tadpoles weighed more and spent less time eating (Hall et al., 2017). Overall, these results suggest that a reduction in growth can have dire consequences on fitness, survival, and reduce mating success if sexual maturity is delayed (Hall et al., 2017).

2.1.4. Objectives

Collectively, published literature highlighting the effects of NaCl on amphibians has mainly fixated on eggs or developing larvae, as these life stages are easily obtainable and thought to be more sensitive to contaminants. Largely, post-metamorphic life stages have been underrepresented in salinity literature, thus, leaving a gap of information on the effects of saline substrates on post-metamorphic amphibians. Except for chemically dominant pesticides, the effects of organic contaminates from terrestrial environments have not been assessed with post-metamorphic individuals.

In addition, the majority of North Dakota's native amphibian species have received minimal attention regarding salinity research. Extensive gaps of information on amphibian response to salinity continue. A full understanding of the effects that saline substrates have on post-metamorphic choice, weight, and consumption rates is a necessity as saline soils persist throughout

the environment. This research examined the effects of extended saline soil exposure on post-metamorphic juveniles, through the measurements of weight and consumption rates. Additionally, this research sought to determine if amphibians actively avoided saline substrates through administered choice tests. An effect on consumption and weight gain from saline soil exposure, along with avoidance of saline surfaces were anticipated to occur.

2.2. Materials & Methods

2.2.1. Species

Canadian toads (*Anaxyrus hemiophrys*), a medium-sized toad, are a widespread species throughout the prairies of the northern mid-west United States and southern Canada. Prominent in a broad range of terrestrial and aquatic habitats, toads spend a majority of their lives in terrestrial environments, only utilizing freshwater habitats during breeding events in early May (Gelder et al., 1986; James et al., 2004). Throughout the summer, Canadian toads disperse into nearby environments but prefer wetter habitats than other species of toads (Constible et al., 2010). Canadian toads utilize their terrestrial environments for shelter, hibernation, protection, and maintaining moisture levels by burrowing into loose soil (Hoffman & Katz, 1989). As a northern species, a large portion of their lives is spent in hibernation, burrowed under the frost line to survive the harsh winters (Breckenridge & Tester, 1961). Given their terrestrial lifestyle, and dermal uptake capabilities, combined with their natural disposition for wetter habitats, extended hibernation period, and the agriculturally dominant nature of the north, this species has the increased potential to regularly encounter saline conditions (Hayes et al., 2010; Smith et al., 2007). Additionally, the effects of salinity on Canadian toads have not been researched.

2.2.1.1. Ethics Statement

All procedures adhered to the protocols approved by the North Dakota State University Animal Care and Use Committee (Protocol # A21001). No animals were euthanized during the duration of this study.

2.2.2. Collection Site

Collection occurred, approximately two miles south of Hawley, Minnesota (46.8225923, 96.3455016) on private property. The site is bordered on the east, west, and south by agricultural fields. Situated on the northern border, is a neighboring homestead. The habitat composition of the collection site is a mixture of deciduous woodlands to the west, with a small non-native prairie grassland to the east. At the time of the collection in late July/early August 2020, the property had 1 shallow seasonal depression and 1 semi-permanent wetland. Using a YSI-85 meter and Quantab Chloride testing strips, the electrical conductivity reading was 306 µScm, while the Chloride levels read at 0.

2.2.3. Collection

Canadian toad juveniles were hand collected from Clay Co. MN between August 2-8, of 2020. Approximately, 140 juvenile toads were hand caught and placed in 5-gallon buckets until transport to North Dakota State University (NDSU). Upon arrival, toads were then split into groups of 15 and placed in Sterilite 32 Qt. (L 60.33cm x W 40.64cm x H 17.46cm) storage containers containing damp paper towels as a hydrating substrate. Holes were created in the lids to allow for proper ventilation. Crickets were fed to each container every other day and paper towels were changed every other day, as well. Toads were held for two weeks before research started. After research concluded, individuals were released back to the site of collection.

2.2.4. Soil Salinity Exposure

Soil treatments were made using tap water, treated with Seachem Prime Freshwater & Saltwater conditioner and API Ammo Lock Ammonia Remover Aquarium Water Conditioner, before adding salt. Electrical conductivity was measured using an HI 9811-5 Portable EC/TDS/°C Meter. Based on the electrical conductivity of the tap water, appropriate amounts of non-iodized Morton Canning & Pickling Salt (no additives) were used to create the various salinity treatments. Appropriate amounts of salt were calculated for 4L of water per treatment, to reach the desired conductivity.

To create the soil needed, blocks of Zoo Med Eco Earth Compressed Coconut Fiber Expandable Reptile Substrate was used. After desired conductivities were reached, one block of coconut fiber was added to 4L of water to create an entire treatment's worth of soil. Individuals were housed in 38oz Snap Pak black rectangular containers with holes punctured in the lids for proper ventilation. Each container contained 8 oz of coconut fiber. Once soil treatments were created, the containers were left uncovered for 48 hrs. to allow for even evaporation to occur from the substrate.

Treatment 1 (hereafter referred to as the control throughout) contained no additional salt and ranged from 576 uS/cm to 810 uS/cm with an average electrical conductivity of 673 uS/cm. Treatment 2 (hereafter referred to as the low treatment throughout) had an electrical conductivity reading of 3625 uS/cm, with 4.21 g of salt added. Treatment 3 (hereafter referred to as the moderate treatment throughout) had an electrical conductivity of 3984 uS/cm, with 8.38g of salt added. Treatment 4 (hereafter referred to as the moderately high throughout) had an electrical conductivity reading of 7523 uS/cm, with 12.73 g of salt added. Treatment 5 (hereafter referred to as the high

treatment throughout) had an average conductivity reading of 9953 uS/cm, with 19.48 g of salt added.

Salinity levels of the soil mixtures were verified by Saturated Soil-Paste Electrical Conductivity tests (Rhoades et al., 1989). These salinity levels were based on previously published literature on multiple species and wetland soil salinity samples collected by the United States Geological Survey (USGS) throughout ND (USGS, 2019). Additionally, anurans were trapped and observed at the higher salinity levels in ND, where tissue samples were collected (Robinove et al., 1958; USGS, 2019).

Each salinity treatment comprised of 20 individuals, for a total of $n = 100$ between the 5 soil salinity treatments. For the duration of the soil research, containers were randomly split between 3 shelves in stacks of 3, with 3 rows per column (approx. 45/shelf). Specimens were housed in a window-less, aquatic-based room, in the basement of Steven's Hall on a 4-tiered metal shelf. The lighting remained consistently on a 12:12 schedule, with a temperature range between 22-24° C.

To ensure proper and consistent consumption before the initial soil salinity exposure, specimens were placed into individual a 38oz Snap Pak black rectangular container 3 days before soil exposure. Moistened paper towels were used as a substrate to prevent dehydration and each toad was fed 4-6 crickets to ensure consumption occurred for all toads before salinity exposure. After the 3-day separation, the toads were weighed to the nearest 0.01 g using an electronic digital balance and measured (SVL, Snout-to-vent length), then randomly assigned to an individual treatment container. The first feeding occurred on the second day of exposure. Every two days thereafter, 4 crickets were fed. After 48 hrs. uneaten crickets were recorded, removed and individuals were fed 4 more crickets. Toads were misted with de-chlorinated, unsalted water every

5 days to ensure proper moisture and humidity levels were maintained. The total duration of salinity exposure was 20 days with a total of 24 crickets given over the duration. After the 20 days, toads were reweighed, remeasured, and then released back to the original collection site.

2.2.5. Choice Tests

Individuals used for the choice-tests continued to be housed in 32 Qt. containers with 15 per container. Following Hatch et al. methods (2010), juvenile toads (separate from soil exposed toads) underwent a 15-minute surface choice test, in a rectangular 6.1q plastic container. Saturated Uline tri-fold paper towels were used as the substrate, with a total of 5 salinity treatments. Using a 10 mL pipette for measurement, each side was completely saturated with 7 mL of water. Half of the container contained 2 saltwater Uline tri-fold saturated paper towels (treatment), while the other half of the container contained 2 Uline de-chlorinated water tri-fold saturated paper towels (control), with 5 cm separating the paper towels. Toads were placed in the middle of the container, in between the saturated paper towels.

After a 10-minute adjustment period, observations were started and continued for 15 minutes thereafter. Observations were recorded using a Canon - VIXIA HF R800 HD Camcorder to minimize human interaction. The location of treatment vs. control (left or right) was randomized for each trial. In between trials, containers were thoroughly rinsed with hot water, sanitized with 2% Chlorhexidine gluconate, and then completely dried. Video footage was reviewed and every minute the side the individual was on was recorded. Toads were considered to be on a "side" if more than half of their body was located on the paper towel. Each salinity treatment had 9 individuals, for a total of $n = 36$.

Treatment 1 (the control) contained no additional salt and ranged from 437 uS/cm to 1042 uS/cm with an average electrical conductivity of 689 uS/cm. Treatment 2 (the low treatment) had

an average electrical conductivity reading of 2253 uS/cm (min/max range: 1659 to 2914 uS/cm), with an average of 1.75 g of salt added. Treatment 3 (the moderate treatment) had an average electrical conductivity of 4001 uS/cm (min/max range: 3126 to 4546 uS/cm), with an average of 3.73g of salt added. Treatment 4 (the moderately high treatment) had an average conductivity reading of 5631 uS/cm (min/max range: 4587 to 6129 uS/cm), with an average of 5.78g of salt added. Treatment 5 (the high treatment) had an average conductivity reading of 7957 uS/cm (min/max range: 7592 to 8260 uS/cm), with an average of 9.50g of salt added.

2.2.6. Analysis

Within the soil exposure experiments, effects of soil salinity between treatments on weight and overall consumption were analyzed. Initial weight was subtracted from the post-exposure weight. The difference in weight and the total crickets consumed were used for analysis. Due to the data breaking normality assumptions of an ANOVA, regardless of transformations, a permutation test (10,000) was used. A permutations test (10,000) was used to determine the effects of soil salinity on mass, and consumption. To assess further differences of NaCl effects between treatments, a permutation t-test (10,000) post-hoc analysis with a Benjamini-Hochberg (BH) correction applied was conducted. The BH correction was chosen to account for the false discovery rate with the multiple comparisons being performed in the analysis. Within each treatment, the time spent on the treatment side was recorded and then compared to 50% (eight would be expected if truly a random choice), using a Mann Whitney Wilcoxon test. The alpha level (α) was 0.05 and P-values less than, were considered statistically significant. Saline soil exposure analysis was performed in RStudio Desktop 1.3.1093 using plyr, RVAideMemoire, coin, ggplot2, tidyverse, devtools, vegan, pairwiseAdonis, and stargazer packages. The choice test statistical analyses were performed in JMP 15.0 (SAS Institute).

2.3. Results

2.3.1. Soil Salinity Exposure

Weight gain and decreased consumption was experienced throughout various treatments during the duration of the experiment (Table 2.1). Saline soil had a significant impact on the mass ($F_{4,95}$=5.16, p = <.001) and consumption rates ($F_{4,95}$=11.6, p=0.004) within the toads (Table 2.2, Figure 2.1 & Figure 2.2). In the control (625 uS/cm), an average of 0.184g (± 0.433, 0.270; standard deviation, median) was gained while consuming an average total of 23.20 crickets (± 1.281, 24) out of 24 offered, throughout the two and half weeks of observation. The low treatment (3593 uS/cm) gained an average of 0.123g (± 0.660, 0.220), while consuming an average of 23 crickets (± 1.026, 23). The moderate treatment (4062 uS/cm) gained an average of 0.225g (± 0.396, 0.270), with an average of 22.80 crickets consumed (± 1.735, 23). The moderately high treatment (7500 uS/cm) had an average weight gain of 0.935g (± 1.058, 0.690), while an average of 21.80 crickets was consumed. The high treatment (9843 uS/cm) had an average weight gain of 0.747g (± 0.877, 0.765), and an average consumption of 18.15 crickets (± 5.204, 20).

Table 2.1: Descriptive statistics for soil salinity exposure consumption rates ($F_{4,95}$=11.6, p=0.004), and mass differences ($F_{4,95}$=5.16, p = <.001) per treatment.

	Consumption				Mass (g)		
Treatments	*n*	μ	*s*	Md	μ	*s*	Md
Control	20	23.20	1.281	24	0.184	0.433	0.270
Low	20	23.00	1.026	23	0.123	0.660	0.220
Moderate	20	22.80	1.735	23	0.225	0.396	0.270
Moderately high	20	21.80	2.331	22	0.935	1.058	0.690
High	20	18.15	5.204	20	0.747	0.877	0.765

All treatments experienced mass increases as they were young of the year, however, salinity levels below 2600 did not affect weight gain (Table 2.2). The low treatment (p = 0.755)

and the moderate treatment (p = 0.592) did not have a significant weight increase and were not statistically different from the control. However, salinity appeared to have a significant effect on an individual's mass (Table 2.2: $F_{4,95}$= 5.16, p = <.001) above 7500 uS/cm. The moderately high treatment had a significant increase of 0.751g in weight compared to the control (p = 0.012), a 0.812g increase compared to the low treatment (p = 0.013), and a 0.710g increase compared to the moderate treatment (p = 0.013). The high treatment also had a significant increase of 0.563g compared to the control (p = 0.023), increase of 0.624g compared to T (p = 0.023), and an increase of 0.522g compared to the moderate treatment (p = .023). The moderately high and the high treatment did not significantly differ (p = 0.731) from each other. Despite the removal of any outliers, a significant difference in mass existed in the high salinity treatments ($F_{4,89}$ = 4.49, p = 0.002; Table 2.2).

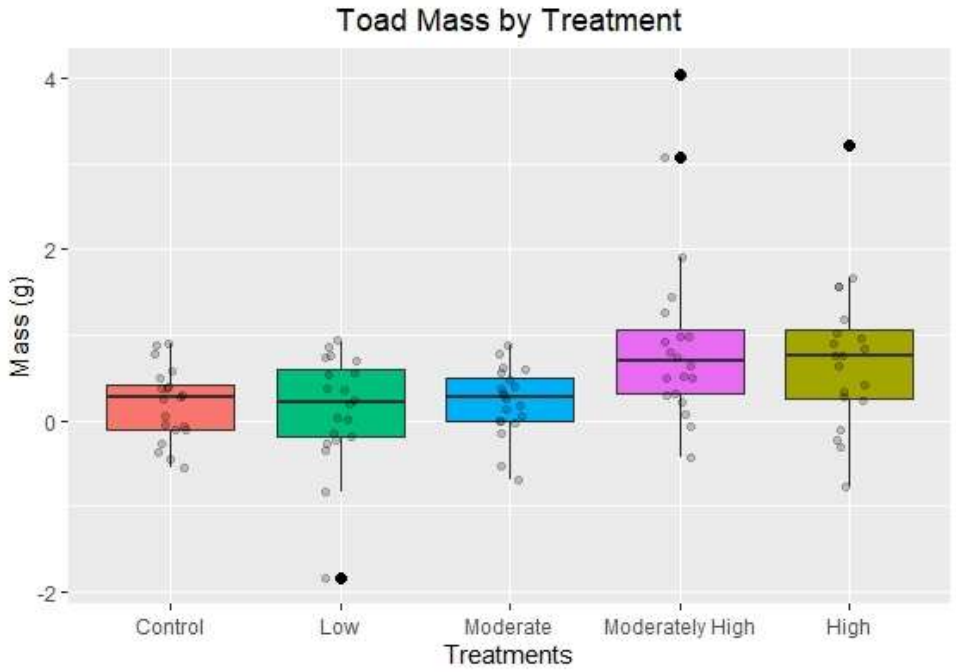

Figure 2.1: Box plot displaying median and IQR mass differences ($F_{4,95}$=5.16, p=.00004) between treatments.

Throughout the treatments, as the salinity levels increased, consumption decreased causing a significant difference among treatments (Figure 2.2: $F_{4,95}$=11.6, p = 0.004). In comparison to the control, the low treatment (p = 0.755) and the moderate treatment (p = 0.592) did not display a significantly different consumption rate. However, the high treatment averaged eating 5 less crickets (p = 0.001) than the control, 4.85 less crickets than the low treatment (p = 0.001), 4.65 less crickets than the moderate treatment (p = 0.001), and 3.65 less crickets than the moderately high (p = 0.009). The moderately high treatment was not significantly different when compared to the moderate treatment (p = 0.207) only consuming 1 less cricket. However, borderline significance existed in comparison to the control (p = 0.055) with an average consumption of 1.4 fewer crickets and 1.2 fewer crickets than the low treatment (p = 0.088). Salinity did not influence consumption rates below 9300 uS/cm, but significantly decreased consumption above that level ($F_{4,95}$= 11.6, p = 0.004). When removing outliers, an effect on consumption remained ($F_{4,89}$ = 14.0, p = 0.004).

Table 2.2: Permutation t-test (10,000) pairwise comparison analysis with a Benjamini-Hochberg correction applied for soil salinity exposure consumption rates (Left: $F_{4,95}$=11.6, p = 0.004), and mass differences (Right: $F_{4,95}$=5.16, p =<.001).

	Consumption				Mass			
	Control	Low	Moderate	Moderately high	Control	Low	Moderate	Moderately high
Low	0.755				0.756			
Moderate	0.592	0.755			0.756	0.731		
Moderately High	*0.055*	*0.088*	0.207		**0.012**	**0.015**	**0.013**	
High	**0.001**	**0.001**	**0.001**	**0.009**	**0.023**	**0.023**	**0.027**	0.731

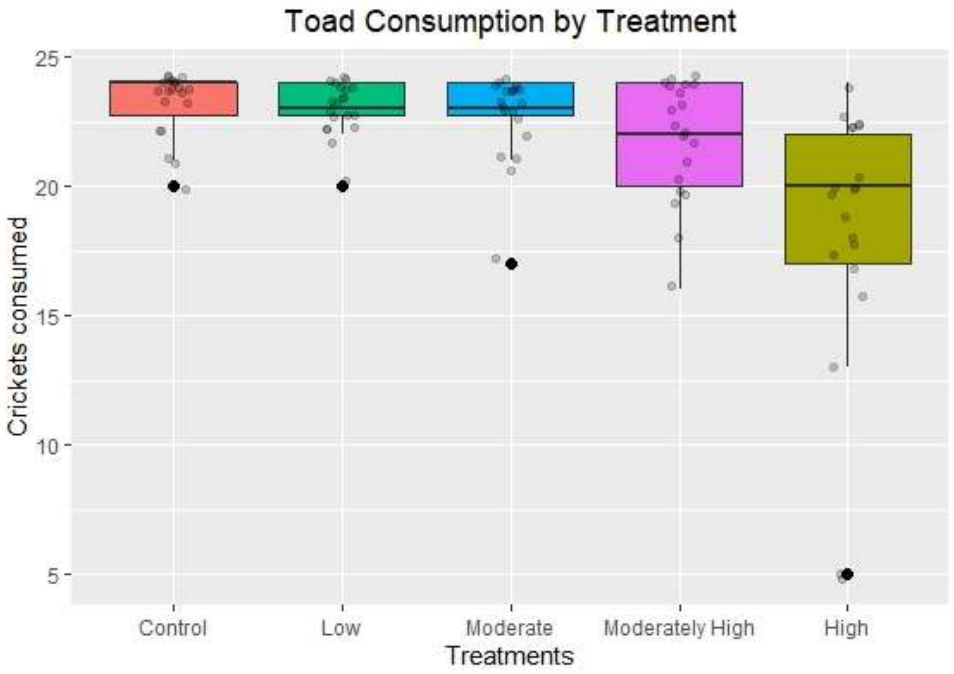

Figure 2.2: Box plot displaying median and IQR consumption rate differences ($F_{4,95}$=11.6, p =.004) between treatments.

2.3.2. Choice Tests

Given that the choice was completely random, and an expected observation for an individual would have been equal for both surfaces, a hypothesized mean of 8 was used to determine significance. However, a Wilcoxon Signed-Ranks Test indicated no treatments significantly deviated from the hypothesized mean. The time spent on the treatment side did not significantly differ from the time spent on the control side (Table 2.3). After running a Wilcoxon Signed-Rank test, the output for the low treatment was Z = -1.85 with a p = 0.101. The moderate treatment was Z = -0.567 with a p = 0.586, while the moderately high was Z = -1.59, with a p = 0.152. The high treatment had a Z = -1.01 with a p = 0.344.

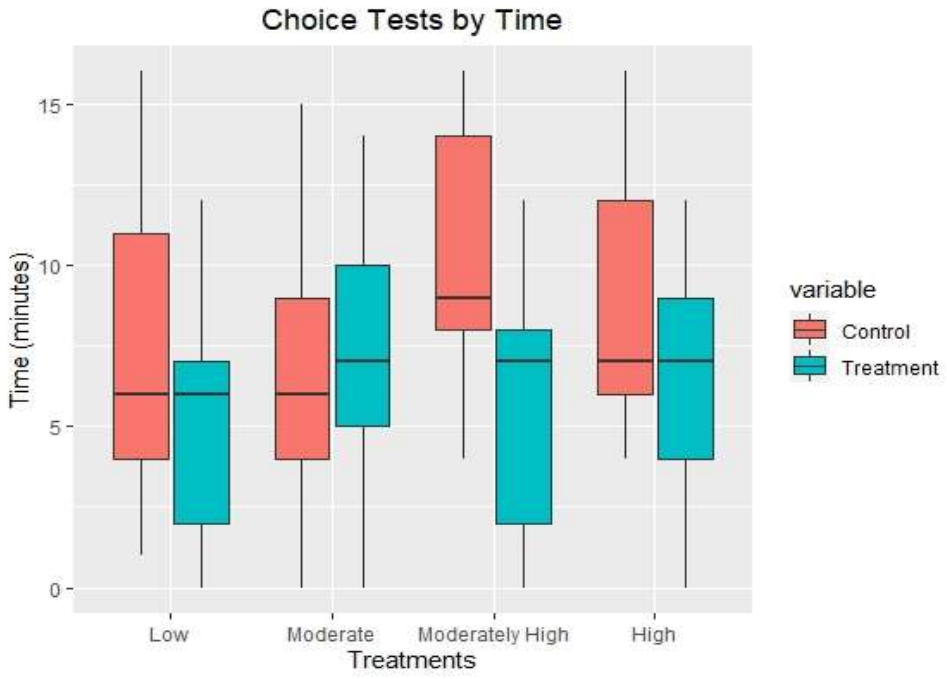

Figure 2.3: Box plots displaying the median and IQR of the time spent on different surfaces between treatments.

Table 2.3: Descriptive statistics for the time spent on the control or treatment surface between treatments.

		Control			Treatment			
	n	μ	*s*	Med	μ	*s*	Med	Rank
Low	9	7.9	5.5	6	5.2	4.5	6	-13.5
Moderate	8	6.6	4.7	6	7.1	4.7	7	-4.5
Moderately high	9	10	4.2	9	5.8	4.2	7	-11.5
High	9	8.7	4.2	7	6.6	4.3	7	-7.5

2.4. Discussion

Sodium chloride effects have primarily been investigated on larval amphibian life stages; while post-metamorphic terrestrial amphibians have been largely neglected. These results provide preliminary insight into the toxic effects of saline substrates on post-metamorphic Canadian toads. The experimental results indicate that high salinity levels in soil decrease appetite and increases

water retention while highlighting that individuals appear not to discriminate between non-saline or saline surfaces.

Although the choice test results were not significantly different, the higher salinity treatments averaged more time spent on the control than they did the treatment sides. This could potentially be indicative of a pattern had the sample sizes been more robust. Nonetheless, the inability to distinguish between saline and non-saline surfaces could have concerning consequences for juveniles if paired with saline soil exposure. Saline soil exposure decreased consumption and increased weight gain, suggesting that the higher saline treatments elicited limiting food consumption and water retention stress responses. Extended exposure to saline soil above 7500 uS/cm likely provoked a response within the toads to retain fluids, causing a weight gain. This weight gain coupled with a decrease in consumption with conductivity readings above 7500 uS/cm, could potentially cause detrimental effects that could lead to a reduction in overall fitness, and survival.

With their semipermeable skin and need for moisture, amphibians can retain fluids to ensure survival. Fluid retention is likely to occur when the normal hydration of the animal is insufficient and the soil water potential is lower than the individual's bodily fluids, to avoid water loss (Cirne et al., 1981; McClanahan, 1972). While the soil in this research remained moist throughout, and food was regularly provided, their stress response mirrored that of experiencing drought-like conditions.

The urinary bladder within an amphibian plays an integral role in salt and water homeostasis, acting as a reservoir of diluted fluids that can be utilized under freshwater limited conditions (Macknight et al., 1980). The storage and fluid retention capabilities of a toad's bladder can account for as much as 30% of its gross body weight, under drought-like conditions (Ruibal,

1972). Previous research has shown that under conditions where freshwater resources are restricted, *Bufo viridis* burrowed, retained urea, and significantly increased plasma osmolality (Katz & Gabbay, 1986). As dehydration progresses, the bladder fluids are reabsorbed, the concentration of urine becomes isotonic to the rest of the body, and the toad is unable to retain moisture/hydration levels (Ruibal, 1962). The time interval in which dehydration occurs is highly dependent on an individual's, environmental variables, and food availability. Fluid retention has been observed in a multitude of anuran species under drought-like conditions (Cirne et al., 1981; McClanahan, 1972; Katz & Gabbay, 1986). While urea or plasma concentrations were not measured in this research, the large increase of mass is likely due to retention of fluids in order to maintain homeostasis under the hypersaline conditions.

If future research were to continue, adult specimens would be collected and salinity exposure periods would be extended for soil and choice experiments. Additionally, lymph and urea concentrations would be monitored and analyzed during the saline soil exposure trials. Previous surface choice tests conducted with chemically intense agriculturally based contaminates have supported that the inability to distinguish between contaminated surfaces persisted, regardless of maturity (Storrs Méndez et al., 2009; Hatch et al., 2001; Jones, 2018). However, it is still suggested to continue salinity surface research with an increased sample size, adult specimens, and longer time intervals in order to see if these results remain unchanged. Variables such as age, pelvic patch development, and the short time interval, could have contributed to the inability to distinguish between saline and non-saline surfaces.

Post-metamorphic anurans spend a vast majority of their life in terrestrial environments. Typically, aquatic environments are utilized for breeding events only. Therefore, moisture absorption, dehydration avoidance, hibernation, and shelter are achieved through burrowing in soil

(Hoffman & Katz, 1989). As the results of this research suggest, the inability to distinguish between saline surfaces could be problematic in selecting beneficial hibernation locations. This result coupled with the potential dehydration risks from saline soil could lead to failed spring reemergence, as saline enriched soils become more prevalent. While saline soils are quickly becoming common occurrences throughout the world, especially in the northern mid-western region of the US, the probability of extended saline soil contact for amphibians is expanding.

Anthropogenic factors are undoubtedly contributing to amphibian declines throughout various locations of the world. While habitat loss is currently a major factor increasing amphibian declines, saline soil compounds the issue, rendering habitats unlivable. Saline soil eliciting dehydration responses for amphibians while concurrently amplifying habitat loss, could cause deleterious effects throughout population and community levels. As salt loading incessantly expands through anthropogenic means, continued salinity research would be beneficial to strategically guide mitigation efforts and management plans for the future of anuran populations. Salt as a contaminate has limited research on post-metamorphic terrestrial amphibians, and further research is suggested.

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