Using Tiger Salamanders (*Ambystoma tigrinum nebulosum*) to Explore the History of the Fungus *Batrachochytrium dendrobatidis* as an Emerging Infectious Pathogen in Arizona

by

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ABSTRACT

Emerging infectious diseases (EIDs) in vulnerable populations are a proposed cause of reduced global biodiversity due to local and regional extinctions. Chytridiomycosis, a fungal disease caused by *Batrachochytrium dendrobatidis* (Bd), is affecting amphibian populations worldwide.

Chapter 1 of this thesis reports using lab-raised larval tiger salamanders (*Ambystoma tigrinum nebulosum*), collected as eggs, to test if Bd infects them. Bd infects metamorphosed tiger salamanders; however, it is currently unknown if larvae can be infected by Bd. Adult frogs tend to host Bd on ventral surfaces and hind legs while tadpoles host Bd in keratinized mouthparts. No research has considered differences in infection between life stages of salamanders. It was hypothesized that Bd can colonize larvae in the same manner as metamorphosed animals. Larval salamanders were inoculated to test if Bd concentrations differ among body regions in larvae compared to metamorphosed salamanders. Larvae can carry Bd with the concentration of Bd varying between body region.

Chapter 2 report using native tiger salamanders (*Ambystoma tigrinum nebulosum*), from northern Arizona and Bd as a study system to test if Bd is native or introduced to Arizona. It was hypothesized that Bd is not endemic to Arizona, but is introduced. There are multiple hypotheses regarding potential routes Bd may have traveled through Arizona and into Mexico. These hypotheses were tested using the Kaibab Plateau in Coconino County, Arizona, as a study site. The plateau is isolated from surrounding areas by the Grand Canyon to the south and the Vermillion Cliffs to the north serving as major biogeographical barriers. It is hypothesized that tiger salamanders are not dispersing into or out of the Kaibab Plateau due to geological

restrictions. Bd, therefore, should not be present on salamanders on the Kaibab Plateau due to geological restriction. Tiger salamanders in stock tanks located on the Kaibab as well as preserved museum specimens housed in the Arizona State University Natural History Collection were sampled. The results indicate that Bd occurs at low levels on Kaibab Plateau tiger salamanders.

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Evan Brus has done above and beyond in supporting me even before I became a graduate student. Everything I know from collecting samples, growing Bd cultures, extracting DNA, analyzing my samples through qPCR, and everything in between is a result of his patience in teaching me. I believe what I learned through Evan allowed me to finish my program efficiently.

Kari Haygood and Lena Meyer from the Collins lab assisted me in extracting many samples. Charlotte Johnston provided me with access to the natural history collection and tools to safely swab museum specimen. Dr. Nico Franz gave me invaluable advice on my presentations. Mahogany Lambert convinced me to get my Master of Science degree, which I see as the triggering factor in starting grad school.

Lastly, I would like to thank my family (Hideyoshi Otsuru, Setsuko Otsuru, Nao Otsuru, and Nayumi Otsuru) for their support and encouragement throughout my studies. I also want to

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Introduction

Emerging infectious diseases (EIDs) may be significant threats to vulnerable populations by causing high mortality rates. As globalization continues, EIDs play a significant role in local and regional wildlife declines and extinction, resulting in reduced global biodiversity (Daszak et al., 2000; Scheele et al., 2019). Pathogen-related biodiversity losses have contributed to high extinction rates in some taxonomic groups, suggesting we are entering a sixth mass extinction (Barnosky et al., 2011).

Of the many species threatened globally with population decline and extinction, amphibians are among the most vulnerable. According to the International Union for the Conservation of Nature (IUCN) Red List, amphibians are the most threatened vertebrates with 552 species critically endangered (2017.3 IUCN Red List Report), outnumbering mammals and birds. Amphibian declines have been observed across all continents except Antarctica (Stuart et al., 2004). Although threats such as habitat alteration and climate change contribute to the losses, numerous amphibian population declines are linked to fungal infectious diseases (Berger et al., 1998; Daszak et al., 1999, Muths et al., 2003; Collins and Crump, 2009; Scheele et al., 2019).

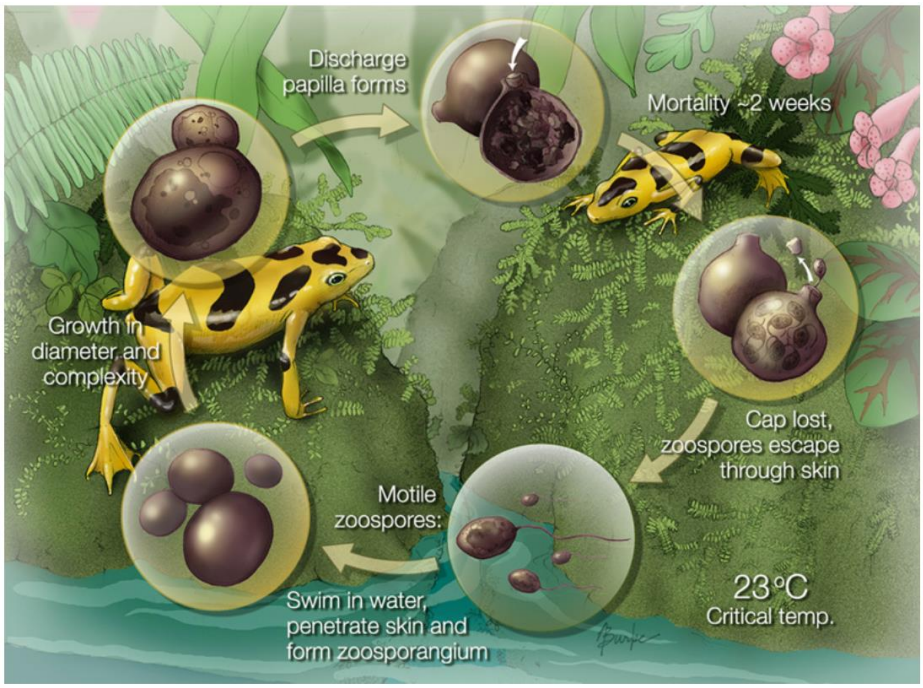

Figure 1: The life cycle of *Batrachochytrium dendrobatidis*. (Adapted from Collins, 2013, drawn by Alison Burke).

Chytridiomycosis is one example of an EID. It is a fungal infectious disease caused by *Batrachochytrium dendrobatidis* (Bd), infecting amphibians at a global scale. Bd belongs to a group of fungi known as chytrids. Bd has two distinct lifestyles, a free-living zoospore and an anchored zoosporangium (Fig. 1). As a zoospore, Bd swims through water using its flagellum to find a suitable host. Upon reaching a host, the zoospore burrows into the host's keratinized epidermal layers forming zoosporangia (Longcore et al., 1999; Berger et al., 2005). In approximately a week, zoosporangia release zoospores into the environment to infect new hosts or move to an uninfected area of its current host (Fig. 1; Collins, 2013). Once infected, Bd releases immune response inhibiting compounds (Fites et al., 2013; Fites et al., 2014), while the infected epidermis

thickens, becoming overly keratinized. As a result, the host's skin permeability and ion transport system are disrupted leading to lethargy, or in the worst case, death (Voyles et al., 2009; Campbell et al., 2012; Carver et al., 2010).

Bd is a generalist pathogen able to infect over 700 species of amphibians (Bosch et al., 2015). Symptoms of chytridiomycosis vary within and among species. The American Bullfrog, *Lithobates catesbeianus,* for example, has a high tolerance to Bd; therefore, Bd infection of American Bullfrogs rarely results in death. Its unusually high tolerance and wide distribution suggests American bullfrogs are a potential Bd reservoir (Garner et al., 2006; Hanselmann et al., 2004). In contrast, other species such as the Panamanian Golden frog and other members of the genus *Atelopus* suffer high mortality, even extinction (La Marca et al., 2005; Gewin, 2008). Other species have mixed outcomes resulting in local extirpation, but not necessarily extinction.

Tiger salamanders have a high tolerance of Bd. They occur across a wide geographical range within the United States and northern Mexico. As juveniles develop into adults, diet and environmental stress may lead to diverse morphs such as terrestrial, aquatic, and cannibalistic adults (Collins and Holomuzki, 1984). Field-collected metamorphosed animals and lab animals both show positive results for Bd infection (Brus, pers. comm.; Davidson et al., 2003; van Rooij et al., 2011). No studies, however, have tested if larval salamanders can host Bd.

Marantelli et al. (2004) described tadpoles hosting Bd in keratinized mouthparts. This contrasts with adult frogs that host Bd on the ventral surface and hind legs (Hyatt et

al., 2007). Like adult frogs, metamorphosed salamanders host Bd on the ventral surface and their feet (Van Rooij et al., 2011; Vasquez et al., 2009).

No tests have compared metamorphosed and larval salamanders regarding Bd occurrence. Since larvae have similar body structures to metamorphosed animals, the potential to host Bd is high. Aquatic larvae are likely to be infected on the ventral surface as reported for metamorphosed animals. Furthermore, the fully aquatic lifestyle of larvae increases the chance of infection by Bd of other body surfaces. I hypothesize that aquatic larvae can host Bd and predict that different body surfaces vary in Bd concentrations.

Materials and Methods

Inoculation of larvae

I used *A. tigrinum nebulosum* larvae from field collected eggs for this experiment. The eggs were collected on March 6, 2017 from the Mogollon Rim. A total of forty animals (twenty infected, twenty control) were placed individually in a container filled with 250mL of water covered with a top to decrease evaporation. Water level was kept low to concentrate Bd in the water bath. A Bd strain isolated on April 12, 2017, from wild-caught *Pseudacris maculata*, (caught March 6, 2017) was used for infecting the animals. I prepared four mTGH agar plates (mixture of tryptone, gelatin hydrolysate, deionized water, and agar) to grow Bd cultures with two additional plates with only mTGH medium as controls. Zoospores were added to the plate on December 24, 2017. I collected Bd zoospores about a week after preparation of plates. I estimated number of spores using a hemocytometer.

Within the collected liquid, I counted approximately 40 million zoospores/ 5 mL. Each animal received 250 microliters of the liquid with about 2 million zoospores. Zoospores were introduced by dropping the liquid into the water of the container where animals were housed individually.

Swabbing Animals

Swabbing areas were slightly modified from Van Rooij et al. (2011). Since aquatic larvae are immersed in water during this life stage, some of the body regions were grouped as the same area. For example, the ventral and dorsal surfaces of the torso were grouped as "body"; similar grouping was done with "head" etc. Initially, the surface inside the mouth was a potential swabbing area just as when swabbing tadpoles, but was dismissed due to unwanted consequences such as stress and possible dislocation of a specimen's jaw. Forcing a larva to open its mouth while alive can be both stressful and dangerous; therefore, I did not use the mouth as a swabbing region.

I swabbed all animals one week after inoculation. Control and Bd-infected animals were swabbed a total of 16 times. Control animals were swabbed with a single cotton swab in the same regions as Bd-infected animals. Bd-infected animals were swabbed in four body regions (head, torso, tail, and limbs) with four separate cotton swabs. Each swab was used on a specific body region. Swabs covered an equal surface area of the body. Identical swab samples were taken on dorsal, ventral, and limbs of each specimen. Swabs on the head were done by moving slowly toward the mouth twice dorsally and twice ventrally. Torso swabs were made in a similar manner, but the swabs

faced toward the tail. Tail swabs were made by moving from the base to the tip of the tail. Lastly, limbs were swabbed only on the ventral side, away from the body. Toward the end of the limb the swab was slightly twisted to gather as many zoospores as possible. Animals were then swabbed after the second week to test for any remaining Bd infection. Swabs taken on the second week ensured that Bd was living on the host. This time, both control and infected groups were sampled with a single swab per animal since I was only testing for presence and not concentration of Bd. All swabs were preserved in 0.5mL 70% ethanol. I changed gloves between handling animals to prevent cross-contamination. Water was not changed nor were the animals moved from their original container during this experiment.

Analysis

I followed the Qiagen DNeasy kit© extraction protocols for animal tissues with modification as suggested by the company representative. Swabs were air dried inside a hood to allow any ethanol on the swab to evaporate. The Qiagen method does not work well with ethanol and may hinder DNA replication. Evaporation of the swabs must be done by leaving the cap of a vial loose while covering the contents to prevent contamination. The DNA extraction process began after two days of air drying.

Extracted DNA samples were analyzed using qPCR. Taqman Universal PCR Master Mix© was used for the reaction. In each well plate, Molecular water was included as a negative control and exogenous Bd samples (ranging from 2x10^2 to 2x10^-2 zoospores) as positive controls. I tested all samples in duplicate wells. After completion

of a reaction, results were checked for logarithmic curves and reaction time indicating Bd presence. Three possible outcomes were expected: “duplicate” (both wells showing a positive number), “singlicate” (one of two wells showed a positive number), or “zero” (neither well showed any response). I interpreted samples with duplicate responses as Bd positive, while samples with zeroes I interpreted as Bd negative. I re-analyzed samples with a singlicate response using qPCR. I interpreted additional positives (singlicates or duplicates) that presented as positive as a Bd positive well. Zeroes in addition to the first run were interpreted as Bd negative.

Results

Initial PCR runs showed the highest number of duplicates in the head region (n = 14 animals), then limb (n = 10), body (n = 6), and tail (n = 3) (Fig. 2). Zeroes were most common on the tail with (n = 7 animals) followed by body (n = 5), head (n = 3), and limb (n = 2). Several singlicates were present per region. Tail, body, and limb all had similar results (n = 10, 9, 8) with head having the least (n = 3). A total of 30 samples among the four regions were re-analyzed by qPCR; 14 showed singlicate and 7 showed duplicate, which I interpreted as Bd positive. Nine samples showed no response and were recorded as Bd negative. All animals showed a positive response to Bd infection in at least one body region after interpretation of qPCR result. The head region was the most infected with 16 animals showing a positive response, while the tail region was the least infected with only 9 animals showing a positive response (Table 1). The body and limb region both had 14 animals showing a positive response (Table 1). Nineteen of the twenty

control animals showed no response. One animal showing a singlicate later tested zero and was interpreted as negative.

Swabs taken from the same 20 animals on the second week after inoculation tested duplicate (n = 13 animals), singlicate (n = 5 animals), and 0 (n = 2 animals). Out of the 20 animals that showed at least one duplicate response, 13 animals continued to display presence of Bd. No additional qPCR was done on singlicates as duplicate results ensured that the animals were infected with the fungus.

PCR results showed that all Bd-exposed animals tested Bd-positive in at least one body region of four. No animal showed clinical signs of chytridiomycosis. This is consistent with previous studies using terrestrial animals. qPCR results suggest this species is tolerant to Bd infection, even as a larva. Such lack of response to infection calls for use of sensitive extraction processes, combined with qPCR, to estimate the smallest Bd load present on their skin. Positive swab results in the second week confirmed that salamanders were not able to clear infections when exposed to higher concentrations of Bd. The data suggest tiger salamander larvae can host Bd, however, the number of positives varied between body regions (Fig. 2). Mortality of salamanders, as a result of chytridiomycosis, was not observed during the 2 weeks inoculation period.

Individual	Head	Body	Limb	Tail
1	-	-	-	+
2	+	+	-	-
3	+	+	+	-
4	+	+	+	+
5	+	+	+	-
6	+	+	+	+
7	+	+	+	-
8	+	+	+	+
9	-	+	+	+
10	-	+	+	+
11	-	-	+	-
12	+	-	+	+
13	+	-	+	+
14	+	+	-	+
15	+	+	-	-
16	+	-	-	-
17	+	+	+	-
18	+	+	+	-
19	+	-	-	-
20	+	+	+	-

Table 1: Larval Samples Showing Positive (blue) or Negative (red) results for Bd

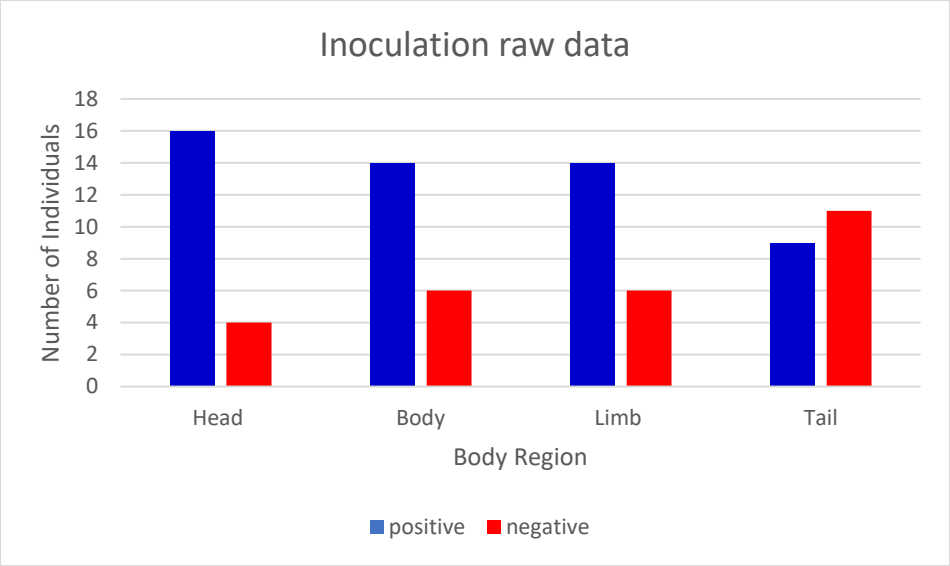

Figure 2: Graph comparing number of individuals testing positive or negative per body region.

Discussion

The interaction of salamander larvae with Bd is understudied. Although past experiments successfully infected metamorphosed tiger salamanders in controlled environments, there are no similar studies with larvae.

My experiment had two objectives: 1) test if tiger salamander larvae can host Bd; 2) test for differences in Bd infection among different body regions. My result suggest that larvae host Bd, but concentration of Bd varied among body regions.

Tiger salamanders are tolerant of Bd infection. As explained earlier, tadpoles, just as some metamorphosed frogs, can host Bd. Presence of keratin is a crucial factor for hosting Bd in a tadpole's mouthparts (Daszak et al., 1999; Marantelli et al., 2004; Longcore et al., 1999). In addition to infecting larvae, I was also interested in whether Bd grew better in certain body regions. Since infection rates differ among life stages in frogs, I hypothesized that salamanders would show similar differences.

Marantelli et al. (2004) confirmed that Bd grows on keratinized surfaces in experiment with tadpoles and metamorphosed frogs. This may suggest that keratin is present in the epidermal layer in all salamander life stages and not limited to metamorphosed individuals. This result contrasts with those for tadpoles in which Bd growth was limited to its oral disc. More research is needed to describe the compositions of the epidermal layers of larvae and test for the presence of keratin and its correlation with Bd infection. No immediate lethargy or mortality also suggests that larval salamanders may host Bd for longer periods in isolated ponds and stock tanks. These results increasingly highlight the significance of tiger salamanders as reservoir species for Bd.

More research should be done on larval stages of amphibians. Movement of salamander larvae for use as fishing bait occurs in western US states such as Arizona (Collins, 1981). The pet trade is a major factor in moving animals across states and country borders (Farrer et al., 2011). I suggest that other salamander and newt species should be tested for Bd and any possible contribution to the spread of Bd across the continent (see also ‘Scheele et al., 2019).

Inoculation of larval salamanders was done to test if larval salamanders can be indicators of Bd in a landscape. Initially, I did not know what life stages of tiger salamanders would be present on the Kaibab Plateau. Although it was certain neotenic adults were absent, it was possible that metamorphosed animals would be rare or absent in the bodies of water sampled. If most animals were larvae, estimating Bd presence would be difficult. In addition to my finding that larval animals can be indicators of Bd, I

found that larval salamanders are not limited to hosting Bd on a specific body region. This indicates that standard swabbing methods for metamorphosed animals can be used for larvae. I recommend that for future studies both larval and metamorphosed tiger salamanders should be sampled to accurately estimate Bd presence in a habitat.

Chapter 2: The biogeography of Bd on the Kaibab Plateau

Introduction

In the 1990's, frogs from the (US) National Zoological Park died from an unknown skin disease. The skin disease was later identified as chytridiomycosis caused by Bd (Longcore et al., 1999). At the time, there were no previous records of parasitism of zoosporic fungi on vertebrate tissue (Sparrow, 1960; Powell, 1993). Despite such an unprecedented event, occurrence of Bd was increasingly reported outside of zoological institutions, most noticeably through loss of amphibians.

Major losses of frog species in particular were reported as Bd spread through Central America leading to a decrease in amphibian diversity (Collins and Crump, 2009). This is one of very few examples documented where an infectious disease is the main cause of species extinction (Skerratt et al., 2007; Collins and Crump, 2009). The movement of Bd is an opportunity to further our understanding of EIDs and their effect on naïve hosts through Bd-related amphibian mortality and population decline (Berger et al.,1998; Muths et al., 2003, Stuart et al., 2004). Bd occurs on amphibians on every continent except Antarctica, where there are no amphibians (Rachowicz et al., 2005; Kilpatrick et al., 2010).

The historical origin of Bd is beginning to come into sharper focus. Although most of the widespread mortality events are associated with one Bd lineage, the global pandemic lineage (BDGPL), other strains are known. There is evidence for a complex evolutionary history of Bd strains with some of the oldest strains in Brazil (Rosenblum et al., 2013; Rodriguez et al., 2014). Other studies suggest origins in China, Korea, Japan,

and Africa (Swei et al., 2011; Bataille et al., 2013; Farrer et al., 2011; Goka et al., 2009). The most detailed study supports a likely role for East Asia, South Korea in particular (O'Hanlon et al., 2018).

Bd's wave-like movement through Central America starting in Mexico is well documented (Cheng et al., 2011; Lips et al., 2006; Lips et al., 2008). Within the United States, Bd has been documented from museum collections and field studies (Padgett-Flohr and Hopkins II, 2009; Talley et al., 2015). Bd has also been confirmed in several counties in Arizona (Annis et al., 2004; Bradley et al., 2002; Schlaepfer et al., 2007). I hypothesize that Bd passed through Arizona as an exotic pathogen before entering Mexico. Currently, there are five hypotheses for potential routes by which Bd may have entered Arizona (Figures 3 and 4).

1. Bd entered with native frogs and salamanders that dispersed south from the Rocky Mountains.
2. Bd entered eastern Arizona with invasive tiger salamanders (*Ambystoma tigrinum mavortium*) originally brought as fish bait (Collins, 1981).
3. Bd entered southwestern Arizona with Rio Grande leopard frogs (*Lithobates berlandieri*) when introduced in Yuma.
4. Bd entered northwestern Arizona with nonnative bullfrogs (*Lithobates catesbeianus*).
5. Bd may have dispersed north on native amphibians from Mexico.

I assume that Bd is nonnative to Arizona arriving on amphibians that dispersed from other parts of the US or were imported into Arizona through the bait trade. The Kaibab Plateau in Coconino county, Arizona, has tiger salamanders (*Ambystoma tigrinum*) (Gehlbach et al., 1969; Collins, 1981). It is, however, now biogeographically isolated as far as salamanders are concerned. The Grand Canyon to the south of the plateau, the Vermillion Cliffs to the north, and its overall higher elevation relative to surrounding arid lands, provide geographical boundaries that limit or entirely prevent dispersal from salamander populations off of the plateau. Assuming that Bd emerged in the western US in the last part of the 20th century I predict that the Kaibab Plateau lacks Bd because its physical barriers prevented the introduction of the pathogen on dispersing amphibians.

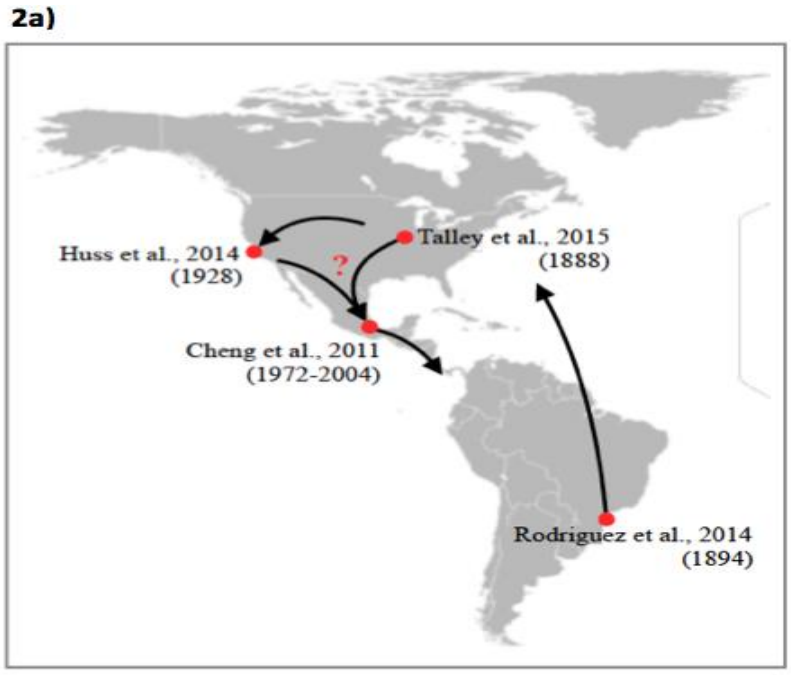

Figure 3. Potential Bd movement, from Brazil to the United States through Arizona and into Mexico.

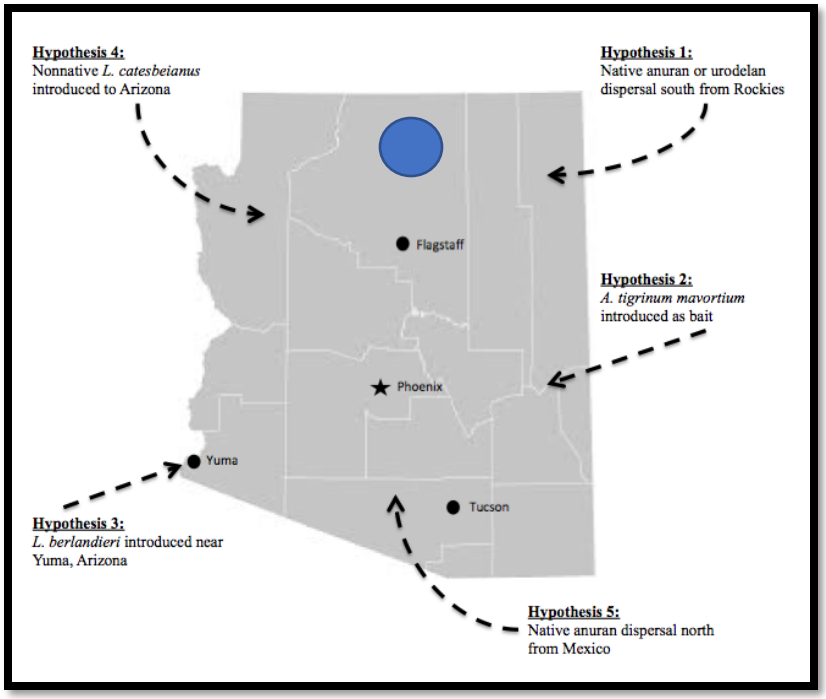

Figure 4. Five hypotheses for how Bd entered Arizona. Blue circle indicates the Kaibab Plateau

Materials and Methods

Collection of field samples

All samples were collected September 2-4, 2017, in the Kaibab National Forest within a 7-mile radius of Jacob Lake. Ponds were chosen before visiting sites using Google Earth Pro©. Different capture methods were used: seine in low vegetation, open ponds; a dip net in ponds with dense vegetation. 20 animals were caught per pond. All equipment used was sprayed with disinfectant Quat-128 after each pond use to prevent contamination between sites. In addition to disinfection, all tools were air-dried away from bodies of water to prevent chemicals from washing into the aquatic habitats.

I collected 81 tiger salamanders (*Ambystoma tigrinum nebulosum*) from five stock tanks on the Kaibab Plateau (Table 3). Different morphological stages were included and treated identically. Each animal was swabbed a total of 20 times. Swabs focused on four areas of the body (head, body, tail, limbs), areas that correspond with the inoculation experiment. Each body area was swabbed a total of five times for consistency of sampling among individuals. All animals were swabbed with one cotton swab. Gloves were changed after sampling each animal to prevent contamination. Swabs were placed in a vial with 1mL of 70% Ethanol to preserve Bd DNA. Each vial was labeled with an identification number to differentiate among pond locations.

Museum Specimens

In addition to field collected animals, I sampled preserved terrestrial tiger salamanders housed in the Arizona State University Natural History Collection. All animals used were from Coconino County, specifically specimens collected on the Kaibab Plateau.

I limited samples to adult metamorphosed salamanders, which host Bd (Davidson et al., 2003). Neotenic adults do not occur on the Kaibab (Collins, pers. comm.). I limited specimens to metamorphosed juveniles and adults as opposed to larval salamanders. By limiting samples to metamorphosed animals, I reduced the time and resources needed to estimate occurrence of Bd.

Jars from Coconino County were separated from other counties and later organized by localities. Jars with specimens collected outside of the Kaibab were disregarded. Specimen jars with aquatic animals were also removed. A total of 63 jars

(without jar-JJ) collected in 23 different locations from 1982-1991 were used (S3). Jar-JJ was not included in the analysis due to reoccurring contamination of the controls.

Before swabbing, specimens were placed in a dissecting tray. To prevent contamination between jars, dissecting trays were thoroughly rinsed in the sink after sampling each jar. Prior to swabbing I rinsed all animals with 70% ethanol to rid them of Bd cells that may have been from other animals sharing the container. Each salamander was swabbed with a single cotton swab on the head, body, limb and tail. Gloves were changed after sampling each animal to prevent cross-contamination between specimens. The swabs were not placed in ethanol but instead were placed in an empty 2mL vial and stored immediately in the freezer. Each jar was labelled with a unique alphabet code and numbers to identify the individual. The frozen samples were quickly transported to the lab where extraction took place.

Analysis

I followed the Qiagen DNeasy kit© extraction protocols for animal tissues with modification as suggested by the company representative. Since the swabs were placed in empty vials, air-drying wasn't necessary. The extracted DNA samples were analyzed using qPCR. Taqman Universal PCR Master Mix© was used for the reaction. In each well plate, Molecular water was included as a negative control and exogenous Bd samples (ranging from 2x10^2 to 2x10^-2 zoospores) as positive controls. All samples were tested in duplicate wells. After completion of the reaction, results were checked for logarithmic curves and reaction time indicating potential Bd presence. Three possible outcomes were expected: duplicate (both wells showing a positive result), singlicate (one

of the two wells shows a positive result), or zero (neither well showed a response). I interpreted wells with the same positive response as Bd positive while samples with a negative response I interpreted as Bd negative. Samples with only one well responding positive, were re-analyzed using qPCR. Any additional positives (one or both wells) were interpreted as Bd positive. I interpreted no response in both wells, in addition to the first run, as Bd negative.

Results

409 museum samples from 1982-1991 in addition to 81 Kaibab field samples (collected in 2017) were analyzed using qPCR (Table 2 and 3). Only 2 out of 409 from museum samples showed a duplicate positive signal after the initial reaction. No field-samples initially responded as duplicates. 385 museum samples and 72 field-collected samples showed no response. The remaining samples were singlicates, with nine from the field samples along with 22 singlicates from museum collections. Singlicate samples from field and museum groups were reanalyzed. Three additional singlicates and one duplicate were recorded in field-collected samples. Four additional duplicates and two singlicates were recorded in museum samples. I interpreted these samples as positive for Bd, increasing the total positive samples to 4 out of 81 field-samples and 8 out of 409 museum samples. A total of 478 samples were recorded as negative (Figure 5).

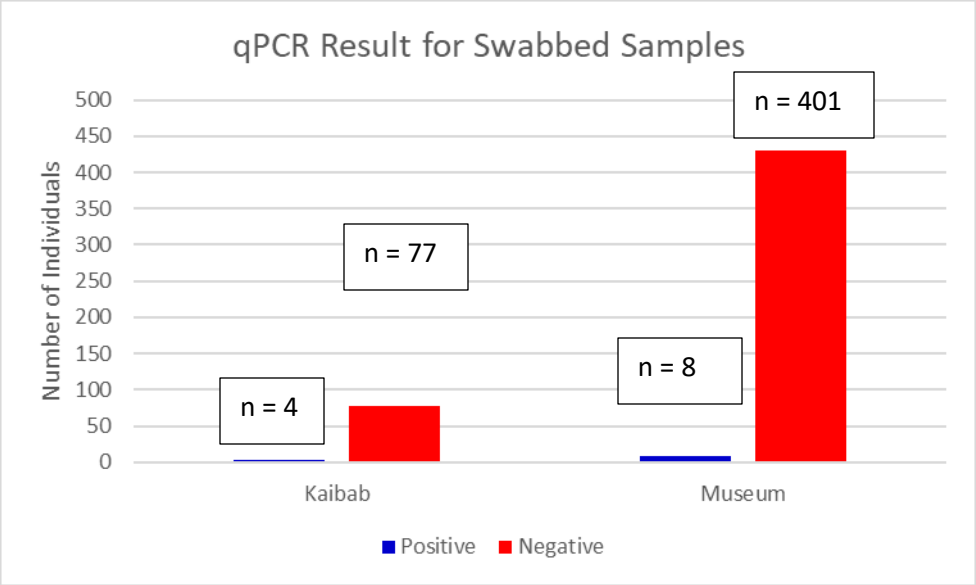

Figure 5: Number of positive and negative animals in Kaibab field collection and museum samples.

Discussion

The Kaibab Plateau has three biogeographically distinct communities separated by altitude: Petran Subalpine Conifer Forest, Petran Montane Conifer Forest, and Great Basin Conifer Woodland (Brown, 1994). Much of the plateau is dominated by the woodlands, which surround the conifer forest at the highest elevation. The biotic communities limited to the Petran Subalpine Conifer Forest are only found in two other locations in Arizona, the San Francisco mountains (with Humphrey's peak, the highest point in Arizona) and the White Mountains, both connected by montane conifer forest on the Mogollon Rim. In contrast, the Kaibab Plateau is isolated in almost all directions. The northern parts of the Kaibab Plateau are isolated by the Vermilion cliffs of the lower Colorado Plateau. The eastern border is isolated by the Colorado River flowing into the

Grand Canyon, which separates the plateau to the south. Lastly, the western border is isolated by the lesser known Kanab Canyon, which includes a tributary leading to the Colorado River. Human access is limited to two main paths, an approach from the north through Utah and crossing the Navajo bridge to the east (Young, 2002).

Likewise, I assume there is no gene flow between populations of tiger salamanders from on and off of the plateau. If introduction of Bd to Arizona is a relatively recent event, then salamanders on the Kaibab should not be exposed to Bd. My result that Bd is present on the Kaibab at very low levels is important. Collection date of salamanders had no effect on Bd presence. Considering the number of localities sampled along with the wide time scale represented, I suggest that the low prevalence of Bd is not ecologically significant.

This finding raises a question as to why Bd is present in such an isolated area. Noting that Bd's emergence as an EID is relatively recent, it is possible Bd is transported as a result of water bird migration. Garmyn et al. (2012) found that wild water fowl in Belgium can potentially act as non-amphibian reservoirs of Bd by hosting the fungus on their keratinous toes. Burrowes and De la Riva (2017) reached a similar conclusion when testing preserved aquatic birds from the Andes. Both studies were done with bird species that do not occur in the United States. Currently, there are no similar studies on the relation of Bd and water birds of the United States. Water-bird populations at elevations and with biotic habitats similar to the Kaibab Plateau are known from the San Francisco Peaks and the White Mountains (Gammonley and Fredrickson, 1998). Studies of naturally occurring water birds dispersing to and off of the plateau would be helpful.

Another possible cause of Bd presence can be due to it being endemic to the region. Historical samples of frogs in Illinois found Bd on several individuals with the oldest sample in 1888 on a *Rana* (*Lithobates*) *sphenocephala* (Talley et al., 2015). Although amphibian population decline has been recorded on all continents (Berger et al.,1998; Muths et al., 2003; Stuart et al., 2004), reports of major amphibian declines have been reported since the late 1960s (Scheele et al., 2019). Despite Bd's presence in samples from over a hundred years ago, reports of Bd epidemics are recent. The pathogenicity of Bd may be a more recent event. Bd has likely been endemic in many habitats across the globe prior to the deadly effects of chytridiomycosis.

The rock layers composing the Kaibab Plateau range from 250 million to 2,000 million years in age (Nations and Stump, 1981). It was mentioned earlier that tiger salamanders reside in stock tanks and ponds on the Kaibab with no recent dispersal on to or off of the plateau. One hypothesis is as follows: Bd has survived on Kaibab Plateau salamanders as a part of the skin microbiome. Salamanders exposed to the pathogen have adapted, just as some frogs have adapted, to Bd and are returning to their original population size before introduction of the pathogen (Collins, 2018). This may explain why such a low number of individuals have any Bd on their skin.

I suggest collecting additional Bd positive animals to isolate the Bd strain. Isolated strains should be compared with other Bd isolates to identify their relationship to other lineages. My research does not strongly support the hypothesis that Bd is a recent introduction to Arizona. It will be important to understand how Bd samples from the Kaibab Plateau relate phylogenetically to Bd lineages elsewhere in Arizona and globally.

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APPENDIX A

MUSEUM COLLECTION RECORDS

S1. Locality data for museum specimen and years they were collected.

Site	Location	Year collected	Latitude; Longitude
Boundary Lake	15.2km NNW North Rim	1984	36.3422; -112.12997
Burnt Lake Canal Tank	26.4km SW Jacob Lake	1990	36.4992; -112.33274
Corral Lake	11.3km S Jacob Lake	1984, 1988	36.61777; -112.25046
Cougar Lake	26.1km S Jacob Lake	1984, 1986, 1988-9	36.483; -112.19088
Crane Lake	21.2km SSE Jacob Lake	1988	36.52992; -112.14916
Deer Lake	35.9km SSE Jacob Lake	1988	36.40208; -112.13051
First Tank	12.3km S Jacob Lake	1989	36.60784; -112.21362
Fracas Lake	9.6km SSW Jacob Lake	1984,1986, 1988-9	36.63068; -112.23859
FSR 282 Tank	8.4km S Jacob Lake	1988	36.62859; -112.22251
Glenn Lakes	17.6km SSE Jacob Lake	1984, 1986	36.56371; -112.17712
Greenland Lake	7.0km NNE North Rim, Grand Canyon N.P.	1984	36.2421; -111.99132
Hidden Lake Tank	7.9km NW Jacob Lake	1988	36.76132; -112.2801
Jimmy and Wayne's Tank	9.5km SSW Jacob Lake	1990-91	36.63716; -112.25557
Johnson Wash Tank	30.6km NW Jacob Lake	1986	36.96083, -112.36992
Jolly Sink Road Tank	4.1km SE Jacob Lake	1990	36.66428; -112.17797
Little Park Lake	7.4km NNW North Rim	1983	36.32467; -112.11222
Mile-and-a-Half Lake	10.8km S Jacob Lake	1990	36.61687; -112.21917
Murrays Lake	9.9km SSE Jacob Lake	1989	36.63087; -112.17553
North Blow Down Tank	32.2km SSE Jacob Lake	1988	36.42943; -112.17351
Oquer Lake	24.1km S Jacob Lake	1988-89	36.50023; -112.223
Road Hollow Tank	27.0km SSW Jacob Lake	1990	36.48181; -112.29846

VT Lake	27.2km NNW North Rim	1988-89	36.44726; -112.12774
Warm Springs Lake	6.9km SW Jacob Lake	1984, 1986, 1988-90	36.69019; -112.28195

APPENDIX B

KAIBAB FIELD-COLLECTED ANIMAL RECORDS

S2. Locality data for field collected specimen and number of individuals caught per location.

Site	Number of Individuals	Latitude	Longitude
Jacob's Reservoir	15	36.74306	-112.246
Buck Lake	20	36.70306	-112.301
Doughnut Tank	15	36.57667	-112.211
Mile and a Half Lake	3	36.61667	-112.219
Murray's Lake	28	36.63083	-112.176

APPENDIX C

KAIBAB MUSEUM COLLECTION RECORD

S3. Assigned jar name with number of individuals and proportion of positive and negative animals.

Jar Name	Number of Individuals	Positive	Negative
A	2	0	2
B	1	0	1
C	6	0	6
D	8	0	8
E	5	1	4
F	1	0	1
G	20	4	16
H	1	0	1
I	6	0	6
J	10	0	10
K	20	0	20
L	20	0	20
M	20	0	20
N	1	0	1
O	5	0	5
P	1	0	1
Q	13	0	13
R	10	0	10
S	10	0	10
T	5	0	5
U	4	0	4
V	6	0	6
W	7	0	7
X	1	0	1
Y	2	0	2
Z	7	0	7
AA	5	0	5
BB	3	0	3
CC	4	0	4
DD	1	0	1
EE	2	0	2
FF	5	0	5
GG	2	0	2
HH	11	0	11
II	11	0	11
JJ	30	0	0
KK	30	1	29
LL	1	0	1
MM	10	1	9
NN	10	0	10
OO	2	0	2
PP	2	0	2
QQ	3	0	3
RR	3	1	2
SS	13	0	13
TT	2	0	2
UU	2	0	2
VV	1	0	1
WW	8	0	8
XX	1	0	1
YY	2	0	2
ZZ	1	0	1
α	6	0	6
Σ	1	0	1
△	1	0	1
□	29	0	29
β	20	0	20
大	3	0	3
αα	4	0	4
ΣΣ	1	0	1
△△	3	0	3
□□	8	0	8
ββ	2	0	2
大大	4	0	4