Determining a safe amount of antifungal treatment for *Rana clamitans* tadpoles to reduce *Batrachochytrium dendrobatidis* infections.

By
Jennifer Diethelm
April, 2015



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. Matthew D. Venesky, Ph.D.

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Dr. Rebecca S. Dawson, Ph.D.

Table of Contents

Acknowledgements……...… 4

Abstract……. 5

Introduction……... 6

Methods…… 13

Results…….. 17

Discussion…… 19

References…… 25

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Abstract:

In recent years, the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* ("*Bd*") has been cause for concern because of its ability to drive amphibian populations to extinction. In response, many zoos and aquariums are attempting to preserve at-risk species by utilizing captive breeding programs for species that are becoming extinct in the wild. However, collecting and clearing amphibians of their *Bd* infections is not straightforward because many antifungal treatments can cause mortality even in the absence of *Bd*. The goal then is to find an antifungal treatment capable of clearing *Bd* infections without causing a significant increase in mortality. My research investigated at what dose the common aquatic antifungal Ick© (active ingredient: malachite green) could clear *Bd* infections in Green frog (*Rana clamitans*) tadpoles. I used 2x4 factorial in which Ick doses (none, low, medium, and high) were crossed with *Bd* exposure (non-exposed and exposed). The experiment lasted for 29 days and started on the day of *Bd* inoculation. Throughout the experiment the number of mortalities were recorded along with the respective tadpole identification number. At the end of the experiment the remaining tadpoles were sacrificed and the mouthparts of all tadpoles were swabbed. These swabs were then subject to DNA extraction and qPCR, the resulting statistics were determined using a survival analysis. As predicted, *Bd* exposure reduced tadpole survival ($P < 0.05$). In addition, I detected a significant main effect of Ick on tadpole survival ($P < 0.05$), where tadpoles exposed to the medium dose had the highest mortality. *Bd* exposure and Ick did not interact to affect mortality. Future research is needed to determine if Ick© is the best treatment for *Bd* infected amphibians.

Introduction:

Amphibians inhabit many different global environments and thrive in a range of ecosystems. Their environments can vary from high altitudes in the mountains to sea level, and in arid deserts to tropical rainforests (Blaustein & Wake, 1995). Many species of amphibians have a complex lifecycle that includes the use of terrestrial and aquatic habitats. This migration between habitats occurs predominately during mating season when the males and females relocate to ponds or streams to breed. In the aquatic environment they produce fertilized eggs, which will eventually develop into tadpoles, where upon they metamorphose and leave their aquatic habitat to live in a terrestrial habitat (Werner, 1986). The migration of young amphibians into a terrestrial habitat is a critical lifecycle stage because it allows for dispersal of the species. Although such migrations serve to proliferate amphibian species, it also results in their exposure to a range of parasitic organisms, including fungi, both on land and in water.

Globally, the vast majority of fungi are harmless and survive on symbiotic relationships by consuming dead matter and creating nutrients that can be used in other ways by the ecosystem. However, some fungi are parasitic to plants and animals as they feed on their host's resources (depleting them of essential nutrients). One example is the water mold *Saprolegnia ferax*, which consumes dead cells and waste produced by fish, but also feeds on live fish and amphibian eggs ("Water Mold" *Encyclopedia Britannica Online*). This mold infects aquatic species by creating a cotton-like film over the body of the host. The mold then becomes a fungal infection, generally called mycosis, by penetrating the epidermal layer causing cellular necrosis (forced cell death) and irreparable epidermal damage, which is usually fatal.

Significant declines in frog populations over the last decade due to a combination of infectious fungi such as *Basidiobolus ranarum* (Taylor et al, 1999) and *Mucor amphibiorum* (Berger et al, 1997) may act as early indicators of environmental decline. Amphibian health is highly sensitive to environmental stressors; consequently, declines in amphibian populations (including their extinction) can be indicative of declining environmental conditions. In response to increased frog mortality, zoos and aquariums have begun captive housing and breeding to protect amphibian species that are at risk due to outbreaks of harmful fungi in the environment. The Captive Survival-Assurance Colony project is one of the few programs capturing frogs from areas known to have been impacted by fungal infections in an attempt to clear all individuals of infection before breeding them to aid in rebuilding the species (Pessier, 2008). This program is beneficial in maintaining the genetic diversity of amphibian species, allowing for favorable inherited traits to progress and decreasing the likelihood of inbred populations. However, it is difficult to effectively treat these infected frogs without damaging their health, especially when aggressive treatments must be used to clear infections. Therefore, the issue at hand is to identify an effective treatment to mitigate harmful fungal infections while maintaining the general health of frogs.

Of the fungal infections that the Captive Survival-Assurance project is attempting to treat, *Batrachochytrium dendrobatidis* stands out as being especially troublesome. The fungal infection *Batrachochytrium dendrobatidis* (referred to as '*Bd*') is among the deadliest fungal pathogens for amphibian species. This fungus causes the disease chytridiomycosis, and is highly infectious during the water-borne zoospore stage. Consequently, chytridiomycosis poses a significant impact on many frog species that live in or migrate to aquatic ecosystems (Berger et al, 2010). As a result, *Bd* has a widespread effect on amphibian populations

because it is easily transmitted in bodies of water. The recent discovery that *Bd* has been shown to infect non-amphibian hosts and is environmentally persistent, even in the absence of infected individuals, adds to its deadliness (McMahon et al, 2011). The ability of the disease to infect the hosts outside the usual realm is cause for concern for frogs and other aquatic organisms. As a result, developing methods to effectively clear chytridiomycosis in ways that will not negatively impact the overall health of amphibians or the environment is critical.

Understanding how chytridiomycosis and the host interact is important for determining an effective treatment. Even though frog skin has a protective mucous membrane, *Bd* can migrate past the mucous membrane and attack the keratinized epithelial tissues of maturing youth and adult amphibians. The *Bd* infection then develops into chytridiomycosis, which prevents the skin from transferring the oxygen necessary for respiration and thereby inhibits the regulation of chemical balances within the body dependent on the skin for regulation. Since the skin is such a vital organ to amphibians and chytridiomycosis effectively destroys its function, the dispersal of *Bd* can be devastating to amphibian populations (Whitehouse & Duffus, 2009). There is, however, a limitation to this disease: because tadpoles have not yet produced keratin in their skin they may be able to temporarily avoid chytridiomycosis. This is shown in a study conducted by Blaustein et al (2005) where tadpoles from differing species were infected with the *Bd* fungus and no treatment was given. It was concluded that the frog species *Rana cascadae* and *Rana catesbeiana* showed no increase in mortality compared to the uninfected control group. Some mouthpart abnormailities were detected in *R. cascadae* but the researchers agreed that not every species will react the same way to chytridiomycosis (Blaustein et al, 2005). The

species used in Blaustein's research share the same genus as the tadpoles being tested in this study. Therefore, the likelihood that the *Rana clamitans* tadpoles will survive the chytridiomycosis is high. In addition, the tadpole developmental stage is ideal for studies of *Bd* fungus clearing treatments because tadpoles have not developed the keratinized tissues that chytridiomycosis infects. This means the tadpoles will be infected with *Bd,* but the infection will not progress into chytridiomycosis. Furthermore, because *Bd* is not fatal in tadpoles testing can be conducted on live animals without the risk of high mortality. If tadpoles are exposed to an antifungal medicine that can clear the *Bd* fungus without increasing the rate of mortaility they should have the opportunity to mature into adults unaffected by chytridiomycosis.

The purpose of antifungal treatments are to clear fungal infections by damaging the membrane of fungal cells and depriving them of organic molecules (essentially starving the cells). Unfortunately the actions of antifungal medicines are not straightforward. In the case of antifungals, differentiating between mammalian and fungal cells is more difficult than distiguishing a mammalian cell from a bacterial cell. This is because the mammalian cell membrane contains the steroid cholesterol, which at the biological level appears very similar to the steroid ergosterol that is found in the fungal cell membrane. As a consequence, antifungal treatments have side-effects because they do not always target the correct cells. This can lead to the harm of mammalian cells instead of fungal cells. The mammalian model is being referenced here because amphibian (ectotherm) research has not yet investigated antifungal treatments in as much depth. Therefore existing amphibian research does not adequately elaborate on the disadvantages of fungal treatments but it is assumed that this cellular interaction is conserved across vertebrates as well. When treating *Bd* infected

amphibians it is important to verify the antifungal is doing more good than harm (e.g. not damaging amphibian cells). It would be counterproductive to use a treatment on amphibians that causes more damage to the amphibian than the fungus.

A recent study tested *Bd* clearance using low doses of itraconazole (0.0025%) in the treatment of *Incilius nebulifer* juveniles (Branelley et al, 2012). Of the groups tested, those treated with higher concentrations of itraconazole (0.01%) and infected with *Bd* had higher mortality rates. This indicated the impact of a combined antifungal treatment with the *Bd* infection may have overwhelmed the innate immune response. This is in part due to the fact that juvenile frogs (at the metamorphic stage) have developed more keratinized epithelial tissues and therefore are more susceptible to *Bd* than tadpoles. Even so, this potentially poses a problem because the tadpoles have to be able to survive the infection long enough for a treatment trial to have an observable effect. Additional side effects of treatments with itraconazole revealed that independent of concentration, itraconazole caused depigmentation in *Alytes muletensis* tadpoles (Garner et al, 2009). Although the treatment proved to be effective at clearing *Bd,* depigmentation of tadpoles makes them easier prey for their natural predators and therefore cannot be considered a viable treatment. All of these results suggest that starting treatment for a *Bd* infection at a lower dose would aid in the clearance of the infection while doing the least amount of harm to the health of the tadpoles.

Research into antifungal treatments has been further conducted using General Tonic® on *Alytes obstetricians* tadpoles (Geiger & Schmidt, 2013). The product General Tonic® is widely available and frequently used to treat captive fish with bacterial infections and parasites. Experiments conducted by Geiger and Schmidt (2013) using General Tonic® to treat *Bd* infections indicated the concentration, 0.625 ml per liter, significantly decreased the

infection burden (determined by number of zoospores) and reduced *Bd* prevalence by 60%. However, similar to the itraconazole treatments, the health of the tadpoles was negatively impacted. Results showed that the lower concentrations had no effect on mortality rates, but high concentrations of the treatment (2.5 ml per liter) using General Tonic® reduced tadpole survival by 60% (Geiger & Schmidt, 2013). Based on these findings, the most beneficial antifungal treatment for tadpoles should be given at doses low enough to decrease the load of the *Bd* infection without causing an increase in amphibian mortality.

Treatment studies have also tested malachite green for clearing *Bd* infecttions. Malachite green is the primary active ingredient in Ick Guard® which is made for the treatment of the aquarium fungus Ick (also known as Ich). In one of the studies conducted on amphibians using malachite green, the results were limited to the successful clearance of *Bd* in adult *Xenopus tropicalis* (Tropical Clawed Frog), but was unsuccessful in the treatment of *Bd* in adult *Hymenochirus curtipes* (Western Dwarf Clawed Frog) (Berger et al, 2010). Based on these mixed results, it is unclear if the chytridiomycosis overwhelmed the frog immune system of the species *H. curtipes*, or if it was the malachite green treatment that failed to effectively clear the *Bd* infection. Since past research using Ick Guard® has been limited to testing on fish and other common aquarium species such as snails and shrimp, there is an opportunity for testing of Ick Guard® on frogs. It is the aim of my study to address whether Ick Guard® can clear *Bd* infections in tadpoles without causing an increase of mortality in the population.

I will be using a 2x4 factorial design, where *Rana clamitans* tadpoles will be placed in the infected or non-infected group and one of the four levels of treatment (none, low,

medium, or high). The resulting data will determine how effective Ick Guard® is at clearing *Bd* infections and it's impact on amphibian mortality.

The main effects of this experiment are concerned with tadpole survival rates, specifically tadpoles' survival as it relates to only *Bd* infections or only fungicide treatments. In terms of *Bd* itself, when *Bd* infections are high I hypothesize that tadpoles will have low survival rates. In addition, when *Bd* infections are low, I hypothesize that the tadpoles will have a lower survival rate than non-*Bd* infected tadpoles, but a higher survival rate than those with high *Bd* infections. When looking at tadpoles in the Ick Guard® treatments, if the fungicide is not toxic to the tadpoles, I hypothesize the survival rate of the tadpoles will be similar to the control group when the fungicide is in high concentration. However, if the fungicide is toxic to the tadpoles I predict the fungicide treatment will result in decreased survival rates, independent of the concentration tadpoles are exposed to.

Delving further into the interactions between *Bd* infections and fungicide treatments, I hypothesize when the fungicide concentration is high and the *Bd* infection is high there will be some clearance of chytrid but a slight decrease in survival rates. Comparatively, when the fungicide concentration is high and the *Bd* infection is low there will be substantial clearance of chytrid with normal (comparable to the control group) survival rates. Furthermore, when the fungicide concentration is low and the *Bd* infection is high there will be minimal clearance of chytrid with a low survival rate. Lastly, when the fungicide concentration is low and the *Bd* infection is low there will be some clearance of chytrid with unaffected survival rates.

Methods:

(a) Collection and Husbandry of Tadpoles

I collected early stage *Rana clamitans* tadpoles (n=100) from a pond in Crawford County, PA, USA on the 18th of October 2014. The pond on this site has not been reported for prevalence of *Bd*. After collection, the tadpoles were promptly taken back to the Steffee Biology Wing at Allegheny College and transferred from their original holding buckets into three separate 38-liter tanks in a temperature-controlled lab. The temperature of the room was kept at 18°C (±1°C) for the duration of the experiment. I waited 24 hours before changing the water to allow the tadpoles to acclimate to room and water temperature. It was crucial that the water used throughout the experiment was de-chlorinated due to the fact that the amount of chlorine used to sanitize public tap water is toxic to amphibians (DeNardo, 1995). To ensure full de-chlorination, tanks of tap water were aerated for seven days. Tadpoles were fed Tetra® fish flakes *ad libitum* every three days and maintained in the laboratory on a 12:12 light:dark photoperiod throughout the experiment. In preparation for the experiment, tadpoles that had similar developmental stages were separated into individual plastic containers filled with 300 mL of aged tap water. Selecting tadpoles of the same developmental stage was important for this experiment because chytrid impacts the different stages of tadpole development in various ways (Marantelli et al, 2004). Therefore, tadpoles within the same stage of development were used to reduce extraneous variables. Once a week all containers were cleaned in the temperature-controlled room by disposing of the old water and replacing it with the aged tap water. This was done by using small mesh filters to hold the tadpoles in their containers while draining the water into a bucket then adding 300 mL of the fresh water into the empty container. In total there were 80 tadpoles, each individual was placed in it's

own container and given a random number between 1 and 80. These containers were then marked with their respective conditions (*Bd* positive or negative and the level of Ick treatment) and indiscriminately placed on three shelves in the temperature-controlled lab to prevent confounding variables due to differences in exposure by location.

(b) Bd Inoculation

Using the protocols of Longcore et al (1999) *Bd* was grown and collected by adding sterile water to cultured plates then gathering the zoospores that were released from the zoosporangia. The count of colonies showed 800,000 zoospores/mL, factoring in that there were 2 mL per container this meant tadpoles in the *Bd*-exposed treatment (n=40) were inoculated with $1.6x10^6$ *Bd* zoospores. In a previous study using toadlets it was found that exposure to $1x10^6$ zoospores for three days was sufficient to cause infection (Carey et al, 2006). On the day of inoculation (Day 0), I used half the amount of water for each container so the zoospore to volume ratio was higher. Twenty-four hours after inoculation aged water was added to each container to bring the volume back to 300 mL (Day 1). I did not immediately replace all of the water from the *Bd* treated containers in order to prolong the exposure time to a week. Tadpoles from the non-exposed treatment (n=40) were given 2mL of inoculate without *Bd* zoospores and had a delayed water change as well to ensure similar treatment of all tadpoles. To prevent accidental *Bd* contamination water changes for the non-infected tadpoles were performed before the infected tadpoles and the wastewater was sterilized using bleach. Additionally gloves were worn at all times and both gloves and mesh filters were replaced between treatments. Two weeks were given after the inoculation of *Bd* before treatments began and during this time mortalities were recorded daily and dead tadpoles were placed in individually labeled scintillation vials that were preserved in a

freezer. These tadpoles were preserved so that mouth swabs could be taken at a later time for use in DNA extractions (see qPCR analysis section).

(c) Ick Treatment

In order to prolong the amount of exposure time to the treatment I delivered the dose of Ick directly into the tadpoles' containers (instead of briefly soaking them in a bath and then returning them to their individual containers). Tadpoles were either given no Ick or one of three different Ick doses depending on their predetermined groups (Day 14). I calculated these doses so that the lowest dose was 25% below the Ick brand's recommended dosage, the medium dose was given at the recommended dosage and the high dose was 25% above the recommended dosage. These amounts were hand calculated so that the dosage could be given using a micropipette into the individual 300mL containers to meet the percentages as mentioned above. The final concentrations used for the experiment were: low dose of 28.1 μl Ick per 300 mL water, medium dose of 37.5 μl Ick per 300 mL water and the high dose of 46.9 μl Ick per 300 mL water. Tadpoles were given their respective doses twice a week, on Mondays and Fridays, starting on the 17th of November (Day 18) and continuing until the 1st of December (Day 28) for a total of 5 Ick treatments. Before each treatment was given the water was changed so that the doses of Ick would not build on the previous concentration causing an increase in the overall dosage of Ick. Once the water change was complete the tadpoles were randomly placed back on one of the three shelves in the temperature-controlled lab. Throughout this portion of the experiment mortalities were recorded daily and dead tadpoles were processed and preserved in a freezer for later DNA extraction.

(d) DNA Extraction and Quantitative PCR Analysis

Quantitative polymerase chain reactions are used in labs to distinguish target DNA via running samples with fluorescent dyes through a thermal cycler. I used it in this experiment to detect the quantity of chytrid DNA present because the amount of chytrid DNA (quantified by the number of zoospores present) equated to the infection load of the tadpole. Meaning that a high quantity of chytrid DNA signifies a high level of infection and a low quantity of chytrid indicates a low level of infection. Prior to collecting samples, tadpoles were euthanized with an overdose of Tricaine-S (Day 29). Afterwards all tadpoles were swabbed by placing fingers on either side of the mouth and applying enough pressure to part the lips, then a sterile q-tip was placed inside the mouth and rotated 360° ten times. Once the mouth swabs were collected in vials, labeled and stored in the freezer all tadpoles were placed in 100% ethanol to clear the remaining *Bd* infections. Mouth swabs were then subject to DNA extraction, for this I used protocols adapted from Boyle et al (2004). After the DNA extractions were diluted to a 1:100 ratio they were processed using qPCR analysis in a step-one real-time PCR system, methods for this were modified from the procedure used in Hyatt et al (2007).

(e) Statistical Analysis

After mortality and qPCR data were obtained a survival analysis was run. The purpose of a survival analysis is to determine whether various treatments affect a separate factor (Miller, 2011). Because few of the *Bd*-exposed tadpoles were infected at the end of the experiment, I focused my analysis on the effects of *Bd* exposure (instead of *Bd*-infection) on survival. I conducted a survival analysis using the “coxph” function in the “survival” package of R statistical software. I used survival to day 29 as a proxy for host fitness and censored all

individuals surviving on day 29 to account for the lack of information about their true times of death (a standard approach to down-weight the influence of these individuals in the survival analysis). I tested for main and interactive effects of *Bd* exposure (exposed, not exposed) and fungicide exposure (none, low, medium, or high) on the duration of time that tadpoles lived. I assessed statistical significance ($P < 0.05$) using the "Anova" function in the "car" package in R.

Results:

My qPCR results showed that only two of the forty tadpoles that were exposed to *Bd* were infected. Due to these results, tadpole DNA from the control and non-infected groups were not run through qPCR as the probability that they were contaminated was highly unlikely. For this experiment, I used exposure to *Bd* (instead of *Bd*-infection) and the effects of exposure to Ick as predictors. I also tested for an interaction between exposure to *Bd* and exposure to the fungicide. Results from the survival analysis revealed a significant relationship between mortality and the *Bd* infection ($P< 0.05$), and additionally mortality and the fungicide treatment proved to be significant ($P< 0.001$). There was no significant interaction between *Bd* infection and the fungicide treatment on mortality ($P= 0.125$). Trends from the graph for mortality and *Bd* exposure show that the infected population had a higher mortality rate than the non-infected population throughout the progression of the experiment (Figure 1). This trend persisted even though only two individuals from the *Bd* exposed population were infected. Trends from the graph for mortality and fungicide treatments exhibited a high percentage of mortality in the medium fungicide treatment, followed by the second highest mortality in the high fungicide treatment (Figure 2). Both the low fungicide

and no fungicide treatments had reduced mortality with the untreated tadpole mortality falling slightly below the low fungicide mortality (Figure 2).

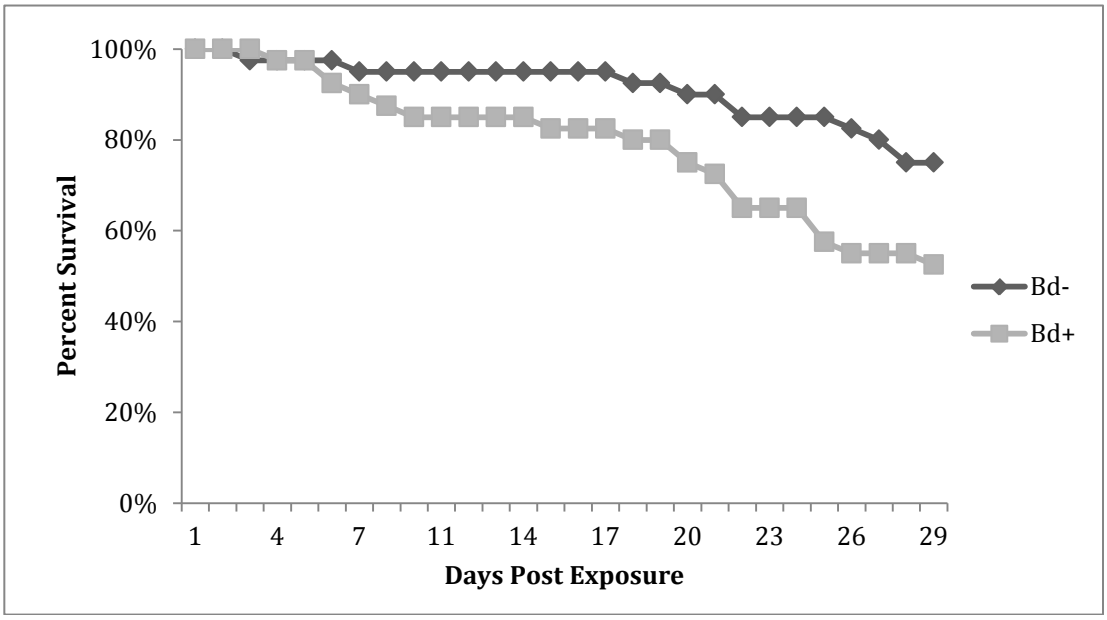


Figure 1. Comparison of percent mortality between Bd infected tadpoles (Bd+) and non-infected tadpoles (Bd-) for the extent of the experiment. Significant differences in mortality were detected between infected and non-infected populations ($P< 0.05$).

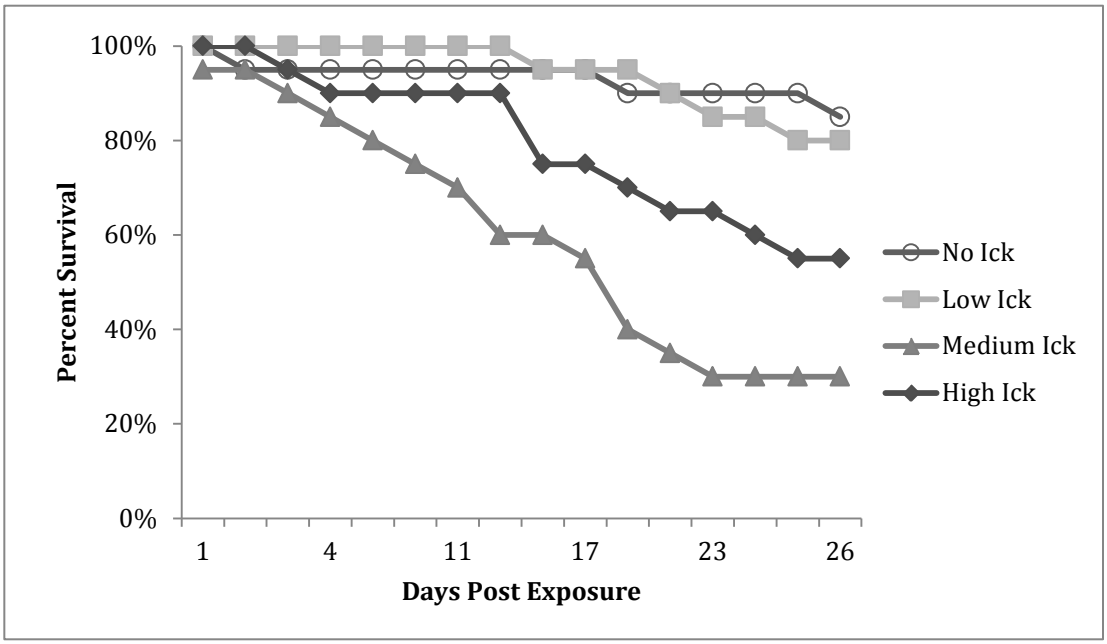


Figure 2. Comparison of significant percent mortality between fungicide treatment levels for the extent of the experiment ($P<0.001$). The medium fungicide treatment had the highest mortality, followed by the high fungicide treatment with the second highest mortality, then the low fungicide treatment and no fungicide treatment, which had the lowest mortality.

Discussion:

The results from this study reveal two major points. First, the *Bd* infections appear to negatively impact more that just tadpole skin respiration. Observations show that tadpoles, which weren't infected, display mortality trends similar to infected tadpoles. Indicating that there are additional health costs associated with exposure to *Bd* zoospores. Second, Ick is lethal at moderate and high doses, as demonstrated by the decrease in the rate of survival of the Ick treated groups, and therefore should not be relied on as a dependable antifungal treatment for *Rana clamitans* tadpoles. Based on these results, the outcome of this experiment exhibits that *Bd* infections have diverse effects on the different species and life stages of amphibians. Additionally, more research needs to be conducted in the field of the toxicokinetics of antifungal treatments (such as Ick) to see if the benefits of the treatment outweigh the cost to the host organism's health.

Although the majority of tadpoles in the *Bd* exposed group were not infected, it is interesting that the rate of survival for this group mirrored what one would expect if they had been infected. As shown by the data, the infected group of tadpoles had a significantly lower rate of survival and overall more deaths than the non-infected group (Fig. 1). In past studies, exposure to *Bd* has also demonstrated high rates of mortality, as found by Tobler and Schmidt (2010) in their "between and within population" studies with *Alytes obstetricans* toadlets, where 90% of the infected specimens died. In a study conducted by Berger et al (2005) all three strains of *Bd* resulted in significant mortality in *Litoria caerulea* metamorphs, although time until death varied greatly. In comparison, my results suggest that even though there was no measurable amount of *Bd* infection, something was negatively

affecting the Bd exposed tadpoles' health to create a similar effect to those that were infected by the BD exposure.

The qPCR sampling data revealed that very few of the tadpoles from the infected groups tested positive for the chytrid infection. This did not match what had been predicted at the beginning of the study; which was that the zoospore count met the required density necessary to cause infections and that the *Rana clamitans* tadpoles would be susceptible to infections (reference dose adapted from Retallick & Miera, 2007). There are several reasons that could justify why there was an over-abundance of non-infected tadpoles in the exposed group.

In a study done by Garner et al (2009b) *Bd* infections correlated with life history trade-offs, such as growth cost and mortality. It was found exposure to *Bd* during the tadpole life stage caused mortality before and after metamorphosis even though these individuals did not test positive for *Bd* infections after they had died. They suggested that in instances of low infection the host might be able to cure itself. However, they also postulated the amount of resources the host uses in order to do this could lead to a compromised immune system being overwhelmed causing the host to die even in the absence of the infection. For my experiment this means that after the period of inoculation (before the start of treatment 2 weeks later) the tadpoles could have fought off their infections. Unfortunately, the addition of the antifungal treatment to their already compromised immune systems could have depleted their resources and caused fatality. Support for this theory comes from research done by Graham et al (2005) on immuno-pathological costs for infections and Pessier et al (1999) for observing that *Bd* infections can lack obvious physical modifications.

Another explanation for the low infection rate could be the tadpoles had low *Bd* infection loads to begin with and the Ick treatments successfully cleared it, so when the DNA swabs were collected at the end of the experiment they would have shown no infection load. Support for this is shown in the study conducted by Garner et al (2009a) where tadpoles infected with *Bd* were successfully cleared of their infections using the antifungal itraconazole. It is possible, but not probable, Ick cleared the infections. However, if the Ick-treated tadpoles were cleared of their infections then the control tadpoles (also infected) should have remained infected.

The low infection rate may also have been influenced by a difference in methods for inoculating the tadpoles with *Bd*. In this experiment a density of $1x10^6$ zoospores was used for the infectious dose while Carey et al (2006) found a density of zoospores between $1x10^7$ and $1x10^8$ was necessary to reach a threshold of infection that caused significant mortality in adult *Bufo boreas*. Carey's research may not be not directly comparable to this study because of the difference in the life stages being tested, but in terms of relevance this study is one of few that has found a zoospore count as low as this to be infectious and fatal.

Lastly, studies have shown that the species *Rana clamitans* may have a low susceptibility to *Bd* infections. As was found by Blaustein et al (2005) in a study conducted on interspecific variation to *Bd*, larvae of *Rana cascadae* and *R. catesbeiana* did not die after exposure to *Bd* whereas larvae from the *Bufo boreas* population showed an increase in mortality because of the infection. Due to the fact that the experimental tadpoles are also from the genus *Rana* this potential immunity could have contributed to the deficiency in the amount of tadpoles that were infected.

Based on the significant results from the fungicide treatment, it seems the increased doses of Ick could be responsible for the high mortality of the tadpoles. Further examination of Figure 2 shows the medium treatment has a lower survival rate than the high treatment. This is inconsistent with a normal dose response curve, but overall the doses follow the expected trend, increased doses of Ick are more lethal than low doses of Ick. This anomaly could be an error due to the fact that a small population is prone to individual differences in susceptibility to infections and consequently results could be skewed. Another possibility, as shown by a study conducted by McMahon et al (2011), is it not unusual for pesticide treatments to result in non-monotonic dose response curves. As tadpoles from their sampling groups demonstrated, both low and high concentrations caused significant mortality while medium doses did not. Within this study they also found additional aquatic vertebrates and invertebrates showed similar sensitivity to the pesticide chlorothalonil, which is thought to have such an effect because it is an endocrine disruptor. The commonly used pesticide, atrazine, was another product determined to have a non-monotonic dose response curve for both early and late developmental stages of *Rana clamitans* frogs. Again, the authors came to the conclusion this pesticide could potentially be an endocrine disruptor because the mechanism causes interference with endogenous enzymes and hormones making it an immunomodulator (Storrs and Kiesecker, 2004). In this case a non-monotonic curve for Ick has the reverse effect with the moderate dose having the worst response (e.g. higher fatality) and the low and high doses producing more favorable responses (e.g. reduction in mortality) (see Figure 3 as reference).

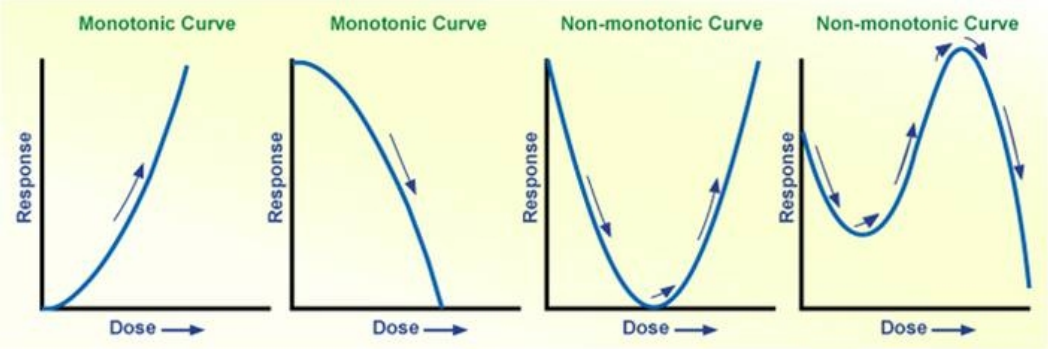


Figure 3. The non-monotonic curve of the fourth chart displays the potentially lethal reaction to the antifungal Ick as the dose increases. Both low and high doses of Ick would cause less mortality and the added benefit of clearing the *Bd* infections whereas a medium dose has an increased rate of mortality. (http://epa.gov/ncct/edr/non-monotonic.html)

Furthering this idea, the non-monotonic effect of Ick doses could be the result of a reaction to a secondary metabolic pathway within amphibians used to process high concentrations of toxins. In this model the higher dose may be "less lethal" because the secondary pathway is more efficient in transforming and/or excreting the toxin out of the body than the primary metabolic pathway. The moderate dose would then cause damage to target organs because of the inability of the host to process it quickly enough using the primary pathway. For this data to be sufficient for a conclusive statement supporting or rejecting these theoretical models, there needs to be further research into these toxicokinetics using a larger population (because smaller populations can have varied results due to less individual variety, as mentioned before).

Recommendations for future research include, in-depth studies on toxicokinetics of antifungal treatments, studies using large populations with variations in life stage and species, and lastly creating a compendium of past research on *Bd* infections. With these advances, not only will researchers be able to access known facts about *Bd* infections, they will be able to conduct more studies on how antifungals are impacting the extensive internal

chemical pathways of the host and if the costs outweigh the benefits. New directions may also be taken in which the host is not treated but the environment that the host lives in is altered. Such as the addition of the *Bd* consuming zooplankton (*Daphnia magna*) into ponds where they can reduce the density of *Bd* zoospores and decrease the probability that amphibians will reach a threshold for infection (Buck et al, 2011). In conclusion, there are still many options available for the treatment of *Bd* infections and researchers should continue to explore these to further protect amphibians worldwide.

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