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# Emerging Infectious Disease and the Imperiled Relict Leopard Frog

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# EMERGING INFECTIOUS DISEASE AND THE IMPERILED RELICT LEOPARD FROG

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## Abstract

The disease chytridiomycosis, caused by the aquatic fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), has emerged as a major contributing factor for worldwide amphibian declines. Although relatively recently described, the impacts from the disease this pathogen causes have been definitively tied to amphibian declines, including some that occurred decades ago. In some cases, declines of individual species occurred with little documentation and are thus poorly understood. The relict leopard frog (*Rana onca = Lithobates onca*) has experienced such a decline and by the latter part of the 20$^{th}$ century only occurred in two general areas in southern Nevada. Recent research has found *Bd* within the historical range of the species and that the species shows evidence of potential resistance to chytridiomycosis. Those authors, however, noted that the *Bd* strains used were not from the local environment and they speculated on possible attenuation. I addressed these concerns by challenging an anuran species known to be susceptible to chytridiomycosis to one of the previously used *Bd* isolates (SLL) that showed hypovirulence towards *R. onca*. I also performed a disease transmission experiment intended to increase virulence in SLL towards *R. onca* in an attempt to elucidate the possibility of attenuation. In other experiments, I isolated *Bd* from anurans in the local environment, and then used these isolates to challenge juvenile *R. onca*, as well as an earlier life-stage thought to be more vulnerable to chytridiomycosis. My results indicate that the SLL isolate is still virulent toward a susceptible host species, but *R. onca* continued to appear resistant toward this particular isolate. My challenge experiments using local isolates of *Bd*, however, showed that *R. onca* is susceptible to

chytridiomycosis from two *Bd* isolates found in southern Nevada, as well as an isolate I acquired from a commercial vender. I found that frogs from a currently *Bd* infected area cleared infections and survived in much higher proportions than those from a *Bd*-free area. This population-level effect, however, was dependent on life-stage and recently metamorphosed frogs from both areas showed low survivorship when challenged with *Bd*.

## Acknowledgments

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## Dedication

I would like to dedicate my thesis to my grandfather, Anthony King (Kulczyk), for taking me on wild adventures in the rural deserts of the southwest and ultimately inspiring my career path. Grandpa Tony, as I called him, was a Great Depression raised, World War II veteran, whose love for the natural world was unmatched. He was a professional photographer whose pictures of Las Vegas celebrities are hung in museums, but it was his images of the outdoors that I will cherish for the rest of my life. He often took my out into the "sticks" to catch lizards, fish, and frogs. These outings would often take us into state or national parks. As I would chase down whatever critter interested me, he would capture the most breathtaking scenic images that I now hang proudly in my home. I credit my sense of stewardship and passion for biological sciences to him and will be forever grateful. I know he would be profoundly interested in the research I have done at UNLV and would have probably ended up out in the field catching frogs with me. Na zdrowie Grandpa Tony!

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# CHAPTER 1

## Introduction

The aquatic fungal pathogen, *Batrachochytrium dendrobatidis* (commonly referred to as *Bd*), is a major driver of declines of amphibians worldwide (Stuart et al. 2004). *Bd* has a broad physiological tolerance (Piotrowski et al. 2004) and is referred to as a generalist pathogen infecting hundreds of amphibian species from all three orders: caudates, anurans, and caecilians (Gower et al. 2013). *Bd* occurs on every continent where there are amphibians and continues to spread to new localities (Olson et al. 2013; Kolby 2014; Bletz et al. 2015). The pathogen is presented with unique challenges from host immune systems and for resource acquisition in different host species, which are generally thought to ultimately impact pathogen fitness in a host-dependent manner (Ellison et al. 2017). The pathogen, however, may respond to different species by changing what genes are expressed, thereby allowing the pathogen to shift resource allocation rather than undergoing evolutionary changes (Ellison et al. 2017).

The *Bd* pathogen has a simple life-cycle consisting of a motile infectious zoospore stage and a reproductive structure called a zoosporangium (Berger et al. 2005). Infections are initiated when the motile zoospore, potentially guided by chemotaxis, attaches to a host's outer surface of the stratum corneum (Berger et al. 2005). Subsequently, a germination tube is produced that extends deeper into the epidermis (van Rooij et al. 2012). The germination tube carries cellular contents that mature into a zoosporangium,

which is a chitin-surrounded sphere with conspicuous hyphae that clonally produces zoospores (Berger et al. 2005; Greenspan et al. 2012; van Rooij et al. 2012).

Infection by *Bd* can lead to the disease chytridiomycosis, which, among other symptoms, is characterized by excess skin sloughing, skin redness (erythema), hyperplasia, hyperkeratosis, and in terminally ill individuals, the loss of righting reflex (Voyles et al. 2009). Chytridiomycosis can be fatal and diseased amphibians are thought to succumb to infection through the loss of vital electrolytes, leading to asytolic cardiac arrest (Voyles et al. 2009). Chytridiomycosis can have severe impacts on amphibian populations and species diversity, which can occur over very short time scales (Rachowicz et al. 2006; Gillespie et al. 2015). For example, in the Sierra Nevada of California (USA), populations of *Rana muscosa* have been decimated by chytridiomycosis (Rachowicz et al. 2006). The disease has driven extirpations of the species from several localities, sometimes within only 1 year (Rachowicz et al. 2006). In the tropical forests of El Copé, Panama, 41% of amphibian diversity was lost shortly after the arrival of *Bd* (Crawford et al. 2010). Similar observations have also been made in Australia where approximately 20% of all amphibians are thought to have declined from chytridiomycosis (Scheele et al. 2017). In many cases, however, the impacts of chytridiomycosis were experienced many years before the disease or causative agent were described (Cheng et al. 2011; De León et al. 2017).

The Monteverde golden toad (*Bufo periglenes*) disappeared from its cloud forest habitat in Costa Rica sometime between 1987 and 1988 (Pounds et al. 1994), which

preceded the description of *Bd* and chytridiomycosis by a decade (Berger et al. 1998, Longcore et al. 1999). Although not fully understood at the time, many thought the species vanished due to abnormal climatic conditions (Pounds et al. 1994). More recent work using museum specimens, however, demonstrated that the decline of amphibians in Costa Rica was likely coincident with the arrival of the *Bd* pathogen (Cheng et al. 2011). Museum specimens taken before observed amphibian declines in southern Mexico, Guatemala, and Costa Rica tested negative for *Bd* infection, while specimens taken during and after the years of declines tested positive (Cheng et al. 2011). The authors also proposed a southern spread of the pathogen, based on the temporal detection of *Bd*, suggesting the pathogen spread from southern Mexico in the 1970's and arrived in Costa Rica by 1987 (Cheng et al. 2011). Similar research has been done in California, first identifying *Bd* in museum specimens of cascades frogs (*Rana cascadae*) in 1978 around the time when declines were first observed (De León et al. 2017). Other species, however, declined with little documentation (Pounds et al. 1994; Skerratt et al. 2007; Fisher et al. 2009).

Sometime during the 20$^{th}$ century, the relict leopard frog (*Rana onca = Lithobates onca*) experienced a dramatic decline that has little documentation (Bradford et al. 2004). Although the species likely declined for many reasons, originally disease was not proposed as a contributing factor. Recently, however, the impacts of disease have been singled out as a possible facilitator for the decline of *R. onca* (Forrest & Schaepfler 2011; Jaeger et al. 2017).

Jaeger et al. (2017) found the *Bd* pathogen in several sites within the historical range of *R. onca*, including one of the persisting natural populations of the species. In laboratory challenge experiments, however, juvenile *R. onca* appeared to be resistant to at least two isolates of the fungus from California known to be extremely virulent toward other anuran species (Jaeger et al. 2017). The authors concluded that *R. onca* may be innately resistant to chytridiomycosis or may have recently evolved resistance. They cautioned, however, that the laboratory *Bd* strains used may have lowered levels of virulence than those in the local environment. Strains of *Bd* in the laboratory that are grown and serially transferred to new growth media have been shown to weaken in their virulence to their original host, referred to as attenuation. (Langhammer et al. 2013; Refsnider et al. 2015). Therefore, Jaeger et al. (2017) speculated that the isolates used may have had weaker virulence, potentially adapting to the culturing environment.

The purpose of my research was to provide clarity on the virulence characteristics of *Bd* from the local environment towards *R. onca,* and to assess the possible confounding factor of attenuation posed in the previous study. I also aimed to evaluate the susceptibility of *R. onca* to chytridiomycosis at a possibly vulnerable life-stage following metamorphosis. To do so, I initiated a set of laboratory challenge experiments with two general aims: determine *R. onca* susceptibility to *Bd* from the local environment, and determine if the *Bd* isolates used in Jaeger et al. (2017) had attenuated (referred to as SLL, described in Chapter 3). I addressed the first aim by challenging *R. onca* to isolates of *Bd* from the local environment at two distinct life-stages. I addressed

the second aim by exposing a group of White's treefrogs (*Litoria caerulea*), a species known to be susceptible to chytridiomycosis (Voyles et al. 2009), to the SLL isolate. If SLL had attenuated, I expected high survivorship in the exposed *L. caerulea*. I also addressed this aim by exposing a group of *R. onca* to SLL, allowing infections to develop, and subsequently cohousing them with naïve, uninfected partners. Prior research by Brem et al. (2013) involving an attenuated laboratory strain of *Bd* showed that after infecting a host, the pathogen can regain some virulence. They infected a group of eastern spadefoot toads (*Scaphiopus holbrooki*) with a strain of *Bd* (JEL 284) that had been maintained in the lab for several years and appeared to have weakened virulence. The authors re-isolated the fungus from an infected toad and evaluated differences in virulence between the original isolate and the re-isolate. The re-isolated JEL 284 showed greater virulence towards hosts, resulting in high mortalities and decreased survival time. In my experiment, I chose to cohouse frogs rather than re-isolate the pathogen to make the experiment more ecologically relevant. If the *Bd* isolate SLL had attenuated, and *R. onca* was in fact susceptible to chytridomycosis, I expected low survivorship in the second exposure group.

A major initial factor in my research was obtaining viable, isolated cultures of *Bd* from the local environment for challenge experiments. While initiating these efforts, I developed a refined protocol for isolating *Bd*. When I began *Bd* isolations, I had some success, but in many cases the microbial media used to grow *Bd* quickly became overrun with unwanted microbial growth. Therefore, working from published methods (Longcore

et al. 1999; Piovia-Scott et al. 2015), I optimized a *Bd* isolation protocol and conducted an analysis of my protocol's efficacy using two common species found in the American Southwest, *Pseudacris regilla* and *Pseudacris triseriata* (Waddle et al. *in review*; Chapter 2). I sampled tissue from three anatomical regions on these animals where the pathogen is thought to concentrate; the abdomen, thigh, and feet (Berger et al. 1998; Brannelly et al. 2017; Ohmer et al. 2017). I then determined which region had the most utility in yielding isolated *Bd*, and evaluated the effects of infection intensity of the sampled frogs on *Bd* isolation success. All protocols involving these animals were approved under IACUC protocol 870994.

Efforts to understand the effects of *Bd* on amphibians in southern Nevada have been limited to only two published studies (Forrest et al. 2015; Jaeger et al. 2017), and only one of these papers (Jaeger et al. 2017) regards *R. onca* and *Bd*, on which I am a coauthor. Jaeger et al. (2017) was the first to ask questions regarding the distribution of *Bd* within the historical range of the species, and the first attempt at determining the susceptibility of the species to chytridiomycosis. I began assisting on this project as an undergraduate and have formulated inquiries as I advanced into graduate school. My development of an optimized *Bd* isolation protocol was written and submitted as a manuscript intended for publication. The version of this work presented in Chapter 2 is written in the plural nominative "we" as it would appear in my publication. My efforts assessing attenuation of *Bd* and further evaluation of *R. onca* susceptibility to

chytridiomycosis is also presented in Chapter 3 as it would appear in a manuscript for publication.

Although my research shows that *R. onca* can be highly susceptible to chytridiomycosis, I remain positive that future research and mitigation efforts will prove successful. My hope is that the information provided here may improve conservation activities for the species, provide opportunities for future inquiries into this system, and ultimately preserve *R. onca* for future generations.

# CHAPTER 2

## A Systematic Approach to Isolating *Batrachochytrium dendrobatidis*

## INTRODUCTION

The amphibian disease chytridiomycosis, caused by the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), has been implicated in declines of amphibian populations and species worldwide (Berger et al. 1998; Longcore et al. 1999; Stuart et al. 2004). Isolating *Bd* from the wild poses some challenges for researchers studying the ecology of this infectious pathogen and attempting to develop mitigation approaches. Protocols for isolating *Bd* from amphibians have been published (Longcore et al. 1999, 2000; Piovia-Scott et al. 2015), and working from these protocols, we developed an approach that included a unique combination of antibiotics to reduce bacterial contamination.

We systematically assessed the isolation success of our protocol on two Hylid frogs in the genus *Pseudacris*. We also evaluated the relationship between isolation success and *Bd* infection intensity of the frogs, and compared isolation success across tissues collected from three anatomical regions where the pathogen is thought to concentrate (Berger et al. 1998, 2005; Puschendorf and Bolanos 2006; Hyatt et al. 2007). Species of these treefrogs are quite common across North America and some are known to be resistant to chytridiomycosis while carrying high *Bd* infection loads (Reeder et al.

2012). Such frogs may provide opportunities for isolating and studying *Bd* across a diverse landscape without damaging populations of rare or endangered amphibians.

## METHODS

*Media preparations* – We used four different antibiotics in growth media when isolating *Bd* from frog tissues: kanamycin, ciprofloxacin, streptomycin, and penicillin G. Both kanamycin sulfate and ciprofloxacin were prepared in separate solutions by adding 10 g and 1 g to 1 L of autoclaved deionized (DI) water, respectively. Streptomycin sulfate (30g) and penicillin G (20 g) were added simultaneously to 1 L of autoclaved DI water. Aliquots of these solutions were kept frozen before use.

We used TGhL and H-broth media for isolation and growth of *Bd*, following preparation protocols from Longcore (2000); however, lactose is not required for *Bd* growth and a 1% tryptone medium should suffice (Piotrowski et al. 2004). We prepared TGhL in 250 ml quantities to ameliorate issues with media hardening prior to pouring. We added antibiotics to both media types once the temperature of the solutions dropped below 50 °C. For each 250 ml of TGhL solution we added 1.25 ml of kanamycin solution (concentration in media = 50 $\mu g \cdot ml^{-1}$), 2.5 ml ciprofloxacin solution (concentration in media = 10 $\mu g \cdot ml^{-1}$), and 2.5 ml of streptomycin-penicillin G solution (concentrations in media = 300 $\mu g \cdot ml^{-1}$ and 200 $\mu g \cdot ml^{-1}$, respectively). For H-broth we added 10 ml of

streptomycin-penicillin G solution (with concentrations in media = 300 μg•ml$^{-1}$and 200 μg•ml$^{-1}$, respectively).

Each 250 ml solution of TGhL media made approximately 25 agar plates (5 cm). We previously experienced desiccation of this medium, so we poured plates (Petri dishes) ~ half-full to maintain appropriate moisture content to facilitate *Bd* growth. Plates were poured under a sterilized laminar flow hood, and once solidified stored in plastic sleeves at 4 °C. We also stored H-broth at 4 °C.

*Animals* – We used 26 *P. regilla* (potentially *Hyliola regilla*, *see* Duellman et al. 2016) and 32 *P. triseriata*. We collected *P. regilla* from Spring Mountain Ranch State Park, Clark County, Nevada, and acquired *P. triseriata* from Arizona Game and Fish Department personnel who collected them at Coleman Lake, Coconino County, Arizona. We temporarily housed each frog individually in clear plastic boxes with clasping lids (20 cm W x 36 cm L x 12 cm H) containing ~ 1 L of aged, dechlorinated tap water, and a small plastic platform for cover and a dry surface. We kept the containers in an environmental chamber set to 19 °C under a 12:12 L:D photoperiod.

*Determining infection intensity* – We determined *Bd* infection intensity of each frog prior to isolation attempts using quantitative real-time polymerase chain reaction (qRT-PCR) of skin swabs as described in Jaeger et al. (2017). Our reactions followed a common assay (Boyle et al. 2004) with infection intensity interpreted as the number of

*Bd* zoospore equivalents (ZE) per swab. To quantify infections, we used representative standards of *Bd* isolated from each of the geographic regions from which the frogs were collected (i.e., Spring Mountain Ranch and Coleman Lake) to reduce the possibility that variations in genomic content among *Bd* strains could affect our zoospore counts (Longo et al. 2013).

*Isolation attempts* – Prior to isolation attempts we euthanized frogs following an accepted protocol, wrapped each in a laboratory wipe, and placed them into small re-sealable plastic bags. We processed all frogs within 24 hours and kept euthanized frogs that were not immediately processed at 4 °C. We processed frogs on an open, sterilized bench. Forceps, small surgical scissors, and inoculating needles were flame sterilized before each use. For each frog, we used several pairs of TGhL-antibiotic plates and an additional 'master plate.' We divided the master plate into sections labeled thigh, abdomen, and foot. The first plate of each pair (referred to as cleaning plates) served as a cleaning substrate (see Longcore 2000), while the second plate eventually held the cleaned piece of tissue (referred to as tissue plates). We placed frogs ventral side up and excised ~ 4 x 4 mm pieces of tissue (see Figure 1), placing each piece into the appropriate section on the master plate. These frogs are quite small, so for foot samples we excised the most proximal ~ 1 mm portion of the largest toe of each foot and associated webbing. We subsequently dissected these samples into ~ 1 x 1 mm pieces, returning each piece to the appropriate section on the master plate.

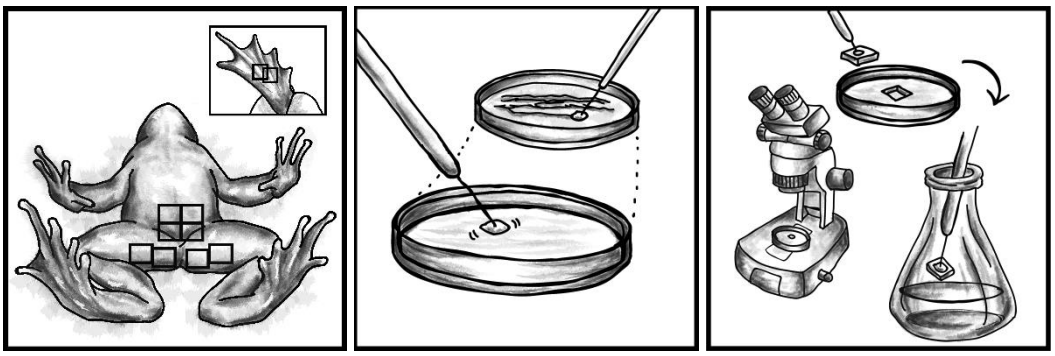

**Figure 1.** *Batrachochytrium dendrobatidis* isolation approach. As indicated by squares in the first panel, tissue is excised in pieces from the thigh, abdomen, and foot. These tissues are plunged and dragged through antibiotic agar plates using a sterile inoculating needle and then left on a new antibiotic plate as indicate in the second panel. The last panel indicates the final steps of assessing *Bd* presence and growth, and then transfer to liquid media.

We individually transferred pieces of tissue from the master plates to appropriately labeled cleaning plates using an inoculating needle. We plunged (submerged) each tissue into the agar and dragged it across and through the agar about ten times, with the intent of removing unwanted fungal spores and bacteria (Longcore 2000). We then spread the tissue sample out in a clean tissue plate, which was then wrapped in parafilm, inverted, and incubated at 23 °C. We took care to ensure that plates were fresh enough to provide a small halo of water around the tissue pieces, as observed under magnification; we believed this was especially important because *Bd* is highly susceptible to desiccation (Johnson et al. 2003). For each *P. regilla*, we collected 8 thigh samples, 4 abdomen samples, and 2 foot samples. For *P. triseriata*, we used 4 pieces of tissue from each of these anatomical regions.

We monitored plates weekly over one month for *Bd* growth under 40x magnification, and noted the number of plates in which *Bd* growth was observed. We also recorded the number of plates that were contaminated and presumed contaminant type; contaminants with hyphae were presumed to be fungal. Once *Bd* zoosporangia were visible, a chunk of the agar (~ 1 x 1 cm) containing the skin sample with *Bd* was transferred to a 125 ml flask containing 50 ml of H-broth with antibiotics. The identity of *Bd* on plates (as opposed to other fungal species) was confirmed with qRT-PCR.

We determined the role of anatomical region (thigh, abdomen, or foot) on the success of *Bd* isolation for each species independently. We calculated proportions of successful plates for each region per individual and then applied a linear mixed multiple regression model. Because frogs were randomly collected from the field, randomness among samples taken from an individual frog were accounted for via variance-covariance structure. Bonferroni adjustments were used for multiple comparisons. The statistical analyses were conducted using SAS version 9.4 (SAS Institute, Cary NC, USA). We determined whether *Bd* infection intensities of frogs influenced *Bd* isolation success by assessing correlations (Spearman's rho) among the proportion of successful plates (those with isolated *Bd* zoosporangia) and the infection intensity (ZE) of the frog from which samples were taken; infection intensity of each frog was divided by its snout-to-vent length (SVL) for size correction.

## RESULTS

We isolated *Bd* from 73% of *P. regilla* and 66% of *P. triseriata* (69% overall). Out of the total frogs used, 78% (45/58) tested positive for *Bd* infection (≥ 1 ZE). Of the *Bd* positive frogs, we isolated the pathogen from 89% of *P. regilla* and 70% of *P. triseriata* (78% overall). We also isolated *Bd* from 5 of 13 frogs that did not test positive for *Bd* infection. We found positive trends between *Bd* infection intensity (ZE/SVL) of frogs and the proportion of plates from which *Bd* was isolated for both species (*P. triseriata*, $\rho = 0.52$, P-value = 0.002; *P. regilla* $\rho = 0.53$, P-value = 0.005).

We isolated *Bd* from all three body regions (thigh, abdomen, foot) of both species (Figure 2). Of these body regions, success at isolating *Bd* was significantly higher for foot samples from both *P. regilla* (foot x abdomen P-value = 0.010; foot x thigh P-value = 0.049) and *P. triseriata* (foot x abdomen P-value < 0.000; foot x thigh P-value < 0.000). Isolation success did not vary significantly between the abdomen and thigh for either *P. regilla* (P-value = 1.00) or *P. triseriata* (P-value = 0.605). The significance of these comparisons were generally consistent even when species were analyzed together.

Contamination from bacteria and unwanted fungi for all plates was low (9.7% of 1554 plates), with remaining plates either containing *Bd* (14.0%) or nothing at all. The most common type of contamination was presumed fungal (77% of total plates with contamination). We observed contamination on 9.5% of cleaning plates and 9.0% of

tissue plates, while 22.4% of the master plates were contaminated. We were able to start *Bd* cultures in H-broth with antibiotics from all the plates with isolated zoosporangia.

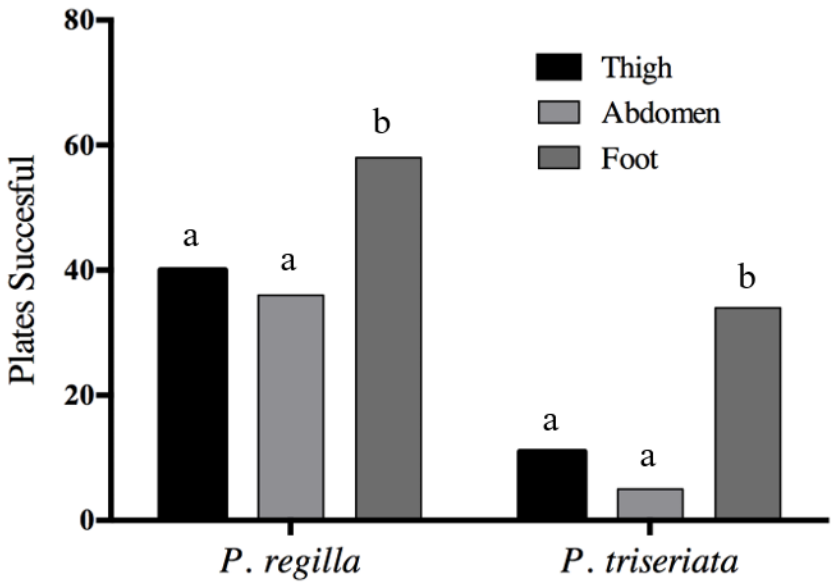


**Figure 2.** Percentage of successful plates (with *Batrachochytrium dendrobatidis*) by body region (thigh, abdomen, foot) of *Pseudacris regilla* and *Pseudacris triseriata*. Letters indicate significant differences between body regions within each species.

## DISCUSSION

We were highly successful using our protocol at isolating *Bd* from populations of *P. regilla* and *P. triseriata* that had high infection prevalence. We isolated *Bd* from each of the three anatomical regions from which we extracted skin samples (thigh, abdomen, foot), but our isolation attempts from the feet (toe and associated webbing) were significantly more successful. Similarly, webbing from hind feet of other ranids have been shown to carry higher *Bd* infections than other body regions, and the webbing of

hind feet has long been suggested as a primary area to sample for *Bd* detection (Longcore et al. 2007). Excitingly, sampling webbing may prove to be an effective, nonlethal approach for isolating *Bd* (as has been suggested to us by Piovia-Scott, J. and Pope, K., pers. comm.).

*Bd* infection intensity of frogs was positively correlated with isolation success, indicating that individuals with higher infection intensities may provide a more successful avenue for *Bd* isolation. We found that once *Bd* infection intensities reach ≥ 40 ZE, we had high levels of isolation success (*Bd* isolated from 74% of *P. triseriata* and 100% of *P. regilla* with such infections). Our success at isolating *Bd* from frogs that tested negative for *Bd* infection was puzzling, but may be due to low intensity infections often being missed using swabbing protocols (Shin et al. 2014). We used an established qRT-PCR method to detect and confirm *Bd* identity (Boyle et al. 2004), but microscopic examination of freshly collected tissue can also be used for detecting *Bd* (Longcore et al. 1999, 2000; Longcore JR et al. 2007). Microscopy may be less costly and quicker then qRT-PCR, particularly if working with only a few frogs, as tissue samples can be excised and viewed immediately (Longcore JR et al. 2007).

Our early preliminary attempts at isolating *Bd* using common antibiotic combinations (e.g., Longcore et al. 1999, 2000; Piovia-Scott et al. 2015) were mostly overrun with bacteria, but our combination of four antibiotics was effective at eliminating most bacterial contaminants. We used concentrations of streptomycin and penicillin as

described in the literature and kanamycin and ciprofloxacin as advised by the manufacturer for eliminating bacterial contaminants in cell cultures. We doubled the concentration of ciprofloxacin above that recommended by the manufacturer for use in cell cultures which did not appear to limit our isolation success, although we did not assess lower concentrations. Using 5 cm plates in pairs allowed us to isolate and monitor each piece of tissue for *Bd* growth (also see Piovia-Scott et al. 2015), and this alteration from earlier protocols using larger plates and multiple pieces of tissue per plate (e.g., Longcore et al. 2000) may keep contaminants contained to one or a few tissue pieces without affecting others. In a few instances, we observed *Bd* on cleaning and master plates which highlights the utility of keeping and monitoring all plates for isolations. Growth of *Bd* to the point of easy observation of zoospores and zoosporangia may also be quite slow, and we observed *Bd* presence on many plates only after 3 to 4 weeks following plating.

Our adoption and refinement of established *Bd* protocols has benefited our research. Since developing this protocol, we have successfully applied it to isolate *Bd* from White's treefrogs (*L. caerulea*) and relict leopard frogs (*R. onca*) used in laboratory experiments and from American bullfrogs (*Rana catesbeiana*) captured from the wild. We believe our alterations will be useful to other researchers conducting biological studies with *Bd*.

*Simplified method*

1. Remove tissue and dissect into ~ 1 x 1 mm pieces using surgical scissors and forceps (see first panel in Figure 1)
2. Clean tissue by plunging and wiping through agar plate (1% tryptone) containing 4 antibiotics (see second panel in Figure 1 and text above)
3. Place tissue on plate with antibiotic agar, cover in Parafilm®, and incubate at room temperature (21–23 °C)
4. Monitor for zoospore activity under 40x magnification (a compound scope at up to 100x magnification and sub-stage lighting has also been suggested)
5. Transfer to H-broth once zoosporangia are visible (see third panel Figure 1)
6. Refrigerate H-broth cultures once clumps of zoosporangia are observed and transfer to new media within 4–6 months
7. For long-term storage, cryo-archive *Bd* isolate as soon as possible following the protocol of Boyle et al. (2003)

# CHAPTER 3

## Effects of Attenuation, Differences in Pathogen Virulence, and Life-Stage on *Rana onca* Susceptibility to Chytridiomycosis

## INTRODUCTION

Amphibians are likely experiencing extinction rates beyond what can be explained as a natural phenomenon (Stuart et al. 2004; McCallum et al. 2007; Wake & Vredenburg 2008). Many contemporary declines of amphibians were initially described as "enigmatic", occurring in pristine or protected environments (Stuart et al. 2004). Eventually, disease emerged as a recognized major explanatory factor (Lips et al. 2006; Wake & Vredenburg 2008; Greenspan et al. 2017). Although many diseases impact amphibians, chytridiomycosis is by far the most detrimental to amphibian biodiversity, driving hundreds of species declines and numerous extinctions (Skerratt et al. 2007). Chytridiomycosis is caused by the aquatic fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*), which now occurs on every continent where there are amphibians (Longcore et al. 1999; Fisher et al. 2009). In many cases the impacts of *Bd* were observed decades before the pathogen was described (Berger et al. 1998; Longcore et al. 1999; Stuart et al. 2004), and *Bd* epizootics drove some species declines and extinctions that originally had little explanation or were poorly documented (Shermann et al. 1993; Pounds & Crump 1994; Young et al. 2001; Stuart et al. 2004; Cheng et al. 2011).

The relict leopard frog (*Rana onca = Lithobates onca*) is a species of conservation concern that suffered a substantial, but poorly document, decline sometime during the 20th century (Bradford et al. 2004). Although a narrow endemic known from springs, wetlands, and rivers in the northeastern Mojave Desert (Olah-Hemmings et al. 2010), by 2001 populations only persisted naturally in two general areas of southern Nevada, both within the Lake Mead National Recreation Area (LMNRA; Bradford et al. 2004). Bradford et al. (2004) summarized possible reasons for the decline of *R. onca* as habitat destruction and alteration, loss of habitat-maintaining disturbance regimes such as grazing animals, and the introduction of non-native competitors and predators. Although *Bd* was known to be present in neighboring regions, these authors did not consider this disease as a contributing factor. Hot ambient temperatures, such as those experienced in the Mojave Desert, were thought at that time to limit *Bd* (Piotrowski et al. 2004; Puschendorf et al. 2009). Recent work on the Amargosa toad (*Anaxyrus nelsoni*), however, has shown that *Bd* can persist in a region of the Mojave Desert. Populations of this toad had high *Bd* infection prevalence and intensities even during summer months, when temperatures were high (24–33 °C) and ambient humidity generally low (Forrest et al. 2015).

The decline of *R. onca* displays some attributes that favor epizootic disease as a contributing factor. Forrest and Schaepfler (2011) noted that all remaining natural populations of *R. onca* were restricted to hot springs, where source water temperatures exceed the thermal maxima for *Bd*. These authors speculated that the thermal waters may

provide refuge for the species from disease. Following this observation, the presence of *Bd* was documented in several anuran species within the historical range of *R. onca,* including one remnant historical population of this species (Jaeger et al. 2017).

In contrast to the hypothesis that *Bd* may have been an important factor in the decline of *R. onca*, Jaeger et al. (2017) noted evidence from limited field observations that adults of this frog can persist with, and even potentially clear, *Bd* infections. Moreover, these authors showed that *R. onca* survivorship was not affected by infections of two *Bd* strains known to be highly virulent in other ranid species and that the juvenile frogs used in their experiments cleared infections at high rates (64% overall). Given these results, Jaeger et al. (2017) concluded that *R. onca* appeared to be resistant to chytridiomycosis. These authors qualified their interpretation by pointing to potentially confounding factors, including attenuation (weakening) of the *Bd* strains they used and that the strains were not those infecting frogs in the local environment.

We followed these previous experiments by isolating *Bd* that is currently infecting frogs in southern Nevada, including from the infected population of *R. onca.* We also obtained a highly virulent isolate from a commercial vender that we inadvertently encountered in a shipment of sick and dying White's treefrogs (*Litoria caerulea*). We subsequently exposed juvenile frogs of *R. onca* to two local isolates of *Bd,* as well as to the isolate from the commercial vender. In separate experiments to assess the possible attenuation of *Bd* used by Jaeger et al. (2017), we exposed young, healthy *L. caerulea* to

one of the previously used isolates (Section Line Lake; SLL). *L. caerulea* is highly susceptible to chytridiomycosis (Voyles et al. 2009) and if the *Bd* isolate remained virulent, we expected low survivorship of infected frogs. We also conducted an experiment using *R. onca* by infecting juvenile frogs with SLL, allowing these frogs to develop infections, and then exposed naïve juvenile frogs through cohousing. Previous research had shown that virulence of attenuated *Bd* increased following infection and re-isolation of the pathogen from hosts (Brem et al. 2013). If SLL had attenuated, we expected little mortality in the initial group of frogs exposed but low survivorship of the second group of frogs exposed by cohousing.

Juvenile frogs well past metamorphosis may not represent the most susceptible life-stage to disease (Rollins-Smith et al. 2011; add cite). During metamorphosis, amphibians go through dramatic anatomical and physiological changes, which includes the immune system (Rollins-Smith et al. 2011). Newly metamorphosed frogs may not have mature and/or functional immunological mechanisms to combat disease until many weeks after metamorphosis (Flajnik et al. 1987; Bakar et al. 2016). To gain insight into the potential impact of *Bd* in wild populations, we assessed the most susceptible life-stage by exposing late-stage tadpoles of *R. onca* to a local *Bd* isolate and then exposed these animals again as they metamorphosed. We then monitored infection intensity and survivorship as these animals developed into young frogs.

The areas occupied by remnant, naturally occurring populations of *R. onca*, referred to herein as Black Canyon and Northshore, differ in current exposure to *Bd* (Jaeger et al. 2017). *Bd* swabbing repeatedly detected the pathogen at a spring site in the Northshore area, but not at sites in Black Canyon (Jaeger et al. 2017). In laboratory experiments, Jaeger et al. (2017) reported that frogs derived from the Northshore area were able to clear *Bd* infections in higher proportions than frogs derived from Black Canyon. Although this pattern lacked statistical rigor, it hinted that previous population-level exposure may have facilitated some level of adaptation to *Bd*. Therefore, we considered source area in all our experiments using *R. onca*, maintaining equal numbers from Black Canyon and Northshore. We hypothesized that animals derived from a population where *Bd* is present would be more resistant than those derived from a naïve population.

## METHODS

*Bd isolates* – In our challenge experiments, we used 4 different *Bd* isolates: SLL, Spring Mountain Ranch (SMR), Lower Blue Point (LBP), and *Litoria caerulea* 63 (LC63). SLL was isolated in 2009 from an infected *Rana cascadae* during an epizootic in the Klamath Mountains, California (Piovia-Scott et al. 2015). This isolate was previously frozen, and was on its 21$^{st}$ transfer (passage) when used in our experiments. In southern Nevada, we isolated SMR from an outwardly healthy adult *Pseudacris regilla* from Spring Mountain Ranch State Park, and LBP from an outwardly healthy juvenile *R. onca*

at lower Blue Point Spring in the Northshore area. As mentioned previously, we isolated LC63 from a deceased adult *L. caerulea* we acquired from a commercial vender; this isolate appeared highly virulent, as we observed mortality of half of the frogs within a month following arrival. We decided to use this isolate because of the evidence of virulence and because we could keep its passage history to a minimum, mitigating the possibility of attenuation. We isolated SMR, LBP, and LC63 in 2016 using an approach similar or identical to that described by Waddle et al. (*in review*; see Chapter 1).

*Experimental animals* – We obtained juvenile frogs and late-stage tadpoles of *R. onca* from a conservation program where they had been raised in captivity from eggs collected in the wild (RLFCT 2016). The juvenile *R. onca* we used in the experiments were 9-12 weeks post-metamorphosis. These frogs were derived proportionately from 6 egg masses, 3 each from Northshore and Black Canyon. We used *R. onca* tadpoles at Gosner stage 31-38, similarly derived from 6 different egg masses collected at these sites. We obtained healthy, young adult *L. caerulea* from a commercial vender. We confirmed that all animals were *Bd* negative prior to the experiments using the protocol described below.

*Challenge experiment with juvenile R. onca* – We placed the 48 juvenile *R. onca* frogs into 4 treatment groups (n=12). Each group was proportionally represented by frogs from each egg mass and area from which the eggs were derived (Black Canyon and Northshore). As summarized below the first two groups were exposed to *Bd* isolates from

southern Nevada, the third group was exposed to the strain associated with mortality in *L. caerulea*, while the last group was an unexposed control.

(1) Exposed with SMR

(2) Exposed with LBP

(3) Exposed with LC63

(4) Unexposed Control

*Challenge experiment with R. onca during metamorphosis* – We used the *Bd* isolate LBP to challenge early life-stages of *R. onca*, because this was the strain infecting *R. onca* in the wild. We placed 72 tadpoles derived proportionately from the egg masses into 4 treatment groups based on source location (Black Canyon and Northshore). We maintained tadpoles in groups of 12 to minimize crowding in the aquariums:

1. Northshore Uninfected Control
2. Black Canyon Uninfected Control
3. Northshore Infected, Aquaria 1 and 2
4. Black Canyon Infected, Aquaria 1 and 2

*Assessing potential attenuation of Bd isolate SLL* – In one experiment, we exposed 12 *L. caerulea* to SLL and maintained an unexposed control group of 13 animals. In another experiment, we exposed 12 juvenile *R. onca* frogs to SLL (1$^{st}$ group) and allowed these

frogs to developed high levels of *Bd* infection (see results). We then cohoused a second group of 12 juvenile *R. onca* ($2^{nd}$ group) with these previously infected animals, keeping 1 or 2 uninfected frogs with an infected frog for a total of 5 weeks. We kept track of individual *R. onca* frogs using photographic references of morphological patterns. Once frogs were separated, each animal was treated identically to the other groups in terms of *Bd* sampling and husbandry (see below).

*Bd growth and exposures* – We prepared *Bd* zoospore inoculum and infected frogs as described in Jaeger et al. (2017). Frogs were exposed to 1,000,000 *Bd* zoospores of their assigned strain for each of 3 days, for a total of 3,000,000 zoospores per frog. We exposed *R. onca* and *L. caerulea* identically. To expose the tadpoles to LBP, we placed tadpoles in groups of 6 into 200 ml baths containing 2500 zoospores of *Bd* per ml for 24 hours (Venesky et al. 2009). Afterwards, the tadpoles were moved back into their respective tanks along with the infected water containing *Bd* zoospores. We repeated this procedure after one week, because the initial exposure resulted in only one of the tadpoles testing positive for infection. The control tadpoles underwent a similar procedure, except we used a sham inoculum derived from sterile agar plates. Prior to exposure, we confirmed tadpoles were *Bd* negative. At metamorphosis, we again exposed each new froglet to LBP, following the protocol for frogs.

*Housing* – We conducted challenge experiments for frogs in an environmental chamber with a 12-hour photoperiod. We maintained temperatures at 19 °C, as this

temperature is environmentally relevant for the frogs and would likely favor *Bd* growth (Piotrowski et al. 2004, Jaeger et al. 2017). All frogs were allowed to acclimate to laboratory conditions for two weeks prior to the start of the study. We housed frogs individually in clear plastic containers with lids (36 cm L x 20 cm W x 12 cm H), which contained ~950 ml aged, dechlorinated tap water. Similar but somewhat larger containers (42 cm L x 33 cm W x 17 cm H) with slightly more water were used for cohousing. We maintained the arboreal *L. caerulea* with a smaller volume of water (~250 ml). All frogs were provided with a platform that served as a dry surface area and cover. We transferred frogs and their platforms each week to clean containers with fresh water. We fed frogs bi-weekly with appropriately sized crickets maintained on a commercial cricket diet.

We housed the groups of tadpoles in 76 L aquaria maintained at room temperature (23 °C) under a 12-hour photoperiod. Each aquarium contained sponge and power filters (250 LPH) to maintain water quality. We monitored ammonia levels using detectors and other nitrogen derivatives (nitrate, nitrite, etc.) using test strips. We conducted 25% water changes once per week or as needed using aged, dechlorinated tap water. We fed tadpoles *ad libitum* a varied diet of organic lettuce leaves, alfalfa (rabbit) pellets, and commercial algae discs. Following metamorphosis, we maintained the frogs in individual plastic containers as described above.

*Infection detection and intensity* – We sampled frogs and tadpoles for *Bd* infection weekly. For each frog, we swabbed 10 times down each ventral side and five times on

each rear foot (Vredenburg et al. 2010; Jaeger et al 2017). We ran all challenge experiments on frogs for 18 weeks. We only swabbed the keratinized mouthparts of tadpoles 5 times per each sample. Once tadpoles underwent metamorphosis, we swabbed the resulting frogs for an additional 16 weeks. We maintained swab tips frozen until testing, and used quantitative real-time polymerase chain reaction (qRT-PCR) to determine infection presence and intensity (Boyle et al. 2004), measured as *Bd* zoospore equivalents per swab (ZE; Vredenburg et al. 2010; Jaeger et al. 2017). To mitigate the potential issue of variable genomic content between *Bd* strains (Longo et al. 2013), we created qrt-PCR standards for each isolate used in experiments following established protocols (Boyle et al. 2004; Longo et al. 2013; Lambertini et al. 2016); this allowed between isolate comparisons (Rebollar et al. 2017).

*Statistical analyses* – For the cohousing experiment, we used a 2-way repeated measures analysis of variance (ANOVA), followed with post-hoc Tukey Tests to analyze the differences in infection intensity between the first and second exposure group of *R. onca*. We also analyzed the effects of collection site on infection intensity for each *R. onca* exposure group in the cohousing experiment using the same statistic. Infection data was log-transformed to meet assumptions of normality.

We used Chi-square tests (two-tailed) to determine the effects of collection site on the proportion of frogs that cleared *Bd* infection. We assumed clearance of *Bd* if a frog tested negative (0 ZE) at the end of the experiment, and only included frogs that tested

positive for *Bd* infection at least once during an experiment. We analyzed survivorship data for all experiments using Mantel-Cox tests. We conducted the statistical analyses in the program Prism 6.0f (Graphpad Software Inc., La Jolla, CA).

## RESULTS

### Challenge experiment with juvenile *R. onca*

*Infectability* – Across all the juvenile *R. onca* frogs we exposed to *Bd,* 92% (33/36) became infected. The frogs we exposed to SMR and LC63 all became infected, but only 9 out of 12 frogs exposed to LBP became infected, with only 5 of these frogs testing positive for *Bd* for more than 1 week. None of the unexposed control frogs tested positive for infection.

*Infection intensities/clearance* – Mean weekly infection intensities for *R. onca* were high across all the *Bd* exposure groups. We observed the highest infection intensities with LC63. The mean weekly infection intensities for this group reached 1,256,231 ± 1,179,612 ZE, with the highest individual measure recorded at 7,150,149 ZE. Frogs exposed to LBP reached mean weekly infection intensities of 882,828 ± 733,110 ZE, with the highest individual infection intensity of 8,868,273 ZE. Frogs exposed to SMR reached a mean weekly value of 225,449 ± 170,812 ZE, with the highest individual value of 1,371,555 ZE (Figure 3).

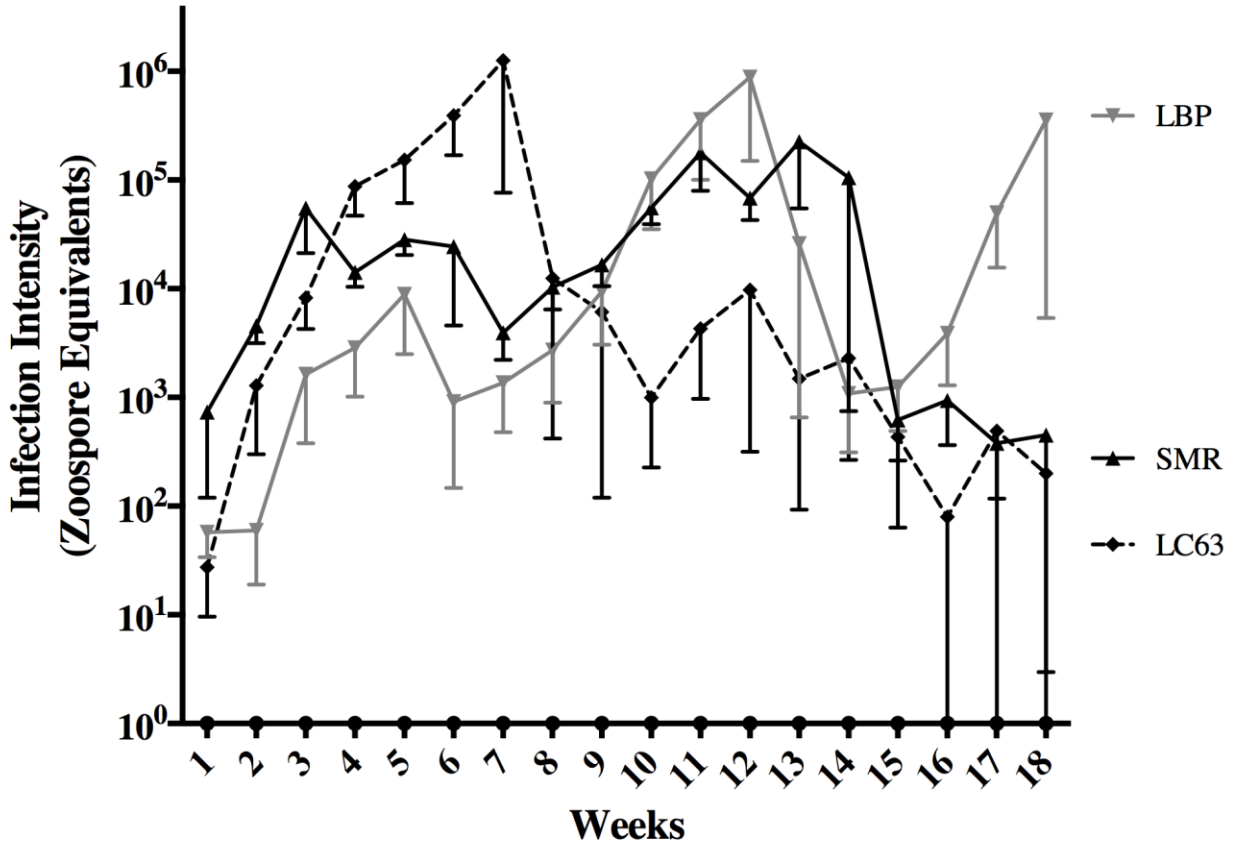


**Figure 3.** Mean weekly infection intensity over 18 weeks for three groups of *Rana onca* exposed to three isolates of *Batrachochytrium dendrobatidis* (LBP, SMR, or LC63). Error bars denote standard error of the mean with only bottom error bar shown for clarity. As frogs died they were not included in subsequent weekly infection data and sample sizes in exposure groups decrease.

Of the *R. onca* that became infected with the various *Bd* isolates we used, 36% (12/33) cleared their infections by the end of the experiment. We observed no difference in the proportions of clearance by isolate ($\chi^2 = 0.35$, df = 2, P-value = 0.84), with 33% (4/12) of the SMR group, 44% (4/9) of the LBP group, and 33% (4/12) of the LC63 group clearing their infections. Across the various groups exposed to *Bd*, the frogs derived from Northshore cleared their infections at higher proportions than those derived

from Black Canyon ($\chi^2$ = 7.64, df = 1, P-value = 0.006; Figure 4), with 59% (10/17) of the frog from Northshore clearing infections compared to only 13% (2/16) of frogs from Black Canyon. The other 21 frogs in the experiment maintained their infections at the end of the experiment or had died.

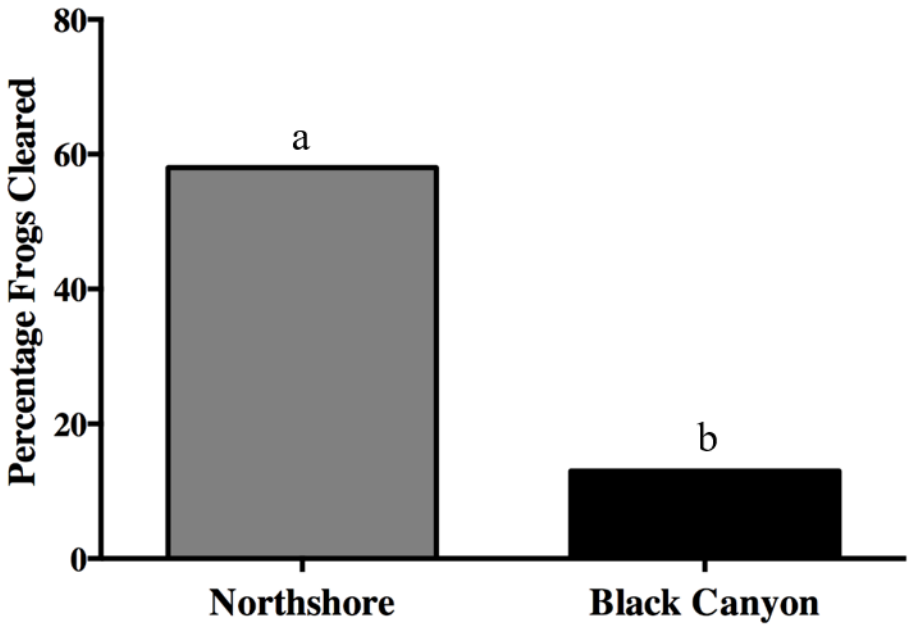


**Figure 4.** Clearance percentages of *Rana onca* frogs derived from Northshore and Black Canyon that were exposed to three isolates of *Batrachochytrium dendrobatidis*. Letters indicate significant differences between groups.

*Survivorship* – We observed that *Bd* strains SMR and LC63 had significant effects on *R. onca* survivorship compared to the unexposed control group (SMR $\chi^2$ = 6.092, df = 1, P-value = 0.014; LC63 $\chi^2$ = 9.62, df = 1, P-value = 0.002). Only 58% (7/12) of frogs exposed to SMR and 42% (5/12) of frogs exposed to LC63 survived to week 18, while all unexposed control frogs survived (Figure 5). We observed clinical signs of

chytridiomycosis (Berger et al. 1998; Voyles et al. 2009) in frogs exposed to LC63 at week 4 when these animals began to die. A similar pattern occurred with frogs exposed to SMR, but not until week 11. We observed mortality in 3 frogs exposed to LBP beginning in week 13, with these frogs also showing clinical signs of chytridiomycosis. As a group, however, the effect of LBP exposure on survivorship was not significant ($\chi^2 = 3.1$, df = 1, P-value = 0.078).

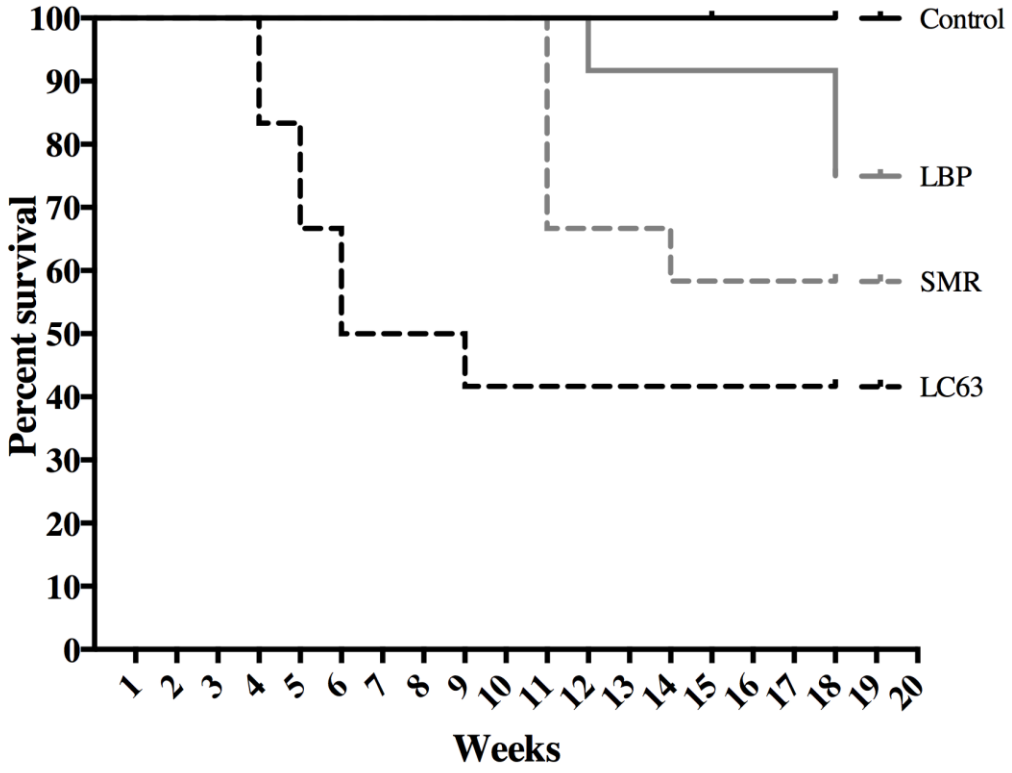


**Figure 5.** Survivorship over 18 weeks of 4 groups of *Rana onca* either exposed to three isolates of *Batrachochytrium dendrobatidis* (LBP, SMR, LC63) or a sham inoculum (Control).

Overall, we observed that 58% (21/36) of juvenile *R. onca* frogs survived *Bd* exposure. When analyzed by source area, frogs derived from Northshore survived at a

significantly higher proportion than those derived from Black Canyon ($\chi^2 = 6.06$, df=1, P-value = 0.014; Figure 6). Of these frogs, 78% (28/36) from Northshore survived, as compared to only 39% (14/36) from Black Canyon. While survivorship of frogs from Northshore did not significantly differ from unexposed control frogs ($\chi^2 = 2.92$, df = 1, P-value = 0.087), survivorship of frogs from Black Canyon was significantly lower ($\chi^2 = 10.6$, df = 1, P-value = 0.0014).

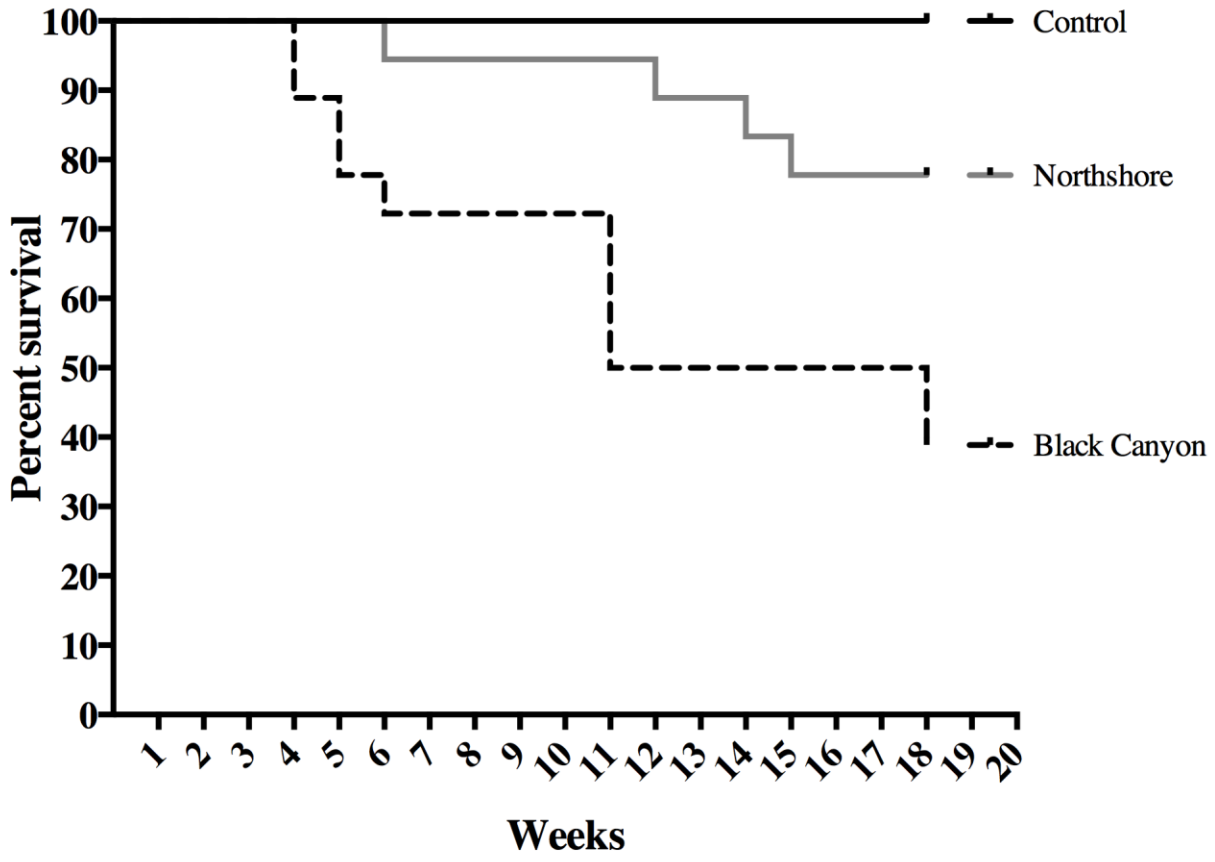


**Figure 6.** *Rana onca* survivorship over 18 weeks after exposure to *Batrachochytrium dendrobatidis* (*Bd*) analyzed by site from which frogs were originally derived. Frogs at Northshore have tested positive for *Bd*, while *Bd* has not been detected in Black Canyon.

**Challenge experiment with *R. onca* during metamorphosis**

*Infectability* – For *R. onca* tadpoles, 23% (11/48) tested positive for *Bd* infection, while 94% (44/47) of those same animals tested positive for *Bd* infection as metamorphosed frogs following additional *Bd* exposure. None of the unexposed control tadpoles and resulting frogs tested positive for *Bd* infection.

*Infection intensities/clearance* – Mean weekly high infection intensity for *R. onca* tadpoles sourced from Black Canyon reached 1 ± 0.5 ZE, with the highest individual infection intensity of only 6 ZE. Mean weekly infection intensity of the resulting metamorphosed frogs reached 66,781 ± 43,338 ZE, with the highest individual infection intensity of 399,584 ZE (Figure 7). Mean weekly high infection intensity for tadpoles sourced from Northshore reached 12 ± 12 ZE, with the highest individual infection intensity of 291 ZE. The mean weekly high infection intensity of the resulting frogs from this group reached 149,859 ± 72,281 ZE, with the highest individual infection intensity of 703,327 ZE (Figure 7). By the end of this experiment, there was no significant difference in infection clearance between Black Canyon and Northshore frogs ($\chi^2 = 0.03$, df = 1, P-value = 0.86), with 13% (3/24) of Black Canyon and 14% (3/21) of Northshore frogs clearing infections by the end of the experiment.

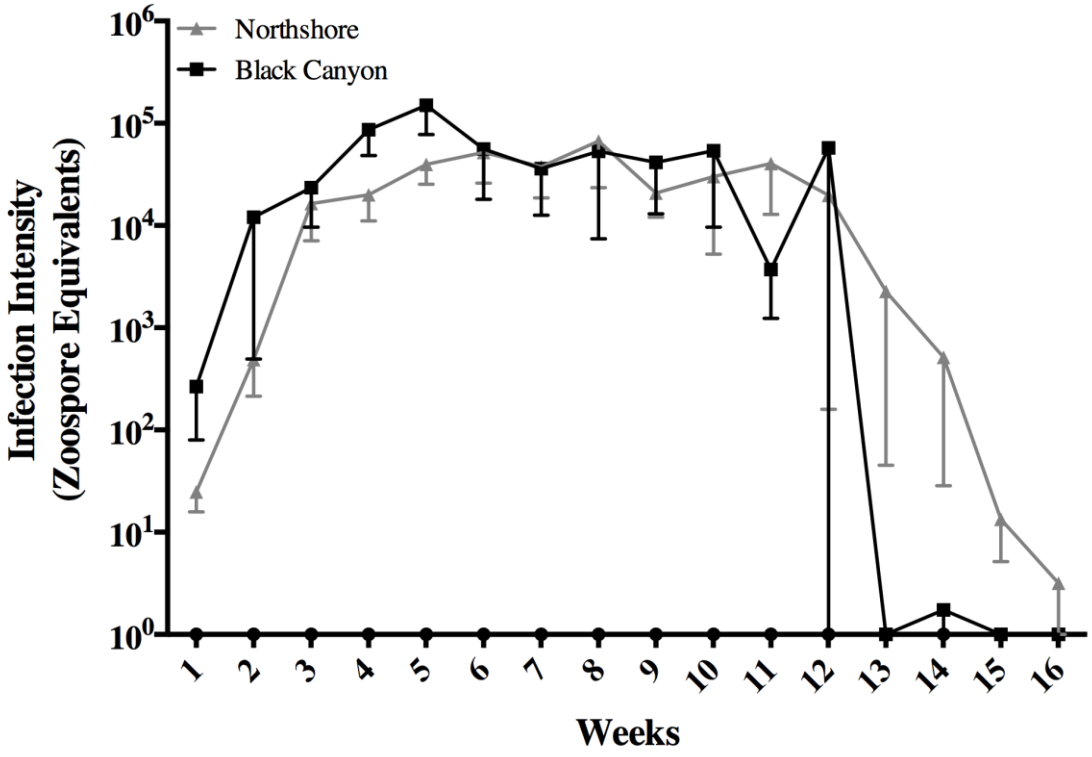


**Figure 7.** Mean weekly infection intensity for newly metamorphosed *R. onca* exposed to LBP isolate of *Batrachochytrium dendrobatidis* as late stage tadpoles and again during metamorphosis. Error bars denote standard error of the mean with only bottom error bar shown for clarity. As frogs died they were not included in subsequent weekly infection data and sample size in exposure group decreased.

*Survivorship – R. onca* survivorship in the tadpole stage was not significantly affected by exposure to *Bd* isolate LBP (47/48 survived); however, survivorship of resulting frogs was significantly affected following the second exposure to LBP during metamorphosis ($\chi^2 = 22.9$, df = 1, P-value < 0.0001; Figure 8). By the end of the experiment, only 21% (10/47) of the newly metamorphosed frogs survived, while survivorship in the unexposed control group was 91% (21/23); one tadpole in the control group from Northshore had not metamorphosed after several months and was not included in these analyses. We observed signs of chytridiomycosis in many of the

infected frogs, while none of the unexposed control frogs showed signs of disease. When analyzed by source population, we found that LBP had significant effects on survivorship of frogs derived from both Northshore ($\chi^2 = 9.59$, df = 1, P-value = 0.0020) and Black Canyon ($\chi^2 = 19.72$, df = 1, P-value < 0.0001). There was, however, no difference in survivorship between Northshore and Black Canyon ($\chi^2 = 0.048$, df = 1. P-value = 0.83), with both groups having low survivorship (26% and 17%, respectively).

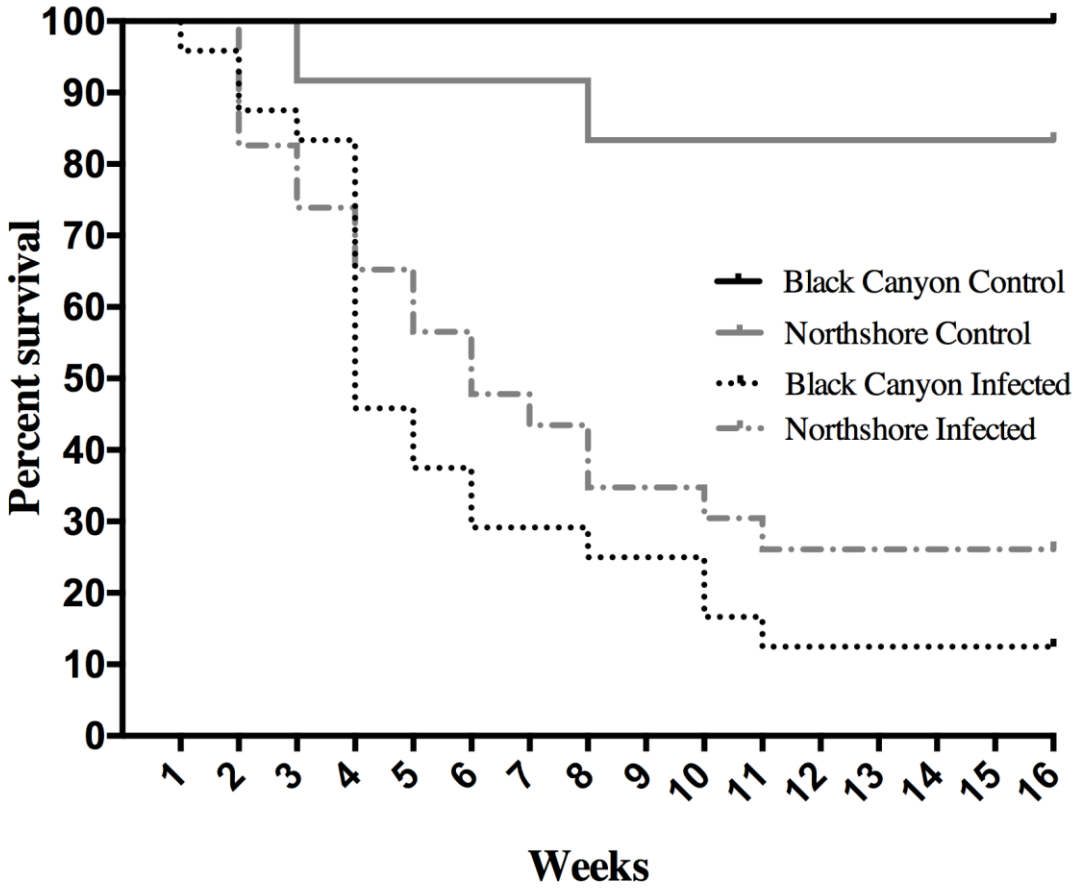


**Figure 8.** Survivorship of *Rana onca* by site from which the tadpoles were derived over 16 weeks after metamorphosis. Frogs were exposed to the LBP isolate of *Batrachochytrium dendrobatidis* as late-stage tadpoles (Gosner 31-18) and again as they were completing metamorphosis. Control animals were exposed to sham inoculum.

**Assessing potential attenuation of *Bd* isolate SLL**

*Litoria caerulea* – All *L. caerulea* exposed to SLL became infected, while none of the unexposed control frogs tested positive for *Bd*. Mean weekly infection intensities of the exposed frogs reached 29,831 ± 27,245 ZE, with the highest individual infection intensity of 7,150,149 ZE (Figure 9). *L. caerulea* survivorship was significantly affected by exposure to SLL ($\chi^2 = 8.35$, df = 1, P-value = 0.004), with only 50% (6/12) survival of exposed frogs, while all of the unexposed control frogs survived. Just 25% (3/12) of *L. caerulea* exposed to SLL cleared their infections by the end of the experiment. We first observed mortalities at week 5 and again from weeks 12 to 15 (Figure 10). Deaths during the latter period followed development of clinical signs of chytridiomycosis.

*Cohousing experiment* – All but one of the juvenile *R. onca* frogs exposed to SLL in the initial exposure group (1st group), and all but one frog in the group exposed by cohousing (2nd group) became infected. The highest average infection intensity for the 1st group was 12,257 ± 5,163 ZE, while the 2nd group reached 123,522 ± 115,721 ZE. The highest individual infection intensities for these groups were 58,818 ZE and 1,395,298 ZE, respectively.

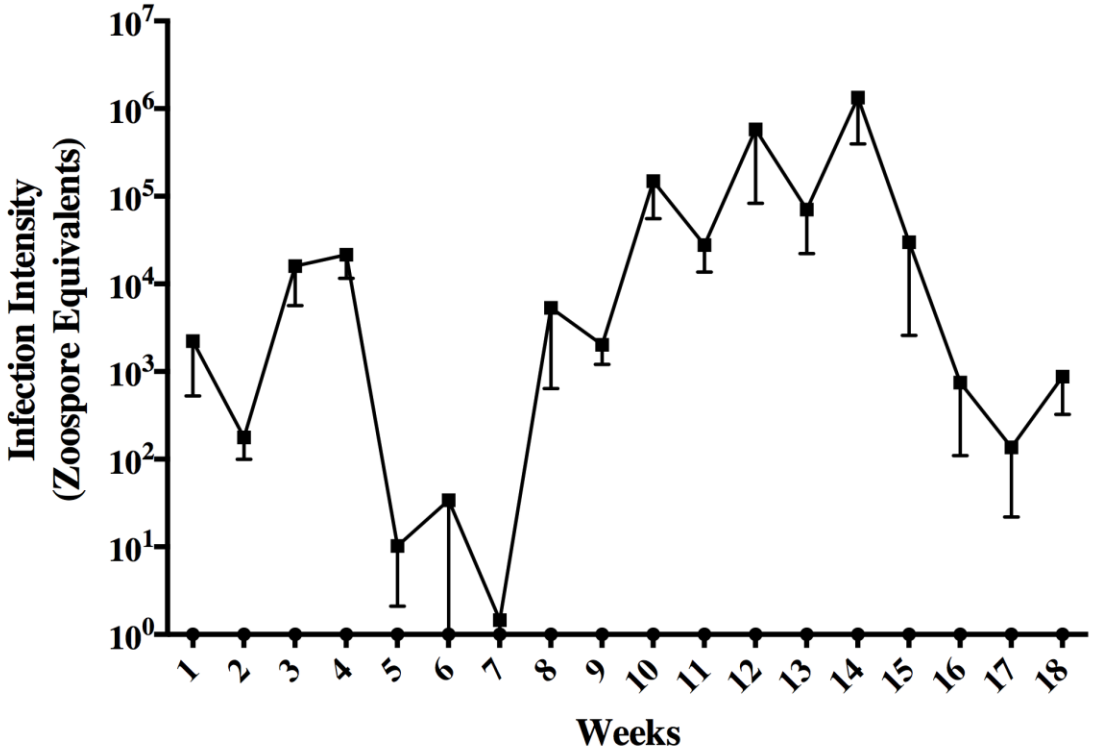


**Figure 9.** Mean weekly infection intensity over 18 weeks after exposure of *Litoria caerulea* to the SLL isolate of *Batrachochytrium dendrobatidis*. Error bars denote standard error of the mean with only bottom error bar shown for clarity. As frogs died they were not included in subsequent weekly infection data and sample size in exposure group decreased.

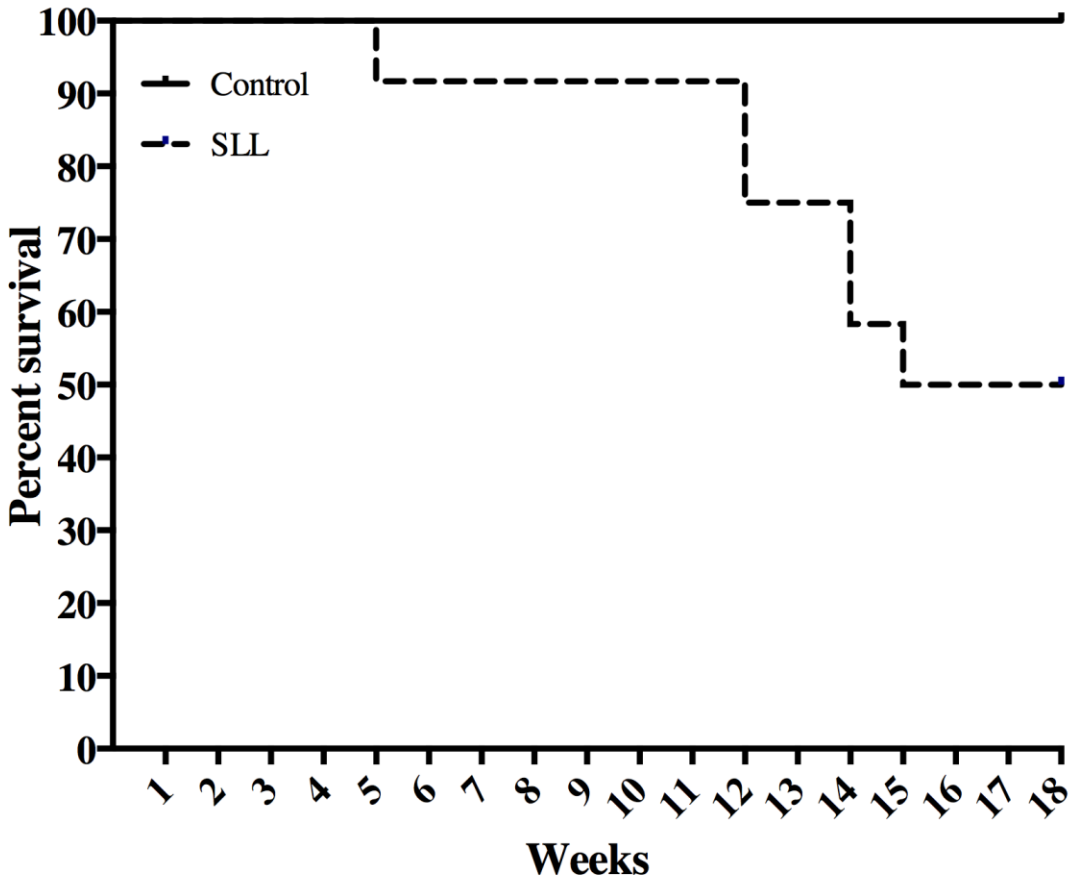


**Figure 10.** Survivorship over 18 weeks for *Litoria caerulea* exposed to SLL isolate of *Batrachochytrium dendrobatidis*.

We found no significant differences in infection intensities between the 1st and 2nd groups (P-values $\geq 0.083$ for interaction, time, and exposure group; Figure 11). When analyzed independently by exposure group, we found significant interactions in both groups for time (weeks) and collection site (1st group $F_{34,255} = 2.85$, df = 34, P-value < 0.0001; 2nd group $F_{34,255} = 3.92$, df = 34 P-value < 0.0001), explaining 9% and 12% of variation, respectively. In both groups, infection intensity was also significantly affected by collection site (1st group $F_{2,15} = 19.68$, df = 2, P-value < 0.0001; 2nd group $F_{2.15} =$

13.89, df = 2, P-value = 0.0004) and time (1st group $F_{17,255}$ = 6.29, df = 17, P-value < 0.0001; 2nd group $F_{17,255}$ = 7.97, df = 17, P-value < 0.0001). In the 1st group, time explained 10% of the variation and collection site explained 41% of variation. In this group, the Black Canyon frogs carried higher average infections than Northshore frogs overall (Figure 12a), with their infection intensities being significantly greater for 11 of the 18 weeks (weeks 6-11, 13-15, 17-18; post hoc test P-values < 0.05). In the 2nd group, 12% of the variation was explained by time, while 34% of the variation was explained by collection site. In this group, Black Canyon frogs also carried higher overall infection intensities (Figure 12b), with significant differences observed at weeks 16 and 17 (post hoc tests P-value < 0.05).

In the cohousing experiment, we observed 100% survivorship of juvenile *R. onca* regardless of how they were exposed to SLL. By the end of the experiment, 55% (12/22) of the frogs had cleared infections, and there was no significant difference in clearance proportions between groups ($\chi^2$ =0.73, df = 1, P-value = 0.40). We observed that 64% (7/11) of the 1st group and 45% (5/11) of the 2nd group cleared their infections by the end of the experiment. Overall, the frogs derived from Northshore cleared their infections in higher proportions than those derived from Black Canyon ($\chi^2$ = 6.60, df = 1, P-value = 0.01; Figure 13). Clearance of infections was 82% (9/11) for the Northshore frogs compared to only 27% (3/11) in the Black Canyon frogs. The 10 remaining frogs maintained infections through the end of the experiment.

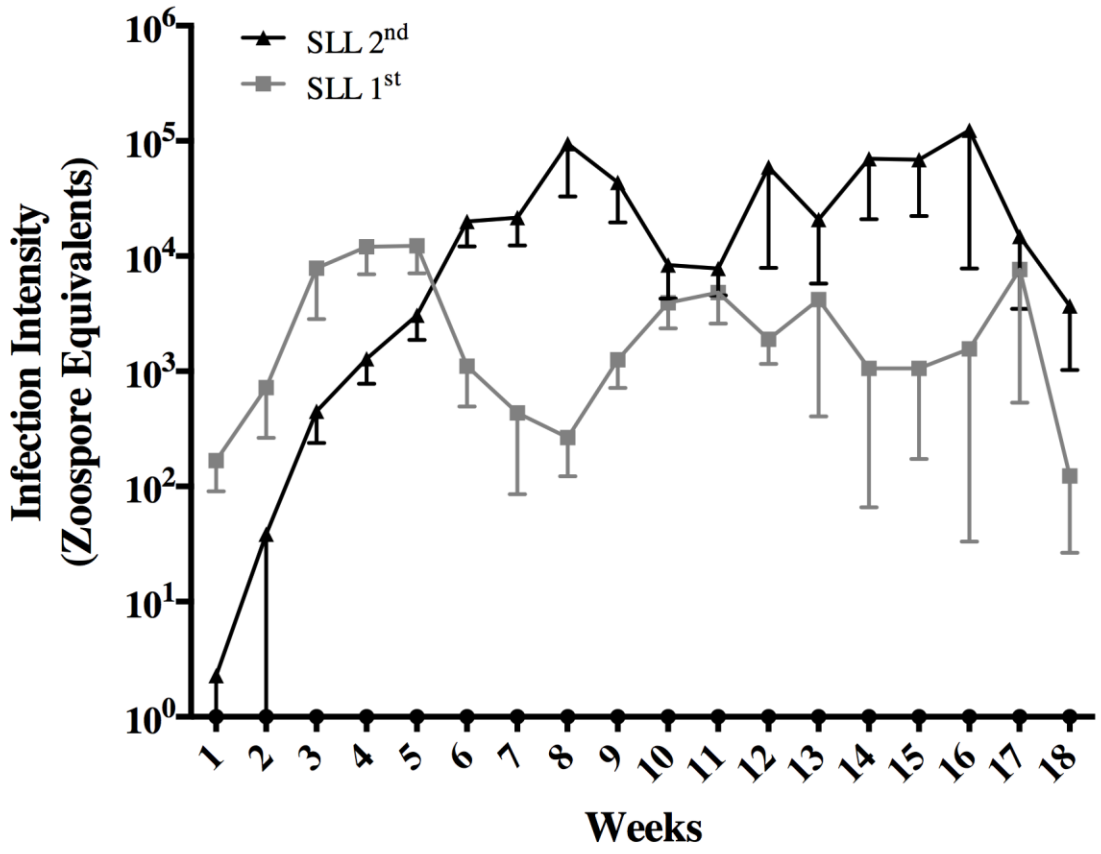


**Figure 11.** Mean weekly infection intensity over 18 weeks for *Rana onca* exposed to SLL isolate of *Batrachochytrium dendrobatidis* (*Bd*) in cohousing experiment. Frogs in first group were directly exposed to the *Bd* isolate by inoculum, while frogs in the second group were exposed by cohousing with infected frogs. Error bars denote standard error of the mean with only bottom error bar shown for clarity.

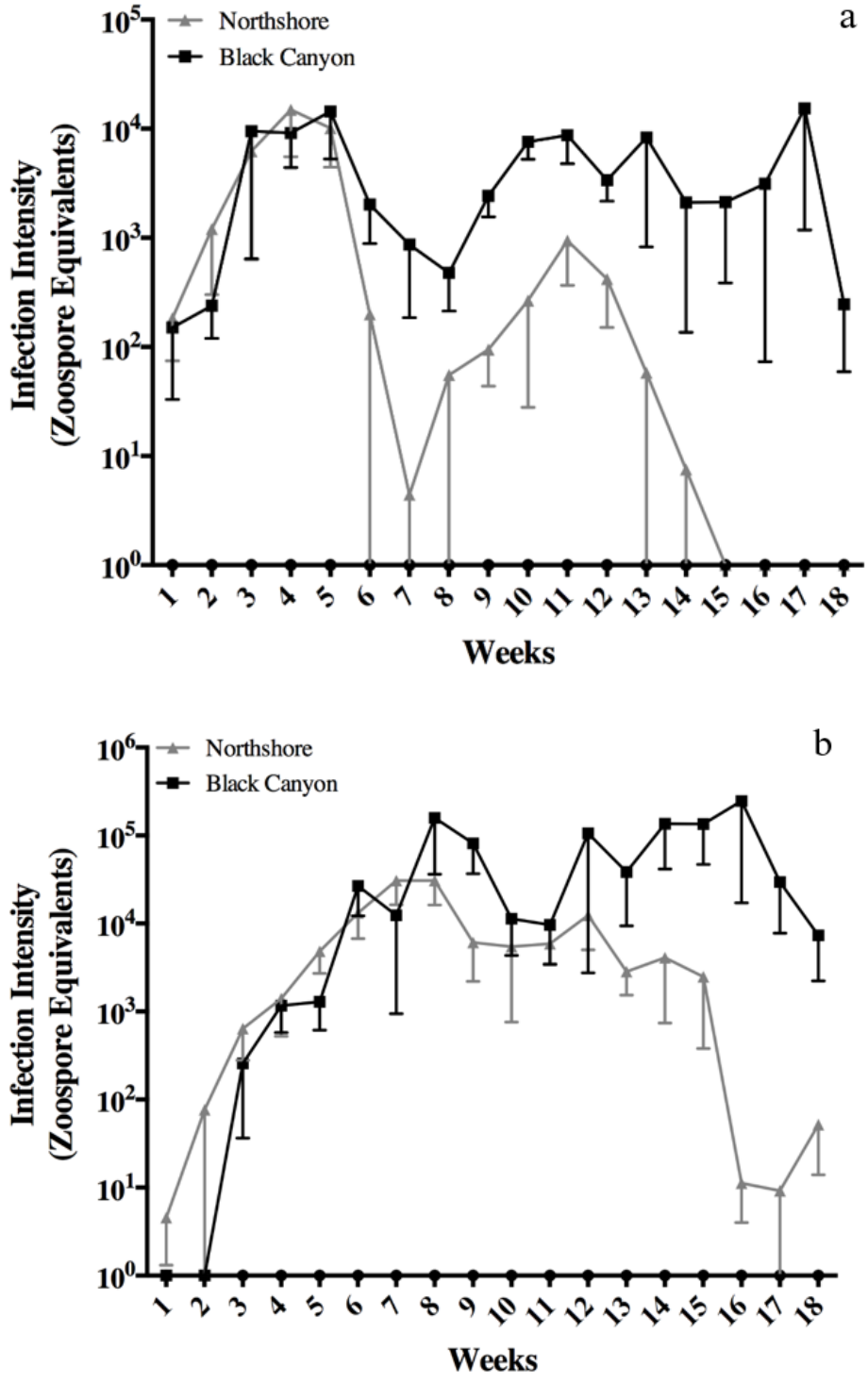


**Figure 12a-b.** Mean weekly infection intensity of *Rana onca* in 1st (a) and 2nd (b) exposure groups of the cohousing experiment separated by site from which frogs were derived. Frogs were exposed to SLL isolate of *Batrachochytrium dendrobatidis*. Error bars denote standard error of the mean with only bottom error bar shown for clarity.

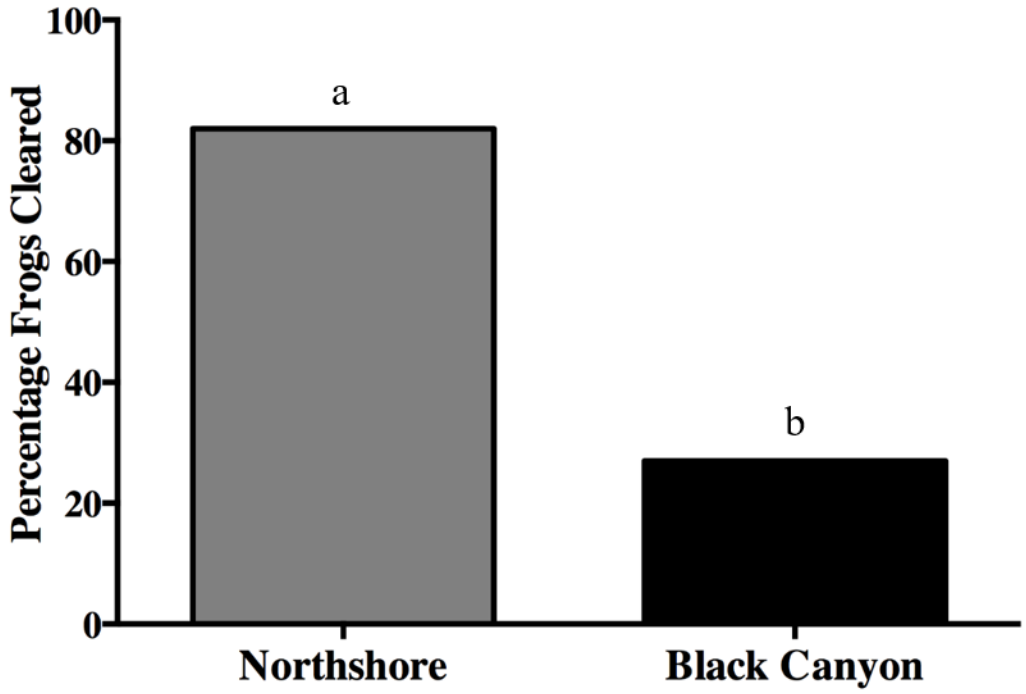


**Figure 13.** Percentages of *Rana onca* in cohousing experiment that cleared SLL isolate of *Batrachochytrium dendrobatidis* grouped by site from which the frogs were derived. Letters denote significant differences between groups.

## DISCUSSION

### Has the *Bd* isolate SLL attenuated?

Previous research on *R. onca* showed evidence of resistant to chytridiomycosis (Jaeger et al. 2017). In that study, juvenile *R. onca* frogs were infected with 2 isolates of *Bd* associated with ranid population declines elsewhere, but survivorship of *R. onca* remained high and not affected by the exposures. One of the isolates used in that study, SLL, had demonstrated high virulence against the original host species, *R. cascadae*, leading to the mortality of over 90% of exposed frogs under laboratory conditions (Piovia-Scott et al. 2015). The other isolate was associated with an epizootic of

chytridiomycosis in *Rana muscosa* (Vredenburg et al. 2010). The lack of mortality in *R. onca* raised the question of whether these isolates had attenuated (Jaeger et al. 2017). We approached this question by challenging *L. caerulea* frogs with the SLL isolate used in the original study on *R. onca*, knowing that *L. caerulea* was susceptible to chytridiomycosis. The resulting low survivorship of these frogs (50%), along with most of the deaths being proceeded by clinical signs of chytridiomycosis, clearly indicates that SLL remained virulent towards this species. We also approached the question of SLL attenuation by passaging the fungus through *R. onca* frogs and then infecting other naïve *R. onca* through exposure to the infected animals by cohousing. If SLL had attenuated, we predicted that the isolate would be more virulent towards the second group of frogs, based on similar experiments with another anuran species (Brem et al. 2013). We did not, however, observe any significant differences in survivorship, infection intensities, or clearance rates between these different exposure groups, and all *R. onca* frogs survived regardless of how they were exposed to the SLL isolate. Given the virulence of SLL we observed in *L. caerulea* and the lack of virulence observed in *R. onca* despite our attempt to increase virulence toward this species, we conclude that SLL is still virulent towards some susceptible host species, but that *R. onca* has tolerance to this particular isolate.

**Is *R. onca* susceptible to *Bd* from the local environment?**

The virulence of *Bd* isolates can vary based on genotype and even between isolates within the same lineage (Fisher et al. 2009; Piovia-Scott et al. 2015). Furthermore, amphibian susceptibility to *Bd* varies widely, with some species showing

infection tolerance (e.g. *Xenopus laevis*, Solis et al. 2010), while others are highly susceptible and succumb to chytridiomycosis (e.g. *Atelopus zeteki* Ellison et al. 2014). Phylogenetic research on *Bd* has currently identified 6 distinct lineages (Bai et al. 2012; Rosenblum et al. 2013), but most amphibian declines have been associated with the emergence of a globally dispersed hypervirulent lineage referred to as the global panzootic lineage (or *Bd*-GPL; Rosenblum et al. 2013). This lineage has been further divided into two main groups, *Bd*-GPL1 and *Bd*-GPL2 (Rosenblum et al. 2013). We do not know which *Bd* lineage (or lineages) occurs within the historical range of *R. onca*, nor do we know when this pathogen colonized the region. Presumably *Bd* arrived with other invasive aquatic species within historical times, and the commercial trade in amphibians provides obvious routes for regional introduction which may be recurring (Picco et al. 2008; Woodhams et al. 2008; Schloegel et al. 2009).

The impact of locally occurring *Bd* strains on *R. onca* was not entirely clear, but the previous exposure of this species to *Bd* isolates associated with ranid declines elsewhere, including SLL, suggested that the impact on *R. onca* was possibly not severe or may have been recently mitigated by adaptation of *R. onca* to *Bd*. As stated by Jaeger et al. (2017, p. 294), "These results may bode well for conservation efforts aimed at establishing *R. onca* populations across a landscape where *Bd* exists." Our exposure of *R. onca* to isolate SMR from *P. regilla* and LBP from *R. onca* in southern Nevada, as well as isolate LC63 from a commercial vendor, clearly shows that the species is susceptible to chytridiomycosis. Isolate LC63 and isolate SMR, acquired from an area of

southern Nevada unoccupied by *R. onca*, both had 100% infectablity in *R. onca*. LC63 had highest mean infection intensities and greatest impact on survivorship we observed (> 1,250,000 ZE), with deaths from chytridiomycosis beginning only 4 weeks after exposure. Isolate SMR also significantly lowered survivorship, with only 58% of frogs surviving to the end of the experiment, although mean infection intensities were somewhat lower than those seen with LC63 and deaths from chytridiomycosis were not seen until 11 weeks after exposure. Interestingly, LBP from a wild caught *R. onca* appeared to have the least impact on *R. onca*. While this local isolate caused chytridiomycosis and death in 3 of the 12 juvenile frogs exposed, and we observed high infection intensities in some frogs (> 8 million ZE), infectablity appeared low, with only 50% of the exposed frogs testing positive for *Bd* infection more than once.

*Bd* can become enzootic, with hosts showing low pathogen loads, survivorship between years, and low population density that may limit disease transmission (Briggs et al. 2010). Although based on very limited field observations, some attributes of an enzootic have been noted at the spring site in Northshore where *R. onca* exists with the *Bd* pathogen (Jaeger et al. 2017). Adult *R. onca* have been observed to either clear *Bd* infection or persist with *Bd* infections over 8 to13 months (Jaeger et al. 2017). In support of this perspective, we observed relatively low infectablity of *R. onca* with the LBP isolate, with only 5 of 12 exposed frogs testing positive for *Bd* more than once. We have, however, observed similar low infectablity with isolate SMR in on-going research challenging *R. onca*. In that experiment, we re-exposed the frogs and all became infected.

The SMR in both these exposures came from the same culture with the same passage history and we used the same exposure protocol; thus, the initial low infectablity was likely caused by factors other than the isolate's innate ability to infect the host. However, the high infection intensities we observe for juvenile *R. onca* in the laboratory, including those sourced from Northshore, appear to be more indicative of epizootic dynamics. Therefore, we caution accepting the conclusion that LBP has low infectability or that the dynamics at Northshore are enzootic. We suspect that had we re-infected frogs during our during our challenge experiment with LBP, infectablity would have been higher and survivorship substantially lower.

Amphibian populations and species in some cases can rebound after *Bd* epizootics (Catenazzi et al. 2017). The mechanisms by which populations recover from epizootics could be a result of local adaptation through the selection of specific immune system genes that confer disease resistance (Savage & Zamudio et al. 2016). We predicted, based on previous observations (Jaeger et al. 2017), that frogs sourced from the Northshore area where *Bd* is present would fare better in their response to *Bd* than frogs sourced from Black Canyon where *Bd* has not been detected. Incorporating all *R. onca* exposed to the various local *Bd* isolates (i.e., LBP, SMR, and LC63), we found that frogs from the Northshore were able to clear their infections in higher proportions than frogs from Black Canyon. More importantly, we observed significant differences in survivorship. While some Northshore frogs were susceptible to chytridiomycosis, as a group, frogs sourced from Northshore survived in much higher proportions than frogs from Black Canyon.

These exciting results suggest that the frogs at Northshore may have adapted to the presence of *Bd*.

**Are recently metamorphosed *R. onca* highly susceptible to chytridiomycosis?**

Newly metamorphosed frogs are thought to initially lack fully functional immune systems required for an effective immune response against *Bd* (Flajnik et al. 1987, Rollins-Smith et al. 2011). In an environment where *Bd* is present, the transition from tadpole to frog (metamorphosis) would likely occur with repeated, if not constant, exposures to the pathogen. We evaluated the susceptibility of *R. onca* exposed as late-stage tadpoles and again as newly metamorphosed frogs, attempting to imitate such conditions. We observed very high survivorship for late-stage tadpoles, with all but one of the tadpoles metamorphosing. In contrast, survivorship following metamorphosis and second exposure to *Bd*, was extremely low (21%). Interestingly, the significant difference in survivorship we observed in the challenge experiments with juvenile frogs between the collection sites (Northshore and Black Canyon) was not repeated in this experiment with metamorphs. Survivorship was similarly low in newly metamorphosed frogs from both sites. The majority of mortalities we observed in newly metamorphosed frogs were within the first 8 weeks post-metamorphosis when immune responses may not yet be mature (Rollins-smith et al. 2011).We speculate that the comparatively low survivorship seen in recently metamorphosed frogs may be due to the lack of immunocompetence at this early life-stage (Bakar et al. 2016).

The observed difference in overall survivorship between our experiments using relatively older juvenile frogs and newly metamorphosed frogs is intriguing, and may indicate an evolved, life-stage dependent mechanism for resistance to chytridiomycosis. Both innate and adaptive immune responses have been shown to be of importance for resistance (Richmond et al. 2009). In *Rana yavapaiensis*, the sister taxon to *R. onca* (Jaeger et al. 2001), certain alleles of the major histocompatibility complex (MHC) class IIB have been suggested to be important for disease resistance (Richmond et al. 2009; Savage & Zamudio 2011; Bataille et al. 2016; Savage & Zamudio 2016). The MHC class II is composed of an alpha and beta chain that together present foreign, lysomally generated antigens to T-cells, which induces acquired immunity (Ohta et al. 2000). Immune suppression at the time of metamorphosis, however, may compromise these activities (Flajnik et al. 1987; Rollins-smith 2011; Bakar et al. 2016), which could explain the lower survivorship and lack of population-exposure effects seen in recently metamorphosed *R. onca*.

**Conservation implications**

*Rana onca* is a species of intense conservation concern and is currently managed under a voluntary conservation agreement and strategy (RFLCT 2016). Establishing new populations through headstarting and translocation has been a successful management strategy for this species. Our results support previous research showing that *R. onca* is resistant to some isolates of *Bd* (Jaeger et al. 2017), but we have also shown that the

species is susceptible to chytridiomycosis from *Bd* isolates found in southern Nevada, as well as an isolate found in frogs from a commercial vendor. Under the current management strategy for *R. onca,* sites where *Bd* is present have generally been avoided for translocations, but sites where *Bd* is not present are very limited. These sites are generally isolated from broader aquatic systems where other anurans known to be vectors of *Bd* (Garner et al. 2006; Reeder & Vredenburg 2012) are present.

When attempting to establish *R. onca* populations at sites where *Bd* is present, we advise not to use tadpoles or frogs younger than 8 weeks post-metamorphosis, since our laboratory results would suggest very low survivorship of these animals. Frogs derived from populations in the Northshore area are more likely to survive at sites where *Bd* is present, but strong selective pressure from *Bd* at Northshore sites, possibly targeting only one or a few genes, could have already reduced the genetic variation present in this area. Low genetic variation could leave these frogs vulnerable to other pathogens (Fu & Waldman 2017) or to other problems associated with inbreeding. An alternative approach, currently being used at one translocation site where *Bd* is present, is to use frogs derived from both Northshore and Black Canyon, thereby capturing as much genetic variation as possible at new sites and letting nature select the most beneficial alleles (J. Jaeger pers. comm.).

Treatments that increase survivorship of frogs in *Bd* infected populations have been attempted with some success. Itraconazole is an effective treatment for clearing

frogs of *Bd* infections (Brannelly et al. 2012) and may serve as a tool to mitigate the impacts of chytridiomycosis outbreaks (Hudson et al. 2016). For example, itraconazole has been shown to increase interannual survivorship of recently metamorphosed *R. cascadae,* allowing frogs to mature past the vulnerable life-stage when immune systems are not fully functional (Hardy et al. 2015).. For *R. onca*, we suggest similar treatments of recently metamorphosed frogs at sites where *Bd* is present, which may allow populations to persist, despite low natural recruitment caused by the pathogen.

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**Anthony W. Waddle**

*Curriculum Vitae*

**Personal Information**

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Email Waddlea3@unlv.nevada.edu

**Education**

2011-2015 University of Nevada, Las Vegas (UNLV)
B.S. in Biology – Cum Laude (3.85/4.0 GPA)

Fall 2015-Fall 2017 (Anticipated) University of Nevada, Las Vegas (UNLV)
Pursuing M.S. in Biology (4.0/4.0 GPA)

**Awards and Academic Achievements**

- Dean's List Honors every semester of B.S. degree (2011-2015)
- 2015 Graduate Recruitment Scholarship ($1600)
- 2016, 2017 Graduate Access Scholarship ($2000)
- 2016 Graduate Professional Student Association Travel Sponsorship ($600)
- 2016 Patricia Sastaunik Scholarship ($2500)
- 2016 Honorable Mention National Science Foundation Graduate Research Fellowship Program
- 2017 Graduate Professional Student Association Research Sponsorship ($1250)
- 2017 Graduate Student Showcase Presentation Finalist ($1500)
- 2017 Rebel Grad Slam 3-Minute Thesis Finalist

**Honor Society Membership**

- Phi Kappa Phi

**Professional Memberships**

- Desert Fishes Council
- American Association for the Advancement of Science
- UNLV Graduate & Professional Student Association
- American Society for Ichthyologists and Herpetologists

**Presentations**

A Questionable Role for Pathogenic Chytrid Fungus in the Decline of the Relict Leopard Frog
Jef R. Jaeger, Rebeca Rivera, Anthony W. Waddle, D. Tyler Harrison, Silas Ellison, Matthew J. Forrest, Vance T. Vredenburg, and Frank van Breukelen
Presented at:

- Amphibian Populations Task Force, University of California, Davis (2016)
- University of Nevada, Las Vegas Graduate & Professional Student Research Forum (2016)
- Colorado River Terrestrial and Riparian Meeting (2016)

Emerging Infectious Disease and the Decline of the Imperiled Relict Leopard Frog
Presented at:

- Joint Meeting of Ichthyologists and Herpetologists, Austin, Texas (2017)
- Friends of Nevada Wilderness Wild Speaker Series (2017)
- UNLV Graduate Student Showcase (2017)

**Publication**s

Jaeger, Jef R., **Anthony W. Waddle**, Rebeca Rivera, D. Tyler Harrison, Silas Ellison, Matthew J. Forrest, Vance T. Vredenburg, and Frank van Breukelen. "*Batrachochytrium dendrobatidis* and the Decline and Survival of the Relict Leopard Frog." *EcoHealth* (2017): 1-11.

**Waddle, Anthony W.**, Marlai Sai, Joshua Levy, Ghazal Rezaei, Jef R. Jaeger "Systematic approach to isolating *Batrachochytrium dendrobatidis*." Diseases of Aquatic Organisms, *in review*

**Conferences**

- Cal/Nevada Amphibian Populations Task Force (2015-2017)
- Colorado River Terrestrial and Riparian Meeting (2016)
- Desert Fishes Council (2015)
- Joint Meeting of Ichthyologists and Herpetologists (2017)

**Current Research Projects**

- Experimental immunizations as a potential to improve translocation success across a landscape where *Batrachochytrium dendrobatidis* occurs: Currently evaluating the utility of two different immunization methods in improving survivability of two species of leopard frogs to chytridiomycosis.
- The effects of disease on different life-history stages of two species of leopard frog: Performing challenge studies to assess the viability of tadpoles and metamorphs that are exposed to *Batrachochytrium dendrobatidis*.

**Past Research Projects**

- A systematic approach to isolating the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, from common disease resistant species of the genus *Pseudacris* (possible *Hyliola*): Systematically sampling wild frogs infected with *Bd* to improve

pathogen isolation success where viable *Bd* cultures are required for downstream study. Also evaluating the efficacy of non-lethal pathogen isolation approaches.
- eDNA early detection of invasive species at Moapa Valley National Wildlife Refuge and Muddy River: Currently optimizing a protocol by comparing DNA acquisition methods and various extraction and PCR protocols to better improve invasive species monitoring.

**Teaching Experience**

- **Undergraduate teaching assistant (UTA) –** UNLV UTA Program, modern biology I. Created and delivered presentations/lectures, set up labs, and assisted students and lab instructor.
- **Substitute Teacher-** Clark County School District. Taught 9$^{th}$ grade biology and 8$^{th}$ grade physical science.
- **Teaching Assistant –** Taught modern biology I & II laboratory. Developed and delivered presentations, designed and administered quizzes, and graded student work.

**Research Mentoring (Undergraduate Research Assistants)**

- Alexandra Zmuda (2015-current)
- Yesenia Vasquez (2016-current)
- Marlai Sai (co-author; 2016-current)
- Josh Levy (co-author; 2016-current);
  Served as an Advisor for Josh's McNair & Title III AANAPISI Summer Research Institute Project "Assessing a Potential Disease-Susceptible Life-History Stage in an Imperiled Anuran Species" Completed Summer 2017
- Ghazal Rezaei (co-author; 2016-current)
- David Miller (2016-current)
- Jessica Hill (2017-current)
- Alexa Krauss (2017- current)
- Emma Keenan (2017-current)
- Hanna Rice (2017-current)
- Shaylene Scarlett (2016-2017)
- Nikhil Pattni (2016-2017)
- Jason Burgins (2016)
- Audrey Bischoff (recent HS graduate; 2016)

**Volunteer Experience**

- **Graduate Student Liaison for the UNLV Ecology, Conservation, and Evolution Club (2015-Present)**
  Facilitate student events involving undergraduate students from all majors in biological research projects. Club was started by one of my undergraduate mentees, Alexandra Zmuda.

- **STEM Ambassador for the Nevada STEM Coalition (August 2015 – Present) –** Through communications with this organization, we have set up a program to allow incoming UNLV freshman to experience original scientific research the summer before their first semester.

- **Undergraduate research assistant (2013-2015) –** UNLV Integrative Physiology Lab. Assisted on several RNAi assays to elucidate the molecular pathway of salivary gland secretion in *Drosophila Melanogaster.*

- **Local Committee Volunteer (2015 – Present) –** California/ Nevada Amphibian Populations Task Force. Volunteer meeting registration.

- **Undergraduate researcher (May 2015 – August 2015) –** Desert Research Institute, Emerging Contaminants and Environmental Microbiology. Developing methods to culture novel microbes, for classical characterizations.

- **Lead undergraduate researcher (February 2014 – August 2016) –** UNLV amphibian disease lab. Studying the effects of a fungal pathogen (*Bd*) on an anuran species of conservation concern. Cared for over 50 frogs housed in laboratory during multiple, 15-week long experiments. Specific tasks included: *Bd* culture maintenance/storage, animal feedings & water changes, protocol development, qrPCR, data collection & analysis, and manuscript writing.

- **Volunteer (February 2015 – June 2015 & September 2015 – Present) –** U.S. Environmental Protection Agency (EPA). Scanning electron microscopy. Imaging of nanoparticles and biological samples.

- **Volunteer (2014 – Present) –** Nevada Department of Wildlife (NDOW). Amargosa Toad Surveys. Assisted biologists in seasonal capture-mark-recapture and survey efforts in support of an anuran conservation initiative.

- **Volunteer (Feb 2015 – Present) –** Bureau of Land Management (BLM). Hummingbird Banding.

- **Volunteer (2001 –2003)** – Gilcrease Bird Sanctuary. The youngest volunteer they ever had. Had a blast with the birds! I worked maintaining aviaries, hatching chicks, and talking to visitors.

**Relevant Employment**

| Date | Employer/Job Title | Description |
|---|---|---|
| Jan 2016 – Present | UNLV Research Assistant | Externally funded from an USFWS grant entitled, “eDNA early detection |

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|---|---|---|
| | | of invasive species at Moapa Valley National Wildlife Refuge and Muddy River." Currently optimizing a protocol by comparing DNA acquisition methods and uniquely assessing the kinetics of DNA degradation in this system. |
| Aug 2015 – Dec 2015 | UNLV Teaching Assistant | Taught two sections of modern biology laboratory (biol196). Created lectures, quizzes, set up labs, and graded assignments. |
| June 2015 – Aug 2015 | U.S. Environmental Protection Agency (EPA)<br>Student Trainee, Biology (Pathways Internship) | Assisted staff scientists with qualitative analysis of a variety of environmental samples. Performed data entry, equipment maintenance, and literature surveys. Utilized scanning electron microscopy (SEM), field flow fractionation (FFF), and single particle-inductively coupled plasma mass spectrometry (SP-ICPMS), to characterize nanoparticles in suspension. |
| May 2015 – Aug 2015 | Desert Research Institute<br>Student Worker II<br>Division of Earth & Ecosystem Sciences | Environmental Microbiology. Assisted with summer projects concerning soil and spring microbiology. Microbial culturing, DNA extractions, sequence analysis. |
| Sept 2013 – Aug 2015 | UNLV Undergraduate Student Worker/Researcher | Assisting on conservation efforts for the Relict Leopard Frog (*Rana onca*)<br>Duties include: Capture-mark-recapture, Nocturnal surveys, habitat maintenance, egg-mass surveys, and translocations. Cultured and cared for fungal (*Bd*) microbial stock cultures used in laboratory studies. |
| Jan 2014 – Feb 2015 | U.S. Environmental Protection Agency (EPA)<br>Student Services Contractor | Characterizing nanoparticles in suspension using a variety of modern technologies including: Scanning electron microscopy (SEM), field flow fractionation (FFF), and single particle- |

inductively coupled plasma mass spectrometry (SP-ICPMS).

**References**


| Dr. Jef Jaeger | Dr. Frank van Breukelen | Dr. Stanley Hillyard |
|---|---|---|
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