PREVALENCE AND GEOGRAPHIC PATTERNS OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN TEXAS

by

Andrea Villamizar Gomez, M.S.

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Committee Members:

Michael R. J. Forstner, Chair

Ivan Castro-Arellano

David Rodriguez

Hsiao-Hsuan Wang

Jamie Voyles

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To Bill, this dissertation would not be in English if it were not for you.

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I take this opportunity to thank my parents, Luz Stella and Juan Ramon and my brother Santiago for always believing in my abilities. When I had doubts, they gave me the praise and support I needed to keep me on track. I thank the friends I made at this school, for their help and assistance, without them I could have never achieved this goal. I also want to thank my committee chair and advisor, Dr. Michael R. J. Forstner, for allowing me the opportunity to conduct this research at Texas State University as well as for his guidance, support.

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ABSTRACT

Over the past 50 years, amphibian populations have undergone dramatic declines worldwide. Several hypotheses have been proposed to explain the causes for the decline of these populations. Contributing factors include habitat loss, shifts in temperature and rainfall patterns, changes in UV-B, and contamination through anthropogenic activities, and Emergent Infectious Diseases. *Batrachochytrium dendrobatidis* (*Bd*) is the pathogen that causes chytridiomycosis, an emerging infectious pathogen, known to be causing declines of amphibians across the globe and threatening overall ecosystem health. While chytridiomycosis in North American amphibians has attracted interest from researchers, the prevalence of this pathogen in Texas remains largely unexplored. To address this deficit, I collected samples during one year from five Wildlife Management Areas across a gradient precipitation. These results suggest that community composition, and differences in environmental conditions affect the prevalence of *Bd* in an area, suggesting that areas with higher species richness and higher annual precipitation have increased pathogen prevalence. Then, to understand *Bd* prevalence in time and its historic distribution in Texas, I examined the temporal range of the pathogen's prevalence, using museum collection specimens dating from 1930 up to 2010. The earliest detection of *Bd* in Texas was confirmed to be in 1936, suggesting that this pathogen is enzootic to the region. We also determined two pathogen hotspot areas in Central and East Texas. With this study, we provide an updated assessment of the prevalence of the historic and current distribution of *Bd* among species as well as across the landscape of Texas. It may also

reveal species that are not susceptible to the pathogen where their presence can help mitigate the spread of this disease and could aid in the conservation efforts for endangered species.

I. PREVALENCE AND GEOGRAPHIC PATTERNS OF CHYTRIDIOMYCOSIS IN TEXAS

Over the past 50 years, amphibian populations have undergone dramatic declines worldwide (Houlahan et al.2000, Scheele et al. 2019a). Several hypotheses have been proposed to explain the cause, or causes, for the decline of these populations (Houlahan et al. 2000, Kiesecker et al. 2001). Potential contributing factors include habitat loss, shifts in temperature and rainfall patterns, changes in UV-B, and contamination through anthropogenic activities (Blaustein et al. 2011, Alton et al. 2012). In addition, two diseases are potential contributors to the declines of amphibian populations: Chytridiomycosis caused by the fungi *Batrachochytrium dendrobatidis (Bd)* and *Batrachochytrium salamandrivorans (Bsal)*, and viral infections by *Ranavirus* sp. (Fellers et al. 2001, Young et al. 2001, Teacher et al. 2010, Price et al. 2014).

Reports that links the presence of *Bd* and *Bsal* to declines in amphibian populations have been published for Europe (Bosch et al. 2001), Central America (Lips et al. 2004, Frias et al. 2008) and North America (Muths et al. 2003, Rachowicz et al. 2006, Vredenburg et al. 2010). Both the *Bd* and *Bsal* fungi belong to the Chytridiomycota family and infect the keratinous parts of the skin of amphibians (Berger et al. 2005a, Martel et al. 2013). Normally, *Bd* thrives at temperatures between 4 and 21°C (Piotrowski et al. 2004) with an optimal temperature for growth can vary among strains (Voyles et al. 2017). The infection of amphibians generally leads to hyperkeratosis, hyperplasia and a disruption of electrolyte transport where levels of sodium, potassium, chloride, and magnesium become abnormally low, leading to death (Berger et al. 2005b, Voyles et al. 2009, 2012). Mortality rate and time to death after exposure and infection

are influenced by pathogen dose, temperature, life stage of the amphibian as well as by the amphibian species (Berger et al. 1999, 2004, 2005a, 2005b, Woodhams et al. 2011, Rachowicz and Briggs, 2007, Kriger et al. 2007a). Pathogenicity has been linked to weather, with temperature as a main contributing factor (Kriger and Hero 2007, Andre et al. 2008). This temperature variation affecting the severity of the infection, being more pathogenic in cool, humid areas (Berger et al. 2005b, Gaertner et al. 2009, Gaertner et al. 2012). Other factors like host population density, drought, tadpole longevity (Rachowicz and Briggs, 2007, Lampo et al. 2008, Briggs et al. 2010, Kärvemo et al. 2018) and a species' behavior, natural history and innate immunity can contribute to the persistence and pathogenicity variation of the disease (Lips et al. 2003, Rödder et al. 2008, Kinney et al. 2011, Savage and Zamudio. 2011, Bradley et al.2019, McDonald et al. 2019).

The surveillance of diseases in wildlife populations is an issue that concerns biologists, veterinarians and public health officers (Wendt et al. 2015, Milholland et al. 2018). Surveillance has been mainly focused on zoonotic diseases that represent a direct threat to human populations such as Ebola, plague, rabies, Hantavirus and SARS (Levins et al. 1994, Lloyd. 2001). While some pathogens occur asymptomatically within a host population, there are occasional epizootic outbreaks that are usually used to determine the presence of a particular pathogen in a population (Wobeser. 1994).

The presence of a disease outbreak in what it is considered a healthy wildlife population has been deemed as an indicator of a habitat disturbance, such as pollution, climate and habitat change, or the introduction of a new species (Mörner et al. 2002). Monitoring pathogen incidence and transmission can aid in the conservation efforts for endangered species. In the specific case of *Bd,* there is not enough information about the

mechanisms that *Bd* uses to spread across the landscape and this is in part due to the incomplete knowledge of *Bd* distribution (Lips et al. 2006, Vredenburg et al. 2010, Olson et al. 2013). An addition factor is that there are several frog species considered to be less susceptible to the disease that may act as pathogen reservoirs (e.g., species from the genus *Telmatobius*; Catenazzi et al. 2011), the African clawed frog (*Xenopus laevis*; Weldon et al. 2004) and the America bullfrog (*Lithobates [Rana] catesbeianus*; Dazak et al. 2004, Weldon et al. 2004, Schloegel et al. 2009, Urbina et al. 2018, Yap et al. 2018). The American bullfrog is considered native in Texas and has been linked to the distribution of the pathogen in the US (Schloegel et al. 2009, Yap et al. 2018).

The state of Texas is the second largest in the U.S. It follows a gradient of decreasing moisture and species richness from east to west but overall it houses over 40 different species of anurans (Brown et al. 2012) The presence of *Bd,* and interactions with the environment in Texas are not well studied (Saenz et al.2010, Garetner et al. 2009, 2012). There are no reports of amphibian declines in the state due to the presence of the pathogen. There are few studies that address the prevalence of the pathogen in the state (Gaertner et al. 2009, Saenz et al 2010, Marshal et al. 2019) but none focus on the interactions of *Bd* with the environment. The main objective of my dissertation is to assess the prevalence of *Bd* in different amphibian populations across Texas, and to link the presence of this pathogen to environmental parameters, geographic distribution, and species abundance and richness. Given the geographic extent of Texas and associated latitudinal and precipitation gradients, this state represents a relevant region for the evaluation of the pathogen environmental associations. Texas also has a rich amphibian diversity that varies among those same gradients in species composition, overall

diversity, and familial representation. Taken together, these factors contribute to the context of my study addressing the interactions of amphibian diversity or biotic parameters to better inform us on patterns of dispersion for the pathogen. Also, I seek to evaluate the presence and prevalence of *Bd* from historically collected amphibian samples from Texas and compare the strains obtained with the samples collected in recent years to evaluate if *Bd* is endemic to the area and might share an evolutionary history with the native host species.

II. PREVALENCE OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN TWO SYMPATRIC TREEFROG SPECIES, *HYLA CINEREA* AND *HYLA VERSICOLOR*

In the past decade *Batrachochytrium dendrobatidis* (*Bd*) has been detected in a variety of amphibian species in North America (i.e., Bradley et al. 2002; Chatfield et al. 2012; Pearl et al. 2009; Schlaepfer et al. 2007; Woodhams et al. 2008), which can be devastating when a species is susceptible to the disease caused by *Bd*, chytridiomycosis (Murray et al. 2009; Pilloid et al. 2010; Rachowicz et al. 2006). The disease disrupts cutaneous function in amphibians and negatively affects electrolyte transport through the skin, causing mortality in some taxa (Voyles et al. 2009).

In the US, common and widespread anuran species, such as American Bullfrogs (*Lithobates* [=*Rana*] *catesbeianus*) are often positive for *Bd*, although some species usually do not exhibit clinical signs of the disease (Garner et al. 2006; Pearl et al. 2009; Steiner and Lehtinen 2008). *Bd*-reservoir species, in this regard, may lead to patterns of higher *Bd* occurrences at sites with increasing species richness (Olson et al. 2013; Xie et al. 2016), and recurrent infections in other species. High *Bd* prevalence in common, reservoir species can pose a danger if they co-occur with disease-susceptible species. Two common North American tree frogs, the Green Treefrog (*Hyla cinerea*) and Gray Treefrog (*Hyla versicolor*) are widely distributed, yet published assessments of *Bd* in these species remain scarce. We argue that these two species should be of particular interest given their wide distribution across the eastern half of the United States and co-occurrence with other species. This scenario is similar to the western US hylid, Pacific Treefrog (*Pseudacris regilla*), which also is hypothesized to be a *Bd*-carrier species

potentially transmitting the pathogen to new ponds and other amphibian species (Reeder et al. 2012). However, the eastern US hylids are popular in the pet trade, which could potentially accelerate the spread of the disease if these commonly traded species are *Bd* carriers (Fisher and Garner 2007; Schloegel et al. 2009).

The occurrence of *Bd* within North American hylid sp. appears to be relatively low, although *Bd* has been detected in several species. For example, Rizkalla (2010) found no *Bd* among 3 Hyla species in Florida and studies conducted in Wisconsin, Minnesota, East Texas, Louisiana, and Georgia found that all Hyla samples were negative for *Bd* (Brannelly et al. 2012; Rodriguez et al. 2009; Sadinski et al. 2010; Saenz et al. 2010; Timpe et al. 2008). However, *Bd*-positive individuals have been detected in the Canyon Treefrrog (*Hyla arenicolor*), Gray Treefrog, Cope's Gray Treefrog (*Hyla chrysoscelis*), and Pacific Treefrog (*Pseudacris regilla*) (Bradley et al. 2002; Muelleman and Montgomery 2013; Fellers et al. 2011). The majority of *Bd* prevalence assessments in treefrogs has been conducted as part of assessments of larger amphibian communities in a particular geographic area; only one study in the eastern US has focused specifically on treefrogs (Brannelly et al. 2012). Consequently, sample size per study normally has been small, ranging from 1 to 42 (mean = ~18), with the exception of Brannelly et al (2012). (2012; N = 258). With a small sample size, *Bd* may not be detected if it occurs at low prevalence (Skerratt et al. 2007).

Texas is home to ~70 amphibian species but the *Bd* occurrence has not been widely studied, with the exception of east and central Texas (Gaertner et al. 2009; Saenz et al. 2010). Thus far, *Bd* has not been assessed in treefrogs of central Texas, but Saenz et al. (2010) tested treefrogs for *Bd* in East Texas and found no positive samples. Central

Texas is of a particular interest since several endemic and endangered amphibian species inhabit this region, such as several species of the salamander genus Eurycea, and the Houston Toad *(Anaxyrus houstonensis*; Dixon 2013). Previous *Bd* research showed 17% of Houston Toads and 83% of the sympatric Gulf Coast Toads (*Incilius nebulifer*) sampled were *Bd*-positive (Gaertner et al. 2010). Gaertner et al. (2012) studied Blanchard's Cricket Frog (*Acris crepitans blanchardi*) and found 89% occurrence whereas Villamizar-Gomez (2013) tested the Houston Toad, Gulf Coast Toad, Blanchard's Cricket Frog, and Hurter's Spadefoot (*Scaphiopus hurteri*) in the same region, and found 3% overall occurrence and only cricket frogs were infected. To date, there have been no clinical signs of pathology from the disease nor mass mortalities recorded in any of the infected species in this region.

In this study, our goals were twofold. First, we assessed *Bd* occurrence in Green and Gray Treefrogs within a pond system in central Texas. Our study site overlaps an area where bufonids and cricket frogs previously tested positive for *Bd* (Gaertner et al. 2009, 2012; Villamizar-Gomez 2013). Therefore, it is important to assess other amphibians that inhabit the area in order to determine which other species might be affected by the pathogen. Second, we conducted a literature review for studies that tested for *Bd* in Green and Gray Treefrogs within the US to examine where the species distribution overlapped with positive and negative *Bd* detections and identify US states that have not been surveyed, to inform future studies of the influence of potential *Bd*-carrier species.

Our study was conducted in Bastrop County, Texas, at Griffith League Ranch owned by Boy Scouts of America (Fig. 1). The ranch lies within the Lost Pines

Ecoregion, dominated by Loblolly Pine (*Pinus taeda*), Post Oak (*Quercus stellata*), Blackjack Oak (*Quercus marilandica*), and Eastern Red Cedar (*Juniperus virginiana*; Brown et al. 2011). Twelve amphibian species occur here, including the endangered Houston Toad (Brown et al. 2011). We surveyed ponds 1, 2, 5, and 9 from March to July 2014 and ponds 1, 5, and 13 from May to October 2015 (Fig. 1). Ponds 9 and 13 were within the burned area caused by the 2011 Bastrop wildfire. *Bd* was previously detected at two ponds in association with Blanchard's Cricket Frog (1 and 5; Gaertner et al. 2012).

As a part of an ongoing survey of Green and Gray Treefrogs, we opportunistically sampled the ponds during night surveys. In 2015, additional frogs were caught by using PVC pipes (Glorioso and Waddle 2014). All frogs were captured by hand; surveyors wore disposable vinyl gloves, changing them between captures. Adult frogs were individually marked by toe clips and tissue samples were placed in individual vials with 95% ethanol for future analyses. Toe clips were stored at -80°C and used for laboratory testing.

We extracted DNA using a DNeasy Qiagen Kit (Qiagen, USA) and the *Bd* detection was assessed using a real time Taqman qPCR assay (Boyle et al. 2004). The probe ChytrMGB2 was used with two species-specific primers ITS1-3 Chytr and 5.8S Chytr summarized in Boyle et al. (2004) and Garland et al. (2010). We ran each sample in triplicate and compared them to a regression line based on a consecutive 10-fold dilution of 5 standards in order to determine any positive detections.

For the literature review portion of this study, we used the Google Scholar search engine to identify any study that involved testing *Bd* in Green and Gray Treefrogs. Key words included broad terms such as "*Batrachochytrium dendrobatidis*" and "Anura", to

more specific “Hyla” and “tree frogs”. We also specifically reviewed the Amphibian Disease section of Herpetological Review in the last 15 years and searched www.*Bd*-Maps.net, an online database that includes geographic data of published and unpublished *Bd* assessments with the goal of providing an available resource for temporal and geographical epidemiologic analyses (Olson et al. 2013). To visually present the data we used ArcMap 10.2.2. We downloaded spatial data layers of species distribution available through International Union for Conservation of Nature (IUCN) and overlaid positive and negative *Bd* detections.

Across our five study ponds we collected 36 Green Treefrogs and 17 Gray Treefrogs in 2014, and 87 Green Treefrogs and 10 Gray Treefrogs in 2015 (Table 2.1). The majority of samples were collected during the highest activity months for Green Treefrogs, June and July (57 and 59, respectively). Zero of 123 Green Treefrog samples were *Bd*-positive. However, 4 of 27 Gray Treefrogs tested *Bd*-positive. *Bd* occurrence in Gray Treefrogs varied from zero to 22% among ponds, averaging 15% overall (Table 2.1). Positive samples were found in ponds 1, 2, and 5 (Fig. 2.1). Our literature review (Table 2.2) demonstrated that *Bd* was not detected in Green Treefrogs in any of the seven states that were sampled within that species’ range (Fig. 2.2), but was detected in 4 of 12 states (Fig. 2.3; Connecticut, Louisiana, Illinois, and Texas [current study]) where Gray Treefrogs samples were analyzed.

Assessing *Bd* in common, widely distributed, and often traded species is important because such species can accelerate spread of the pathogen and pose a greater risk to more vulnerable species. In this study, we showed with a relatively large sample size (N = 123), that Green Treefrogs tested negative for *Bd*, which is consistent with

previous studies. At the same localities, we were able to detect *Bd* in Gray Treefrogs with a much smaller sample size (N = 27). What is particularly intriguing is that the Houston Toad and Gulf Coast Toad in 2006, Blanchard's Cricket Frogs in 2009 and 2012, and Gray Treefrogs in 2014/2015 tested *Bd*-positive at the same localities where Green Treefrogs tested *Bd*-negative (Gaertner et al. 2009, 2010, 2012). Similarly, Brannelly et al. (2012) did not find infected Green Treefrogs among 258 samples collected from the wild in Louisiana but were able to infect Green Treefrogs in the laboratory, although they did not subsequently show any clinical signs of the disease.

Research has shown that seasonality and temperatures can have a great influence on *Bd* occurrence among Anurans (Pullen et al. 2010; Retallick et al. 2004; Sapsford et al. 2013). In general, prevalence is negatively associated with high air temperatures. For example, Kriger and Hero (2007) showed that individual frogs are capable of acquiring *Bd* and clearing their infections, which was closely tied to changes in climatic conditions. More relevant to our study, Gaertner et al. (2009) found no infection in the month of July. In our study, 116 of 150 (77%) samples were collected in June and July, usually the hottest months in central Texas. While those are the months when Green Treefrogs are most active, the high air temperatures could be responsible for no *Bd* detections in Green Treefrogs in our study and testing Green Treefrogs during the predicted high-prevalence months in this region is of future interest. However, Brannelly et al. (2012) collected samples year-round and Saenz et al. (2010) collected samples from January through May and still failed to detect *Bd* in this species.

In comparison, the four *Bd*-positive detections in Gray Treefrogs were found in March (N = 2), May (N = 1), and June (N = 1), and a high occurrence of ~89% for these

ponds in late spring was also found by Gaertner et al. (2009). The Gray Treefrog breeding season usually starts earlier in the year than the Green Treefrogs (Saenz et al. 2006) which would then correspond with lower air temperatures. It is also worth noting that the only ponds with no *Bd* infections were pond 9 and 13, the uplands of which catastrophically burned in a wildfire. Surveying these ponds for *Bd* throughout the year is warranted in order to address the hypothesis that the changes wrought by wildfire might be contributing to the lack of *Bd* occurrence. Since the *Bd* life cycle is closely tied to water, highly aquatic species might be the most vulnerable (Berger et al. 2005b). Given that treefrogs spend the majority of the breeding season calling in the surrounding vegetation and not at the pond edge, this might provide less exposure to *Bd*, which could explain the generally low occurrence.

Overall, there is no apparent pattern in the *Bd* distribution across the Gray Treefrog geographic range (Fig. 3). Southern, central, and eastern regions detected positive individuals in recent years, but at the same time northern, eastern, and central regions also failed to detect *Bd*. Filling in these gaps and conducting research that increases the sampling effort will help understand not only the *Bd* distribution in these frogs but other factors that may influence the patterns in prevalence, such as seasonality, latitude/longitude, elevation, as well as biotic factors and prevalence in co-occurring species. With the fungus being potentially transmitted among populations inhabiting the same aquatic habitat, it is important to continue to address *Bd* prevalence in different taxa and across various geographic regions in order to identify both vector and susceptible species.

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TABLES

Table 2.1: Green and Gray Treefrogs (*Hyla cinerea* and *Hyla versicolor*, respectively) sampled for *Batrachochytrium dendrobatidis (Bd)* during 2014 and 2015 field seasons in Bastrop County, Texas, USA.

Site	Species	No. *Bd*-positive/No. sampled		Prevalence (%)
		2014	2015	
Pond 1	*H. cinerea*	0/8	0/27	0
	H. versicolor	2/7	0/2	22
Pond 2	*H. cinerea*	0/9	0/0	0
	H. versicolor	1/6	0/0	17
Pond 5	*H. cinerea*	0/1	0/55	0
	H. versicolor	0/4	1/7	9
Pond 9	*H. cinerea*	0/18	0/0	0
	H. versicolor	0/0	0/0	0
Pond 13	*H. cinerea*	0/0	0/5	0
	H. versicolor	0/0	0/1	0
Total	*H. cinerea*	0/36	0/87	0
	H. versicolor	3/17	1/10	15

Table 2.2: Studies that assessed *Batrachochytrium dendrobatidis (Bd)* prevalence in Green and Gray Treefrogs (*Hyla cinerea* and *Hyla versicolor*, respectively) across US states. "+" represents *Bd*-positive detections and "-" represents *Bd*-negative detections.

State	Species	+/-	Citation
Florida	*H. cinerea*	-	Rizkalla et al. 2009, 2010; Rothermel et al. 2008
Georgia	*H. cinerea*	-	Green and Dodd 2007; Rothermel et al. 2008; Timpe et al. 2008;
Louisiana	*H. cinerea*	-	Brannelly et al. 2012
North Carolina	*H. cinerea*	-	Bd-maps 2007; Rothermel et al. 2008
South Carolina	*H. cinerea*	-	Daszak et al. 2005; www.Bd-maps.net 2007; Rothermel et al. 2008
Texas	*H. cinerea*	-	Current study; Saenz et al. 2010
Virginia	*H. cinerea*	-	Pullen et al. 2010
Connecticut	*H. versicolor*	+	Richards-Hrdlicka et al. 2013
Louisiana	*H. versicolor*	+	Rothermel et al. 2008
Illinois	*H. versicolor*	+	Mulleman and Montgomery 2013
Massachusetts	*H. versicolor*	-	Longcore et al. 2007
Maine	*H. versicolor*	-	Longcore et al. 2007
Minnesota	*H. versicolor*	-	Rodriguez et al. 2009
Oklahoma	*H. versicolor*	-	Bd-maps 2007
Pennsylvania	*H. versicolor*	-	Glenney et al. 2010
South Dakota	*H. versicolor*	-	Brown and Kerby 2013
Tennessee	*H. versicolor*	-	Rollins et al. 2013
Texas	*H. versicolor*	+/-	Current study; Saenz et al. 2010, respectively
Virginia	*H. versicolor*	-	Pullen et al. 2010

FIGURES

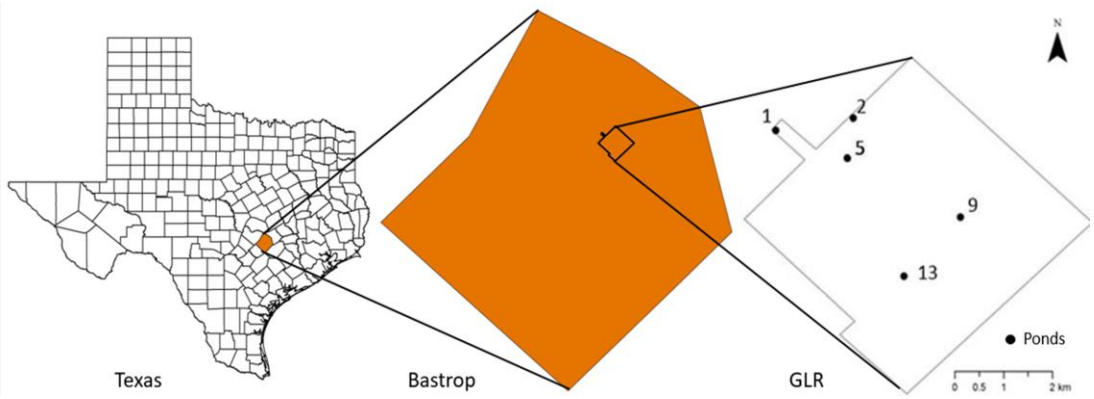

Figure 2.1: Map showing USA with Texas highlighted in black (top) and more specifically the map of Texas with outlined counties (bottom left). Current study was conducted at Griffith League Ranch (GLR) in Bastrop County (highlighted in orange). From 19 ponds at GLR, we assessed ponds 1, 2, 5, 9, and 13 where Green and Gray Treefrogs (*Hyla cinerea* and *H. versicolor*, respectively) occur.

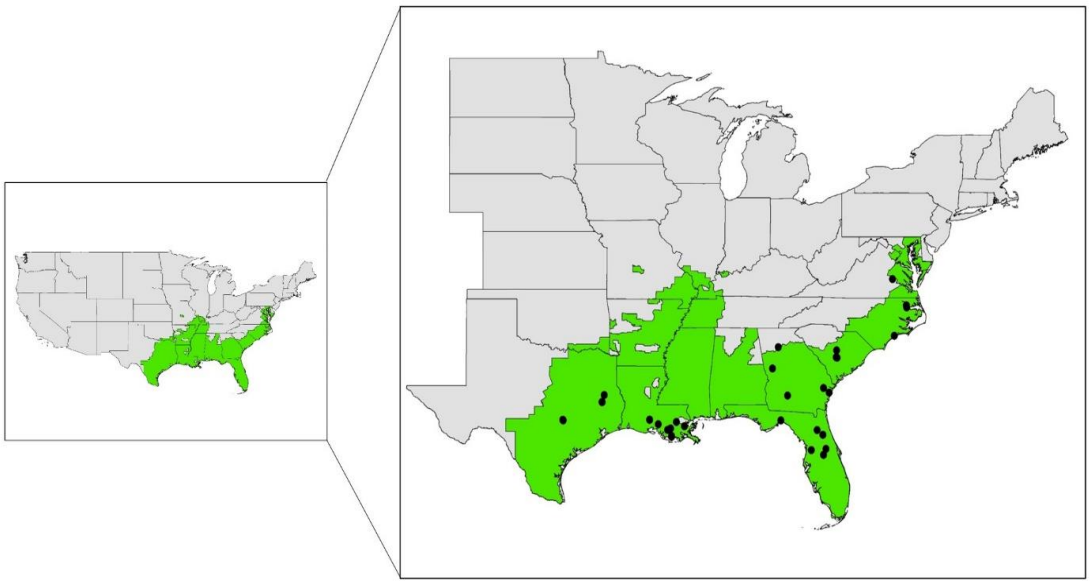

Figure 2.2: United States map outlining the Green Treefrog (*Hyla cinerea*) distribution (green). Circles represent the regions where treefrogs were surveyed for *Batrachochytrium dendrobatidis* (*Bd*) and found to be *Bd*-negative. To date, no Green Treefrogs have been found to be *Bd*- positive.

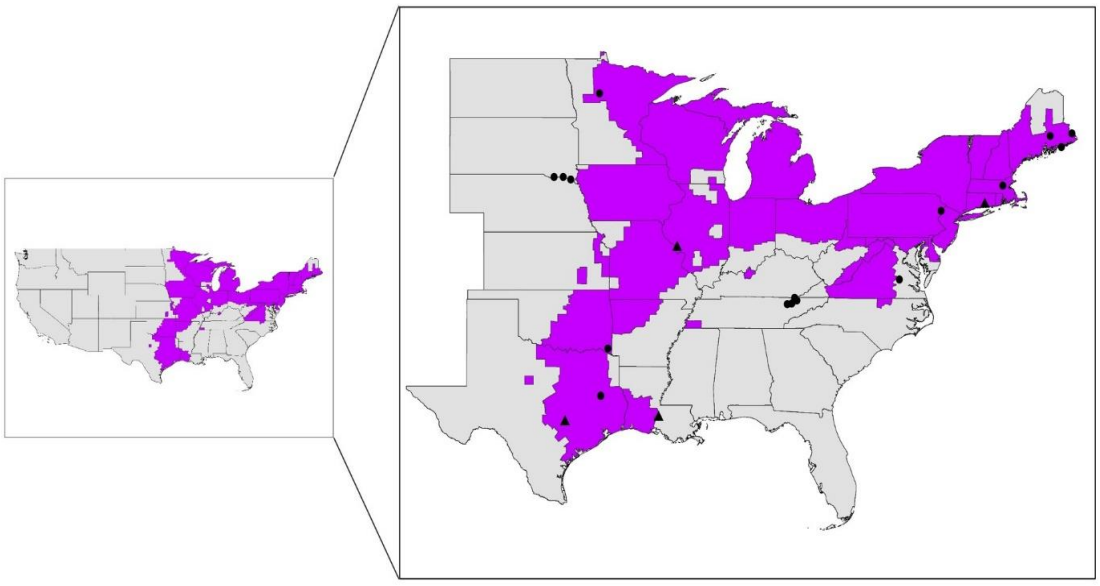

Figure 2.3: United States map outlining the Gray Treefrogs (*Hyla versicolor*) distribution (purple). Circles represent the regions where treefrogs were surveyed for *Batrachochytrium dendrobatidis* (*Bd*) and found to be *Bd*-negative. Triangles represent the regions in which the frogs were found to be *Bd*-positive.

III. HISTORICAL GEOGRAPHIC DISTRIBUTION AND POSSIBLE ENZOOTIC PRESENCE OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN TEXAS

ABSTRACT

The causal agent of chytridiomycosis, *Batrachochytrium dendrobatidis* (*Bd*), is a fungus known for infecting over 300 different species of amphibians globally. This study sought to determine the historical prevalence and distribution of *Bd* in Texas, drawing results from museum specimens. We sampled one of the largest museum collections in Texas, the Biodiversity Research and Testing Collections (BRTC (formerly the Texas Cooperative Wildlife Collection (TCWC)) at Texas A&M University. Our sampling plan focused on the three main families of Anurans, and nine species which have the widest geographical distribution within the state. These included: *Acris crepitans*, *Hyla cinerea*, and *H. versicolor* from Hylidae, *Lithobates [Rana] catesbeianus* and *Lithobates [Rana]. berlandieri* from Ranidae, and *Bufo debilis*, *B. nebulifer*, *B. woodhousii*, and *B. houstonensis* from Bufonidae. Of the total number of specimens found in the collection, we sub-sampled 30% of each species per decade from 1930 to present, via skin swabs totaling 1,510 independent sampling events. We analyzed these samples for the prevalence of *Bd* with a Real-Time PCR (qPCR). We detected the first record of *Bd* among the samples to be in the 1930s and higher prevalence during the 1990s. Our generalized mixed effect models showed that the highest prevalence occurred during the 1980s and 1990s, and that *A. crepitans* had the greatest likelihood of hosting the pathogen. We used the program SatScan to generate a spatio-temporal clustering analysis. We determined 2 clusters of significant hotspots, one in central Texas, and the

second one located in east Texas. This study may provide insights into the dynamics of this disease and any correlations with changes in the environment over time in Texas.

INTRODUCTION

Chytridiomycosis, a skin fungal disease caused by *Batrachochytrium dendrobatidis* (*Bd*), has been called the worst emerging infectious disease recorded among vertebrates (Gascon. 2005). Infection by *Bd* can cause skin to hyperkeratosis, hyperplasia, a disruption of electrolyte transport and an osmotic imbalance due to the loss of electrolytes, particularly sodium and potassium, eventually resulting in the death of the host (Berger et al. 1998, Voyles et al. 2011). To date, this fungus has been found to affect over 500 different species of amphibians (Lips et al. 2004, Scheele et al. 2019a), with documented occurrences around the globe (Berger et al. 1998, Bosch et al 2001, Lips et al 2006, Vredenburg et al. 2010). Further, it has been discovered that there are multiple strains of the pathogen (Farrer et al. 2011, Rodriguez et al. 2014, Schoegel et al. 2012, Bataille et al. 2013, Jenkinson et al. 2016). This genetic diversity (Farrer et al. 2011, Rosenblum et al. 2013, O'hanlon et al. 2018), extensive geographic range (O'hanlon et al. 2018, James et al. 2015), and varying host assemblage compositions of infected areas (Bosch et al 2001, Lips et al 2006, Vredenburg et al. 2010) have been suggested to be the major causes of the pathogen's heterogeneous virulence.

As research into the impacts of *Bd* in amphibian assemblages has grown in the past decade, occurrences of the pathogen across the U.S. have been detected from museum specimens dating back to the late 1800s and early 1900s (Ouellet et al. 2005, Padgett-Flohr and Hopkins 2009, Huss et al. 2013, Benavides et al. 2012). The

exponentially increasing number of cases reported for this pathogen in the past decade has led to two competing hypotheses that attempt to understand this sudden emergence based on the interactions between the hosts within an assemblage, the pathogen, and the environment, the endemic or enzootic pathogen hypothesis and the emerging pathogen hypothesis (Rachowicz et al. 2005). The endemic or enzootic pathogen hypothesis suggests that the pathogen has always been present in the environment, but recent environmental changes, habitat disturbance, and stochastic events are responsible for recent disease outbreaks (Rachowicz et al. 2005, Pounds et al. 2006, Rodriguez et al. 2014, Becker et al 2016). Conversely, the emerging or epizootic pathogen hypothesis suggests that the disease outbreaks are caused by the recent exposure of *Bd* to naïve hosts.

One possibility that would support the emerging pathogen hypothesis is the global transport of amphibian species that can tolerant *Bd*. Two example species that would support the emerging pathogen hypothesis are the American bullfrog, *Lithobates [= Rana] catesbeianus*, which is cultivated for food in Asia and America (O'hanlon et al. 2018, Yap et al. 2018, Byrne et al. 2019), and the African clawed frog, *Xenopus laevis*, which were exported world-wide for use in pregnancy tests (Weldon et al. 2004, Lips et al. 2008, Schoegel et al. 2012, Lips 2016). Both hypotheses have been supported in various publications, with phylogenetic analyses suggest that the current chytridiomycosis is both enzootic to certain regions, and novel to others (Rosenblum et al. 2013, O'hanlon et al. 2018, Lips 2016). Specific enzootic *Bd* strains have been identified, such as *Bd*ASIA-1 (which includes *Bd*CH isolate) in Asia, *Bd*-Cape which is found in South Africa and Spain (Farrer et al. 2011), *Bd*-Korea (Bataille et al. 2013), and

*Bd*ASIA-2/*Bd*BRAZIL (Rodriguez et al. 2014, Schoegel et al. 2012, Morell 1999). Each of these *Bd* strains have a restricted global occurrence, and in areas that show declines but lack an enzootic strain, phylogenetic analyses have identified a more widespread strain coined the Global Panzootic Lineage (GPL), to which most of the major global disease outbreaks have been attributed (Rosenblum et al. 2013, O'hanlon et al. 2018, James et al. 2015).

In the United States, it has been thought that *Bd* represents an epizootic pathogen, linked to the amphibian declines in the western United States of *Lithobates [Rana] pipiens* (Morell 1999), *Rana chiricahuensis, Rana yavapiensis* (Berger et al. 1998), and *Lithobates [Rana] muscosa* (Rachowicz et al. 2006). The outbreaks in some of these species have not been linked to environmental changes or enzootic pathogen interactions but rather the introduction of *Bd* into host populations (Morell 1999, Rachowicz et al. 2006). Studies on *Bd* spread within these communities has been documented as a wave, spreading rapidly through an area, sometimes with no single reservoir providing an explanation for outbreaks (Lips et al. 2006, Vredenburg et al. 2010).

While *Bd* has been detected in amphibian communities across the U.S., there have been no documented reports of *Bd*-related declines of amphibians in the central or eastern U.S. (Longcore et al. 2007), all declines linked to *Bd* have been reported in the west (Huss et al. 2013). Some studies suggest that the first appearance of *Bd* occurred in the early 1960's, with the earliest detection of the pathogen recorded from the histological sampling of two specimens of *Lithobates [= Rana] catesbeianus* from California in 1961 (Padgett-Flohr et al. 2009, Ouellet et al 2005), suggesting that the amphibian pet trade of the 1940s is the most likely explanation for the widespread introduction of the pathogen

throughout the U.S. (O'hanlon et al. 2018, Lips et al. 2008, Schoegel et al.2012, Ouellet et al. 2005). However, there are contrasting reports from Illinois that date from 1888 (Talley et al. 2015), and California in 1928 (Huss et al. 2013), that might indicate that *Bd* presence in the U.S. may predate 1888.

While *Bd* has been suggested to be a major contributor to global amphibian declines, its prevalence, and impact within various amphibian communities, as well as its environmental correlates in Texas, have not been well studied. While there have been no reported amphibian declines in the state directly linked to *Bd*, relatively few studies have sought to address the explanatory mechanisms for the prevalence patterns of this pathogen (Gaertner et al. 2009, Saenz et al. 2010, Gaertner et al. 2012), or even the specific strains of *Bd* that are affecting the amphibian communities in central Texas (Marshall et al. 2019). The objective of this study is to assess which hypothesis best describes the emergence of *Bd* in Texas through a retrospective sampling of the Biodiversity Research and Teaching Collection (BRTC) of Texas A&M University. We predict that *Bd* behaves in Texas like an enzootic pathogen, with low prevalence among amphibian assemblages.

RESULTS

Of the 1,510 amphibians sampled, 116 specimens tested positive for *Bd* across 4 of the 9 various species examined including, *L.* [=*R.*] *catesbeianus*, *A. crepitans*, *R. berlandieri*, and *H. versicolor*, yielding an overall prevalence of 7.68% across the state. None of the toad species, nor any individual of *H. cinerea* tested positive for *Bd*. Further analyses omitted the bufonid samples to prevent statistical bias in the predictive

modeling. This restricted this study to a total of 859 samples with an overall prevalence of 13.5% (Table 3.1, Figure 3.1).

The earliest detection of *Bd* was found in a *Lithobates* [= *Rana*] *catesbeianus* specimen collected in 1936. This species showed the highest overall prevalence of those sampled, with an average prevalence of 17.9%. A peak in the prevalence of the pathogen in this host spp. was observed in the 1950s where 36.3% of those specimens sampled showed quantifiable amounts of *Bd* (Table 3.1). Detections of the pathogen were also found in both *Acris crepitans* in 1941, and *Lithobates [=Rana] berlandieri* in 1949. *Acris crepitans* specimens showed the second highest overall prevalence rate (15.7%), with a spike in the 1980s where 48.9% of the surveyed specimen tested positive for the pathogen. *Lithobates [=Rana] berlandieri* were found to have an overall prevalence of 10.1% with the highest reported rate found in the 1990s at 22.2% of those sampled. *Bd* was also found in *Hyla versicolor* in 1969, but this species showed the lowest prevalence of all the species which tested positive for the pathogen at only 2.9%, and only one positive specimen detected in the 1960s, '70s, and '90s (Table 3.1).

The Generalized Mixed Effect Model analysis showed that *L.* [=*R.*] *catesbeianus* (*p- value* = 0.006) and *A. crepitans* (*p-value* = 0.016) had a significantly higher chance of hosting the pathogen compared to other species. In a temporal analysis it was also identified that the 1980s (*p-value* = 0.0006) and 1990s (*p-value* = 0.002) had significantly more detections of the pathogen than the other decades sampled.

The spatio-temporal analysis showed five distinct outbreak points of pathogen dispersal across the state throughout all observed decades. The earliest detection of *Bd* occurred from specimens collected in both Grimes and Walker Counties in the 1930s,

where spread to the neighboring counties was observed in the following decades. Nearly 20 years later, another outbreak point was identified in northeast Texas, around Henderson County, in which the same spread to neighboring counties was observed. Another two epicenters of the pathogen were identified in both central and northeastern Texas beginning in the 1970s, followed by one in the 1980s in South Texas (Figure 3.2).

The spatial-clustering analyses identified a total of four clusters, or hotspots, of *Bd* prevalence. Of the four clusters identified, two of them proved to be statistically significant predictors of increased prevalence of the pathogen (Clusters 1 and 2). Cluster 1 had an approximate ratio of 106.8 km located in central Texas (*p-value* = 0.00094) and was significant throughout the decades of 1990 - 2000 , and cluster 2 had an approximate ratio of 50.61 km and is located in east Texas (*p-value* = 0.0011) and was significant throughout the decades of 1980 - 1990 (Table 3.2, Figure 3.3). While not statistically significant, there are two more clusters that could be identified as "potential hotspots". These clusters were found in northeast and west Texas respectively (Clusters 3, and 4). The analysis also identified three clusters of "coldspots" where there was a lower overall predicted prevalence. Clusters 5 and 6, found in central and south Texas were significant, with ratios of 198.28 and 142.5 km and *p*-values of 0.002 and 0.0047, and were significant throughout the decades of 1980 – 1990, and 1970 - 1980 respectively (Table 3.2 and Figure 3.3). We also run a temporal analysis that suggested one single significant (*p*-value > 0.0001) hotspot during the decades of 1980 – 2000. It originates in Erath County (north-central region) with a radius of 176.44 km, that include the cities of Wichita Falls to the north, Waco to the south, Abilene to the east and Dallas to the west. It overlaps with Clusters 1 and 6 of the Spatio temporal analysis, and the time frame

overlaps with the occurrence of cluster 1.

DISCUSSION

This study confirms that *Batrachochytrium dendrobatidis* has been present in Texas since at least 1936. To our knowledge, this represents the earliest detection of *Bd* in Texas, and is among the earliest detections of *Bd* in the U.S. (Huss et al. 2013, Talley et al. 2015). Additionally, three samples collected in the 1940s, and six collected in the 1950s predate recorded detections of *Bd* published in the U.S. (Padgett-Flohr et al. 2009, Ouellet et al. 2005) for other species other than the American Bullfrog (*A. crepitans, R. berlandieri*). While we do report the earliest confirmed detection of the pathogen in Texas, it should also be noted that, despite the use of a more sensitive detection methodologies than traditional histological records (Ouellet et al. 2005, Kriger et al. 2006, Retallick et al. 2004), older specimen preserved in museum collections are subjected to significant rates of DNA degradation. This could potentially limit the number of detections of the pathogen, making the estimation of overall prevalence difficult. But there are reports from studies in the US that show earlier detections, Illinois in 1888 (Talley et al. 2015) and California in 1928 (Huss et al. 2013). As well as Korea in 1911, (Fong et al. 2015) and Brazil in 1894 (Rodriguez et al. 2014). This suggest that short fragments can still be detected despite degradation.

One of the current hypotheses for *Bd* spread in the U.S. suggests that it was first introduced in 1949 by the export of both the African clawed frog, *Xenopus laevis,* from South Africa (Weldon et al. 2004) and American bullfrog, *Lithobates [=Rana] catesbeianus*, in the food and pet trades (O'hanlon et al. 2018, Padgett-Flohr et al. 2009,

Weldon et al. 2004). Suggesting that the wavelike spread of the pathogen in some frog species in California would fit the movement characteristics of an epizootic pathogen (Lips et al. 2006, Vredenburg et al. 2010, Padgett-Flohr et al. 2009), and even though this spread has been shown in some frogs species, a report of *Bd* in newts in California suggest a non-epizootic behavior, with low prevalence, and low mortality rates (Chaukulkar et al. 2018) . This non-epizootic behavior seems to be the case for the central and eastern regions of the U.S. Studies of *Bd* in Texas have found no amphibians presenting clinical signs of infection or reported die-off events linked to the pathogen, despite relatively high prevalence in several taxa (Gaertner et al. 2009, Saenz et al. 2010, Gaertner et al. 2012). Further, our spatio-temporal analysis would support a radial expansion of the pathogen from the counties where it was first detected in the 1930s (Figure 3.2). If interpreted as indicative of overall occurrence, it further shows the presence of the pathogen starting in northeast and central Texas in the 1960s, and another during the 1980s in south Texas. The sudden onset of pathogen outbreaks followed by periods of seeming low prevalence suggests an unknown point and time of introduction of the pathogen into Texas. This lack of discernible wave-like dispersal pattern, typical of an epizootic pathogen, suggest that the pathogen's spatio-temporal print follows the pattern of a enzootic pathogen, or there might be presence of both invasive and native strains in the same area, as is the case in Brazil (Rodriguez et al. 2014, Bovo et al. 2016, Carvalho et al. 2017). These types of pathogen movements show small outbreaks of the disease though time and those could be linked to regional changes such as, changes in the composition of the amphibian assemblages already in contact with the pathogen (Rachowicz and Vredenburg 2004, Briggs et al. 2010, Urbina et al. 2018, Bovo et al

2016), as well as changes in virulence of strains (Fisher et al. 2009, McDonald et al. 2019, Urbina et al. 2018) .

While our model appears unable to pinpoint specific decades of outbreaks in some areas of the state, it should be noted that there is inherent sampling bias in museum collections as researchers typically collect samples in readily accessible areas, as well specific times of the year and sometimes focus on a particular species. Subsequently creating geographic gaps throughout various decades (Padgett-Flohr et al. 2009, Laduc et al. 2010). This sampling bias could help explain the deficit of positive samples collected from the Texas panhandle. False positive amplifications can occur due to cross contamination of specimens that share the same collection jar. This was mitigated by a thorough EtOH wash.

The prevalence of a pathogen in an assemblage can be affected by different pathogen-host interactions, where the host, the species relative abundance and species richness play an enormous role in pathogen-host dynamics, leading to two contrasting outcomes: the “amplification effect” and the “dilution effect” (Keesing et al. 2010, Hartfield and Alizon 2013, , Zargar et al. 2015). In either case, there is a gradient rule linked to the competence of the host (ability to transmit an infection to another susceptible host). An assemblage with high species richness or low relative abundance might “dilute” the pathogen prevalence if most of the hosts in this assemblage are considered “noncompetent,” thus decreasing the transmission events and infection rates (Becker and Zamudio 2011, Keesing et al. 2010, Zargar et al. 2015, Benavides et al. 2012, Searle et al.2011b). The contrasting outcome would occur when a pathogen is present in an assemblage of “competent” hosts. In this scenario, assemblages that have

hosts with a high pathogen competency can “amplify” or increase the infection rates and transmission events if they have high richness, or high relative abundance (Zargar et al. 2015, Searle et al.2011a, Clay et al. 2009, Huang et al 2016).

This scenario seems to become apparent in our spatio-temporal clustering analysis. In cluster 1, the geographic distribution of the four species tested overlaps, thus potentially increasing the risk of exposure of competent hosts that are not only sympatric but also syntopic. The remaining hotspot (Cluster 2) does not have a complete distributional overlap of the four species like cluster 1, (*R. berlandieri’s* geographic distribution does not reach east Texas). However, this area presents the highest herpetofauna density per hectare in the state (Brown et al. 2012), and both species with the highest overall prevalence of *Bd* (*A. crepitans*, and *L.* [=*R.*] *catesbeianus*) are widely distributed in this area. In contrast, the cluster 4 (potential hotspot) has an overall low biodiversity (Brown et al. 2012) but within the amphibian assemblages are competent hosts that help maintain the prevalence of the pathogen and could potentially increase incidences of the pathogen in this area. Such potential increase in total prevalence of the pathogen could help explain the larger overall predicted approximate ratio of this hotspot to the others, as the pathogen may thrive in this lower overall biodiversity environment (Table 3.2).

One potential explanation for the predicted coldspots identified in the spatio-temporal analysis could be attributed to anthropogenic effects. Identified in the 1970s to 1990s these two cold spots extend across rapidly developing agricultural land, and the rapidly urbanizing metropolitan areas of Houston and Dallas. As these areas developed and urbanized, Anuran habitat was subsequently lost, limiting potential areas in which the

pathogen had access to suitable hosts, resulting in a drop in overall prevalence. However, increased sampling efforts and a further review of urbanization and development patterns in these areas throughout the 1970s to 1990s is needed to make a definitive conclusion.

In conclusion, we have determined that *Bd* may have been present in Texas since 1936, suggesting that amphibian populations in Texas have been coexisting with this pathogen for almost 100 years. It is unclear if there are ongoing amphibian declines in Texas that could be related to *Bd*, because currently there have been no massive die-off events attributed to *Bd* reported across the state of Texas. In addition to the early detection of the pathogen in amphibian communities, its dispersion does not show a clear wave-like expansion typical of an introduced pathogen (Lips et al. 2006, Vredenburg et al. 2010, Padgett-Flohr et al. 2009), but instead fluctuates among decades like of an enzootic pathogen, with the highest peak found in the 1980s-2000s, which could indicate the introduction of other more virulent strains (Talley et al. 2015, Bovo et al. 2016). These sudden and random outbreaks could also be linked to environmental changes, habitat fragmentation, changes in the assemblage composition and/or even stochastic events that could increase the probability of infection (Rachowicz et al. 2004, Becker and Zamudio et al. 2011, Benavides et al. 2012, Talley et al. 2015, Bovo et al. 2016).

MATERIALS AND METHODS

SAMPLING

We sampled 1,510 whole, post metamorphic anuran specimens collected across the state of Texas from 1930 to 2010. These specimens had been collected and cataloged in the Biodiversity Research and Teaching Collections (BRTC) of Texas A&M

University. We focused our study on the three main Anuran families (Hylidae, Ranidae, and Bufonidae), which together are abundant within the state. Within these three families, we were able to sample nine different species which have an extensive geographic range across the state, including *Acris crepitans, Hyla cinerea,* and *Hyla versicolor* from the Hylidae family, *Lithobates [= Rana] catesbeianus* and *Lithobates [=Rana] berlandieri* from the Ranidae family, and *Bufo debilis, Bufo nebulifer, Bufo woodhousii*, and *Bufo houstonensis* from the Bufonidae family. We randomly sub-sampled 30% of individuals from each of these nine species collected per decade in order to evaluate the pathogen's prevalence per decade and species. Geographic locality data was readily available from the BRTC's records, but for those specimens cataloged without precise GPS data, we used the centroid of the county of collection as an approximation.

MOLECULAR ANALYSIS

All specimens at the BRTC collection were preserved with formalin and stored in 70% ethanol (EtOH). We used non-invasive *Bd* sampling methods to collect DNA (Marshall et al. 2019). Since the specimens are stored in communal jars by species, each specimen was thoroughly rinsed with 70% EtOH, and allowed to air dry prior to sampling to mitigate cross contamination (Rodriguez et al. 2014, Boyle et al. 2004, 2007). We extracted DNA using the Prepman Ultra (Applied Biosystems) kit, following the standard protocol provided by the manufacturer (Boyle et al. 2004). The detection of *Bd* was assessed via a Taqman Fast Advanced Master Mix on a StepOne Plus Real Time PCR System (Applied Biosystems, Inc). We used a MGB2 probe specific for *Bd* as well as ITS1 and 5.8S *Bd* specific primers (Garland et al. 2010, Kilburn et al. 2010, Rebollar

et al.2014). Each sample was analyzed in duplicate and compared to a serially diluted standard curve ranging from 0.1 to 100 genomic equivalents (g.e.) with the JEL423 strain used as a genomic reference (Cheng et al. 2011). Reactions producing an amplification curve above 40 cycles (g.e. < 1 zoospore) were considered negative for the pathogen (Kriger et al. 2007b). Each reaction required an overall reaction efficiency between 90 - 110%, and an R^2 value greater than 0.990. Samples in which only one of the two duplicate reactions positively amplified the diagnostic marker were re-analyzed on a subsequent run for verification.

DETERMINING PATHOGEN PREVALENCE

Samples were categorized by both species and the decade they were collected in. Prevalence, both by decade and species, was calculated by dividing the number of total positive samples by the grand total sampled, with a 95% Jeffrey's confidence intervals (CI). Generalized linear mixed models (GLMs) were used to make comparisons across the established categories. We used ArcMap 10.5 to generate maps of all sampled localities. We generated spatio-temporal maps using all positive samples and classified them in seven temporal classes that represented the 7 decades that were analyzed (1930s-2000). A kriging analysis was used to predict dispersion trends across the landscape. Subsequently we ran a Bernoulli analysis conducted using the presence and absence data to test for spatial and temporal autocorrelation among the samples using SaTScan v9.4.2 software (Kulldorff et al. 2009). This allowed for the prediction of "hotspots" where statistically significant clusters of samples evidenced an increased prevalence of the pathogen. Simultaneously, the Bernoulli analysis identified areas within the state where

there were statistically significant clusters of samples devoid of the pathogen (coldspots). These hot and coldspots were input into a Getis-Ord GI* analysis using ArcMap 10.5 to generate a geographic map of these areas across the state of Texas.

TABLES

Table 3.1: *Batrachochytrium dendrobatidis (Bd)* detection and prevalence per decade in Texas with 95% Confidence intervals (CI 95%) for the taxa: *L. [=Rana] catesbeianus*, *A. crepitans*, *L.[Rana] berlandieri*, *H. versicolor* as tested from specimens in the BRTC Collection of Texas A&M University. A total of 859 specimens were included in the analyses. An additional 651 samples from the anuran species, *B. debilis, B. woodhousii, B. houstonensis and Hyla cinerea*, tested negative for the pathogen and were not included in the analyses or statistics.

	Lithobates [Rana] catesbeianus			***Acris crepitans***			***Lithobates [Rana] berlandieri***			***Hyla versicolor***			**TOTAL**		
Decade	***Bd+***	**Prevalence %**	**CI 95%**	***Bd+***	**Prevalence %**	**CI 95%**	***Bd+***	**Prevalence %**	**CI 95%**	***Bd+***	**Prevalence %**	**CI 95%**	***Bd+***	**Prevalence %**	**CI 95%**
1930	1/6	16.7	0.02, 0.56	0/11	0	0.0, 0.20	0/10	0	0.00, 0.22	0/3	0	0.00, 0.54	1/30	3.3	0.00, 0.15
1940	1/18	5.6	0.01, 0.23	1/48	2.1	0.0, 0.09	1/11	9.1	0.01, 0.35	0/11	0	0.00, 0.20	3/88	3.4	0.01, 0.09
1950	4/11	36.3	0.14, 0.65	2/58	3.4	0.01, 0.11	0/10	0	0.00, 0.22	0/22	0	0.00, 0.11	6/101	5.9	0.03, 0.12
1960	6/35	17.1	0.07, 0.32	10/131	7.6	0.04, 0.13	2/30	6.7	0.01, 0.20	1/21	4.8	0.01, 0.20	19/217	8.7	0.06, 0.13
1970	1/12	8.3	0.01, 0.33	8/38	21.1	0.10, 0.36	1/16	6.3	0.01, 0.26	1/13	7.7	0.01, 0.31	11/79	13.9	0.08, 0.23
1980	0/13	0	0.0, 0.17	23/47	48.9	0.00, 017	4/17	19	0.09, 0.47	0/6	0	0.00, 0.33	27/83	32.5	0.23, 0.43
1990	9/34	26.5	0.14, 0.43	19/48	39.6	0.27, 0.54	2/9	22.2	0.05, 0.54	0/9	0	0.00, 0.24	30/100	30	0.22, 0.39
2000	4/16	25	0.09, 0.49	9/79	11.4	0.06, 0.20	5/46	9.8	0.04, 0.22	1/20	5	0.01, 0.21	19/161	11.8	0.08, 0.17
All	26/145	17.9	0.12, 0.25	72/460	15.7	0.13, 0.19	15/149	10.1	0.06, 0.16	3/105	2.9	0.01, 0.07	116/859	13.5	0.11, 0.16

Table 3.2: Spatio-temporal clustering analysis of Hotspot (High) and Coldspot (low) cases of *Batrachochytrium dendrobatidis (Bd)* in Texas from 1930 to 2010, where decade represents the time frame where sampling was most significant. Samples were analyzed in SaTScan using a Bernoulli model. Significant clusters are indicated by *.

Hotspot (High) clusters					
Cluster	**Radius (Km)**	**Observed Cases**	**Expected Cases**	**Decade**	***p*-value***
Cluster 1	106.8	8	1.42	1990 – 2000	0.00094*
Cluster 2	50.61	10	2.75	1980 – 1990	0.0011*
Cluster 3	170.29	8	2.57	1970 – 1980	0.195
Cluster 4	387.13	11	4.67	1970 – 1980	0.388
Coldspot (Low) clusters					
Cluster 5	198.28	0	7.5	1980 – 1990	0.002*
Cluster 6	142.50	0	7	1970 – 1980	0.047*
Cluster 7	187.78	0	3.5	1980 – 1990	0.082

FIGURES

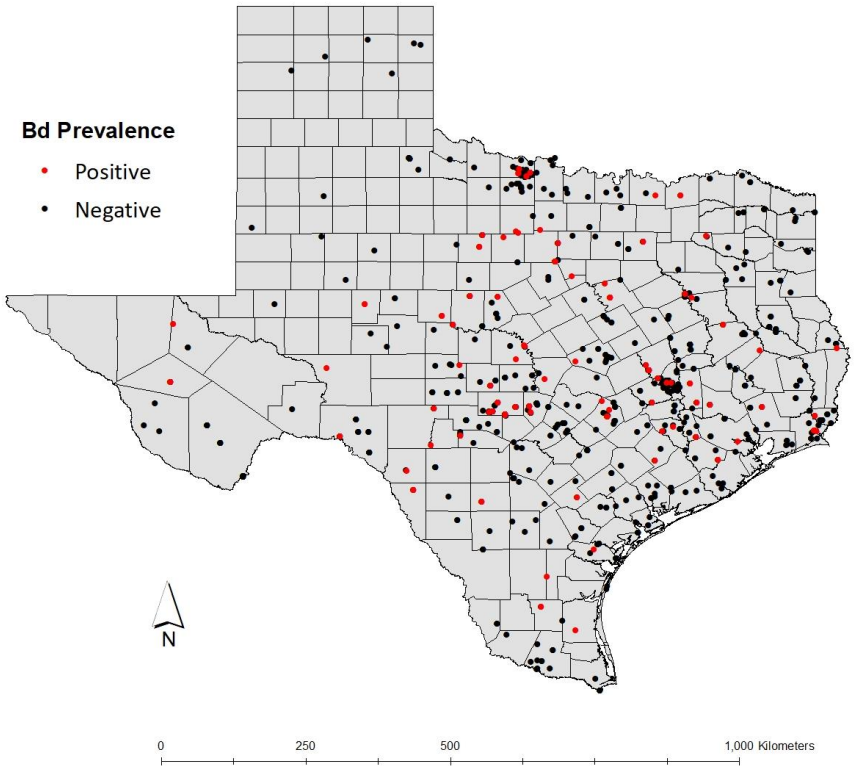

Figure 3.1: *Batrachochytrium dendrobatidis* (*Bd*) detection results from specimens in the BRTC collection from Texas A&M University. The taxa *L. [=Rana] catesbeianus, A. crepitans, Lithobates [Rana] berlandieri, H. versicolor* provided positive results from 1930-2010 (n = 859). Specimens that tested positive for the pathogen are shown in red (n = 116), while specimen that tested negative are shown in black (n = 758).

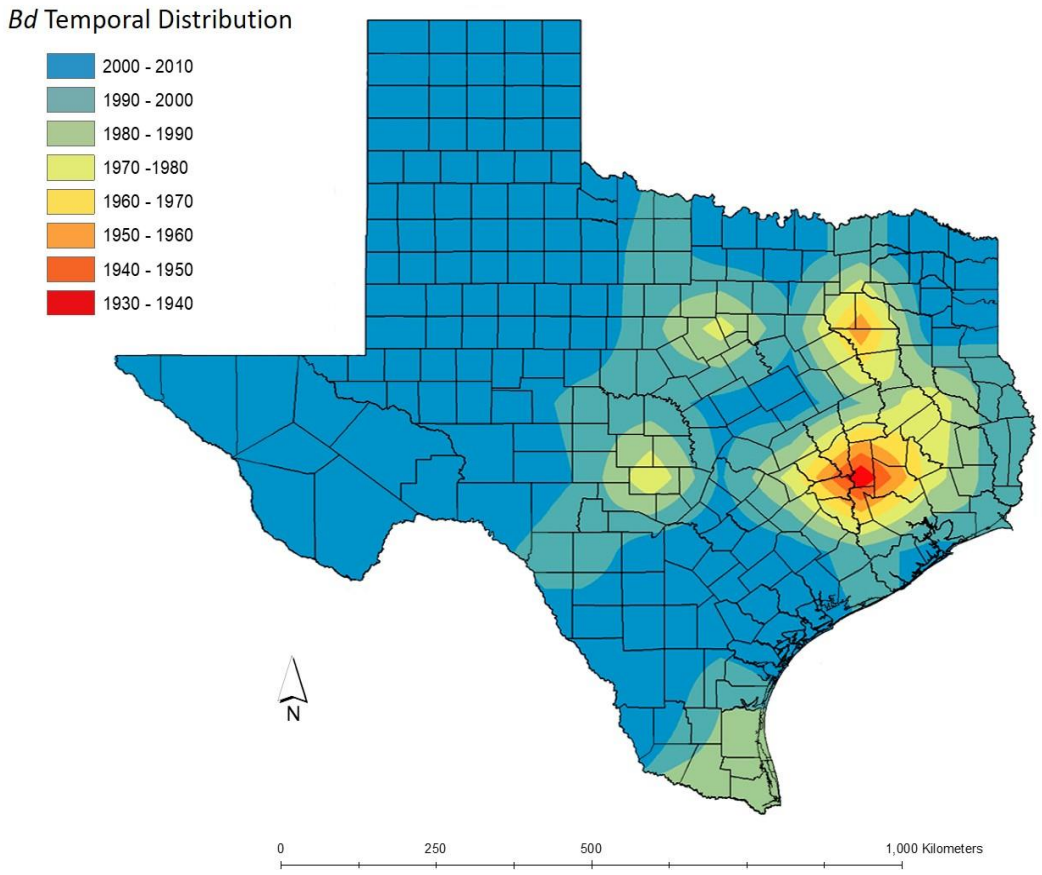

Figure 3.2: Spatio-temporal distribution of *Batrachochytrium dendrobatidis* (*Bd*) in Texas from 1930-2010. A kriging analysis was applied to all positive samples (n = 116), across the eight surveyed temporal classes. Areas across the state are shown by the decade in which the first positive case of *Bd* was detected.

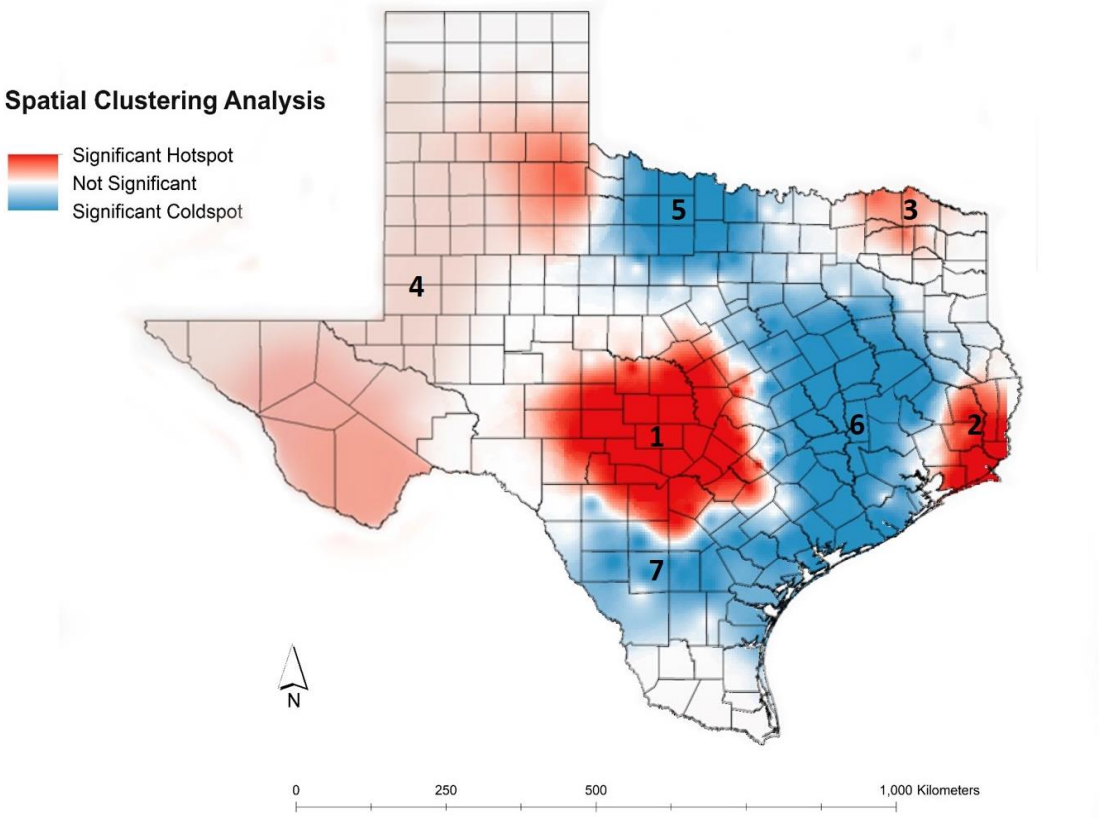

Figure 3.3: Spatio-temporal clustering analysis of *Batrachochytrium dendrobatidis* (*Bd*) in Texas shows four hotspot clusters in red (clusters 1-4) where clusters 1 and 2 are significant. In addition, there are three coldspot clusters (clusters 5-7) in which clusters 5 and 6 have significant values, and cluster 7 approaches significance.

IV. AMPHIBIAN ASSEMBLAGES AND THE PREVALENCE OF *BATRACHOCHYTRIUM DENDROBATIDIS* ACROSS THE TEXAS ENVIRONMENTAL GRADIENT

ABSTRACT

The driving factors that help sustain Chytridiomycosis, a disease affecting amphibians caused by the pathogen *Batrachochytrium dendrobatidis* (*Bd*), likely include both environmental factors and host assemblage composition as contributing to the prevalence of the pathogen. We sought to evaluate the current prevalence of *Bd* in Texas across precipitation gradient. In total, we collected 562 samples from different species in five Wildlife Management Areas (WMA) across the state, and evaluated trends that provide predictive indicators of increased prevalence of *Bd*. We detected an overall prevalence of 26% throughout the state, with highly variable prevalence among species. We determined that amphibian host assemblage composition is the main driving factor for *Bd* prevalence in Texas. Finally, we found that *Bd* positively correlates with different environmental factors such as precipitation and temperature, but these correlations are not constant across all sampled WMAs. This represents the first evaluation of the spread of *Bd* across the state of Texas, as well as the first study to evaluate the effect that both assemblage composition and environmental factors play in predicting areas of high susceptibility to increased prevalence of this pathogen.

INTRODUCTION

Batrachochytrium dendrobatidis (*Bd*) is the pathogen that causes the fungal disease chytridiomycosis. It was first described in the 1990s (Longcore et al. 1999) and has since been documented as a cosmopolitan amphibian pathogen, occurring in every continent with amphibian populations (Olson et al. 2013). Given an almost global distribution, *Bd* is suggested to be the worst infectious disease ever recorded among vertebrates both in terms of the number of species it impacts as well as its nature to drive infected species towards extinction (Gascon et al. 2007). The behavior of this pathogen is unique. Although *Bd* is nearly ubiquitous, and affects a variety of different species of anurans, the outcome of that infection can vary within and among species. In some cases, it can lead to a rapid decimation of an affected population (Daszal et al. 1999, Woodhams et al. 2005, Crawford et al. 2010) while in other instances a species or population may not develop any clinical signs, and/or not experience the characteristic die-off events that have been associated with this pathogen (Woodhams et al. 2008, Schoegel et al. 2010, Reeder et al. 2010). This variation is not clearly understood, but has been attributed to factors such as host-pathogen interactions, assemblage composition (Smith et al. 2007, Becker and Zamudio 2010, Voyles et al 2011), climatic variables (Ruggeri et al 2018, Sonn et al. 2019), innate host immunity (Savage and Zamudio. 2011, Bradley et al.2019, McDonald et al. 2019) and even habitat variation (Brem et al. 2008, Fisher et al. 2009, Becker and Zamudio 2010).

Studies have demonstrated that the severity of infection significantly varies by species. For instance, the Mountain Yellow-legged frog (*Rana muscosa)* is a prime example of the pathogen driving a species towards extinction (Voyles et al. 2012).

Conversely, the American bullfrog (*Lithobates [=Rana] catesbeianus*) and Green Tree frog (*Hyla cinerea*) both fail to develop clinical symptoms of chytridiomycosis (Dazak et al. 2004, Weldon et al. 2004, Schloegel et al. 2009, Rizkalla 2010, Urbina et al. 2019, Yap et al. 2019). This variation in symptomatic response is an example of the host-pathogen interaction effect. There are a variety of mechanisms for these differences in susceptibility including innate immunity, symbiotic relationships, and even behavioral patterns (Lips et al. 2003, Rödder et al. 2008, Kinney et al. 2011, Voyles et al. 2011). Regardless of the mechanism, the host-pathogen interactions observed in different amphibian assemblages are likely to impact the overall prevalence of the disease at the assemblage level.

Assemblage composition has a major effect on the relationship a disease can have in a population. There are two contrasting hypotheses that address host-pathogen dynamics based on host species richness and pathogen prevalence; these are the dilution effect and the amplification effect. The dilution effect suggests that, in assemblages with high biodiversity, the prevalence of the pathogen appears diluted if the density of individuals from susceptible species is low (Searle et al. 2011a, Zargar et al. 2015). A dilution effect may be observed in certain assemblages where the biodiversity is low, as an increase in the relative abundance of incompetent hosts leads to a reduction in the total number of transmission events (Searle et al. 2011b, Zargar et al. 2015). In contrast, the amplification effect describes instances where the prevalence of the pathogen increases in assemblages with a disproportionately higher number of susceptible individuals (Zargar et al. 2015). In the case of *Batrachochytrium dendrobatidis*, studies have shown instances where the pathogen has exhibited both a dilution effect (Searle et al. 2008) and an

amplification effect (Becker and Zamudio 2010), suggesting that amphibian assemblage composition is a key factor in overall prevalence.

In addition to assemblage composition, research has also identified the pathogen's reliance on climatic variables, and influence of habitat variability. *Bd* has been shown to have an optimal temperature range of 17-21°C, with an effective range of 4-25°C (Piotrowski et al. 2004). As it is a water-borne pathogen, it is also reliant on the prevalence of water bodies and moist areas, preferring ponds to streams (Longcore et al. 1999). This creates a wide geographic range in which the pathogen can survive. However, there are also habitat factors that have been shown to contribute to its prevalence such as canopy cover (Padgett-Flohr et al. 2010, Becker et al. 2012). The number of suitable water bodies in a given area for amphibians also contributes to the prevalence of the pathogen by providing either an indirect dilution or amplification effect. In areas in which an amphibian population has access to multiple water bodies the pathogen may appear to exhibit a dilution of the prevalence, as the probability of interacting with a susceptible host is lower. Conversely, in areas of limited water bodies the probability of interacting with a host is higher, and as a result it may demonstrate an apparent amplification effect.

Texas is the second largest state in the U.S. with an area of 695,621 sq. km. Studies show a gradient in average year temperature from north to south, which tends to increase and stabilize on the southern areas with a mean temperature of 12˚C in the north and 23˚C in the south (Owen 1989, Owen and Dixon 1989). Reports of variation in precipitation suggest that in Texas annual rainfall follows an east to west gradient with the highest average annual rainfall near the Louisiana border being around the 139 cm

contrasted westward to places such as El Paso that have an annual average of 20.5 cm (Owen 1989, Owen and Dixon 1989). Due to this variation in environmental conditions previous studies have suggested that amphibian species' richness should follow the same gradients, being greater in areas with a decreasing variability in temperature and high precipitation volume being the most important predictor affecting amphibian species' richness (Owen 1989, Owen and Dixon 1989).

Understanding the factors that characterize the prevalence and severity of this pathogen are of key importance in preserving amphibian communities around the world. While the study of this pathogen has developed quickly in the past decade, Texas remains relatively data depauperate in the study of these types of interactions. Pragmatically, given the number of naturally occurring amphibians in the state, the variability of climatic factors and distinct ecoregions, which create unique habitat landscapes, Texas provides an interesting region for the study of these factors play in the prevalence of *Bd* in amphibian communities. This study seeks to provide the first in-depth review of key factors related to the prevalence of *Bd* in amphibian communities across the state of Texas.

MATERIALS AND METHODS

SAMPLING

We divided the state of Texas into five different geographic zones based on average precipitation and temperature (Figure 4.1), as previous studies suggest that precipitation is the most important factor that accounts for species richness in amphibians (Owen 1989, Owen and Dixon 1989). We selected a state-owned Wildlife Management

Area (WMA) in each geographic zone and sampled the WMA once per season for a year (i.e. quarterly). From east to west the samples WMA were: JD Murphree, Gus Engeling, Kerr, Matador and Black Gap. Each sampling trip consisted of three consecutive nocturnal surveys in which we did one two-hour time constrained survey and collected anurans opportunistically. We collected a total of 562 anurans and each amphibian was placed in an individual Ziplock bag to prevent cross contamination between animals. Individual amphibians were swabbed with sterile cotton tips with a plastic handle, after which the tips were placed in PrepMan tubes and were stored in the dark until analyzed at the lab. All captured amphibians were released in the area where they were collected (Kriger et al. 2007b). To prevent cross contamination across the WMAs, all field equipment including footwear was treated with commercial bleach (final conc. 5% NaOCl) after each field sampling event.

MOLECULAR ANALYSIS

DNA was extracted using PrepMan® Ultra Sample Preparation Reagent (Gaertner et al. 2009; Kilburn et al. 2010). The presence of *Bd* was assessed using a real time Taqman qPCR assay (Boyle et al. 2004; Kilburn et al. 2010). The probe ChytrMGB2 was used with two species-specific primers ITS1-3 Chytr and 5.8S Chytr summarized in Boyle et al. (2004) and Garland et al. (2010). We ran each sample in triplicate and compared the results to a regression line based on a consecutive 10-fold dilution of five standards. Using the JEL423 strain as a genomic reference and following the suggestions of Rebollar et al. (2014), we considered that one copy of the amplified gene fragment (ITS1 – 5.8S) was equal to 0.045 zoospore equivalents. Samples with

values above this zoospore number were considered positive for the presence of *Bd*. We ensured that the method was valid by measuring metrics for both efficiency (95-110%) and linearity ($R^2 \geq 0.997$). Duplicates of the positive samples were averaged to estimate zoospore equivalents per sample.

STATISTICAL ANALYSES

We evaluated the prevalence of *Bd* (proportion of infected individuals) both within species, and among Wildlife Management Areas by dividing the number of infected individuals by the total number of animals sampled using a 95% Jeffrey's confidence interval (CI) (Table 4.1). We used RStudio to run Generalized Linear Mixed Effect Models with Maximum Likelihood (glmmML), with a binomial distribution. We compared our sampling efforts with the Chao estimate for species richness to generate a diversity estimation (Chao 1984, Chao and Lee 1992). The relationship of *Bd* prevalence, against seasonality changes and target species were treated as fixed factors, with clustered WMAs serving as a random effect. A model was generated for each WMA, without clustering, in which we evaluated the prevalence of *Bd* against environmental parameters (the monthly average maximum and minimum temperature, and total precipitation) as well as mass, snout-vent length (SVL), sex and species of every animal collected during a sampling event. The best models were selected based on the lowest AIC scores.

RESULTS

We collected 562 skin swabs from 12 different species of anurans, 103 were confirmed infected with *Bd*, yielding an overall prevalence of 26%. *Pseudacris crucifer* (Spring peeper) presented the highest prevalence within species (86%) followed by *L. [R.] clamitans* (Green frog) (30%) and *Acris crepitans* (Cricket frog) (27%), with several amphibian species, including all of the bufonids, presenting a 0% prevalence(n=70)(Table 4.1). The WMA with the highest prevalence was Kerr WMA at 26% (n=170), while the lowest prevalence was found at Matador WMA (0%; n=64) in which no positive animals were detected across the entire study (Table 4.2).

The maximum likelihood generalized linear mixed effect model (glmmML) in which WMAs were clustered as a random effect suggested that the only significant predictor of *Bd* prevalence was species. Specifically *(i.e., A. crepitans (p-value = 0.05), P. crucifer (p-value = 0.03), L. clamitans (p-value = 0.03.)* were all indicative of higher rates of *Bd* prevalence.

Subsequently, generalized linear mixed effect models generated for each of the five WMA suggest different predictive variables. The J.D. Murphree WMA showed temperature (*p-value = 0.04*) and species *L. [R] catesbeiana (p-value = 0.02)* were significant predictors. The Kerr WMA suggested that precipitation (*p-value =>0.001*), temperature (*p-value = 0.002*), and species *A. crepitans (p-value = 0.001), L. berlandieri (p-value = 0.01)* were significant. Finally the Gus Engeling WMA predicted that SVL (*p-value =0.02*), and species *H. versicolor (p-value = 0.01),* were significant predictors, with temperature approaching significance (*p-value = 0.06*). Models for the Black Gap

WMA showed no significant predictors, and the Matador WMA was excluded as it was found to have an overall prevalence rate of 0%.

DISCUSSION

The number of reports of *Batrachochytrium dendrobatidis* infecting a variety of species globally have greatly increase in the past decade (Chatfield et al. 2012, Pearl et al. 2009, Schlaepfer et al. 2007, Woodhams et al. 2008). In addition, *Bd* has been found to be devastating for certain amphibian communities (Rachowicz et al. 2006, Murray et al. 2009) while other species, such as the American bullfrog, seem to be relatively immune, and instead act as a reservoir fort the pathogen (Woodhams et al 2008, Schloegel et al. 2010, Reeder et al. 2012, Urbina et al. 2019, Yap et al. 2019). This heterogenous species response confounds clear approaches to managing the disease when is enzootic to an area (Puschendorf et al. 2009, Stockwell et al. 2015, Skerratt et al. 2016, McDonald et al. 2019) or understanding the risk factors that lead to outbreaks when it is a resident pathogen (Rachowicz et al 2005, de Queiroz Carnaval et al.2006, Tarrant et al. 2013, Becker et al. 2016). Among the factors that have been studied, amphibian assemblage diversity may act as a buffer (dilution) preventing the transmission to the pathogen among individuals (Schmidt and Ostfeld 2001, Searle et al. 2011b) or if depauperate, it might amplify the disease among communities composed of sensitive species (Pillot et al. 2013, Scheele et al. 2019b). Likewise, environmental conditions, as well as the availability of the aquatic habitats needed for the pathogen to survive, can lead to simple increases in the case of water scarcity leading to more hots-pathogen contact (Becker et al. 2007, Becker and Zamudio 2011).

Texas uniquely provides both the temperature and water availability gradients that could assist with examining these factors. Those same structured gradients from north-south for temperature and east-west for water availability also drive amphibian assemblage diversity (Owen 1989, Owen and Dixon 1989). Somewhat uniquely there are taxa that remain present the near full breadth and depth of Texas (*Lithobates [Rana] catesbianus*, *Acris crepitans*) while others have a more restricted geographic range in the state (*Pseudacris crucifer*, *Lithobates [=Rana] clamitans*) while still having distributions nearly the full depth of the state temperature gradient (Dixon 2000). Texas as a study area then enabled us to sample amphibians, test for the pathogen, and then seek to better understand how those previously identified factors may play a role in its prevalence.

In examining the overall prevalence of *Bd* across the WMAs we begin to see an example of an amplification effect. The species with the highest prevalence of the pathogen were *P. crucifer* (86%), *R. clamitans* (30%) and *A. crepitans* (27%) all primarily caught at the three WMA locations in East and Central Texas, which had the highest overall prevalence of all surveyed sites (22-26%), and are located in areas that present the highest species diversity and relative abundance in Texas (Brown et al 2012.). This likely suggests that these three species represent competent host species, and their proportionately higher occurrence in the surveyed areas led to an increased prevalence of the pathogen (Keesing et al. 2006, Scheele et al. 2019b). These are also geographically areas with high water availability seeming to indicate that given a high pathogen prevalence, widely available water does not dilute its prevalence on the amphibian assemblages.

Conversely, when we examine the prevalence rate in North and West Texas we see both sampling sites present a lower overall detections of the pathogen, as well as the lack of the previously mentioned competent hosts species, which might suggest that species such as *Lithobates [R] catesbeiana* and *R. berlandieri* which have a prevalence of 14% and 12% respectively are the most competent hosts in the area, but are surrounded by noncompetent host (i.e. s *B. speciosus* or *B. woodhousii*) that have a prevalence of 0%, and might suggest that a dilution effect is taking place (Keesing et al 2006, 2010, Cohen et al 2016). Therefore, based on the host-pathogen dynamics in the area, including species diversity and relative abundance, we were able to observe instances of both an amplification and dilution effect across the state of Texas.

When we examine this host-pathogen dynamic at a smaller geographic scale, we begin to see the impact of assemblage composition as a predictive variable for the occurrence of *Bd*. In our maximum likelihood generalized linear mixed effect model, in which we clustered all sampling locations as a random effect, we found the only significant predictor of prevalence to be species. This helped identify both the most competent (*P. crucifer, A. crepitans,* and *R. clamitans*) and incompetent (*H. cinerea, R. blairi,* and *B. speciosus*) hosts surveyed throughout our study (Table 1). In addition, when we examined the individual models generated for each WMA, we found that the surveyed locations with the highest overall prevalence (Gus Engeling, J.D. Murphree, and Kerr) (Table 2) also had the highest occurrence of competent hosts, while the sites with the lowest overall prevalence (Black Gap, and Matador) had the highest occurrence of incompetent hosts. The models also suggested that climatic variables, such as precipitation and temperature, were significant predictors. However, we saw these

variables become non-significant in areas with lower overall prevalence of the pathogen (i.e., Black Gap and Matador WMAs), suggesting that the even though environmental factors have a role in the prevalence of *Bd*, assemblage composition is the biggest driver in the pathogen incidence in Texas . Therefore, while we observed significant climatic variables in some of our WMAs more sampling should be conducted in North and West Texas to identify which variables are most relevant.

Due to the wide geographic distribution of *Batrachochytrium dendrobatidis* (Fisher et al. 2009, Olson et al. 2013) research is needed to better understand key variables to identify communities with increased risk for outbreaks. While some studies have begun to focus on the impact of climate and habitat landscape (Ribas et al. 2009, Murray et al. 2011, Olson et al. 2013), others seek to evaluate how different amphibian communities are impacted by the pathogen (Searle et al 2011a, Volyles et al. 2011, Gervasi et al. 2013). These pathogen-host interactions are key to explaining the variability in the severity of infection as some species remain seemingly unaffected while others suffer characteristic die-offs.

This study provides one of the first such evaluations of *Bd* prevalence across the state of Texas and across its various amphibian communities. Analysis of overall prevalence, and statistical tests suggests that assemblage composition, and subsequently host-pathogen interactions, are the most significant predictors of overall prevalence in an area. While significant climatic predictors were also observed, the lower overall prevalence of the pathogen in North and West Texas suggest that more sampling is required to fully identify the most important factors. This is also the first documented study in which both an amplification and dilution effect have been observed in the same

state, demonstrating the impact of assemblage composition on the pathogen's prevalence. It also demonstrates that management or planning for pathogen outbreaks will require robust amphibian monitoring programs and surveillance of the pathogen among those populations in order to understand those effects at the largest geographic scales. While Texas remains relatively data depauperate, this study provides the first broad evaluation of *Bd* prevalence across the state in a variety of amphibian communities and provides such a first step in the suggested baseline monitoring and surveillance necessary for managers to understand the disease dynamic in their regions.

TABLES

Table 4.1: Prevalence of *Batrachochytrium dendrobatidis (Bd)* by species, using 95% Jeffrey's confidence intervals (CI). Collected from five different Wildlife management areas across five different gradients of precipitation.

Species	Positive	Negative	Total	*Bd.* Prevalence (%)	CI (95%)
Pseudacris crucifer	12	2	14	86	0.62 - 0.97
Lithobates[R] clamitans	14	32	46	30	0.19 - 0.45
Acris crepitans	52	143	195	27	0.21 - 0.33
Lithobates [R] sphenocephala	3	13	16	19	0.06 - 0.42
Lithobates [R] catesbeianus	5	32	37	14	0.05 - 0.27
Lithobates [R] berlandieri	13	98	111	12	0.70 - 0.19
Incilius nebulifer	1	9	10	10	0.01 - 0.38
Hyla versicolor	3	52	55	5	0.02 - 0.14
Lithobates [R] blairi	0	9	9	0	0.00 - 0.24
Gastrophyne carolinensis	0	10	10	0	0.00 - 0.22
Hyla cinerea	0	17	17	0	0.00 - 0.14
Scaphiopus couchii	0	7	7	0	0.00 - 0.29
Bufo debilis	0	1	1	0	0.00 - 0.85
Bufo punctatus	0	5	5	0	0.00 - 0.38
Bufo speciosus	0	22	22	0	0.00 - 0.11
Bufo woodhousii	0	7	7	0	0.00 - 0.29
TOTAL	**150**	**426**	**576**	**26**	**0.15 - 0.22**

Table 4.2: Prevalence of *Batrachochytrium dendrobatidis (Bd)* by the Wildlife Management Area (WMA) within Texas sampled, using 95% Jeffrey's confidence intervals (CI). Spp. Richness is the total number of species collected, the Chao Index used to calculate the species prediction and diversity estimation.

WMA	Total	Spp. Richness	Chao Index	*Bd.* Prevalence (%)	CI (95%)
Matador	64	8	6.32	0	0.00 - 0.04
Black Gap	73	4	10.31	3	0.01 - 0.08
JD Murphree	123	8	15.01	22	0.15 - 0.3
Gus Engeling	134	7	12.89	24	0.17 - 0.32
Kerr	170	6	14.58	26	0.2 - 0.33

FIGURES

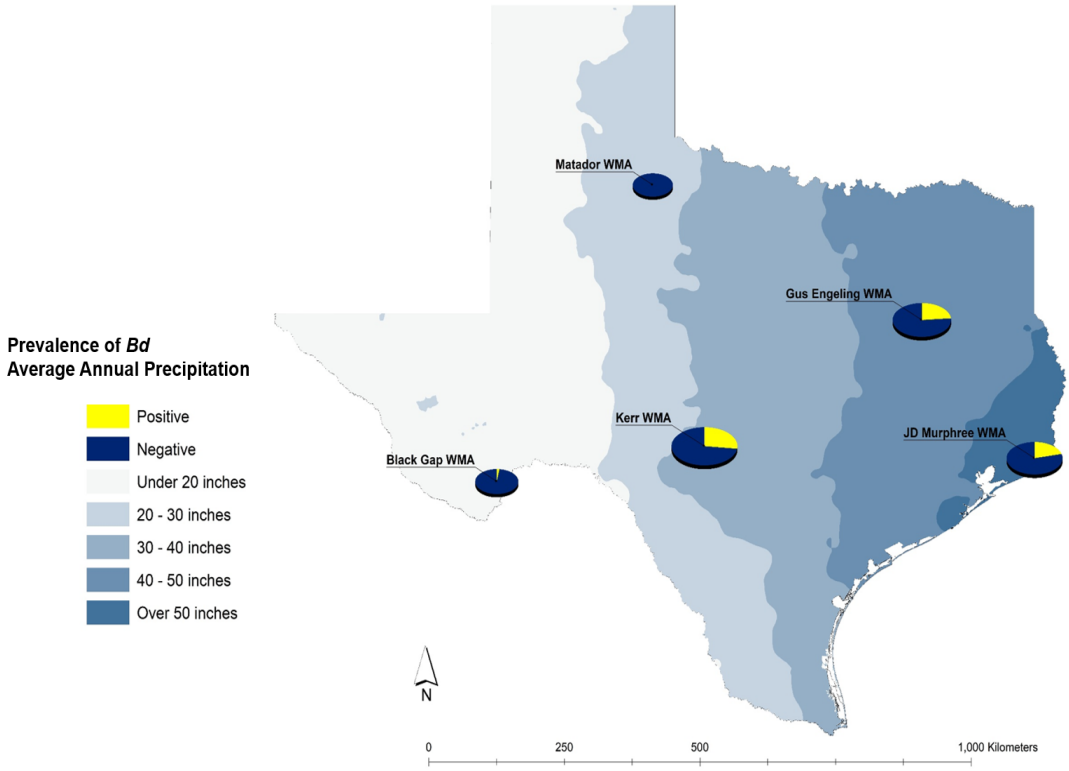

Figure 4.1: Wildlife Management Areas (WMA) sampled in Texas, across a precipitation gradient. Circle size represents the total number of animals caught at each location (N). The yellow wedges show the proportion of individuals positive for *Batrachochytrium dendrobatidis*. Black Gap WMA 3% N=73, Matador 0% N=64, Kerr WMA 24% N=170, Gus Engeling WMA 24% N=134 and JD Murphree 22% N=123.

V. CONCLUSIONS

Since chytridiomycosis was first described in the 1990s studies have shown its occurrence in various amphibian communities around the globe. The prevalence of this pathogen is driven by a both a variety of environmental factors, such as temperature, precipitation, and canopy cover, as well as the specific host-pathogen interactions present in the amphibian assemblages. The variability of the host-pathogen interactions have been attributed to specific behavior, or even an innate immunity. These specific interactions affect the prevalence of the pathogen due to the availability of competent hosts (animals that effectively transmit the infection to other susceptible individuals) over non-competent hosts. Due to the lack of documented outbreaks and reports of amphibian declines in Texas, we expected to find low overall prevalence of *Bd,* with the highest peaks in humid areas such as east Texas as well as the areas with the largest species diversity.

We used different approaches to analyze the prevalence and geographical patterns of *Batrachochytrium dendrobatidis (Bd)* in Texas. First, we evaluated its relationship between two sympatric tree frogs (*Hyla cinerea* and *Hyla versicolor*), where we were able to determine that there is a low occurrence of the pathogen in *H. versicolor* (~15%) across its entire geographic distribution. Moreover, there were no positive detections of *Bd* in *H. cinerea* in our entire study, nor have there been any reports of positive amphibians caught in the wild throughout the species' geographic distribution. Further sampling, as well as environmental and specific innate immune analysis should be conducted to evaluate if this is a true non-competent host.

In another study, we used museum collections to sample specimen collected from the 1920s to present day. Our sampling efforts showed that *Bd* has been present in Texas since at least the 1930s. We also reported the first detections of *Bd* in the U.S. (1940s) for *Acris crepitans*, and *Lithobates [Rana] berlandieri*. This allowed us to create temporal models for each decade sampled and allowed us to show that Bd might be enzootic in Texas. In evaluating our data in a spatio-temporal model we also showed decades in which outbreaks occurred (hotspots), as well as a predictive model for future outbreak events. Future analyses should be focused on the areas of the spatio-temporal models in which coldspots, or areas where the pathogen is less likely to occur, and evaluate which factors drive these low prevalence rates, especially in areas with such high biodiversity.

Finally, amphibians were sampled across five different Wildlife Management Areas (WMAs) around the state in each season spanning one year. The current prevalence of *Bd* across the state of Texas is 26%, with species assemblage composition appearing to be a significant factor to predict areas of high prevalence. Hotspots previously identified in east and central Texas were confirmed as having some of the highest overall prevalence rates in the state. In addition, individual models created across a precipitation gradient suggested that there is a variation in regards to other driving factors that might affect the prevalence of *Bd* in different areas of Texas. While this suggests that a generic management plan for the prevention of outbreaks is unfeasible, our models may help the creation of site-specific plans catered to the specific host-pathogen interactions, and environmental factors for each location.

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