The Effect of Aquatic Surface Area on Frog Resistance to a Fungal Pathogen

By
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April 24, 2017



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.

_____
Pledge

Approval by the Senior Thesis Committee:

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Dr. Venesky

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Abstract

Climate change is an ongoing issue around the world over the past two decades, especially in regards to how it changes the habitats of wildlife. For aquatic environments, this climate and resulting habitat change could be detrimental and poses as a potential stressor that decreases an organism's ability to resist disease. As global temperature rises, aquatic environments get smaller. Smaller surface area of such environments decreases dissolved oxygen content, causing breathing rate to increase and thus could be a potential stressor. This study investigates how variance in surface area size affects the resitance of African Clawed frogs' (*Xenopus laevis*) to the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). Frogs were randomly distributed to aquatic environments that had either a small, intermediate, or large surface area with the same depth and volume. After 14 days, frogs were put into individual containers and exposed to *Bd.* Thirteen days after exposure, I swabbed the skin of each frog and used qPCR analysis to determine the infection intensity (genomic DNA equivalents of *Bd*). Mass was also taken prior to and after infection. Only 7 of the 24 frogs exposed to *Bd* were infected upon completion of the experiment and 22 of the 24 frogs experienced a decrease in mass. Surface area size was not a significant predictor of *Bd* infection intensity. This study suggests that, against my original hypothesis, surface area size does not increase *X. laevis* frogs' susceptibility to *Bd* but a larger change in surface area or a more immediate introduction of the pathogen may increase disease intensity. This implies that this specific change in aquatic environment shape does not decrease frogs' ability to resist *Bd*.

Introduction

Organisms are subjected to stressors on a daily basis and are impacted greatly by them. In order to combat potential damage, a series of hormones are released in order to bring the organism back to homeostasis. This process, which occurs in the hypothalamic-pituitary-adrenal axis, begins with the release of corticotropin-releasing hormone (CRH) from the hypothalamus. CRH then binds on the anterior pituitary, promptin the release of adrenocorticotropic hormone (ACTH). ACTH travels to the adrenal glad, where it binds and releases glucocorticoids (GCs) (Tsigos and Chrousos, 2002). Specifically for vertebrates, the GC corticosterone (CORT) functions to mobilize energy resources by altering behavior, brain function, physiology etc. in order to return to homeostasis. GCs are a type of defense mechanism, allowing the organism to allocate energy to what is needed (Sapolsky et al., 2000). These hormones are released in every organism, although duration and elevation of the levels of the GCs differs between animals and the stressor applied.

There are two types of stress: acute stress and chronic stress. Acute stress is moderate and short and can promote a better response to important traits such as reproduction, resistance to disease, and growth. This type of stress can be immune enhancing by improving dendritic cell, neutrophil, macrophage, and lymphocyte trafficking, maturation, and function and it has also been shown to supplement innate and adaptive immune responses (Dhabhar, 2008). These immune enhancing properties are found to mainly facilitate reproduction by improving energy storage and increasing amphibians' ability to attract mates during breeding (Moore and Jessop, 2003, Emerson and Hess, 2001). In some frogs, predator attacks, an acute stressor, stimulate the release of

antimicrobial skin peptides, which immediately increase their ability to fight off disease (Ramsey et al., 2010). Long-term stressors often cause chronic stress, which is long term and unhealthy for the organism. This type of stress is typically immunosuppressive because it deregulates the innate and adaptive immune responses and suppresses immunity by decreasing leukocyte numbers, trafficking, and function (Dhabhar, 2008). Long-term temperature change showed that amphibians increase in susceptibility to infection due to a decrease in immune function (Raffel et al., 2006). Depending on the type and duration of stress, it can either slightly benefit or harm an organism.

Stress can influence susceptibility to disease, making organisms more likely to be infected, trigger a response to the pathogen already inside of them, or maintain a pathogen already in the body (Cohen and Williamson, 1991). In a study done on humans by Cohen and Williamson (1991), researchers found that stress causes greater risk of getting upper respiratory infection symptoms, higher risk for bacterial infections, and that stress may reactivate herpes viruses. Overall, it is shown that negative stress in humans has a significant negative impact on the ability of the immune system to fight off pathogens and infection. Likewise, in other mammals such as mice, social stress has been shown to have a detrimental effect on health. This in turn, makes them more vulnerable to disease due to lowered immunity (Bartolomucci, 2007). Although this study does not involve parasites, it does show that different types of stress can cause a greater decline in the immune system, causing a greater risk of disease. Higher levels of GCs can also be found in mice with higher levels of intestinal parasites than those without (Brown and Fuller, 2007). This indicates that stress could be a factor in their ability to rid the body of these parasites. Branching off of experiments and observations made with humans and other mammals and stress, we

can begin to visualize how stress may affect the immune system of amphibians, which is not as well researched.

Infectious diseases and parasitic invasions are becoming a more widespread issue in organisms around the world to the point where no major ecosystem on earth remains unaffected. These often result in declines of many wildlife species, affecting biodiversity and declines in population sizes. For example, the population of seals in the North Sea has decreased by about 30%, lion populations have declined by 20%, and amphibian populations are threatened all due to the presence of infectious diseases (Dobson and Foufopoulus, 2001). Although pathogen populations have increased, the interaction of environmental factors and a resulting decrease in health of organisms has potentially paved a way for them to grow. As resistance to disease decreases, naturally the prominence of that pathogen will increase due to availability of susceptible hosts. The combined effects of environmental changes, resulting organismal health and how it affects disease prominence is rarely studied but is a potential factor in the rise of infectious diseases.

One specific way that amphibians have more recently been experiencing stress has been directly through their environment, specifically climate warming and pond drying, which is a type of chronic stress. Global warming can have a negative effect on amphibians due to change in their immediate environment, largely including a decrease in availability of water and a resulting limited dispersal ability to other areas (Araújo et al., 2006). Some studies have shown that decreasing water levels to simulate pond drying and infection slow development and body size and weight and lower the survival rate (Székly et al., 2010, Kiesecker and Skelly, 2001). Accelerated development is a stress-induced defense mechanism, indicating that this situation was agitating. The combination of pond drying

and infection had an even deadlier effect on organisms as compared to either separately. One reason pond drying could be stressful is because it potentially could create different shaped ponds for the amphibians, altering the surface area of the ponds the frogs live in.

As ponds change shape and decrease in surface area and depth, they reduce the amount of possible oxygen consumption for the amphibians living in them. For amphibians that mainly utilize aquatic respiration, this poses a large threat to their survival and prompts them to increase their dependence on air breathing (Calich and Wassersug, 2012). The effect of decreased surface area has been studied in *Xenopus laevis* larvae and tadpoles in reference to their development and growth but very little research has been done on adult frogs or the interaction between this stressful situation and the ability to resist disease. Therefore, in my study, *X. laevis* adult frogs were used to better understand this interaction. They are suitable for this experiment due to their common use in laboratory research and their utilization of aquatic respiration. They also contract the fungal pathogen *Batrachocytrium dendrobatidis* (*Bd*) and the resulting infection, called chytridiomycosis, and are a potential vector of the disease to native frogs in other countries (Solís et al., 2010), therefore it is important to study what conditions heighten the likelihood of increased infection intensity.

One particular fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*), has plagued amphibian populations around the world. When amphibians are infected with *Bd*, they often develop chytridiomycosis, an infection that targets the keratinized epidermal skin cells resulting in sloughing and reddening of skin, convulsion and lethargy. These fungi have motile, flagellate zoospores, which actively seek out the keratinized, living skin

(Fisher et al., 2009). This disease has resulted in many declines of amphibian populations, even driving some to extinction.

There is a lack of knowledge in the area of amphibians and stress, which could be very important in the overall understanding of the relationship between amphibians and disease, specifically *Bd*. Some research states that stress could have a positive correlation with disease intensity, meaning that as stress increases so does disease intensity (Peterson et al., 2013, Fonner et al., 2017), while others claim that stress does not affect susceptibility nearly at all (Haislip et al., 2012, Searle et al., 2014). Most studies positively correlating stress and disease measure levels of stress after or during infection. Peterson et al. (2013) shows that infection increases baseline plasma GC levels, specifically corticosterone (CORT). But in the study done by Fonner et al. (2017), plasma CORT levels were increased daily for 9 days, a way to induce stress, and then the frogs were inoculated with *Bd* and compared to those not treated with CORT. Overall, they had increased infection abundance compared to *Bd*-inoculated animals not being stressed but they did not experience an increase in clinical signs of chytridiomycosis. Induced stress overall had a significant affect on the amphibian's ability to resist *Bd*. On the contrary, the experiment done by Haislip et al. (2012) crossed natural stressors and disease in larval anuran species and found that exposure to predator cues did not increase the likelihood of disease. Although none of these studies are particularly done on aquatic frogs, they present interesting and opposing results that prompt a question as to which is likely to happen in aquatic frogs.

In the present study, interactions between *Xenopus laevis* frogs and their environments are observed to help us better understand their ability to resist *Bd*. Frogs were put into three different containers with identical depth and volume but differing

surface area. The hypothesis for this study is that the smaller the surface area, the more stressed the frogs would be and subsequently more prone to disease infection and loss of mass. As surface area decreases, dissolved oxygen content also decreases, causing the frogs' respiration rates to increase. They must work harder in order to get the oxygen needed and therefore would be more stressed and potentially more susceptible to disease and loss of mass.

Materials and Methods

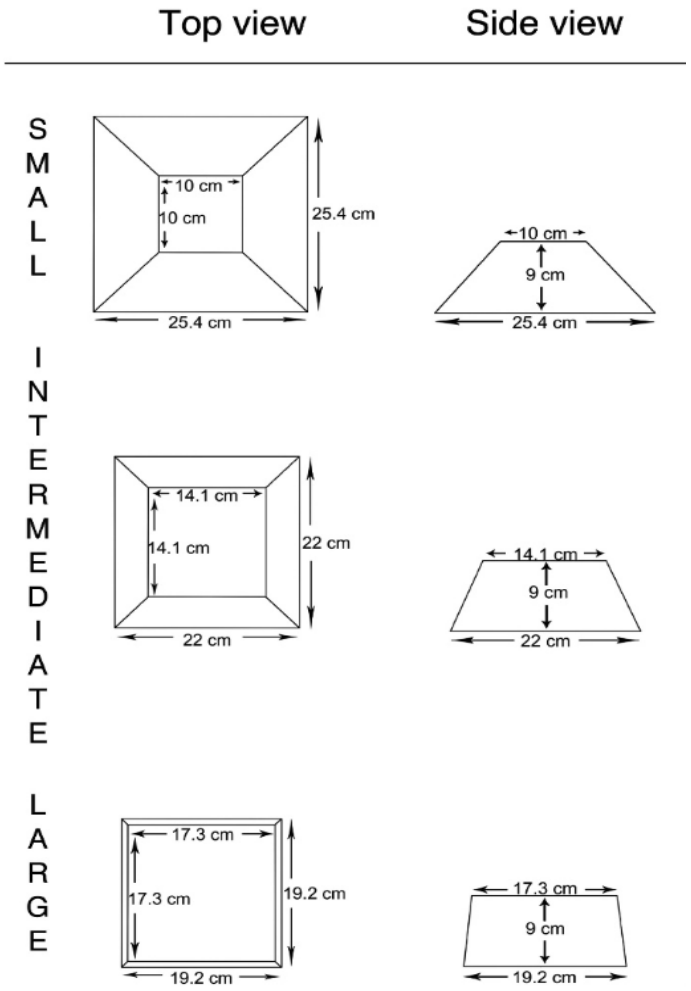
Animal Collection and Husbandry

The 27 *Xenopus laevis* frogs were bred in the lab and kept in a temperature-controlled environment specifically for amphibians. To avoid size variances, only the smallest frogs were chosen for the experiment (1.339 g-2.872 g). The frogs were fed 1-2 pellets of Nasco Frog Brittle per frog every 2-3 days by placing about 6 pellets in each container each time they were fed. Throughout the duration, the light cycle was 12 hours of light followed by 12 hours of dark each day and the temperature of the room was 19°C.

Experimental Design

To test the interaction between surface area size and disease resistance via aquatic environment shape manipulation, frogs were randomly distributed into the treatment groups. Treatments included small surface area, intermediate surface area and large surface area (Figure 1). Three containers for each size were used with three frogs per container, totaling 9 frogs per treatment and 27 frogs overall. The containers each had the same depth and volume but differing surface areas. Each held 3 L of water with a depth of 9

cm. The surface areas of the containers were 100 cm^2, 199 cm^2, and 299 cm^2. Wire mesh (approximately 4 cm) was placed at the top of the containers to prevent them from escaping. Water was cleaned by siphoning approximately 75% of the water from each container at least once a week, as feces and food pieces accumulated. An equivalent volume of water was replaced into each container. The frogs stayed in these containers for 2 weeks, were transferred to identical individual containers and infected and remained in those containers for 13 days before being swabbed and euthanized. The experiment ran for a total of 30 days.



(Calich and Wassersug, 2012)

Figure 1. Diagrams of the surface area manipulation containers. They differ in air-water interface but maintain the same depth (9 cm) and volume (3 L). Surface areas are 100 cm^2, 199 cm^2, and 299 cm^2, from top to bottom.

Bd Inoculation

Once the frogs spent two weeks in their respective containers, they were weighed and transferred into plastic cylindrical containers with a height of 7.5 cm and a diameter of 11.2 cm. To infect the frogs, they were initially placed in 200 mL of aged tap water and were given 2.5 million zoospores of *Bd* directly into the water via a plate and a broth. The frogs stayed in the 200 mL of *Bd* infected water for 2 days before

cleaning the water by discarding 2/3 of the initial water and replacing it with clean, aged tap water until it was about ¾ full (approximately 700 mL). Containers continued to be cleaned once a week in this manor. There was no mortality at the end of this portion.

Amidst the first part of the experiment, on day 7, one frog jumped from a large container, leaving this treatment with just 2 frogs. After infection, on days 18 and 28 a frog from a small container and an intermediate container, respectively, escaped, leaving 24 frogs at the experiment's end.

Swabbing

At the end of the experiment, 13 days after infection, all frogs were swabbed, weighed, and euthanized. The ventral skin surface and hind limbs of the frogs were swabbed according to the procedures of Hyatt et al. (2007). They then were placed in approximately 50 mL of tricaine methanesulfonate (MS-222) for about 5 minutes until the frog was no longer moving or breathing. After confirmed death, the frogs were massed and frozen. Swabs were stored at -20 °C until analyzed with qPCR.

DNA Extraction and qPCR

Bd DNA was extracted with PrepMan Ultra and then analyzed via Real-time Taqman PCR assay according to protocol by Boyle et al. (2004). Standard genomic quantities of *Bd* DNA were used as DNA markers and positive controls in order to confirm success of qPCR. All 24 samples were analyzed via qPCR and the number of genomic DNA/zoospore was used to measure infection intensity in frogs.

Statistical Analyses

I conducted a series of statistical models to test how surface area (the predictor variable) affected frog mass and two metrics of *Bd* infection. In each statistical model, I nested individual frog within container to account for the fact that there were multiple, non-independent, measures within each replicate. In my first statistical model, I tested whether surface area (small, intermediate, or large) was a significant predictor of mass change (the response variable) throughout the experiment using mixed-effects model. To test whether surface area affected *Bd* prevalence, I used a generalized linear mixed-effects model with a binomial error distribution. Lastly, I used linear mixed-effects model with a normal error distribution to test for differences in average *Bd* infection intensity (log-transformed to meet the assumptions of the test) between surface area treatments (the predictor variables).

Results

Change in Mass

The mass of the frogs in each treatment decreased from prior to the experiment to after (Table 1). However, there was no effect of surface area size on average mass change ($p=0.74$). In opposition to my hypothesis, frogs in the intermediate and large surface area containers showed the largest qualitative change in mass while those in the small surface area container showed the least change in mass (n=8, n=9, n=8, respectively) (Figure 2).

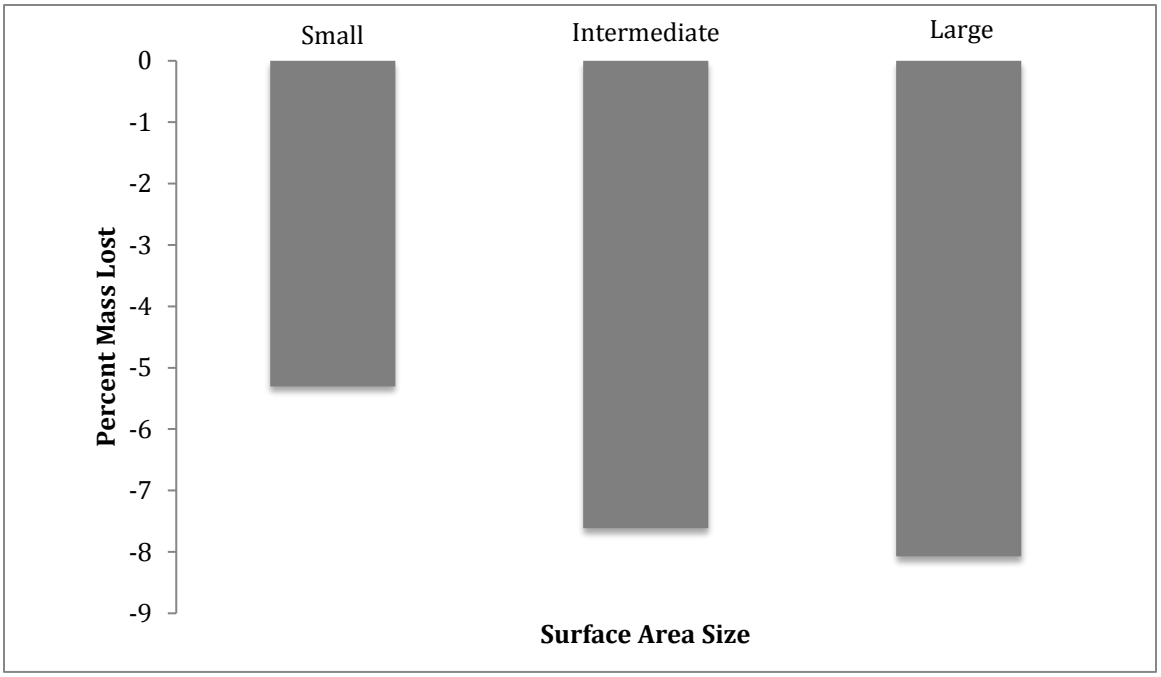


Figure 2. Mean percent change in mass for frogs in differing surface area size from before infection to after infection. All treatment experiences loss in mass. Error bars represent standard error with n=8, 8, 9, respectively. Frogs that experienced the intermediate surface area showed the largest change in mass.

Bd Prevalence and Intensity

No mortality was observed throughout the experiment. After qPCR was run on all 24 samples for infection intensity, seven frogs yielded positive infection results while the remaining 17 had zero infection. Of the seven, two were in the small treatment, two were in the intermediate and three were in the large (Figure 3). The prevalence of infection between treatments was not significant ($p=0.82$). The average log infection intensity was highest in frogs housed in the small surface area treatment with 2.485 average zoospores, followed by large with 2.448 average zoospores and intermediate with 2.277 average zoospores (Figure 4). There was no significance found between treatments and *Bd* infection intensity ($p=0.79$).

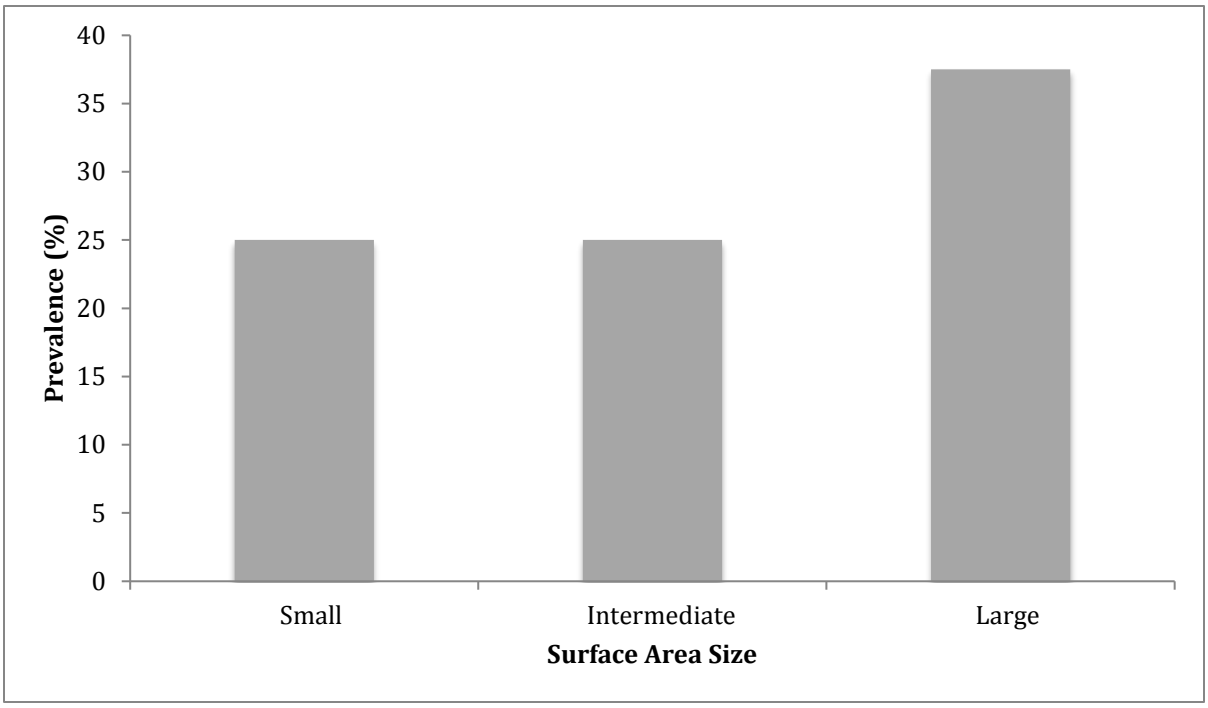


Figure 3. Percentage of frogs infected in each treatment. Overall number of infected is 7 (n=24).

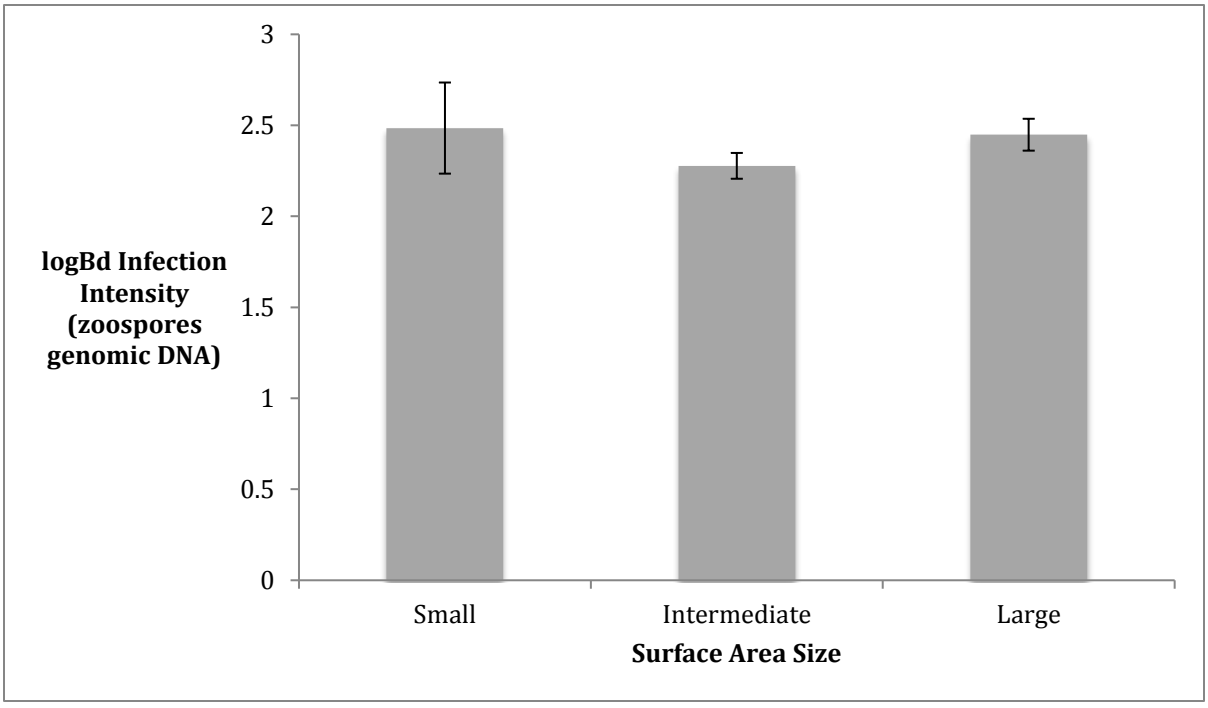


Figure 4. Average logBd infection intensity of frogs in small, intermediate and large surface area containers measured by average zoospores/genomic DNA per swab of those infected. Error bars represent standard error.

Discussion

My results indicate that the shape of the aquatic environment for *Xenopus laevis* frogs does not have an effect on *Bd* infection, in opposition to what I had originally predicted in my hypothesis. Although the infected frogs with the highest intensity were in the small surface area treatment, the large surface area treatment had the highest prevalence of infection. The largest percent change of mass was in the intermediate surface area followed by large surface area and finally the small. Overall, there is no consistency with the treatments and no correlation between surface area size and infection shown.

The prevalence of infected frogs in this experiment was 29%. This is a fairly low percentage considering all frogs were exposed to *Bd* and projected to be infected. This was not expected because, although *Xenopus laevis* frogs have been shown to be rather resistant to *Bd* due to specific antimicrobial skin peptides, incidence among populations is still on the rise. One study showed that naïve frogs became infected in the presence of zoospores after just 11 days, although infection is low at approximately 7.4 ± 3.4 zoospore equivalents. At 20 days, these same frogs increased to an average of 272.5 ± 124.2 zoospore equivalents followed by a sharp decrease around 44 days after exposure (Ramsey et al., 2010). The frogs in my experiment were only measured for infection intensity 13 days after exposure. The antimicrobial skin peptides secreted when alarmed or in danger are effective in fighting off infection, keeping *X. laevis* healthy and free of disease shortly after exposure (Ramsey et al., 2010), potentially being extremely effective in preventing infection. Had they been exposed to the infection for a longer period of time or measured for infection intensity further from the date of exposure, infection may have been higher and a more prominent correlation could have been found.

One other reason the frogs may not have responded how I hypothesized is due to becoming acclimated to their environment. As a response to a chronic stressor, animals often adapt and eventually do not perceive the stressor as debilitating to their overall health (Romero, 2004). *Xenopus laevis* specifically have been found to acclimate to their surroundings over time. One study showed that exposure to lower temperatures did not have an effect on locomotor skills due to their ability to thermally acclimate (Wilson et al., 2000). These frogs were able to modify muscle properties in response to a change of temperature over a period of time while many other species were not able to. Also unlike other frog species, *X. laevis* frogs have effective adaptive immune responses including lymphoid organs, major histocompatibility complex, antibody classes, cytokines and chemokines, and T Cell-mediated immunity (Rollins-Smith et al., 2009). Not only do they have innate immune defenses to help them acclimate to stressors and threats to their health, they also use these adaptive immune responses as these threats may persist. Since the frogs remained in the containers for two weeks, they potentially acclimated to the change in surface area and the resulting decrease in the dissolved oxygen concentration (Calich and Wassersug, 2012). If the change in surface area were presented for a shorter period of time, the frogs may have been less resistant to *Bd* due to the sudden change in environment. The adaptive immune response may not have the opportunity to activate, leaving the frogs more susceptible to a lowered immune system and a higher likelihood of contracting chytridiomycosis. The most successful method may be to infect the frogs after the antimicrobial skin peptides are secreted and before they become acclimated to the stressor or continually decreasing the surface area so they do not have a chance to acclimate.

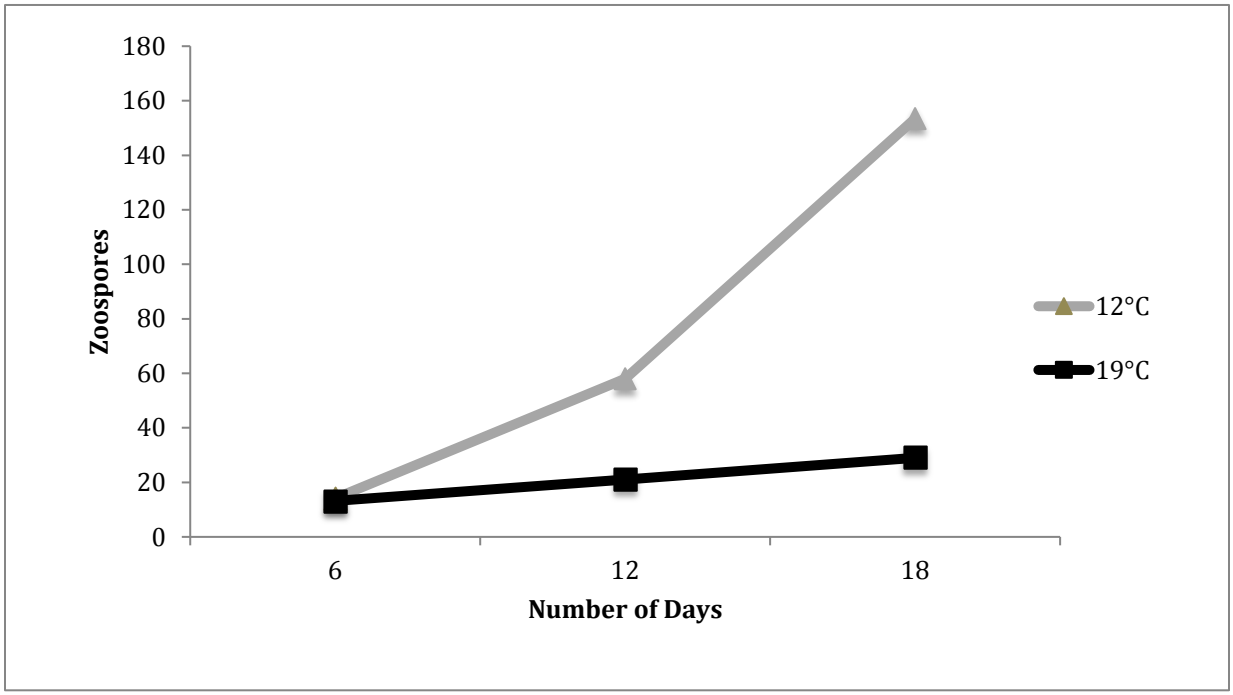
The change in surface area also may have not been a stressor to the frogs. Although the previous study that used these containers showed that breathing rates increased as surface area decreased (Calich and Wassersug, 2012), this is not typically listed as a defense mechanism to stress. This study was also done on tadpoles, which may be more inclined to have higher breathing rates due to allocating energy to both development and stress management whereas the adult frogs can allocate more energy to only stress management and thus better handle it. A more dramatic decrease in surface area could present more substantial results because the frogs then may not have a strong enough adaptive immune response to eliminate the potential damage of the stressor. Another way to stress them out more would be to add a second variable such as a change in depth or volume of water, which could also be altered with climate change. Also, if glucocorticoid levels, specifically corticosterone (CORT), had been measured for each treatment, the effect of the surface area may have been clearer. If CORT levels were measured periodically, when the frogs are stressed would be clear and help understand why or why not and how much the frogs are infected. At this time, there is no way to tell whether the change in environment actually caused the frogs stress or not, and these variables would have given a better idea of the effect of the treatments.

Due to the lab conditions and resources, it is possible that the frogs did not experience higher infection intensity because of the isolate of *Bd* used. Previous research has shown that *X. laevis* frogs are often used as test subjects for *Bd* and prevalence is rarely an issue when doing so. To account for the lack of prevalence in this experiment, it could be due to the specific strand of *Bd* and how it has adapted to its environment. This particular strand of *Bd* was found to respond to temperature differently than expected. Appendix 1

shows that over a period of 18 days, the *Bd* isolate used in this experiment had a significantly higher increase in infection intensity at 12°C than at 19°C (Appendix 1). The frogs in this experiment were kept in a 19°C room, therefore it is possible that the isolate of *Bd* was not strong enough in the temperature provided to infect the frogs as expected. Subsequently, because infection was not significantly prevalent or intense between the treatments, no conclusions can be made on how surface area size effects *X. laevis* frogs' ability to resist *Bd* infection.

Further research would be beneficial to develop a better understanding of the effect of pond shape on amphibians' ability to resist *Bd*. A larger sample size would help amplify the results and potentially alter the outcome. To get a more complete picture of how climate change may affect frog's ability to survive disease, it would be beneficial to test how depth and volume would affect their capability to survive and resist infection. Although this particular study did not produce biologically significant results, how the changing environment affects wild life, like amphibians, is important to research for it can be detrimental to populations of frogs and ultimately on the ecosystem. As the climate changes and temperature rises, habitats will change and the ability to survive could decrease for specific species of animals. Gaining an understanding of climate change and disease will allow us to be more proactive in preventing the seemingly inevitable damage made by it.

Appendix 1



In a separate senior comprehensive project, Victoria Rollin examined the isolate of *Bd* used in this experiment under two separate temperature treatments, 12°C and 19°C. This study showed that the number of zoospores was much higher when inoculated at 12°C as compared to 19°C. This means that this isolate is more virulent at low temperatures. This is abnormal because *Bd* typically thrives in 17°C-25°C but due to being kept in a 4°C refrigerator for over a year, this isolate adapted to the colder temperature and now has higher infection intensity in the lower temperature.

(From Victoria Rollin's paper titled Effect of Temperature on *Batrachochytrium Dendrobatidis* growth in culture)

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