Spring 5-6-2024

Measuring the Effects of Selenium Exposure on Batrachochytrium dendrobatidis (Bd) Growth in vivo in Larval American Bullfrogs (Rana catesbeiana).

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Measuring the Effects of Selenium Exposure on *Batrachochytrium dendrobatidis* (Bd) Growth *in vivo* in Larval American Bullfrogs (*Rana catesbeiana).*

By

Taylor Morrison

A Thesis Submitted in Partial Fulfillment
Of the Requirements for the
University Honors Program

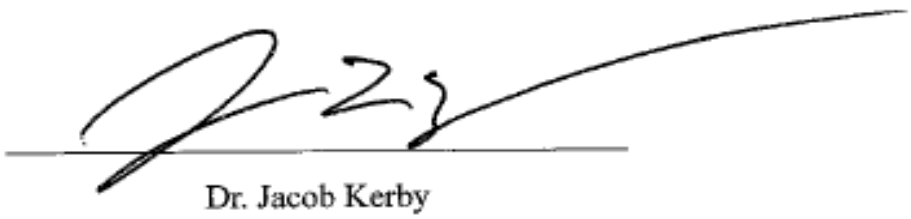
Department of Biology
The University of South Dakota
May 2024

The members of the Honors Thesis Committee

appointed to examine the thesis of Taylor Morrison

find it satisfactory and recommend that it be

accepted.



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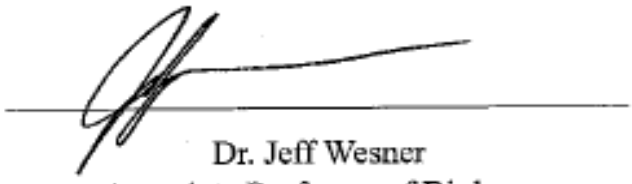

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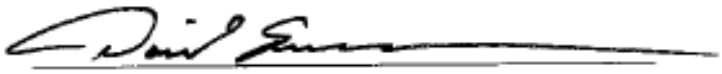

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ABSTRACT

Measuring the Effects of Selenium Exposure on *Batrachochytrium dendrobatidis* (Bd) Growth *in vivo* in Larval American Bullfrogs (*Rana catesbeiana).*

Taylor Morrison

Director: Jacob Kerby, Ph.D.

Most amphibians in today's world are exposed to a variety of environmental stressors. This project's main objective was to determine any effects of selenium on Batrachochytrium dendrobatidis (Bd) and infection levels in South Dakota amphibians. I selected American bullfrogs (Rana catesbeiana) as they are susceptible to Bd and are found throughout eastern South Dakota. The secondary objective of this project was to measure any impact of Bd and selenium, combined, on growth measures of the frogs. The third objective was to see if survival rates were reduced when amphibians were exposed to Bd and selenium. Tadpoles were collected (n=225) from the Gavins Point Fish Hatchery in Yankton, SD and were separated into nine groups (25/treatment). Ventral-cloacal swabbing was performed and analyzed using qPCR to determine Bd levels. Growth was recorded (mass and SVL) via the use of electronic calipers. Survival data were collected daily throughout the experiment. I found the highest levels of Bd infection where individuals were exposed to both selenium and Bd, yet no impact of time was found (Table 1 and Figure 1). Tadpoles decreased in mass while increased in SVL across all treatment groups. I found no effects of treatment on either measure of growth or on survival, yet do highlight that the survival rates was lower overall (~50%). These data indivate that low levels of selenium might not otherwise impact American Bullfrog

tadpoles, but do not appear to lead to increased infection levels when exposed to Bd. This finding suggests that further attention should be paid to this important contaminant in understanding its role in disease dynamics in South Dakota.

Keywords: selenium, *Batrachochytrium dendrobatidis*, American bullfrogs

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INTRODUCTION

Amphibians around the world are exposed to environmental stressors that are contributing to an extinction crisis (Warne et al. 2016). It is suspected that at least 100 species of amphibians have gone extinct in recent decades (Stuart et al. 2004). Environmental stressors are factors that cause a strain on the fitness of an organism (Koprivnikar 2010). The importance of environmental stressors is emphasized by the statement on 100 possible species being extinct as it shows the current impact of environmental stressors (to date). In addition to the 100 species suspected to be extinct, many more species are considered endangered (Koprivnikar 2010). In 2004, 1856 species of amphibians or 32.5% of amphibian species are listed as threatened (Stuart et al. 2004). These stressors may impact reproduction, the development of ecosystems, and survival. Common environmental stressors are pesticides, parasites, and disease (Koprivnikar 2010). A factor may be considered an environmental stressor when it impacts the organism and the ecosystem surrounding the organism. Different environmental stressors have differing intensities, which means that the duration and impact of each stressor vary (Koprivnikar 2010). Some amphibians may be affected by specific types of stressors more often than other species. This variation in response to stressors contributes to the likelihood of an amphibian species to suffer from mass die-off events or even extinction (Blaustein and Kiesecker 2002). The potential environmental stressors an amphibian population may be exposed to can vary by location, with some stressors being more prominent in different geographic regions due the stressor adapting to the location's

environmental conditions (Stevenson et al. 2013). For example, in agriculture, pesticides are used on crops. During irrigation, these pesticides run off and go into nearby wetlands and tile drainage sites. Amphibian populations that are in these locations are exposed to higher concentrations of this environmental stressor. Other environmental stressors that vary from location to location include temperature, precipitation, and soil type (Gupta and Gupta 2017). The variety in environmental stressors can make it difficult for researchers to predict what specific environmental stressors each species will face and how these stressors may interact to impact each species.

There are numerous different kinds of environmental stressors, with some common stressors including pesticide drainage, parasites, and climatic changes (Campbell 2021). The pathogenicity of different environmental stressors can be influenced by temperature and transmission methods. During different times of the year, amphibians are more susceptible to stressors as pathogen development and transmission methods are affected (Stevenson et al. 2013). This occurs as the presence of certain pathogens has been recorded more in cooler temperatures than in warmer temperatures (Kolby et al. 2015). Additionally, humidity also plays a factor (Kolby et al. 2015). Some pathogens, like Bd, are known to be more prevalent in high humidity due to the pathogen being able to be spread easier (Kolby et al. 2015). This is due to the high humidity in certain environments, like forests, preventing direct sun exposure, which causes pathogen prevalence to be higher. These common environmental stressors are often driven, in part, by anthropogenic activities (Kolby et al. 2015). The impacts of these stressors on the local ecosystem are often poorly understood or ignored in many contexts.

Selenium

Selenium is an environmental contaminant that can be found in many bodies of water throughout South Dakota (Henry and Wesner 2018). Wetlands are important for maintaining a healthy environment as they remove pollutants and excess nutrients from the water that flows through. The Prairie Pothole Region includes grasslands and wetlands that are located in Iowa, Minnesota, and the Dakotas (Johnson et al. 2008). Selenium is a naturally occurring heavy metalloid and essential nutrient (Campbell 2021). At the amino acid level, selenium can replace sulfur to be incorporated into proteins (Hoffmann and Berry 2018). Selenium is an essential nutrient that is needed for cellular function. In our cells, it is used to regulate oxidative stress and processes involving the immune system (Hoffmann and Berry 2008). Environmental concentrations of this metalloid have been steadily increasing in South Dakota wetlands due to the expansion of agricultural practices. Agricultural practices such as irrigation, causes selenium in the soil to be released and washed away into nearby wetlands (Lemly 2004). The selenium in the soil has most likely come from the previous weathering of rocks (Lemly 2004). Tile drainage systems also promote an increase in selenium concentrations. Tile drainage systems are used in agriculture to remove excess water from fields, so that the soil does not retain a large amount of water (Tangen and Finocchiaro 2017). Tile drainage systems cause selenium release by removing water, which can contain anything else used on the land (fertilizers, pesticides, etc.), into nearby wetlands (Campbell 2021). The ultimate impact of these drainage systems is that the amount of selenium released into the wetlands increases (Schwarz et al. 2018). Non-agriculture ways that release selenium into the environment include mining and the combustion of fossil fuels (Lemly 2004). These are all known ways that selenium is released into the environment.

Some wetlands in South Dakota contain relatively large amounts of selenium, which poses a threat to amphibians and other animals that utilize the wetlands, including humans who rely on or use these bodies of water (Gerberding 2020). While selenium is required in the diets of many species for proper development, there can be negative effects from consuming too much or too little selenium (Espinosa-Ortiz et al. 2015; Janz 2010). Selenium toxicity can also be called selenosis. The narrow concentration range of selenium when it is essential versus when it is toxic varies from species to species (Janz 2010). Additionally, it varies by development stage and age. Thus, it is important to know the effects of when selenium is considered too little or too much. When too much selenium is accumulated in an organism it can result in selenium toxicity, which often has negative effects on development, particularly in larval amphibians. The effects of too much selenium in amphibians include apoptosis of cells, unregulated oxidative stress, and inhibition of growth (Espinosa-Ortiz et al. 2015). It can also cause damage to digestive and excretory systems by increasing the hepatosomatic index and causing necrosis of tissues (Sorensen et al. 1984). This occurs due to the accumulation of selenium in the amphibian's body causing the cells not to function correctly (Sorensen et al. 1984). More physical impacts from selenium toxicity include deformation of keratinized mouthparts and spinal malformations (Janz 2010). These physical impacts listed also cause an impact on what the amphibian can do. Capabilities of amphibians that are impacted include predator avoidance, survival, swimming, and feeding habits (Janz 2010). Due to the physical impacts of selenium toxicity, amphibians are not able to survive and act as normal amphibians. Selenium deficiency occurs when there is not enough selenium in the diet, which impacts amphibians by causing the immune system to not work as well and

an increased mortality rate when infected with a disease (Moya et al. 2013). One important takeaway from selenium toxicity is that fast-growing organisms are more sensitive to varying levels of selenium (Lanctôt et al. 2017). This is because their body is in a highly permeable state, which causes excess selenium and ions in the environment to enter their body (Lanctôt et al. 2017). Although selenium is beneficial for the diet of all animals, it can become toxic at different levels for different animals. The impacts of selenium are great independently, but does it increase or decrease the fitness of an organism when combined with disease?

Batrachochytrium dendrobatidis

Bd is a fungal pathogen that impacts amphibians all over the world (Warne et al. 2016). In 2012, Bd was detected in 52 countries around the world with the United States being one of them (Olson et al. 2013). This fungal pathogen demonstrates low host species specificity and is known to infect 516 species in 52 countries (Olson et al. 2013). Bd infection may result in chytridiomycosis, a condition that has been linked with mass die-off events around the world (Warne et al. 2016). This is due to the Bd targeting susceptible animals (Jani and Briggs 2014). Tadpoles are susceptible to Bd as they use a lot of energy to go through metamorphosis. There are a variety of symptoms linked to Bd with differing severity of each. Some common symptoms of Bd include red skin, abnormal feeding behavior, and discoloration around the mouth (Jani and Briggs 2014). The threat of Bd infection is ongoing, with many researchers devoted to the mitigation, management, and eradication of this pathogen in wild and captive amphibian populations (Stevenson 2013). This allows researchers to learn more about the disease and how it moves between amphibians. These specific characteristics highlight what Bd does to

amphibians, but different environmental conditions influence its presence in nature. Knowing these characteristics allows researchers to understand the pathogen better and allows them to keep track of its presence in nature.

Bd has many specific characteristics that promote its pathogenicity with the environment being a determining factor if Bd is spread or not (Stevenson 2013). One characteristic is temperature, as Bd grows better in cooler temperatures and moist environments (Harvell et al. 2002). With these cooler temperatures, Bd can produce zoospores and spread to other amphibians who are not infected. Another key aspect of Bd is its mode of transportation. The primary mode of transmission of Bd occurs through zoospores in aquatic environments, allowing it to be easily spread from one amphibian to the next (Stevenson 2013). By contaminating aquatic environments, Bd can grow and infect different hosts easily as many amphibians utilize water during their life cycle (Olson et al. 2013). For frogs specifically, aquatic environments are used primarily when they are considered tadpoles. Once they mature, many utilize the land. In aqueous environments, aquatic zoospores are produced and infect the keratinized skin of juvenile and adult amphibians (Rohr et al. 2017). However, Bd can also use dry environments to infect amphibians. This occurs when an infected frog leaves the water and goes to land (Kolby et al. 2015). The infection stays with the amphibian and infects the environment that the frog touches. It can also occur through contact with contaminated water. This mode of transmission occurs through the use of uniflagellate zoospores that are either carried by water currents or swim short distances (Rachowicz and Vredenburg 2004). Transmission can also occur through direct contact with another infected animal (Rowley and Alford 2007). This occurs through direct touching of two frogs where one is infected,

and the other is not infected. Bd zoospores infect amphibians by burrowing into the skin, disrupting osmoregulation (Gervasi et al. 2013; Jani and Briggs 2014). Osmoregulation is the regulation of osmotic pressure in animals through the use of osmoreceptors (Jani and Briggs 2014). When osmoregulation is disrupted, amphibians are unable to control the amount of solute and water concentration. The lack of osmoregulation causes Bd infection to often be fatal to amphibians. Fatality occurs when water and salt concentrations are not in homeostasis causing blood pressure to drop (Jani and Briggs 2014). Additionally, water and/or toxic waste can accumulate in the body disrupting all cellular functions. Bd has been detected in South Dakota amphibian populations, although there have not yet been any documented mass mortality events (Campbell 2021).

Continuing Research

It is important to note that a lot is still unknown about emerging infectious diseases (EIDs) and pathogens. One factor that is still unknown in the research field is determining the susceptibility levels to different pathogens for different individuals or species. By determining what causes the high susceptibility, researchers would be able to help limit the spread of different pathogens. A crucial aspect of pathogens is that they maintain animal population levels, so eradicating a pathogen can be harmful to the world as population levels would increase. Another unknown area of interest is how different pathogens interact with other pathogens or environmental stressors. While research about the interaction of pathogens with environmental stressors is still ongoing, not much is known about how different pathogens interact with other environmental factors. This topic is important to learn about so that a better understanding can be reached about

specific pathogens. For Bd, very little is known about how Bd impacts the environment and animal populations while interacting with other pathogens and environmental stressors. Through research, this area of interest can be understood better by examining a specific disease and determining its impact on other stressors in the environment.

It is important not only to understand the effects of these stressors individually but to also understand the effects of interacting agents, such as pathogens and the environment, on the hosts in the environment and their survivability (Koprivnikar 2010). By considering the interacting stressors in the environment with the decline in many species, a better understanding can be reached of the impact of the combined effect of environmental stressors. The bigger picture that can be reached is determining the overall relationship between different environmental stressors. This relationship can either be beneficial or harmful to the pathogen and its disease-causing abilities in animals. Additionally, knowledge about these kinds of relationships allows for a better understanding of the impact of environmental stressors on amphibians to be seen. It is important to use this view to see how environmental stressors can be combined to cause a larger impact. It is important to learn how these two environmental stressors can impact species of amphibians because they are some of the top environmental stressors that have a huge impact. By learning the combined effect of these stressors, we are allowed to figure out ways to help different species of animals.

One specific animal taxon that is impacted by environmental contaminants and disease is amphibians. Recently, it is thought that over a hundred species of amphibians have gone extinct with many other species endangered due to environmental contaminants (Stuart et al. 2004). By looking at the combined effects, researchers can

help keep the endangered species alive and learn how best to help them. Although there are several combinations of these categories of stressors in the wild, we are particularly interested in the combined effects of selenium and *Batrachochytrium dendrobatidis* (Bd) because these stressors are often found together in South Dakota wetlands, which are home to many amphibian species (Gerberding 2020; Henry and Wesner 2018). It is important to research the impact of these environmental stressors together as they are currently impacting amphibian species in South Dakota and can impact more animals that are not amphibians. Evaluating the effects of a chemical stressor on the growth of a fungal pathogen can provide insight into how fungal pathogens may, potentially, be mitigated through chemical methods. It also provides an opportunity to learn how chemical stressors may impact fungal pathogens. This specific combination of stressors is also interesting because research has shown that selenium can slow the growth and shorten the lifespan of Bd zoospores *in vitro* (Campbell 2021).

Campbell's study has led me to my project to determine if the relationship between Bd and selenium relationship still exists when performed *in vivo*. To address this topic, I used the model amphibian species the American bullfrog (*Rana catesbeiana*) and the chemical stressor selenium and the fungal pathogen Bd. The American bullfrog is typically found in the eastern portions of northern United States, but also can be found throughout South Dakota and commonly are exposed to selenium and Bd (Jordon 2024). This work is the first to examine the combined potential effects of selenium and Bd on American bullfrogs. I predict the Se will negatively affect Bd on the skin of the frogs and that those in the combined treatment will exhibit the lowest amount of Bd as compared to

Bd only groups. I also predict that Se will have a negative effect on tadpole growth and survival despite reducing the Bd load.

MATERIALS AND METHODS

Data Collection

Field Collection

In May 2023, I collected American bullfrog (*Rana catesbeiana*) tadpoles from a pond at the Gavins Point Fish Hatchery near Yankton, SD, USA. These tadpoles were transported back to the laboratory in Vermillion, SD, USA in fish bags that were stored inside of coolers with ice. All tadpoles were housed together in 10-gallon aquariums filled with reconstituted reverse osmosis (RRO) water. In the aquarium, bubblers were placed, and the tadpoles were fed with algae wafers and shrimp pellets *ad libitum*. The tadpoles were in these tanks until they were done with metamorphosis, which took approximately 3 weeks. Once they completed metamorphosis, the individuals were transferred to 16-ounce deli containers with 50 mL RRO water. The individual deli containers were held under a light-dark cycle, where there were 12 hours of light and 12 hours of darkness. The water of each individual deli container got changed and replenished with food every 3 days. I recorded daily survival by monitoring the movement in each container.

Experimental Design

To examine the effect of selenium (Se) on Bd exposure, I randomly assigned them to one of four treatment groups. These treatment groups varied based on Se and Bd exposure concentration (control, 0 µg/L Se/100 zoospores/mL Bd (Bd only), 3.5 µg/L Se/0 Bd (Se only), or 3.5 µg/L Se/100 zoospores/mL Bd (Bd and Se) (n=14, 16, 16, 17, respectively)). For the exposure to Bd and selenium, all animals were exposed individually and never came into contact with each other. Individuals were exposed to Bd for a period of 72 hours via a water bath and then were removed and placed in water (either with or without

Se, depending on treatment) without added Bd. Individuals who were randomly assigned to be exposed to Se (3.5 µg/L) were exposed to selenium via a water bath. Each individual in a treatment group containing selenium were re-dosed with selenium when the water was changed every 3 days. The value selected for these selenium exposures (3.5 µg/L) reflects a concentration between the concentration for selenium deficiency (2 µg/L) and the concentration for selenium toxicity in (5 µg/L) (Beckon 2003). Selenium exposure lasted the entire length of the experiment (28 days). The average water temperature was 20.2°C (±0.44) and the air temperature was 21.9 °C (±0.26). At the conclusion of this 28-day experiment, the remaining individuals (Control n=7, Bd only n=10, Se only n=9, Bd and Se n=10) were euthanized via rapid decapitation, and the liver was removed from all remaining individuals. The remaining tissues were preserved in 95% ethanol and frozen for future analysis to evaluate the accumulation of selenium throughout the body.

Growth

To analyze changes in growth rate based on the treatment group, I recorded snout-vent length (SVL; mm) and the mass (g) of each three times per week. The SVL was measured using electronic calipers instead of a ruler to give a more detailed measurement. The mass was measured using an electronic scale and was measured to the nearest 0.01g.

Bd Growth

Beginning on day 0 and again on days 7, 14, 21, and 28, all individuals were sampled for chytrid using ventral-cloacal swabbing. I performed ventral-cloacal swabbing by using a swab and running the swab over the anterior surface of the individual's body and the individual's limbs. The swabs were then put in sealed containers and analyzed later. To

monitor the effect of selenium exposure on Bd growth and chytrid progression, individuals were monitored daily for signs of chytrid infection. Specific signs that I looked for are redness and thickening of the skin, lethargy, and gross lesions on the skin. For the swabs used in the qPCR, each Bd swab used a single swab. From each of the individuals used, there were 3 different swabbing events (control n=32, Se-only n=40, Bd-only n=39, Bd and Se n=40). By using all of the swabs, a better representation is seen with no concern of bias.

Survival

To examine the effect of exposure to Se and Bd on survival, I collected survival data every day for the duration of this experiment. Individuals were checked once per day to see if they were alive or dead. All deceased individuals were removed from their individual deli containers and immediately frozen to preserve the tissue.

Statistical Analysis

Statistical analyses were conducted using Python (Version 3.11.8), using libraries including Pandas (1.5.3) for the manipulation of data, NumPy (1.24.0) for numerical operations, and Statsmodels (0.13.5) for statistical testing. I examined the data for normality and variance homogeneity to satisfy the assumptions of parametric statistical tests. Given the nature of the data, a logarithmic transformation (log(x+1)) was used to transform the response variable to fit the normality assumptions of the ANOVAs. An ANOVA was performed to examine the effects of treatment, time, and their interaction on the log-transformed gene copy numbers. The treatments included four categories: control, selenium only, Bd only, and selenium and Bd combined. The time points measured for estimates of Bd load were day 0, day 3, day 7, and day 28. The time points measured for

estimates of growth (mass and SVL) were on days 0, 7, 14, 21, and 28. After examining the effects of time, a simpler one-way ANOVA test was performed on the data that corresponded to the final day, 28. For Bd load, the log transformed data on day 28 were used for analysis. For growth data, those remaining on day 28 were examined for their difference from day 28 – day 0. Tukey LSD tests were used for post-hoc comparisons across the four treatment groups.

Survival analysis was conducted to evaluate the differences in survival rates across four treatment groups. I employed the Kaplan-Meier estimator to calculate the survival probabilities for each treatment group over the study period. Survival curves were generated to visually represent the survival distribution of subjects in each group. Differences between the survival curves of the treatment groups were statistically evaluated using the Log-rank test.

RESULTS

Bd load

The ANOVA showed an effect of treatment type ($F_{3, 182.}$= 5.03, p = 0.0023), but no effect of time ($F_{1, 182.}$= 0.087, p = 0.786). Table 1 displays the estimates of infection over time. As there was no clear pattern due to time, I examined only the differences at the end of the experiment (Table 1 and Figure 1). The ANOVA showed an effect of treatment type ($F_{3, 33.}$= 5.58, p = 0.0033).

TREATMENT	DAY 0	DAY 3	DAY 7	DAY 28
CONTROL	2.61	3.94	3.69	1.15
SELENIUM ONLY	3.26	4.09	1.45	1.08
BD ONLY	0.56	2.18	0.00	1.04
SELENIUM AND BD	4.15	2.86	2.90	6.65

Table 1: Table showing the results from the log-transformation and averaging the gene copy numbers. No clear pattern is seen in Bd infection as time continues.

The Tukey's LSD post hoc test showed significant differences between the combined group and all others, but no differences amongst the other three groups (Table 2, Figure 1).

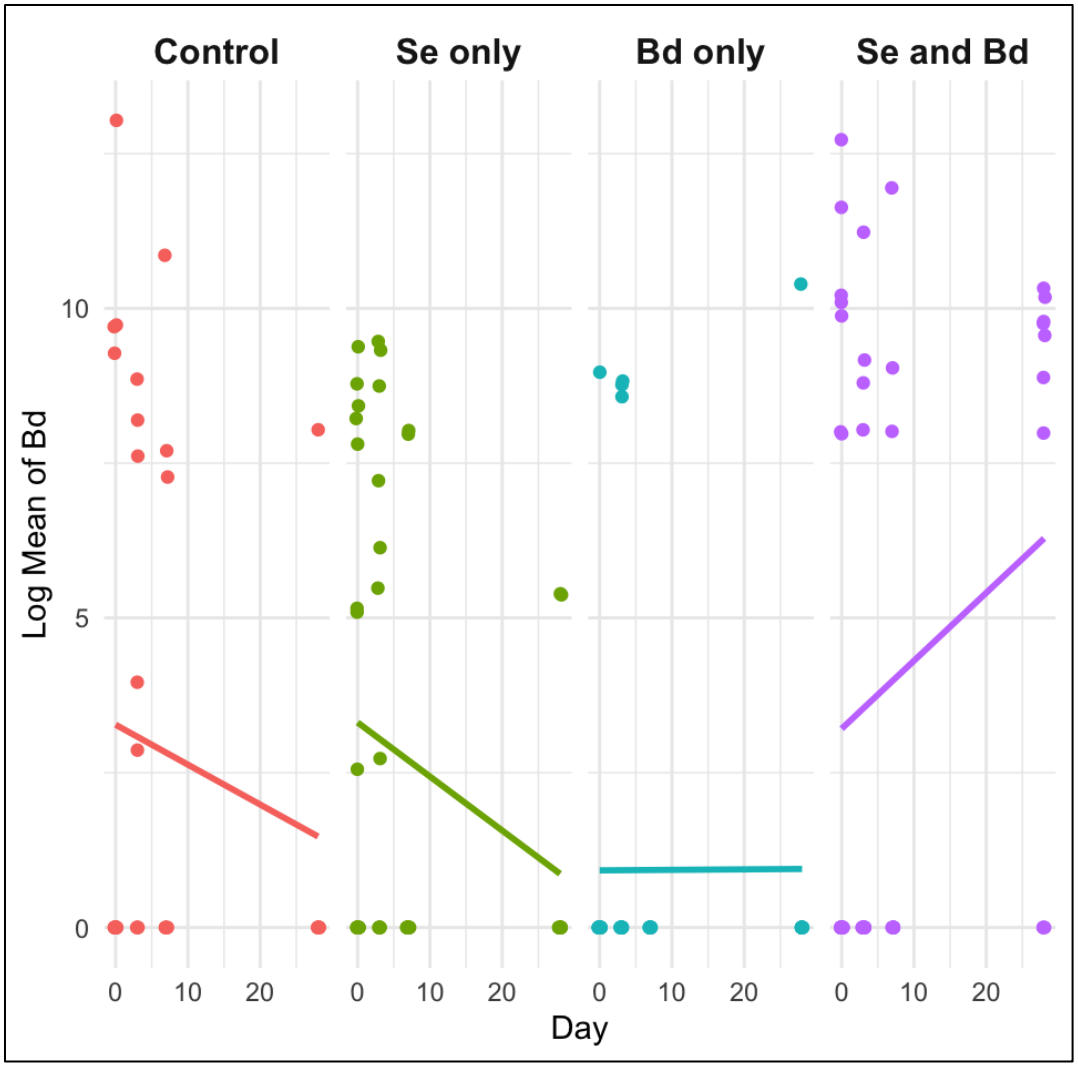

Figure 1: Mean log-transformed gene copy numbers (±1 SE) for the four treatment groups using data collected on day 28 post-exposure.

Treatment Pair	Mean Diff	Std. Error	P-adj
Bd only- control	-0.10925	1.69925	0.99990
Control-Se only	-0.07214	1.69925	0.99997
Bd and Se- control	5.49982	1.69925	0.01397*
Bd only-Se only	-0.03711	1.54205	0.99999
Bd only- Bd and Se	5.60906	1.54205	0.00482**
Bd and Se-Se only	5.57195	1.54205	0.00518**

Table 2: The results from Tukey's HSD using day 28 mean log-transformed gene copy numbers from different treatment pairings. Standard error for each treatment pair is shown. (* = significant; ** = highly significant)

Growth

The ANOVA showed no effect of treatment type on either mass difference ($F_{3, 32.} = 0.812$, $p = 0.497$; Figure 2), or SVL difference ($F_{3, 32.} = 0.053$, $p = 0.984$; Figure 3).

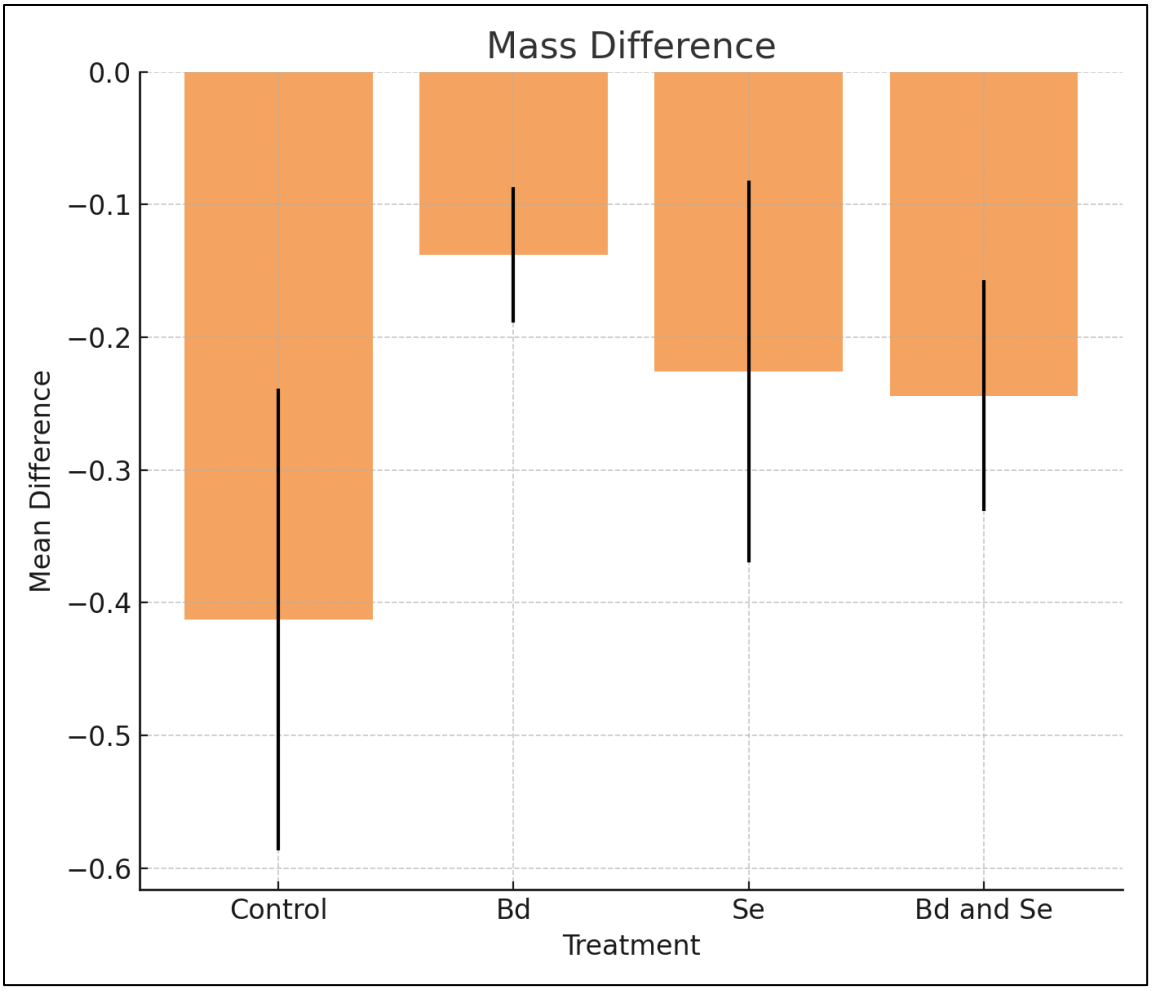

Figure 2: Mean difference of mass (g) (±1 SE) for the four treatment groups using data collected on day 28 post-exposure minus day 0 measurements. Shown is a bar graph to illustrate the differences in loss of mass between each treatment group.

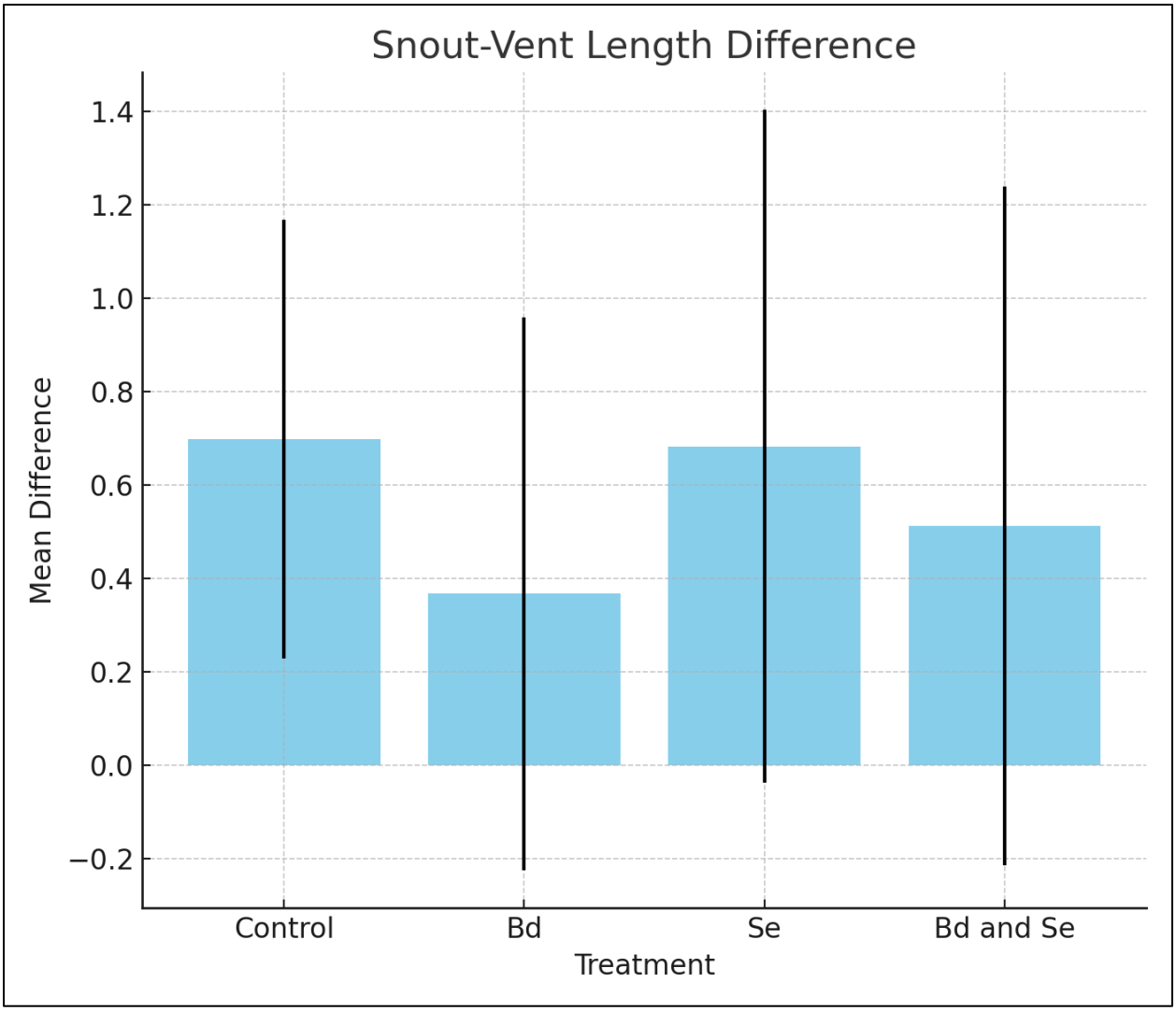

Figure 3: Mean difference snout vent length (mm) (±1 SE) for the four treatment groups using data collected on day 28 post-exposure minus day 0 measurements. Shown is a bar graph to illustrate the differences between each treatment group.

Survival

The Log-rank test revealed no statistically significant differences in survival rates among the treatment groups (Bd only, Bd and Se, Control, Se only) over the study period (x^2 = 0.3, df = 3, p = 1.00; Figure 4).

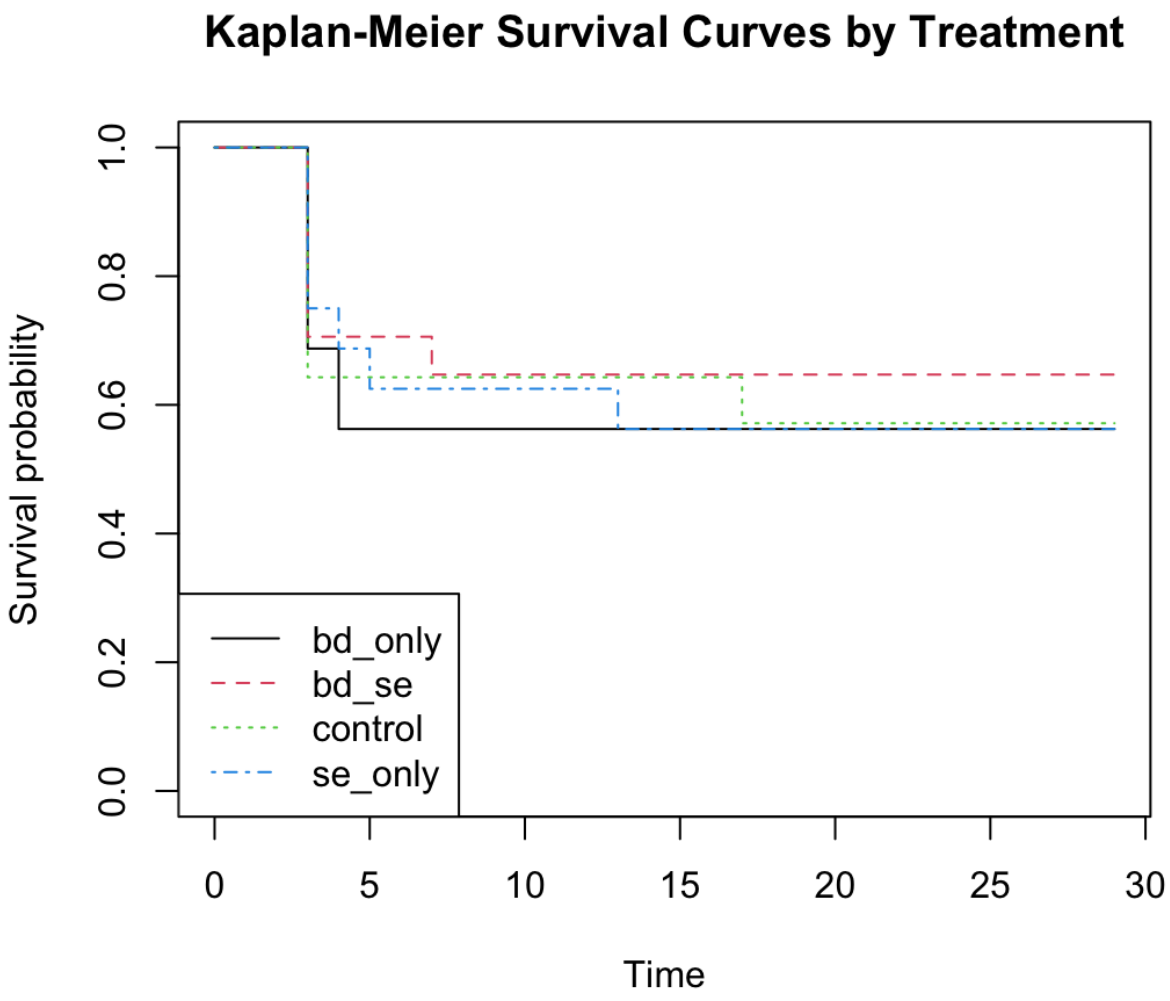

Figure 4: Kaplan-Meier survival curves for each treatment group. The plot displays no significant differences in survival probabilities over time for Bd only, Bd and Se, Control, and Se only groups.

DISCUSSION

This project's main objective was to determine the effects of selenium exposure on Bd exposed tadpoles. Our results did show that there is a relationship between Bd and selenium, but not as I originally hypothesized. The presence of selenium increased Bd's presence on tadpoles instead of reducing it. Additionally, I found no effects of either treatment on growth or survival measures.

I hypothesized that the treatment group containing Bd and selenium would show fewer Bd zoospores present. However, it appears that the opposite is occurring (Figure 1). The Bd and selenium group contained the most zoospores out of all the treatment groups. This is surprising as the Bd and selenium treatment group contained more zoospores than the Bd-only treatment group. This result suggests that selenium is reducing the ability of the amphibian to fight off infection. However, this may not be the case as the Se-only treatment group had the greatest decrease in Bd load during this experiment. These results are important as they suggest an interactive effect of Bd and selenium, which clearly can have important implications in wild populations. It is important to note that I used wild caught tadpoles which likely were exposed to Bd in their native habitats. This is indicated by the detection of Bd in both control and selenium only treatments (where there should have been no infections). While this can be problematic, nonetheless it is intriguing that despite low level infections occurring, when tadpoles are dosed with higher levels of Bd and combined with selenium they do exhibit significantly higher levels by day 28. As data are log transformed, these effects are large, over 5 orders of magnitude higher concentrations. One big question raised from this result is how the

results would have differed if all tadpoles were cleared of Bd infection prior to the experiment. This opens up a door for further research to determine if that would have an impact on the results. Another concern that can be raised is that the Bd and Se treatment group started off on having the highest Bd load (Table 1). To determine if this impacted the result, I could account for it by rerunning the one-way ANOVA test by using day 28 data minus day 0 data. This would cancel this concern and show if it had any impact. In regards to the data shown in Table 1, no pattern was seen as time went. For any of the treatment groups, Bd load did not clearly increase or decrease, which tells us that time did not play an important role. This can be applied to the concern raised on the Bd and selenium treatment group containing the highest Bd load in day 0 as we can see from Table 1 that time did not have an impact. Further research should still be conducted to examine if starting off with a high Bd load impacted the data recorded from the Bd and selenium treatment group.

One previous study in our lab, although done *in vitro*, was used to form the basis of my original hypothesis that selenium would negatively affect Bd. This study used a similar strain of Bd and exposed it to selenium as well (Campbell 2021). Well microplates were used to estimate Bd growth, and a spectrophotometer was used to measure absorbance values. The results showed that selenium negatively impacted the growth of Bd by altering the disease dynamics in reproduction (Campbell 2021). This result was seen when there was a decrease in optical density with an increasing concentration of selenium. The main takeaway from Campbell's study is that there was a negative relationship between selenium and Bd *in vitro*. When comparing this result to my results a big difference in the terms of the studies was seen. Campbell's study

recorded optical density, while I recorded the presence of actual Bd zoospores on the frog's skin. Potentially the concentrations of actual selenium that Bd was exposed to was different between the two exposures. Or it could simply be that the effects of selenium on the amphibian were much greater than on the Bd itself. Previous work done in the laboratory has shown these types of concentration-based effects on Bd and amphibians, although it was using a different contaminant and species of amphibian (Brown and Kerby 2012). Low concentrations of triclosan resulted in better survival of Bd exposed amphibians, while higher concentrations of triclosan killed the amphibians. Clearly, the context of exposure is critical to consider when examining multiple stressors.

The secondary objective of this project was to determine the impact of Bd and selenium on growth. Despite the clear differences in Bd load of the combined treatment, no significant differences in the change of mass or SVL were detected across any of the treatments (Figures 2, 3). One surprising result that was found was the control treatment group lost an average of 0.4 g (Figure 2). This is a significant amount of weight as the individuals in this experiment averaged around three to four grams. All treatment groups lost mass throughout this experiment with the Bd-only treatment group losing the least (0.12 g). The Bd and selenium and selenium only treatment groups lost a bit more than 0.2 g through this experiment (Figure 2). The average amount of mass lost through all the treatment groups was around 0.24 g (Figure 2). The loss in mass can be explained by the growth seen in SVL. As mass declined through this experiment, SVL increased in all treatment groups. The groups that had the highest increase were control and selenium only as they grew around 0.7 mm in SVL (Figure 3). The results from the Bd only treatment group showed that these individuals grew almost 0.4 mm, while the Bd and

selenium treatment group grew 0.5 mm (Figure 3). The average SVL increase from all the treatment groups was around 0.55 mm (Figure 3). At the end of this experiment, the average mass was 3.26 g whereas the SVL was 33.15 mm across all groups. The results taken from the data on growth indicate that individuals in all treatment groups were allocating an approximately equal amount of energy toward growth. One important takeaway from this is that the combination of Bd and selenium stressors does not cause individuals to allocate fewer resources to growth than the other groups. Recently metamorphosized individuals were used in this experiment, however, larval amphibians may yield different results as they spend a lot of resources during metamorphosis (Wilbur and Collin 1973). While I did not use larval amphibians in this experiment, analyzing the impact of Bd and selenium across multiple amphibian life stages can provide better predictions and understanding of the impact of these combined stressors. These results show that Bd and selenium do not cause a difference in growth rate in American bullfrogs (*Rana catesbeiana*).

The third objective of this experiment examined the probability of survival over time to determine if Bd and selenium have an impact on survival. The results on this objective found no differences in the probability of survival among each treatment group (Figure 4). This could be due to the stressors interacting with the amphibians, but it is more likely that the amphibians were stressed in their new environments. This can be seen as the drop-in survival rates stopped at around day 4 to 5 (Figure 4). Around days four and five, the individuals should have adjusted to their new environment, which explains the constant survival rates from these days forward (Figure 4). At the end of this 28-day experiment, the probability of survival was recorded as 56.7-57.2% across all

groups. These results indicate that the combination of Bd and selenium does not increase or decrease the probability of survival when compared to the other treatment groups.

Future Work

Further research is required to understand the effects of Bd and selenium on wildlife in South Dakota. This research is valuable to understanding the combined effects of Bd and selenium in South Dakota wetlands and shows there are more Bd zoospores in individuals who were exposed to Bd and selenium than individuals in the other treatment groups. However, the selenium only treatment group had a high initial load of Bd and experienced a decrease in Bd load at the end of the experiment. This can influence conclusions by needing more research to be done. To determine the relationship between Bd and selenium, this research needs to be repeated to figure out if the results are the same. A lingering question from this is to determine if a higher amount of selenium to Bd ratio caused this result in the selenium only treatment group. I would also suggest looking at different environmental stressors in combination to compare the results with this experiment. Beyond examining metamorphosis, it would also be important to examine impacts to tadpoles. By using larval amphibians, it would allow for an understanding of the impact of Bd and selenium on a variety of amphibian life stages. I would also adjust the selenium concentration to determine if a varying level of selenium interacting with Bd would produce different results. I would also suggest collecting more individuals to obtain results that apply to the large majority. This would allow for a better representation to be made of environmental stressors that amphibians are currently exposed to. Nonetheless, this work contributes to a growing body of research that

highlights the complexities of multiple stressors and the need to study them in a variety of contexts.

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