

Effects of salinity on amphibian disease outcomes

**By
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May, 2025**



**Allegheny College
Department of Biology**

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**A Senior Comprehensive Project in Partial Fulfillment of the Requirements
for a Bachelor of Science Degree from Allegheny College**

*I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code
and to maintain the integrity of both myself and the College community as a whole.*

Mitchell Dugan
Pledge

Approval by the Senior Thesis Committee:

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Dr. Becky Dawson

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Abstract

One important contaminant that is affecting freshwater ecosystems and the wildlife living in them is salt used to deice roads. Salt can act as an energetic stressor on amphibians, as they could struggle to osmoregulate themselves and maintain ion balance. Amphibian kidneys are adapted to conserve ions by producing diluted urine. Increases in ions can be taxing as amphibians try to rid themselves of excess ions. Salinity can also trigger a stress response which typically helps amphibians cope with the stimuli. But when the stress response is active for too long, it can be harmful to individuals, seen in a decrease in immune function, which can cause animals to be more susceptible to disease. It is unclear whether salt is acting as a stressor and harming amphibian disease outcomes, or if salt is having positive effects on disease outcomes. To investigate this, *Xenopus laevis* adult frogs were exposed to the fungal pathogen *Batrachochytrium dendrobatidis* (Bd) and housed in three possible salt treatments: no salt (0 mOsm/L NaCl), low salt (235 mOsm/L NaCl), and high salt (340 mOsm/L NaCl). It was hypothesized that frogs in the highest salinity treatment would have the highest Bd load and worst outcomes from disease. qPCR results revealed that no frogs were infected with Bd. Based on these results, my hypothesis could no longer be investigated, but instead I looked into how salinity and Bd exposure affected the survival of frogs. Salinity and Bd exposure both had statistically significant effects on frog survival; however, no interaction between salt and Bd on frog survival was observed. Frogs in the highest salinity group had a 0% survival, while frogs in the other two groups both had 100% survival. My conclusion based on my results was that sudden exposure to high salt could cause mortality, in contrast with the same level being survivable after gradual acclimation over time. The Bd exposed group had a 68% survival rate while the non exposed group had a 63.2% survival rate. Further studies are needed to understand

the implications of my findings regarding salt and mortality, as well as to form a connection between these effects of salinity and amphibian disease outcomes.

INTRODUCTION

Human activities are affecting and shaping ecosystems in many ways. Some harmful activities include inappropriate dumping of waste materials, excessive burning of fossil fuels, and destruction of natural habitats. The effects of these activities can include the continuing progression of climate change and global warming. Global warming and climate change have largely been driven by the burning of fossil fuels and in all have led to a 1.4°F increase in the earth's temperature since 1880, as well as 3.2mm per year rises in sea level (Sivaramanan, 2015). The effects of human activities are seen on smaller scales as well. Specifically, aquatic ecosystems are frequently disrupted due to human interference. Freshwater ecosystems have been contaminated by a mixture of chemicals as a byproduct of oil extraction, these chemicals include acids and friction reducers (Robert et al., 2018). In Pakistan, heavy metals such as lead and mercury are entering water sources from the wastes of urban and agricultural areas (Afzaal et al., 2022). The world's oceans are also becoming increasingly more acidic due to influxes of CO₂ from the burning of fossil fuels (Guinotte & Fabry, 2008). All of these contaminants are widely known to cause harm to ecosystems. Studying the effects of human activities and their contamination of aquatic ecosystems is an important topic that can impact the conservation and protection of aquatics worldwide.

A specific contaminant that could affect aquatic ecosystems is salt. Road salt that is used in the winter to reduce icing on roads, can end up running off into freshwater communities. Through the years, road salt use has increased to almost 25 million tons in the United States

alone(Solomon et al., 2023). This rise in use has been linked to an increased chloride concentration in freshwater ecosystems (Solomon et al., 2023). This suggests that road salt increases are causing increases in the salinity of the surrounding water. This is also supported in a previous study, which found a direct correlation between the amount of road salt purchased and used on roads by the state of Minnesota to the salinity of surrounding freshwater ponds (Novotny et al., 2008). This further supports the idea that human activities are associated with increases in salinity of freshwater sources. Roadside salt entering freshwater ecosystems was found to cause a decrease in biodiversity of plants and animals (Szklairek et al., 2022). This idea of a loss of biodiversity is evidence that animals are impacted by salinity and could even suggest salt is contributing to their death. In Nova Scotia, salinity increases have been shown to have negative impacts on nearby ecosystems, with some species experiencing toxic levels of salt and in turn dying from exposure (Collins & Russell, 2009). Salinity is having a large effect on ecosystems in the fact that it is decreasing biodiversity and leading to deaths and this makes studying it so important. Understanding exactly what increasing salinity is causing can help in prevention of salinity rises as well as conservation efforts. This contamination of water sources is often unintended, but also unaccounted for as a consequence of human activity.

Freshwater aquatic vertebrate physiology provides some processes to adjust to and to tolerate salt in their water. In a typical freshwater community, the water has a lower concentration of ions than that of within organisms bodies and blood. Due to this concentration gradient, the organisms need to frequently rid themselves of excess water through a process called osmoregulation in which organisms excrete dilute waste fluids via the renal or kidney system (Griffith, 2016). The role of the kidneys in this process is to both excrete the dilute waste fluids and maintain salt concentrations by actively transporting ions against concentration

gradients (Griffith, 2016). While this process of ion uptake and reabsorption tries to minimize energy usage, changes in external salt concentration can cause an increase in energy expenditure as organisms try to cope with this stress (Griffith, 2016). In order to suit the above needs, among others, the amphibian kidney has specific structures. The kidney of *Xenopus* for example has many structures similar to that of mammals, such as nephrons which are responsible for filtering the water and ions from the bloodstream to be excreted in the urine (Lienkamp, 2016). Mammals' nephrons however have structures that help them to conserve water such as a long loop of Henle to make more concentrated urine in order to conserve more water for the body (Yu, 2016). However the amphibian kidney, specifically those that are aquatic, tends to have a very short loop of Henle in order to conserve ions, this produces less concentrated urine (Rhodes, 2016). This shorter loop of Henle is adapted for use in freshwater with low concentrations of ions. If the concentration of ions rises in the freshwater, it can be energetically taxing for amphibians to produce more concentrated urine to excrete excess ions. Though some aquatic vertebrates are able to handle changes in salt levels and ion concentrations, others may succumb to its effects. *Monopterus albus* or Asian swamp eels have been found to have their osmoregulation disrupted in waters with high salt concentrations, this caused higher rates of mortality in the organism (Pedersen et al., 2014). Osmoregulation is a constant process for most aquatic vertebrates. Extreme changes in environmental ion concentrations can cause a stress response or even death in the organisms.

On top of ion concentration changes, the stress response can also be triggered by changes in temperature, water pH, or food insecurity. Essentially any conditions that could be less than ideal for an organism, will trigger a stress response to help an organism cope with the conditions. The effects changes of salt have on aquatic organisms have been studied in great detail, with

organisms consistently being found to have entered a stress response as a result of salinity changes. A main part of the stress response that is being triggered in aquatic organisms are glucocorticoids which are hormones that they release, one of which is corticosterone (CORT). CORT and other glucocorticoids are released by the hypothalamic-pituitary-adrenal axis and act to regulate energy balance, growth, and maintaining homeostasis (Tornabene et al., 2024). When an organism faces minor disturbances that trigger a stress response CORT works to redirect energy usage, mediate energetic trade offs, and alter behaviors all in an effort to increase an organism's fitness in response to the disturbance (Tornabene et al., 2024). In all, CORT is acting to help organisms survive physical burdens in the short term. However over the long term, high CORT levels from the stress response lead to immune suppression in *Rhinella icterica* (de Assis et al., 2015). This prolonged stress response is often referred to as chronic stress, and can be harmful for amphibians as it is also energetically taxing. In developing amphibians, chronic stress has been shown to reduce size of the animals and cause altered behaviors such as increased food intake (Denver, 2009). The reduced size of the developing animals could be attributed to the increased energy need for the chronic stress response. Stress, especially chronic stress, can have serious effects on amphibians and can negatively impact whole populations.

Amphibians like most animals rely heavily on their immune system to protect themselves from pathogens of many kinds. Their immune system comprises multiple organs including the thymus, spleen, and liver, these organs develop immune cells such as B cells and T cells or lymphocytes (Ruiz & Robert, 2023). These lymphocytes produce antibodies to fight off pathogens and protect the body. An immune system can be affected by the stress response, the immune system's functions can be inhibited or decreased due to being in a stress response. An inhibited immune system as a result of the stress response, has been marked as having lower

numbers of lymphocytes to fight off infection (Raffel et al., 2006). This further supports that the stress response can hinder immune functions and leave organisms vulnerable to pathogens with decreased lymphocytes. Amphibian tadpoles exposed to stressors had lower levels of T cells and a decreased ability to combat infections (Robert et al., 2018). If amphibians are not forced into a stress response they may have better likelihood at surviving disease as their immune system may not be inhibited. Pathogens have already shown to cause global declines in amphibians.

Amphibian declines have largely been attributed to habitat loss, but increasing disease spread is also a huge factor that is leading to huge die offs of amphibians (Brown et al., 2013). This is just showing how important maintaining proper immune function is to individuals, but also populations as a whole. Since immune function is vital to amphibian survival, reducing unnatural stressors can help to protect populations.

Deicing salt could cause a stress response and affect the immune system of freshwater amphibians, which is such a concern because a hindered immune system can leave amphibians susceptible to pathogens. The decrease in immune response can happen in a variety of ways, each playing a part in how likely an amphibian is to succumb to disease. *Lithobates sylvaticus* larvae raised in higher salinity environments had suppressed immune function and were more likely to die of ranavirus infection, this was attributed to the salinity levels acting as a stressor and increasing glucocorticoid levels (Hall et al., 2020). This is in support of salinity levels acting as stressors on amphibians, this also supports that amphibians under stress are more susceptible to disease. Salinity from road salt has also been shown to leave *Lithobates sylvaticus* and *Lithobates pipiens* larvae more susceptible to parasitism due to decreased levels of white blood cells (Milotic et al., 2017). This also supports the idea that salinity is decreasing immune function, this in turn can cause harmful effects in amphibians and their outcomes from pathogen

exposure. This is so important because salinity combined with pathogens is leading to die offs of entire populations and throwing off the balance of some freshwater communities.

Many pathogens are harming and killing populations of amphibians. One that is massively important is *Batrachochytrium dendrobatidis* or Bd. Bd is a fungal pathogen that is causing amphibian species declines and extinctions across the globe, the pathogen has contributed to one third of amphibian species being considered as imperiled or in jeopardy (Olsen et al., 2013). Bd affects the epidermal cells of amphibians and causes the deadly disease of chytridiomycosis (Fisher et al., 2009). Bd infects the skin cells of amphibians which contain keratin, the infection causes a disruption in the skin's normal function and can lead to difficulties with osmoregulation, which in turn leaves the animal with imbalances of water, oxygen, and electrolytes such as sodium and can lead to death (Berger et al., 2005). Bd has become an alarming problem for ecosystems across the globe. This makes studying this pathogen important, to aid and improve conservation efforts and begin to protect amphibians. Something that is unclear from previous studies is how road deicing salt affects the Bd outcomes of amphibians. Some studies have found salt to improve outcomes, for example, one study found that *L. aurea* exposed to elevated salinity had lower levels of Bd transmission in a community, and they found that animals that did become infected had lower mortality rates (Clulow et al., 2018). This is in support of salinity improving Bd outcomes. This can be attributed to amphibians being more tolerant to the salt levels than Bd. However, other research has found salinity to act as a stressor. To investigate this phenomena, *Xenopus laevis* or African clawed frogs will be used as the study organism as they are available and widely used. *Xenopus laevis* are relatively resistant to Bd infection and its symptoms (Rollins-Smith et al., 2009). Since the frogs may be more resistant to Bd than other species, it will be easier to discern whether the salinity stress causes more negative

Bd outcomes. Understanding how salinity stress affects Bd outcomes could help in conservation efforts of amphibians afflicted with this pathogen.

Objectives

Based on how salt used to deice roads, is getting into freshwater ponds and causing frogs to be energetically stressed at higher levels, the objective of this study was to determine how different levels of salt affect disease outcomes in *X.laevis* exposed to *Batrachochytrium dendrobatidis* or Bd. Based on this objective, I hypothesized that frogs in higher salinity environments would be found to have higher infection loads and worse outcomes from infection. Specifically I predicted that frogs exposed to Bd and high salinity would have the lowest survival rates and would have the highest infection load compared to any other groups. I also predicted that exposed frogs in low or no salinity would have lower infection loads.

Methods

Animal purchase and basic husbandry

Xenopus laevis were purchased from Xenopus.com, each with an approximate size of 2.54cm measured from snout to vent. A total population of 60 frogs was purchased, with an additional 10 sent in case of mortality during shipping. Animals were housed individually, in polystyrene containers with the dimensions 19.3675cm L x 16.51cm W x 11.43cm H. The containers were filled with approximately 600mL of dechlorinated water. The animals were stored in an environmental chamber held at 16° C +/-0.5° C. The individual containers had a loose plastic grate on top to limit tank jump outs and deaths. Animals were fed Xenopus express™ Premium Sinking Frog Food 0.16cm, twice per week. Initially, animals were fed 4

pellets and increased to approximately 8 pellets beginning on day four of having the frogs. Water changes occurred twice per week, in which approximately three quarters of the current water volume was dumped out and replaced with fresh dechlorinated water to fill the containers back to the original volume. The water changes occurred the day after each feeding day.

Bd exposure

Frogs were randomly chosen to either receive Bd exposure or receive a mock exposure without any Bd. Bd zoospores were collected from an available culture. Bd zoospores on an agar plate were flooded with freshwater to make the Bd solution. The same process was followed to create the Bd- solution, however these plates were not filled with Bd. 25 frogs were exposed to Bd and 19 frogs received the sham exposure. The exposure procedure was adding 3mL of either the Bd solution or Bd- tryptone water sham solution to the current water of each individual. 1mL of the Bd solution had approximately 2×10^6 zoospores, meaning that each Bd exposed frog was exposed to approximately 6×10^6 zoospores. The number of zoospores used was higher than a previous experiments at 10^4 zoospores, in an effort to ensure infection occurs (Boyle et al. 2007). Feeding and water change schedule continued as normal, as previous works found that 20 hours of Bd inoculation was enough (Boyle et al. 2007). However, now that the water was exposed to Bd, any old water removed from frog containers during water changes had bleach added and at least 10 minutes passed before disposal in order to kill the Bd. This was in an effort to prevent accidental infections from water contamination in the disposal area. The current experiment held the frogs in the Bd inoculation for approximately 3 days before the next water change, again in an effort to ensure infection would occur. The day of Bd exposure and sham exposure was treated as day 1 of the experiment as the treatment began in the morning and the

day was counted as a full day. This also began the count of days alive for the frogs, again with this marking day 1 of being alive.

Salinity treatment

On day 13, the Bd exposed and mock exposed groups were split further into three approximately equal groups. These groups would be used for the three different salinity treatments, ensuring each group was random and contained approximately equal numbers of both Bd exposed and mock exposed individuals respectively. The salinity treatments were created by adding NaCl to dechlorinated water. The salinity treatments were 0 mOsm/L NaCl for no salt, 235 mOsm/L NaCl for some salt, and 340 mOsm/L NaCl for high salt level (Pena-Villalobos et al. 2016). The frogs were left in their salinity treatments for an additional 12 days. The levels of salt remained constant by performing water changes with the correct level of salt for the remainder of the experiment.

Death and euthanasia

Any animal that died before the beginning of experimental manipulations was discarded via a biohazardous waste bin. In the event of death after the commencement of the experiment, frogs were placed into small plastic screw topped vials and frozen, to be examined and have data collected at the conclusion of the experiment. At the conclusion of this experiment the animals needed to be euthanized as they cannot be entered into the wild after being exposed to a contagious pathogen. On day 24 which was the conclusion of the experiment, all remaining frogs were euthanized. The frogs were euthanized with an overdose of MS-222 (Webb et al., 2005). This anesthetic was used as it is the only known anesthetic that does not affect the viability of Bd

DNA. The individuals were held in the same containers from the rest of the experiment and had a small amount of MS-222 added to their water and then were swirled around. After waiting for a 15 minute period they were examined for any signs of life via the visual examination of the heart. The individuals were examined by both the naked eye and through a microscope to ensure death had occurred. If signs of life persisted, more MS-222 was added to the water. When frogs were deemed dead after examination, they were again entered into the plastic screw top vials and frozen to have data collected at a later date.

DNA extractions and Measurements of Bd infection

Individuals were removed from the freezer and thawed for at least 10 minutes. Individuals were swabbed 10 times from the hip to the tip of their toes, on each leg, using a cotton tipped swab. Gloves were changed after handling each frog to limit cross contamination between individuals. The tips of the swabs were stored in 1.5mL microcentrifuge tubes and frozen until the date of DNA extraction. Swab vials had 40 μ L of Prepman Ultra added, and were heated at 100° C for 10 minutes and then centrifuged for 3 minutes at 13,000 rpm. The vials were then micropipette from, for as much liquid as possible, to be added to a snap top vial of pure DNA. The DNA vials then had 2 μ L of liquid extracted and added into another snap top vial filled with 18 μ L of sterile, ultrapure reagent water, to create a dilution to be used for quantitative polymerase chain reaction or qPCR. The samples were finally centrifuged briefly and frozen for qPCR. DNA extractions were analyzed using real time TaqMan qPCR assays using standard protocols from previous works (Boyle et al., 2007). The qPCR was carried out using an Applied Biosystems Step One Real Time PCR system (Applied Biosystems, Foster City, CA USA). Initially, qPCR was set to be run on all Bd exposed individuals, as well as randomly selected

mock exposed individuals. After all Bd exposed individuals were analyzed, it was determined that there was no need for analysis of mock exposed individuals because all Bd exposed individuals were negative for Bd DNA at the time of analysis.

Statistical Analysis

None of my Bd-exposed frogs tested positive for Bd. As such, I am unable to directly assess my hypotheses related to the effects of salt on metrics related to Bd infection. However, because ~36% of my frogs died throughout the course of the experiment (and because there was variability in the number of days that they survived), I was able to test whether my experimental manipulations affected frog survival. For my statistical test, I used a cox proportional hazards model, a common statistical model to analyze survival data. Unlike using a binomial statistical model to test whether experimental manipulations affect the probability of mortality (a binomial event) occurring, this statistical model tests whether, and how, experimental treatments affect the time that it takes for mortality to occur. As such, I tested for the main, and interactive effects, of the categorical variable "Bd exposure" (exposed, not exposed) and the continuous variable "salt" (0 mOsm, 235 mOsm, or 340 mOsm) on the number of days frogs survived.

Results

As stated above, after completing qPCR, none of the animals were found to be infected with Bd at the conclusion of the experiment. The qPCR results indicated that all methods were proper and the plates were run correctly, a standard curve made from the plate was shown and indicated the plate results were valid (Figure 1).

Figure 1.

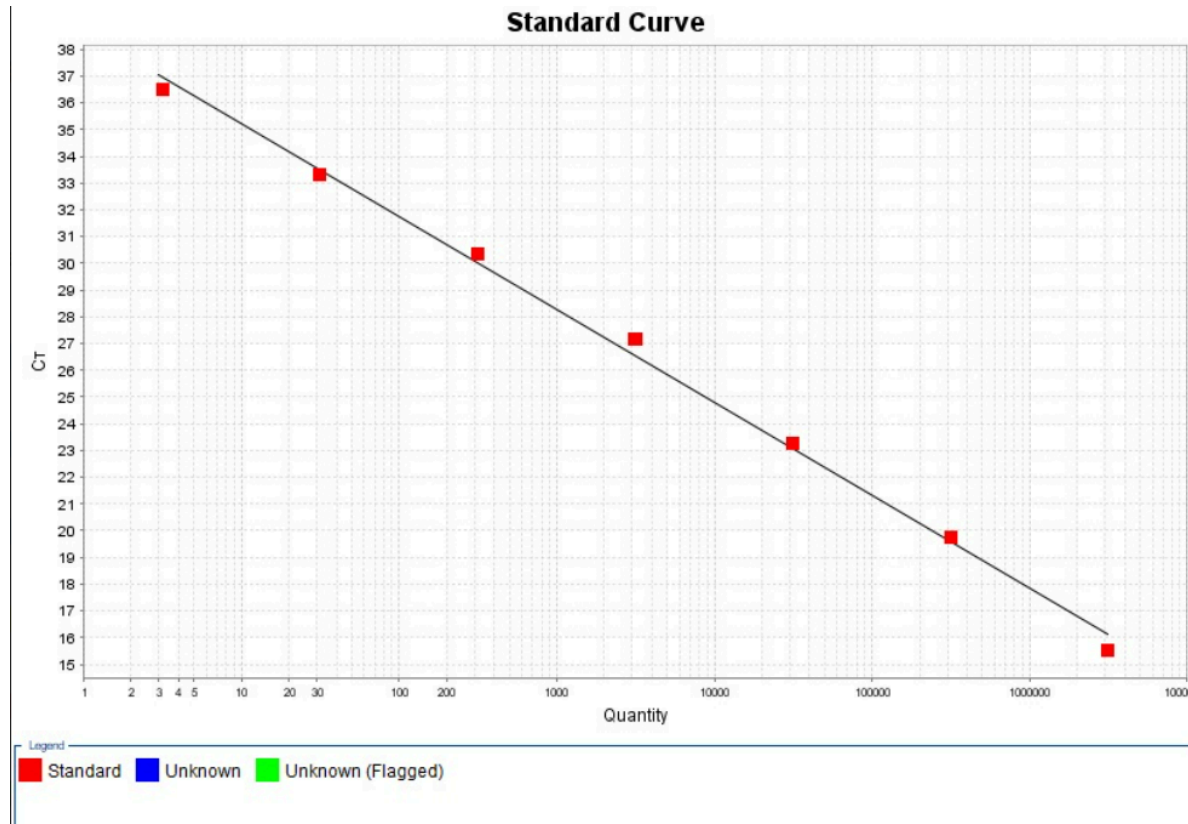


Figure 1. Standard curve created from results of qPCR. The plate was run with samples from two different projects, both looking for *Batrachochytrium dendrobatidis* or Bd infection and infection intensities. The x-axis indicates the quantity of Bd DNA that was detected, and the y-axis represents the threshold cycles or Ct values which are single data points from the qPCR results. The Ct values essentially indicate how many cycles it took the machine to replicate a traceable amount of DNA, so the lower the Ct value, the more DNA was in the sample. This standard curve from the plate run indicated that proper techniques were followed and accurate results were obtained. With this information, we are confident that no frogs were infected with Bd at the conclusion of the experiment.

Due to the results obtained from qPCR that no frogs were infected with Bd at the time of death, the results must shift focus. Instead the data used to quantify what occurred during the experiment was the number of days alive. The factors investigated as affecting the number of days alive for frogs were salt concentrations, Bd exposure, and an interaction between salt and Bd exposure.

The three salinity groups had total survival calculated regardless of Bd exposure status, as well as the number of days alive for each individual, with day 1 being the day of Bd exposure and day 24 being the maximum number of days alive as euthanasia occurred on this day. The salt groups of no salt and 235mOsm or low salt both had 100% survival rates to day 24 (Fig.2). It is worth noting that one animal in the 235mOsm group died on day 24, due to this being the day of euthanasia the individual was still counted as living for 24 days despite dying before euthanasia. The 340mOsm population had a total survival of 0% by the conclusion of the experiment (Fig.2). Of the 15 individuals in this group, 9 died by day 14 or one day after salt treatments began, bringing the percent survival of this group on day 14 to 40%. An additional two frogs died on day 15 and population survival was brought down to 26.66%. An additional frog died on day 17 bringing survival to 20%, and on day 18, the remaining three frogs in this treatment died, leaving the group with a 0% survival rate. So within 5 days of salt exposure, all frogs in the high salt group had died. Salt was found to have a statistically significant effect on days alive ($F=59.745$, $p<0.0001$).

Figure 2.

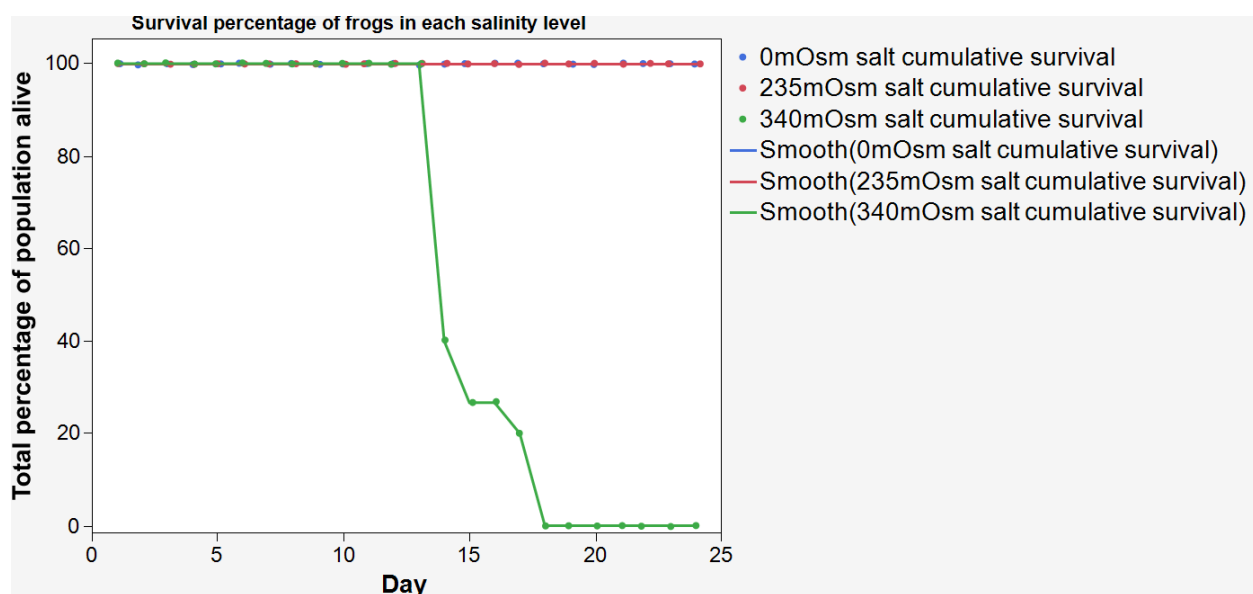


Figure 2. A scatter plot of data with trend lines to better see trends in the data. The graph represents the total survival percentage of frogs in each salinity group. The x-axis is the day of the experiment, which also represents the days alive of the frogs in the groups. The y-axis represents the total percentage of the population that is still alive on each day with the dots representing the exact percentage on each day. The no salt or 0mOsm group is represented by the blue dots and blue line. The low salt or 235mOsm group is represented by the red dots and red lines. The high salt or 340mOsm group is represented by the green dots and green lines. Both the no salt and low salt groups remained at 100% survival for the duration of the experiment. The high salt groups saw declines beginning on day 14 or one day after salt treatment began, and the entire group died and reached a 0% survival at day 18. Again salt had a statistically significant effect on the amount of days alive for the frogs and the high salt group saw a complete die off before the conclusion of the experiment.

Bd exposed and sham exposed groups had a total survival percentage calculated regardless of the salt concentration of each individual, as well as the total number of days alive for each individual. Again with day 1 being the day of Bd exposure and day 24 being the conclusion of the experiment. The Bd exposed group had deaths beginning on day 14, with 2 frogs dying on this day, of the 25 total in this group, and survival dropped to 92% (Fig.3). On day 15, two additional frogs died and survival was reduced to 84%. An additional one frog died on day 17 bringing survival to 80%. The last deaths of this group occurred on day 18 with 3 frog deaths and survival percentage dropped to 68% for the remainder of the experiment. The sham exposed group had deaths also beginning on day 14, in which 7 of 19 total frogs died, bringing survival percentage to 63.2% (Fig.3). The survival percentage would remain at 63.2% for the remainder of the experiment as no additional frogs died. Bd exposure was determined to be statistically significant and had an impact on the number of days frogs lived ($F=5.99$, $p=0.01438$).

Figure 3.

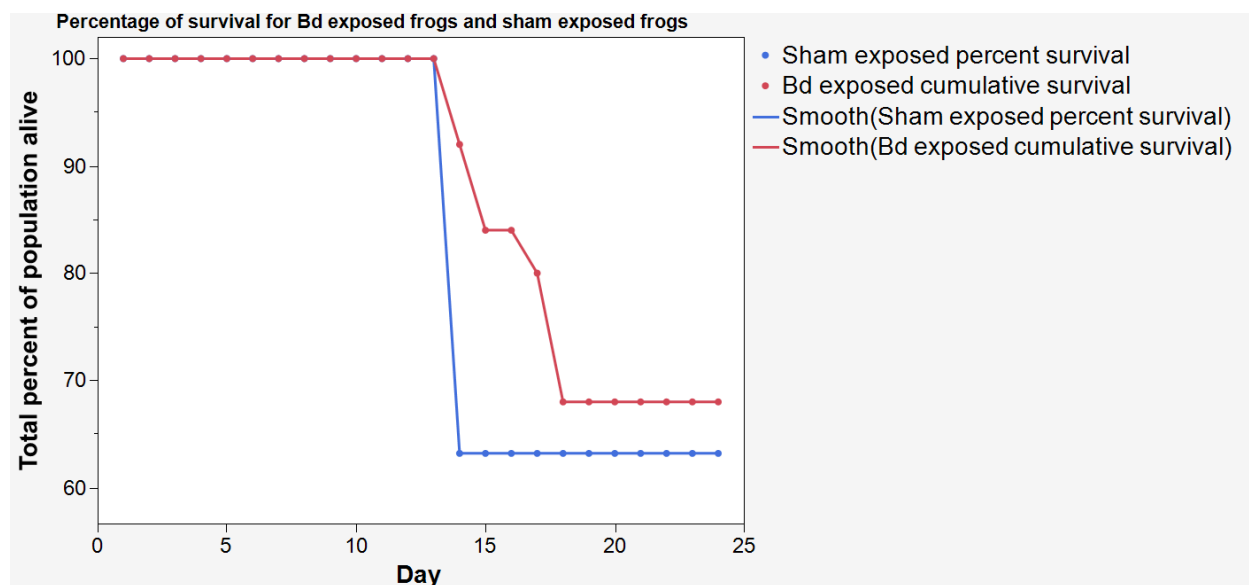


Figure 3. A scatter plot with trend lines to better see trends in the data. The plot represents the total survival percentage of frogs either exposed to Bd or the sham exposure. The x-axis represents the day of the experiment and also represents the number of days alive for frogs. The y-axis represents the total percentage of the population that is still alive, with dots representing the exact percentage of the population alive on any given day. The red line and red dots represent the Bd exposed group, while the blue line and blue dots represent the sham exposed group. A trend of the sham exposed or blue group was that it saw a large die off on day 14, but no further deaths. The Bd exposed or red group had more gradual die offs across more days, and eventually ended up with a higher percent of the population surviving than the sham exposed group.

Both salt levels and Bd exposure individually had statistically significant effects on survival. However, upon testing for the interaction between salt and Bd exposure on survival, it was found that they were not statistically significant ($F=3.259$, $p=0.19601$). This means that there was no statistically significant interaction between salt levels and Bd exposure together on survival.

Discussion

Understanding how human contaminants can affect the balance of ecosystems is an important area of study, which can help us to better protect the organisms present in our

ecosystems. Specifically, one contaminant that is often overlooked is salt used to deice roads. Salt is particularly interesting to look at because its effects both generally on an organism as well as its effects on organisms with disease, are lacking consensus in the current literature. The current study had no *Xenopus laevis* test positive for Bd infection at the conclusion of the experiment. Due to this I could not investigate the original hypothesis which was that frogs in the highest salt treatment would have the highest infection load and worse outcomes from disease. However based on our results I could test a new metric, how salt treatment, as well as Bd exposure affects the days alive for individuals. An interaction between salt and Bd exposure can also be tested on its effect of survival for frogs.

In the current study I found salt to have a significant effect on survival of frogs. Interestingly, the salinity amounts used in a previous study, which were tolerated by the *Xenopus laevis* (Pena-Villalobos et al., 2016), were lethal in this experiment. A possible explanation is that in this experiment, the frogs were immediately given the highest salt treatment, as opposed to increasing the salt over time to allow for better acclimation to the salinity change. One study found that acclimation to low levels of salt as juveniles increased the salinity tolerance later in life of amphibians (Venancio et al., 2019). This further supports the idea that the frogs in this study died from the high salinity level due to not being acclimated first at lower levels of salinity. Another study found osmotic shock, or a sudden influx of ions caused amphibian muscle cells to form vacuoles to hold excess salt, they also found that the muscle cells had a decreased ability to fire action potentials to move (Devlin et al., 2001). This is another possible reason death could have occurred in the study, as the frogs could have been unable to obtain food or surface if the salt caused decreased ability to move. Another study found that high enough salinity would also cause decreases in survival in tadpoles (Chinathamby et al., 2006). Possible implication of the

current results is that if a sudden influx of salt entered into a freshwater ecosystem, there could be an increase in mortality of both tadpoles and post metamorphosis frogs of multiple species. This result would support the prediction of high salinity frogs experiencing worse outcomes. Further experiments could be conducted investigating sudden changes in salinity versus gradual changes in salinity, to further form a conclusion regarding the effects of salinity on amphibian survival.

Of course when salinity is better understood it will be easier to study its effect on amphibian disease outcomes. One past study found that increased salinity decreased transmission of Bd between individuals and led to increased survival of individuals (Clulow et al., 2018). In order to investigate this, the present study could have incorporated a baseline qPCR run to ensure infection did occur after exposure. Then the study could look into any possible curative effects that the salt may have had. Due to no frogs in the present study being positive for Bd at the conclusion of the experiment, it is impossible to determine if they ever were infected at any point. Another study found that additional salt in the environment aids frogs in maintaining electrolyte balances after being infected with Bd, this in turn could increase their survival of Bd (Berger et al., 2010). Salt has some promise in limiting transmission of Bd and increasing survival. However, salinity could also act as a stressor and decrease survival of animals. Understanding this is important and finding a balance between possible curative effects, but also unintended harm from salt can transform conservation efforts for many species of amphibians (Stockwell et al., 2015).

Again with no Bd positive frogs after qPCR, I had to shift conclusions to simply how Bd exposure affects survival of amphibians. Bd exposure had a significant effect on frog survival. However, an interesting result is that a higher percentage of frogs given the sham exposure died

than the Bd exposed frogs. This could be attributed to the lower amount of sham exposed frogs in the highest salinity than the amount of Bd exposed frogs in the lowest salinity. The frogs were determined to truly be Bd negative because qPCR results were good, with a good standard curve and other samples on the same plate of another student coming back as positive (Fig.1). This indicates that the frogs were truly Bd negative at the conclusion of the experiment. The method for Bd exposure followed that of a similar paper that found a lower amount of zoospores used than the amount used in the current experiment caused infection (Boyle et al., 2007). It is unclear why the frogs may have been not infected, as *Xenopus laevis* are resistant to death from Bd, but are however still susceptible to infection with a high enough number of viable Bd zoospores (Rollins-Smith et al., 2009). The Bd zoospores were viable in this case as another student exposed salamanders to zoospores from the same colony and had infections in their case. To further investigate if it was the species that caused the lack of Bd infection, the current experiment could be replicated with a different species. Bd exposure and salinity were found to have a statistically non-significant interaction on the survival of frogs. This could be attributed to the lack of Bd positive individuals. Further studies are necessary to truly clarify the effect of salinity on Bd infection intensities and outcomes. Though this study can't investigate and add clarity to the question of whether salt is helping or hurting animals infected with Bd, it can however add to the idea that salt can cause detrimental effects and even death when concentration reaches a certain level, especially if it's a sudden influx of salt.

One metric not tested in this study was the weight of frogs to track their growth over time. This metric was not captured because *Xenopus laevis* are aquatic organisms. Due to them being aquatic, it is difficult to capture accurate weight data for individuals as excess water on them will affect the results. A previous study found that salt exposure can cause a growth

survival tradeoff in amphibians, which caused the animals to exhibit higher levels of CORT and had less growth, as well as higher mortality than amphibians in lower or no salt concentrations (Tornabene et al., 2021). This is in support of salinity affecting both growth of individuals and their survival. Salinity was also found to affect development in a variety of ways in *Anaxyrus terrestris*, these included reduced survival of tadpoles, earlier metamorphosis at smaller sizes, and sublethal effects which could impact the success of different life stages of frogs exposed later in development (Welch et al., 2019). These are more meaningful effects that can occur in amphibians due to salt exposure. If there is clarity from research on salinity having a curative effect on Bd infection, these other harmful downsides must be considered. Downsides of salinity include decreased growth, developmental problems, and even lethality in amphibians. The current study further supports the idea that sudden changes in salinity can cause quick deaths in populations. Other species including fish and plants could also be impacted by increased salinity. More research is needed to understand each possible downstream effect of increasing salinity. Based on these effects, it is important to be mindful of how much salt is used each winter to deice roads, as well as other sources of salinity which are increasing the salinity of freshwater ecosystems. Based on this, solutions must be implemented to decrease this runoff of salt, to limit negative impacts on populations. If salt is an effective treatment for Bd, studies can be performed to find a balance between helping individuals with Bd and limiting harmful effects to the ecosystem. A possible solution is removing Bd positive amphibians from the ecosystem and giving them a salt solution for a period of time to see if this will aid them in fighting off Bd infection, and returning them to the ecosystem after becoming Bd negative. Other studies could determine a best salt level for the ecosystem, and add salt to maximize positive effects and limit

negatives. This would however also require constant monitoring of salinity to ensure excess salinity does not make its way into the ecosystem from other human activities.

Overall, the present study did not aid in the understanding of salt and its effects on Bd infection. However it did provide evidence of salinity's possible deadly effects on amphibians. Also, it could point towards how important time is, in relation to salt exposure, again with what is a survivable amount of salt when acclimation is allowed with increasing increments of salt over time as opposed to the lethality observed when this salt concentration is hit in an instant. Additional studies on more species will help our understanding of salinity's effects on Bd infection. All studies will contribute to the goal of preventing amphibian death and extinction.

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