# PREVALENCE OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN AMPHIBIAN COMMUNITIES OF CENTRAL TEXAS AND TAMAULIPAS, MEXICO

by

Andrea Villamizar Gomez, V.M.D.

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Committee Members:

Michael R. J. Forstner, Chair

Ivan Castro Arellano

Dittmar Hahn

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## ABSTRACT

*Batrachochytrium dendrobatidis (Bd)*, a fungus that causes chytridiomycosis on infected amphibians, and has been implicated as a potential causative agent in the amphibian population declines of the past 50 years. This study seeks to assess the prevalence of *Bd* in amphibian communities in Central Texas and the state of Tamaulipas, Mexico. In Central Texas two counties were evaluated during the spring of 2012, and for the Tamaulipas assessment samples of 18 amphibian species were collected between 2004 and 2008 at 16 different localities that ranged from 100-2900 meters in elevation. All the samples were obtained from non-consumptive toe clippings and swabs. The presence of the pathogen was assessed by using a Taqman quantitative real time PCR (qPCR) assay in a highly sensitive approach to detection. Interestingly, there is a low prevalence for *Bd* in Central Texas in comparison with previous studies conducted in this area. All samples tested from Tamaulipas were negative for the presence of the pathogen. This study implies that monitoring the pathogen in both southern Texas and northern Mexico requires explicit changes to normal surveys protocols. To better understand the dynamics of the fungus in these environments, will require increased efforts during periods that accommodate pathogen thermal preferences. Further exploration in this regions and adjacent areas will help to inform of the prevalence, widespread and epidemiology of *Bd* and would help prioritize conservation efforts.

# CHAPTER I

## Introduction

*Background*

Amphibian populations have been shown to be declining over the past century (Berger *et al.* 1998, Houlahan *et al*.2000). Areas with declining populations in America include apparently pristine enviroments in Central and North America, including the cloud forests of Guatemala (Campbell 1998), Honduras (McCranie and Wilson 2002), Costa Rica's tropical mountains (Pounds *et al*. 1997, Lips 1998, Lips *et al*. 2003), and the Western United States (Knapp and Matthews 2000, Jaeger *et al*. 2001, Bradley *et al*. 2002).

Several hypotheses have been proposed to explain the cause, or causes, for the decline of these polulations. Declines are likely the result of habitat loss, but also of changes in environmental conditions such as shifts in temperature patterns, changes in UV-B radiation or rainfall, or contamination through anthropogenic activities. Two diseases are meant to contribute to the declines of amphibians populations: chytridiomycosis caused by the fungus *Batrachochytrium dendrobatidis,* and infections by *Ranavirus* sp. may each be part of a cumulative impact on these populations (Fellers *et al*. 2001, Young *et al*. 2001, Blaustein & Kiesecker 2002, Collins & Storfer 2003, Bread & O'Neil 2005). Reports that correlate the presence of *B. dendrobatidis* to declines in amphibian populations have been published for Europe (Bosch *et al.* 2001), Mexico (Lips *et al*. 2004, Frias *et al.* 2008) and North America (Green and Sherman 2001, Muths *et al.* 2003, Rachowicz *et al.* 2006). *B. dendrobatidis* is a fungus that belongs to the chytridiomycota family and parasites the keratinous parts of the skin of amphibians. Most

members of the chytridiomycota family are free living or commensal organisms that develop in aquatic environments and soils (Longcore *et al*. 1999). Normally, *B. dendrobatidis* occurs at temperatures between 4 and 21°C with an optimal temperature for growth that ranges between 17 and 21 °C (Piotrowski *et al.* 2004). It has two life stages represented by a motile zoospore, and a reproductive zoosporangium. Zoospores are uniflagelated organisms that can live in the water for a short period of time, and travel distances of 1-3 cm (Garner *et al.* 2006) attracted mostly by chemotaxis of amino acids and sugars present in the amphibians skin (Moss *et al.* 2008). Once the zoospore penetrates the skin, it forms a cyst and initiates the reproductive stage, developing into zoosporangia, which produces more zoospores that can either reinfect the host, or be released back into the environment (Berger *et al.* 2005). *B. dendrobatidis* infection of amphibians generally leads to hyperkeratosis, which causes a disruption of electrolyte transport where levels of electrolytes (sodium, potassium, chloride and magnesium) are abnormally low, leading to animal mortality (Berger *et al*. 1998, Voyles *et al*. 2007, 2009). Amphibians exhibit few clinical signs of chytridiomycosis until they are heavily infected and close to death. In severe chytridiomycosis, the clinical signs include anorexia, lethargy, abnormal posture with hind legs extended, and lack of righting reflex (Berger *et al*. 2005). Mortality rate and time to death after *B. dendrobatidis* exposure and infection are influenced by the pathogen dose, the temperature, the life stage of the amphibian as well as by the amphibian species (Berger *et al*. 1999, 2004, 2005, Lamirande & Nichols 2002, Woodhams *et al*. 2011, Rachowicz & Vredenburg 2004, Kriger *et al*. 2007). The pathogenicity of *B. dendrobatidis* has been linked to weather variation with temperature as the mainly contributing factor, but there are also geographic

differences. Thus, while seasons may affect the severity of the infection, being more pathogenic in cool, humid areas (Berger *et al.* 2005, Gaertner *et al*. 2009, Gaertner *et al*. 2012) other factors like population density, drought, tadpole longevity (Briggs *et al*. 2010, Lampo *et al*. 2008, Rachowicz *et al.* 2007) and a species' differences in behavior, natural history and immunity can contribute to the persistence and pathogenicity variation of the disease (Lips *et al.* 2003, Rödder *et al*. 2008, Kinney *et al*. 2011).

*B. dendrobatidis* has been studied in North America for almost 50 years as a source of possible amphibian declines using historical samples or wild caught animals (Bradley *et al*. 2002, Ouellet *et al*. 2005, Rachowicz *et al*. 2006, Gaertner *et al*. 2012). Initial studies focused on outbreaks and sudden mortality events such as the *Rana muscosa* massive die-offs in Sierra Nevada, California (Rachowicz *et al*. 2006) or the *Rana yavapaiensis, Rana chiricahuensis, Hyla arenicolor* mortalities in Arizona (Bradley *et al*. 2002). These acted as a catalyst for studies examining the prevalence of the disease in North America. Recent studies have focused on the presence of *B. dendrobatidis* in locations where no apparent mortalities have been detected. The results obtained present the real distribution of *B. dendrobatidis*, and show a high prevalence of the disease in anuran and salamander populations that exhibit mild to non-clinical signs of the of the disease (Ouellet *et al*. 2005).

Even though *B. dendrobatidis* has been the focus of many studies in North America, there are areas that have been given more attention than others. Figure 1 shows a map of all the *B. dendrobatidis* tests done in North America, where states like California and Oregon have over 1000 samples analyzed, but on the other hand Texas and northern Mexico have just a few hundreds. Interestingly there are a no reports of

positive samples in east Texas and central Mexico (Gaertner *et al.* 2007, Saenz *et al.* 2010), but the pathogen is present in central Texas (Gaertner *et al*. 20012a, 2012b, 2009).

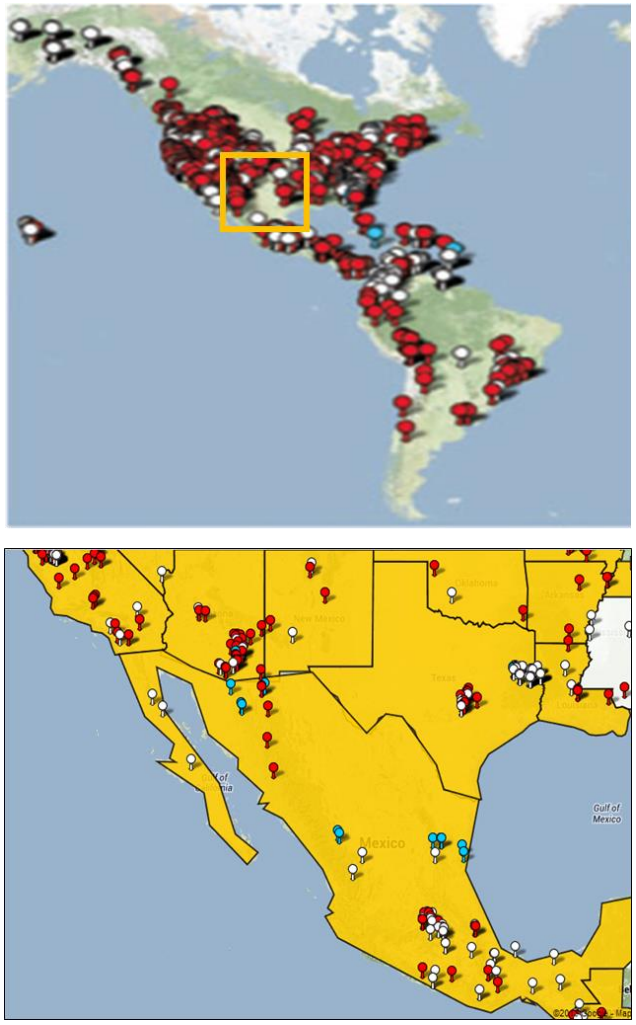

Figure 1: Distribution of *B. dendrobatidis* samples published in America, and more specifically Texas and northern Mexico.

Among the causes that may favor the pathogen presence to a specific site are temperature, humidity, population density, and tadpole longevity (Briggs *et al*. 2010, Lampo *et al*. 2008, Rachowicz *et al.* 2017). Also species' differences in behavior, natural history and immunity can contribute to the persistence as well as the pathogenic variation

of the disease (Lips *et al.* 2003, Rödder *et al*. 2008, Kinney *et al*. 2011). Climatic variation is often listed as one of the primary factors for increased detection or presence of the pathogen, with temperature being one of the main contributing factors enabling pathogen growth. Thus, detection of *B. dendrobatidis* can be linked to the geographic differences from a biotic province, resulting in the common hypothesis that seasonal variations may affect the presence and severity of the infection, being more pathogenic in cool, humid areas (Berger *et al.* 2005, Gaertner *et al*. 2009, Gaertner *et al*. 2012).

A biotic province is a geographical area, characterized by particular differences in soil, physiography and ecological climate supporting a flora and fauna specific of that region. Particular biotic provinces are often anthropogenically modified, producing new habitats due to alterations of the landscape and local vegetation. This is in addition to the normal disturbance and regeneration processes that place the biotic provinces in a constant state of change (Vestal 1914, Dice 1949). I have selected to evaluate the presences of *B. dendrobatidis* in two provinces (Figure 1), the Texan province where all the Central Texas samples from Bastrop County and Comal County were collected, and the adjacent Tamaulipan province located in the state of Tamaulipas, Mexico.

The Texan biotic province has sandy soils and is covered mostly by grassland and patches of deciduous trees. It is also common to find forests of post oak (*Quercus stellata*), black jack oak (*Quercus marilandica*), Texas hickory (*Carya buckleyi*), and Loblolly pine (*Pinus taeda*).

This province has high seasonal variation, with long summers and temperatures that as can reach 40 °C. The winters are mild and relatively short with temperatures that

reach the -10 °C. Precipitation is most pronounced during the long growing season (McBryde 1933, Clements & Shelford 1939, Gaertner *et al.* 2010, Abbot 2011).

The Tamaulipan biotic province (Figure 1) is a semiarid region, characterized by deciduous and thorny plants interspersed with grassland (Muller 1939, Blair 1950, Johnston 1963). The majority of the province is confined to the Gulf coastal plain, with mountains rising to an elevation of 2250 m above sea level in the southwest corner on the state of Tamaulipas . The temperature of the province is relatively high throughout the year, with averages highs around 32°C, and lowest temperatures at 15°C. The vegetation of the plains consists mostly of semiarid tropical thorn scrub, with thorn forests are predominant in the central region with acacia, mesquites, and cactuses although riparian areas can support lush gallery forest (Cram *et al*. 2006, Martin 1958, Alvarez 1963, Flores-Villela 1993, Morrone *et al*. 2002). The mountains, south east of the state, are surrounded by tropical deciduous forest and montane scrub at elevations between 600-900 m, and pine-oak forest at 800-1400 m (Martin *et al*. 1954). A transition from the tropical deciduous forest of the coastal plain, to predominantly oak forest occurs in the southwest of the mountain region, with additional fragments of tropical evergreen forest appearing irregularly at lower elevations (ca. 500-1000 m) on the eastern slopes, and cloud forests on karst formations occurring at elevations of 900-1700 m. The summits and higher interior slopes support dry oak and day pine-oak forest, and temperate Madrean pine-oak woodlands characterize highest ranges >2250 m (Martin 1958, Gómez-Hinostrosa & Hernández 2000).

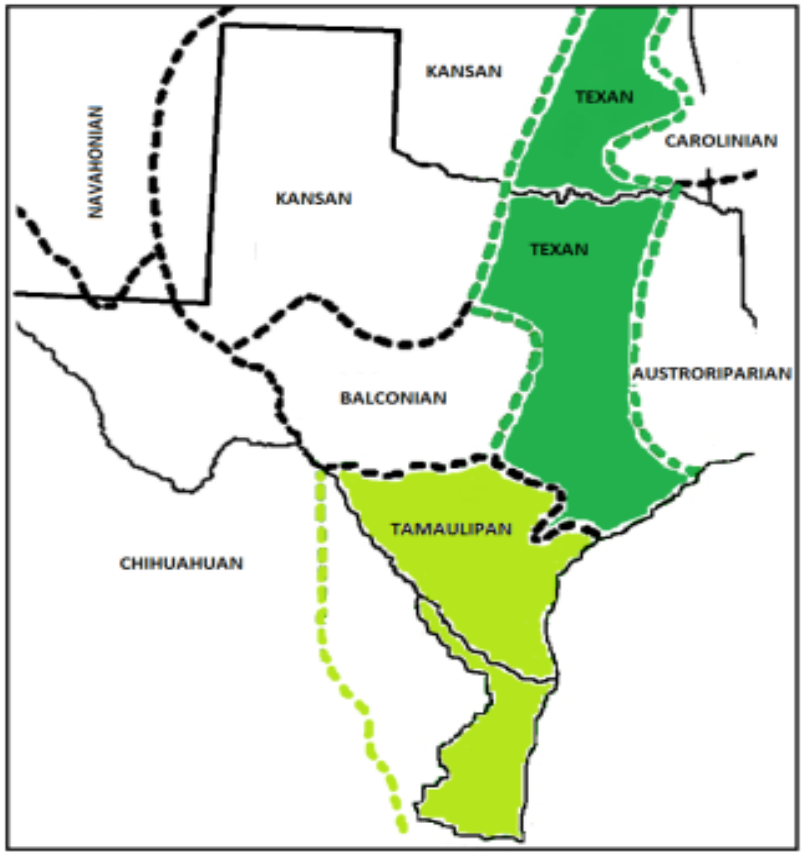


Figure 2: Biotic provinces of Texas according to Blair, W. F (1950).

The impact to an amphibian population infected with *B. dendrobatidis* can vary from persistence with low to no mortality to massive die-offs without any recovery (Dazack *et al*. 2003, 2004, Retallic *et al*. 2004, Schloegel *et al*. 2006). There is very little known about this disease in the Texan biotic province, and all of the reports are from Bastrop County (refs). There is no assessment of the presence of the disease in Comal County, except for a report of a possible outbreak in 2010, where only 4 samples were viable enough to be analyzed and were positive for *B. dendrobatidis*. There is also no known study done in the state of Tamaulipas that assess the prevalence of this disease.

Detection is only one issue, as the pathogenicity of *B. dendrobatidis* varies among specific strains of the fungus (Retallick *et al*. 2006). Amphibian populations may be used to one particular strain that causes little to no distress among individuals, but when faced with a novel strain it may result in massive die-offs, decimating the populations (Berger *et al*. 2005). *B. dendrobatidis* is known to infect over 350 different species of amphibians and there is evidence that it has been one of the major causes of extinction in three species of frogs (Retallic *et al*. 2004, Schloegel *et al*. 2006, Gewin *et al*. 2008).

There are many different detection methods that are available, and have been used for the past 20 years. Necropsies and histopathology normally look for the most common lesions that suggest the presence of the pathogen, as well as the damage cause such as infiltration of the keratin layers of the skin, edema, epidermal hyperplasia and hyperkeratosis. They also detect the actual presence of the spores in the keratinized layers of the skin (Fellers *et al*. 2001, Bradley *et al.* 2002, Berger *et al*. 2002, Ouellet *et al*. 2005, Daszac *et al.* 2009). Immunohistochemistry instead focuses mainly on the detection of antibodies for the pathogen (Berger *et al.* 2002, Olsen *et al*. 2004). While all of these types of detection prove to be sensitive in the detection of *B. dendrobatidis*, they are also time consuming, and demand expert histological experience to perform the analysis (Boyle *et al*. 2004, Kriger *et al.* 2006a, 2006b). Polymerase chain reaction (PCR), and quantitative (real-time) Taqman polymerase chain reaction (*q*PCR), which detect the presence of a DNA fragment from the fungus (Boyle *et al.* 2004, Kriger *et al* 2006a, 2006b, Gaertner *et al.* 2012) has been demonstrated to be another very reliable mean to detect *B. dendrobatidis* in both wild and captive amphibians (Kriger *et al*. 2006a). It is less expensive than a histological exam and less time consuming. The sensitivity of *q*PCR

tests shows a quantification of the number of zoospores present in each sample; this allows comparing the level of infection variability among samples collected, which could be translated in different rates of infection among a specific population (Boyle *et al*. 2004, Kriger *et al*. 2006a, 2006b).

For this study, I seek to gather occurrence data of *B. dendrobatidis* for south-central Texas spanning our recent drought and from historical samples from just prior to the drought from northern Mexico. A motivation of this work is to assess the prevalence of this organism among a variety of Central Texas localities in order to obtain a potential explanation of an amphibian die-off event that occurred in Comal County. A secondary goal is to assess prevalence of *B. dendrobatidis* among the amphibians in Tamaulipas, Mexico using historically collected samples, to provide a baseline for comparison in future studies of those areas analogous to prior work in Central Texas.

*Objectives*

The main objective of the project was to evaluate the presence of *Batrachochytrium dendrobatidis* in the amphibian population in Tamaulipas, Mexico and in two counties (Bastrop and Comal) of Central Texas. These two provinces areas are among those in North America with relatively few studies on the subject. A second objective was to associate the possible factors that may have led to the positive prevalence of this organism in relation to these two different biotic provinces. The basic tasks of this work included to:

- Determine the prevalence of the disease for several amphibian species using *q*PCR.
- Examine historical occurrence for an, as yet, untested region of northern Mexico just south of the Texas border

- Conduct an evaluation of an amphibian mortality event in Comal County to assess an outbreak of *B. dendrobatidis* as a potential explanation of the mortality.
- Seek to refine the existing knowledge of how seasonal changes contribute to an increase or decrease in the presence of the disease.

# CHAPTER II

## Materials and Methods

*Sampling*

Two different counties in Central Texas were sampled: Bastrop and Comal. In Bastrop County two sampling sites were; Griffith League Ranch and Bastrop State Park. In Comal County, 2 sites adjacent to the area of the mortality event from 2010, were selected for sampling. In Figure 3, the yellow circle represents the site of the mortality event from 2010 and the green circles are the sampling sites in 2012 for both Bastrop and Comal Counties.

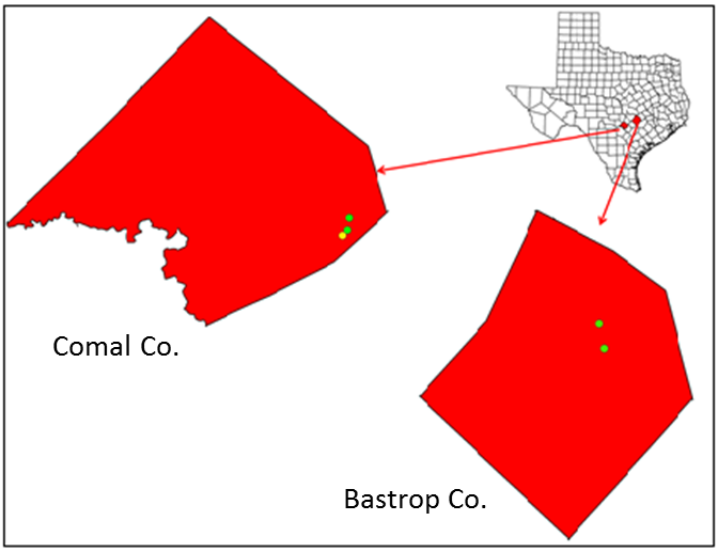


Figure 3: *Batrachochytrium dendrobatidis* sampling sites in Comal and Bastrop County, Texas. The yellow circle represents a possible outbreak site in Comal Co. The green circles represent sampling sites.

For the analyses of prevalence of *B. dendrobatidis*, toads were sampled opportunistically in Comal County and Bastrop State Park by using drift fences around small semi-permanent reservoirs on the Griffith League Ranch. All samples were obtained from non-consumptive toe clippings during the spring of 2012 and were stored in 95% ethanol at -80°C for posterior DNA extractions. The species analyzed in Bastrop County were: *Bufo houstonensis, Bufo nebulifer, Acris crepitans* and *Scaphiopus hurteri* with a total of N= 188 samples (Table 1), for Comal County the species analyzed were *Bufo nebulifer, Acris crepitans* and *Pseudacris clarkii* with a total of N= 122 samples (Table 2).

Fifty six historical samples were retrieved from the M. R. J. Forstner Frozen Tissue Catalogue held at Texas State University (Table 3), from wild caught amphibians, from 2004 to 2008 in 16 different sites across the state of Tamaulipas (Figure 4).

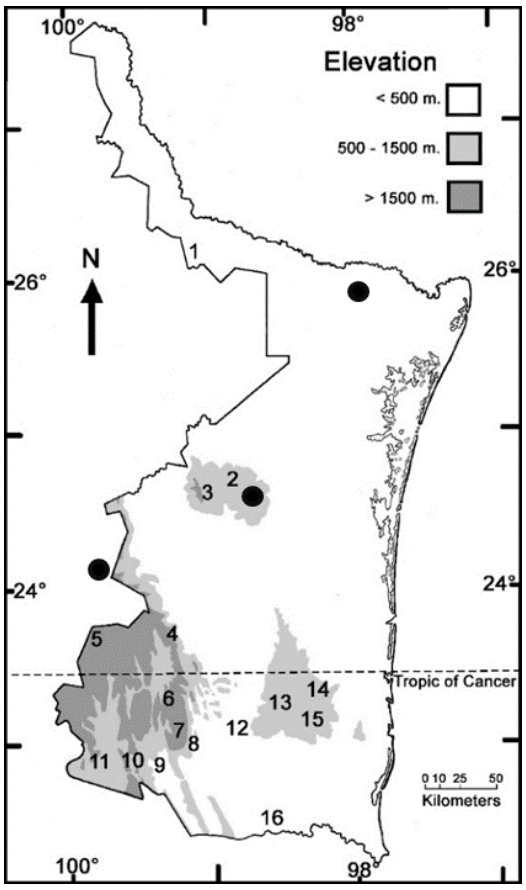


Figure 4: Localities sampled in Tamaulipas: 1) Miguel Aleman, 2) San Nicolas, 3) San Carlos, 4) Victoria, 5) Miquihuana, 6) Jaumave, 7-8) Gómez Farías, 9) Ocampo, 10-11) Tula, 12) Xicotencatl, 13) Llera, 14-15) Aldama, and 16) Ciudad Mante. The black circles represent the location of the 3 weather stations.

*Molecular Detection*

The DNA extractions were made using a DNeasy Qiagen Kit, tissue protocol (Qiagen, USA). The detection of the disease was assessed by using a real time Taqman *q*PCR assay (Boyle *et al*. 2004). This protocol uses the probe ChytrMGB2 with two species-specific primers ITS1-3 Chytr and 5.8S Chytr. The assays were set in triplicates

for each sample, using a regression line based on a consecutive 10-fold dilution of 5 standards (known positive samples with high numbers of spores), to which the samples are compared to (Figure 5). Each study had an efficiency average of 98.15% and an $R^2$ average of at least 0.997. It was considered a positive result if two of the triplicates showed presence of the disease.

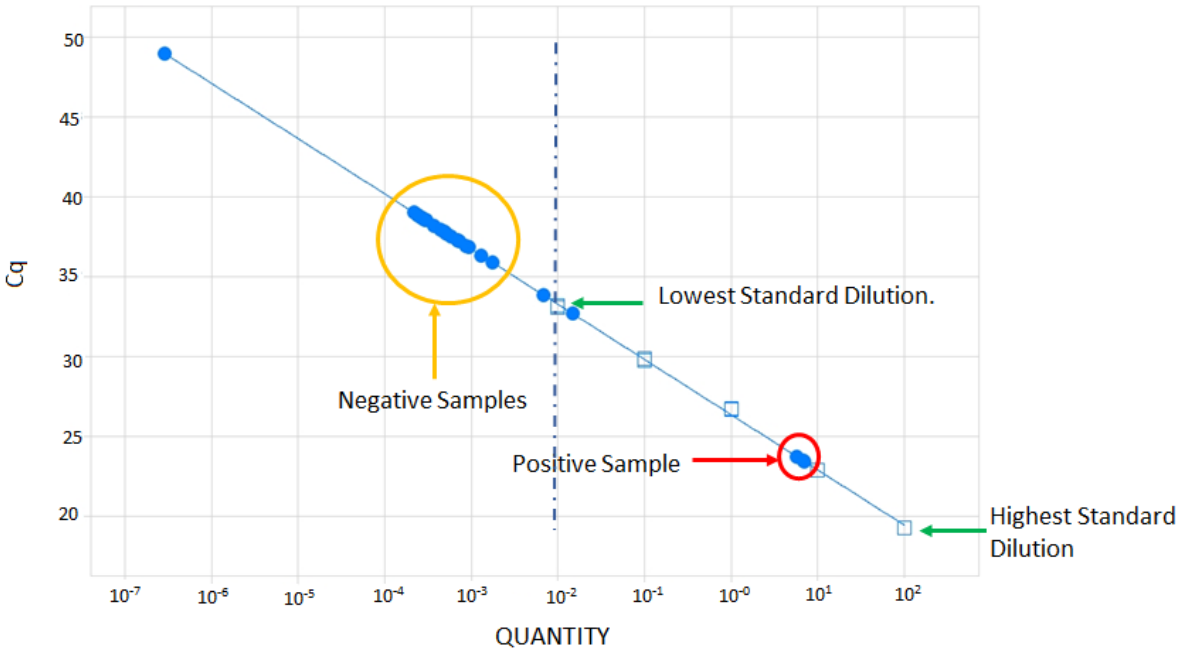


Figure 5: Example of a regression line result of a *q*PCR assay. Where the X axis represents the consecutive dilutions of the standards and the Y axis the quantification cycle.

*Environment Analysis*

Temperature, wind speed and humidity were recorded using a kestrel 3500 during the sampling dates and then were compared to the temperatures posted on the National Oceanic Atmospheric Administration web page (NOAA) using the Elgin TX station for the weather in Bastrop and the Comal TX station to obtain weather in both counties. The

reports by the INIFAP (Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuaruas) were used to evaluate differences in temperatures according to the dates of the samples obtained from Tamaulipas, Mexico. 2007 was the year that had the most complete data for the 3 weather stations that were used (Figure 4). The weather stations were chosen due to their closeness to the sampling sites and each one of theme represents one of the three gradients of elevations previously depicted (Figure 4)

Analysis of variance and paired t-tests were used to determine differences of temperature and precipitation during the last five years previous to this study for the Bastrop and Comal Counties samples.

# CHAPTER III

## Results

*Bastrop County*

One of the most recent studies published in Central Texas by Gaertner *et al*. (2012) showed the presence of *B. dendrobatidis* in Bastrop County. Results show a prevalence of 89% average across the year 2009. With this in mind the same property was sampled opportunistically, where 188 samples were collected during the spring season (Table 1). As shown in the figure 6, the red bars show that the prevalence for *B. dendrobatidis* in 2012 was significantly lower than the detection done in 2009, with some detection of *B. dendrobatidis* early in the season (March and April) only, with all positive samples belonging to 3 *Acris crepitans* frogs, while all samples in May and June (N=101) remained negative.

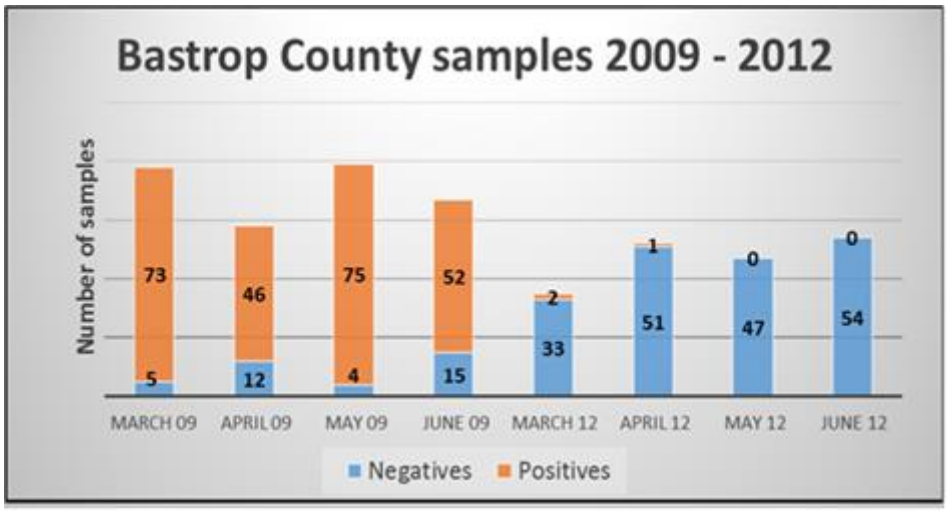


Figure 6: Bastrop County positive and negative samples collected during the spring in 2009 (Gaertner *et al.* 2012) and 2012.

To further explore the weather factors that affect the presence of *B. dendrobatidis* in this population, temperature and precipitation measurements from the years 2008-2012 were taken from the Elgin weather station – 412820, and an analysis of variance was used to account for differences between years. Figure 7 shows no significant differences among the monthly temperature means across the 5 years with a p-value of 0.965, df= 11.

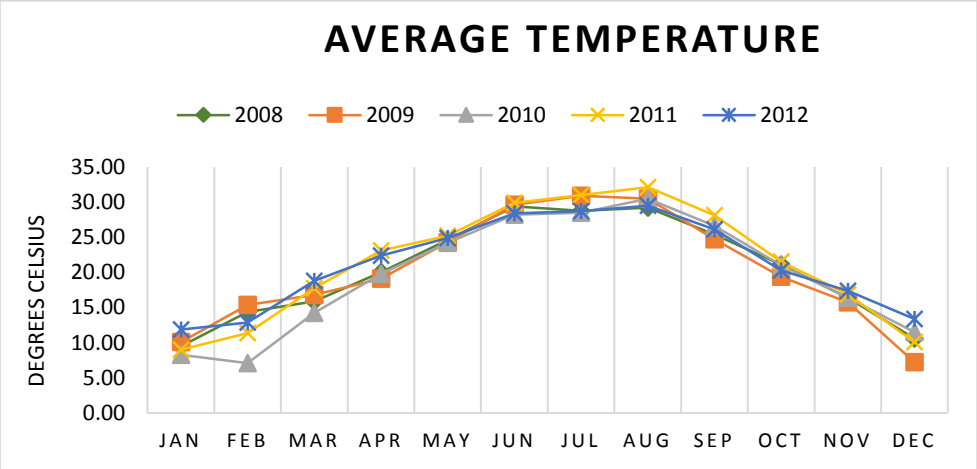


Figure 7: Monthly temperature means from 2008 to 2012, in Bastrop County, TX.

The same analysis of variance was determined among the monthly precipitation data for the years 2008 to 2012 (Figure 8) which also shows no significant difference, with a p-value of 0.608 df = 11.

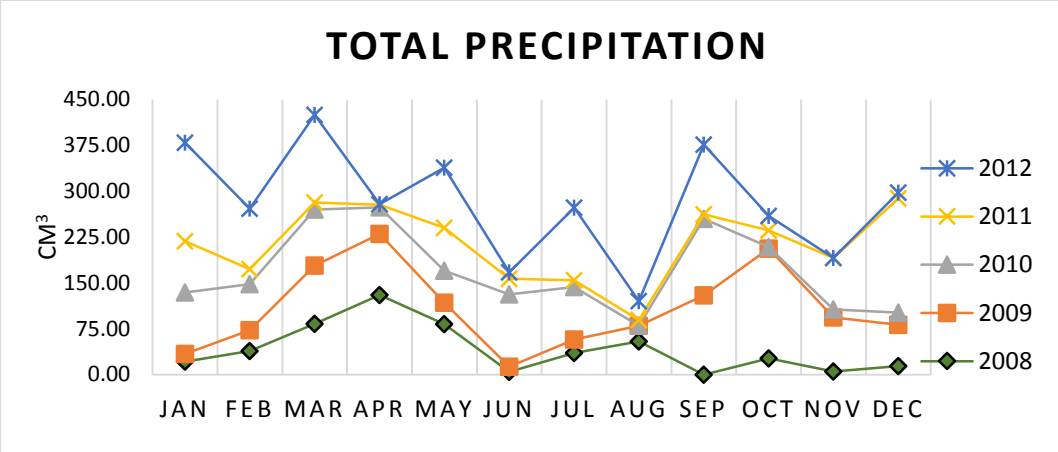


Figure 8: Monthly Precipitation from 2008 to 2012, in Bastrop County, TX.

A paired t-test was conducted to compare the average precipitation of each month for the years when the sampling was done (2009 and 2012) where the p-value = 0.9569 showed that there were no significant differences between those two years.

*Comal County*

In 2010 in the city of New Braunfels a mortality event of 20 leopard frogs (*Rana berlandieri*) occurred. It was speculated to be due to a *B. dendrobatidis* outbreak. Four individuals, which were not decomposed and remained in suitable condition to be analyzed for the presence of *B. dendrobatidis* were all found to be positive for the organism. There was no record of the presence or absence of *B. dendrobatidis* in this County prior to this detection. , However, subsequent sampling, alongside anecdotal and incidental evidence from the site were not sufficient to enable a clear answer regarding whether this particular amphibian mass mortality event was a disease outbreak, or resulted from other causative factors. It is apparent that the organism is present in this

County due to the positive samples that were obtained from the mortality event. It remains unclear if this particular mortality event was caused by *B. dendrobatidis.*

One hundred and twenty two samples were collected in Comal County during the spring of 2012 (Table 2) as a representative of the amphibian populations that were close to the site where the mortality event occurred. There were no *Rana berlandieri* individuals present in 2012 at any of the sites surveyed, including the original site of the 2010 mortality event. During the sampling time the prevalence of the pathogen was found to be the highest in March with a 3.7% of prevalence (N= 2 *A. crepitans*), as compare to April and May where the prevalence of the pathogen was of 1.69 % (N= 1 *A. crepitans*), and 0.00% respectably as shown in figure 8.

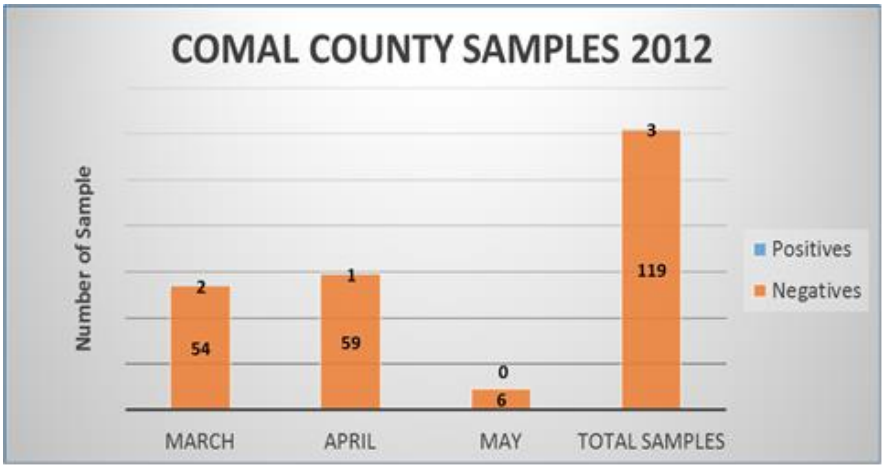


Figure 9: Number of positive and negative samples from Comal County in 2012.

Considering the low prevalence of the disease it was presumed that a variation in temperature or precipitation could be a possible explanation for either an outbreak of *B. dendrobatidis* or serve as another cause of death that could have caused the 2010 mortalities. A pair t-test was conducted in order to compare precipitation and temperature

between the years of 2010 and 2012 but both results showed no significant differences with p-values of 0.661 and 0.636 respectably (Figures 10, 11).

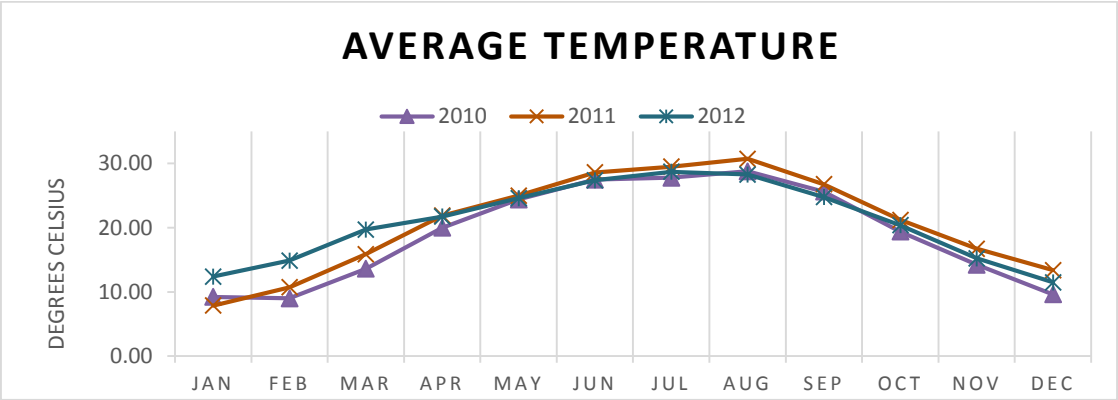


Figure 10: Monthly temperature means from 2010 to 2012, in Comal County, TX.

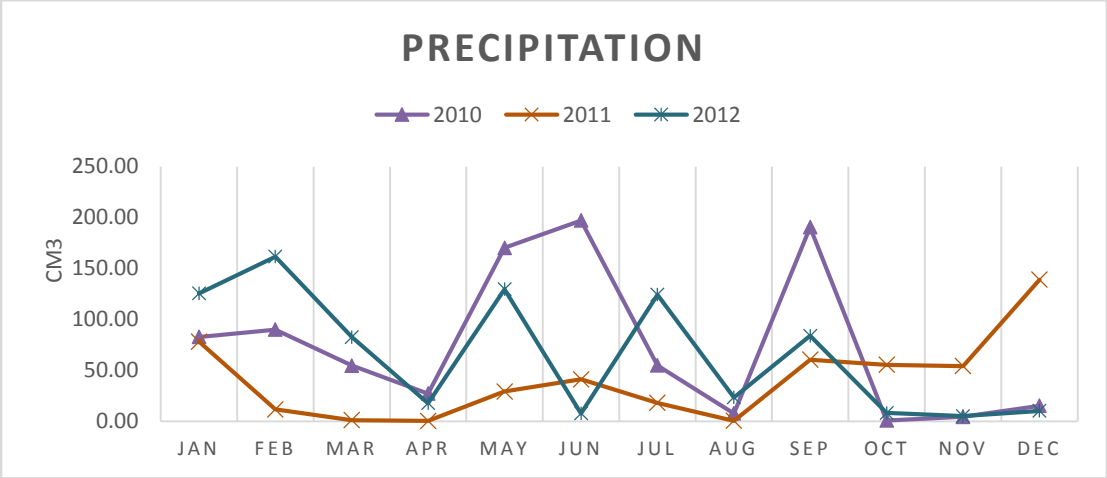


Figure 11: Monthly precipitation from 2008 to 2012, in Bastrop County, TX.

The results also show that for the 2012 sampling, both counties, Comal and Bastrop, have the presence of the pathogen during the months of March and April but during the sampling of May and June, all the samples collected where negative. This may be due to temperature variation between those months and that it might be too high for *B. dendrobatidis* to develop (Gaertner *et al*. 2012a, 2012b, 2009; Voyles *et al.* 2012)

*Tamaulipas, Mexico*

There have been no published evaluations of *B. dendrobatidis* in northern Mexico as yet, and the 56 historically collected samples were negative for *B. dendrobatidis* among the population of amphibians in Tamaulipas, Mexico. In order to evaluate if temperature was a factor that negatively impacted the pathogen presence during the time when samples were collected, temperature data from 2007 was obtained. During this period the temperature was higher on average during the months of collection for the elevations from 0 – 1500 m above sea level than what it is reported as optimal for *B. dendrobatidis* growth (Piotrowski *et al.* 2004). However, the temperature was between 5°C and 20°C for elevations above 1500m, well within the optimum temperature range for the fungus (Figure 12)

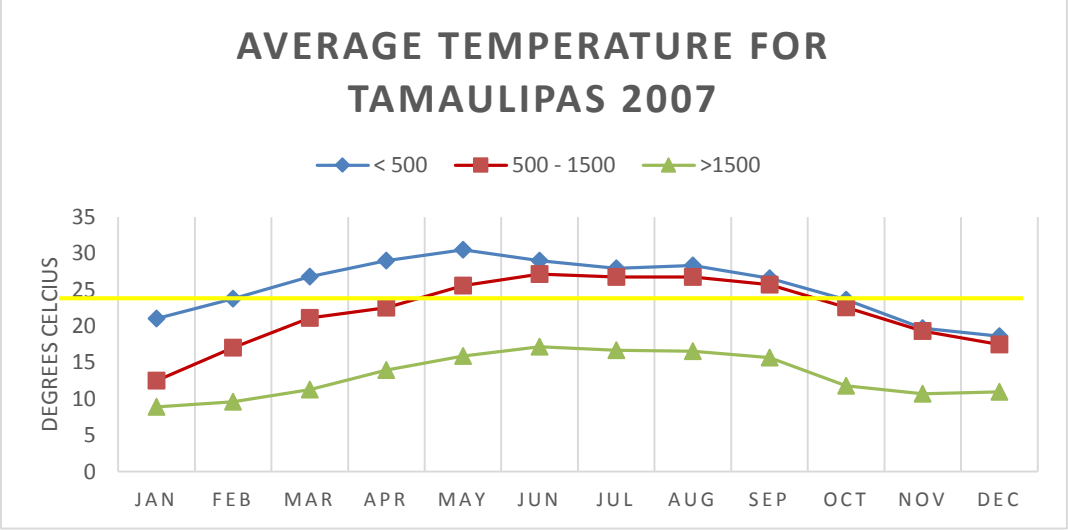


Figure 12: Average monthly temperatures at 3 different elevations during the year 2007 from Tamaulipas, Mexico.

# CHAPTER IV

## Discussion

In comparison with the amphibian declines that are positively linked to *B. dendrobatidis* infection in South and Central America (Pounds *et al*. 1997, Campbell 1998, Lips 1998, McCranie & Wilson 2002, Lips *et al*. 2003), North American amphibian communities seem to be less susceptible to the disease (Olson *et al.* 2013). This occurrence could be due to seasonal climate variation, particularly temperature and humidity. It may also be a consequence of species richness and the complexity of amphibian communities in contrast from northern to southern increases in species richness. The possibility of infection increases with species richness and the complexity of the ecosystem, where multiple different species can act as vectors or passive carriers (Olson *et al.* 2013). Also, humid zones with average temperatures between 15 - 25°C are more likely to maintain the disease due to the specific pathophysiology of *B. dendrobatidis* where most of the infection takes place in water (Berger *et al*. 2005). Despite the spring and autumn season in Central Texas providing temperatures within the optimal rage for the growth of *B. dendrobatidis*, seasonal variation above this range or below it, especially during the summer and winter affects the prevalence of the disease (Olson *et al.* 2013).

In 2009 the prevalence of *B. dendrobatidis* in Bastrop County was in average 89% (Gaertner *et al*. 2012). During the 2012 analysis the prevalence reported was of 0.55%. Giving that there was no significant variation in temperature and precipitation, which are the two common weather constants that can affect the presence of the pathogen, it was

assumed that there were other causes that could cause such a dramatic decline (Figure 6). On September 4th, 2011 a high-intensity wildfire began in Bastrop County as a result of strong winds caused by the tropical storm "Lee" and extreme drought conditions that occurred during the months previous to the fire. After being mostly contained in late September, and finally extinguished on October 29, the fire had burned a total area of 13,406 ha which represents 39% of the Lost Pines ecoregion (Brown *et al.* 2013a). The drought and high temperatures that lead to the 2011 wildfire may have been the cause for such low prevalence of *B. dendrobatidis* in Bastrop County by directly affecting the fungus on the environment by killing all the zoospores, as well as affecting the dispersion and numbers in the amphibian communities that were in the area (Brown *et al.* 2012, 2013a).

Animals that are affected by the disease usually have clinical signs that vary from ataxia, lethargy and skin lesions (Berger *et al*. 2005) that would have made it less likely for them to escape a fire with that magnitude, which also may result in the low prevalence of the disease due to the fact that there are less infected animals that can spread the disease post-fire. After the fire, the habitat suffered different changes that may also contribute to the low prevalence of the disease such as near complete canopy loss that not only increases the temperature in exposed areas but also increases the probability of exposure to UV-B radiation. This increase to UV-B has been reported as a cause of amphibian declines and may also affect the presences of the disease by affecting the fungus itself (Braustein *et al.* 1994, Alton and Franklin. 2012). The loss of the breading ponds (Figure 13) or changes on water quality of the ponds could also be a contributing factor. It is possible that the abundance of the amphibian species present may have

varied (Brown *et al.* 2012, 2013a, 2013c), favoring the ones that are less susceptible to *B. dendrobatidis* infections or less likely to disperse widely, which could also be another cause for the decline of the pathogen after the fire.

Figure 13: Pond #9, a breeding pond in GLR, Bastrop County: a) year 2012. 1 year before the fire, b) in year 2011, one month after the fire, and c) in year 2012, 1 year after the fire.

Bastrop County houses the largest population of the endangered toad *Bufo (Anaxyrus) houstonensis,* which was listed as federally endangered in the 1970s, and the populations have been declining since then. Environmental factors, habitat reduction and invasive species are among possible causes of the declines (Brown *et al.* 2012, 2013a, 2013b, 2013c). *B. dendrobatidis* could also be a contributing factor. There is not yet enough information of the behavior or occurrence of *B. dendrobatidis* in Bastrop County to make definitive statements about the effects of this pathogen. The earliest studies that have been conducted are from 2009 (Gaertner *et al.* 2009, 2012) and the results that are obtained now are going to be affected by the changes resulting from the wildfire. I think it is important to repeat the studies made in 2009 with cricket frogs (*Acris crepitans*) to have a more explicit replication that could evaluate the occurrence, prevalence, and species host diversity of the pathogen after the fire. Future monitoring that seeks to track the presence of *B. dendrobatidis* in other species that have not been part of any previous

studies and are known to be affected by the disease such as *Bufo* sp., and *Rana* sp. is an obvious and important future project. If such analyses include annual evaluations it would help to evaluate the time of recovery for the disease after a natural disaster such as a wild fire and it would also help create management plans that might help reduce the widespread of the disease using prescribed fires as a landscape level approach to control.

In Comal County the first report of *B. dendrobatidis* was related to a mortality event in a residential area in 2010, where 20 amphibians of 2 different species; 16 *Bufo nebulifer* and 4 *Rana berlandieri* were found dead. The 4 *R. berlandieri* were in good enough condition to be tested for chytridiomycosis and were positive to *B. dendrobatidis*. The remaining 16 found dead *B. nebulifer* were judged to be too decomposed, or skeletal for such analyses. The study conducted in 2012 was assessing the prevalence of the disease in the surrounding areas where the previous event happened, and if it was possible to relate the presence of the disease with the mortality event from 2010. The amphibians collected in 2012 (table # 2) show a low prevalence of the pathogen, and no *R. berlandieri* were found at this sites. It is possible that *B. dendrobatidis* killed all the leopard frogs and that is why there was no subsequent evidence of the species occurring at this site. This is a normal prediction of an SIR graph (Figure 14), where an outbreak of a pathogen leads to an epizootic peak in species that are highly susceptible. In a period of time the disease kills all the susceptible animals, leading to the extinction of this population. Which could be the case of *Rana berlandieri* in Comal County.

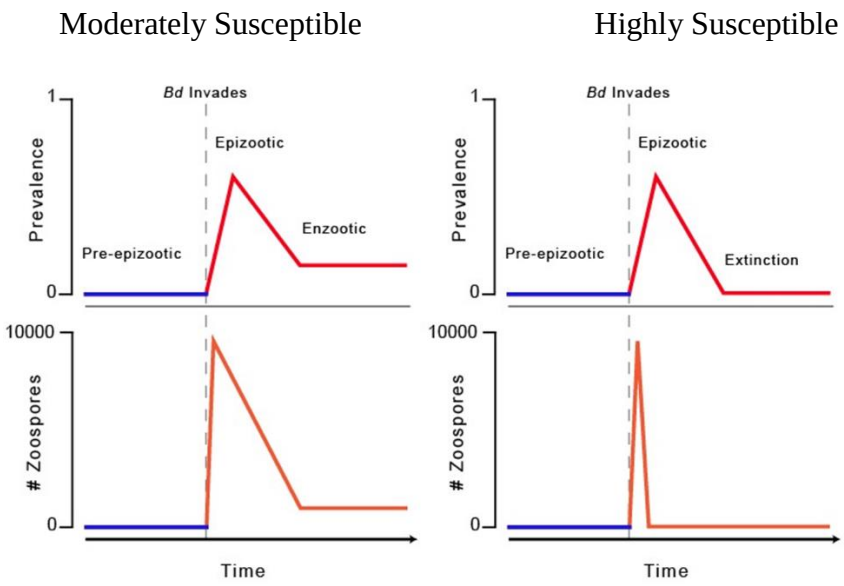


Figure 14: Graph of a *B. dendrobatidis* SRI outbreak in Moderately and Highly susceptible species. In relation with prevalence of the disease and number of zoospores.

The fact that there were no *R. berlandieri* in 2012 is not solid evidence that it was a *B. dendrobatidis* outbreak. From the 122 samples analyzed in 2012, 33 were *Bufo nebulifer*, the other species present in the mortality event from 2010, and all of the samples tested for this taxon, in the years following the 2010 mortality event were negative for the disease, which does not follow the predicted trend of an EID.

Unfortunately there are many different parameters that could have caused the mortality event that were not taken into consideration such as; temperature, water quality, presence of toxic chemicals or any other anthropogenic contamination (Figure 15), and other diseases like *Ranavirus*. In order to conduct a full epidemiological assessment all the possible causes of death should be analyzed, which was not the case in the 2010

event. All that I can conclude from our study in Comal County is that *B. dendrobatidis* is present in a low percentage of the population (Figure 9), and that the evidence for the mortality event in 2010 is inconclusive to be cataloged as a *B. dendrobatidis* outbreak.

The dynamics of *B. dendrobatidis* with respect to temperature, humidity and population numbers has not been extensively evaluated, but it has been established that there is an interaction among these factors (Olsen *et al.* 2013). The sampling sites in Comal County are located in an urban and semi-urban area with low density of amphibian communities and water sources that dry out during the dry seasons of the year. These conditions reduce the probability of transmission and infection just because of the small richness of species, and because the physiology of the disease prevents its development in elevated temperatures. This could then have been exacerbated by the lack of available surface water for both amphibians and the pathogen as was the case during the drought of 2011 (Figures 10 and 11).

Due to the low occurrence shown in this studied, it is not possible to imply that *B. dendrobatidis* was the cause of death and that indeed it was an outbreak of the disease. However this is the first time an assessment of this pathogen has been done in Comal County and given its proximity to other areas that are known as prevalent for the disease it would be a wise suggestion to continue sampling amphibians to obtain data that might show the cyclical occurrence of the pathogen throughout Central Texas. Since there is a low prevalence of the disease (Figure 9) in an urban area I would also suggest sampling in rural areas to assess differences of occurrence in areas that are not heavily impacted by human populations where the species richness is significantly lower.

There have been several reports (Pounds *et al*. 1997, Campbell 1998, Lips 1998, McCranie & Wilson 2002, Lips *et al*. 2003) that investigate the "spread" of the disease southward out of Central America and into South America. There have not been complementary investigations that seek to track the pathogen northward out of Mexico if that is still possible. Surveys in Mexico conducted so far consist mostly of species abundance in the southern regions where *B. dendrobatidis* has been found as a possible cause for populations declines (Lips *et al*. 2004). This investigation of historical samples from Northern Mexico show no positive samples for *B. dendrobatidis.* One of the possibilities for these results is that the samples were collected in months when the temperature was higher than the optimal temperature for *B. dendrobatidis* to developed (Figure 12).

According to Olsen *et al.* (2013), altitude is not considered as a critical factor in the growth and spread of *B. dendrobatidis* if factors such as temperature, precipitation and species richness are favorable. To my knowledge that one reference is the only paper that includes altitude into the model and found it to be non-significant. In this study it is not possible to determine if altitude has significant effect on infection variation, but it is possible to infer if temperature variation has an effect in the absence of the pathogen. Figure 12 shows the temperatures from 2007, previous climatological information is not complete in the three weather stations that were used from the INIFAP website. The average temperatures during the sampling periods were higher than what it is reported as optimal for *B. dendrobatidis* growth and development (Piotrowski *et al.* 2004) for the altitudes that range between 0 up to 1500 m, which could be one of the reasons why the samples are not positive for *B. dendrobatidis*. However that does not explains why at

altitudes above 1500 where the temperatures are between the optimal we still find no positive samples. The small sample size might be a factor affecting the results but if the prevalence of the disease from the closest adjacent studies is used as a guidance, in that there is an 84 % prevalence of the disease in South Mexico (Frias- Alvarez *et al.* 2008) then from 56 samples collected in Tamaulipas State at least 48 of them (84%) should be positive. There is also another study done in North-west Mexico where the prevalence of *B. dendrobatidis* was of 60 %. If this prevalence is extrapolated to our results then at least 34 samples should be positive. Further sampling with larger number of animals needs to be done to assess if Tamaulipas is free of *B. dendrobatidis,* also the sampling months shouls account for seasonal variation and elevation due to the fact that temperature as well as elevation are contributing factors that might help prevent the widespread of the disease, abundance studies and species richness are also on the parameters to take into account to make an assessment of possible declines on the amphibian population..

Research on *B. dendrobatidis* is relatively new and there is still much to be done. The most common hypothesis is that *B. dendrobatidis* moved among countries via international trade, originating from Africa (Daszak *et al.* 2003, Mazzoni *et al.* 2003, Pasmans *et al.* 2004) and spreading as an epidemic wave (Laurance *et al.* 1996, 1997). The occurrence of *B. dendrobatidis* in amphibian communities from scattered areas worldwide indicates an epidemic that spreads amazingly fast, unless the organism has long been enzootic and previously unrecognized. Whether it is an enzootic organism or an epidemic, detecting and monitoring the presence of *B. dendrobatidis* is a helpful diagnostic tool when a mortality event occurs.

## APPENDIX

Table 1: Samples obtained from Bastrop County in 2012 (N=188).

| Species | # of Samples | Date | qPCR (Bd) |
|---|---|---|---|
| *Bufo nebulifer* | 86 (toe) | Mar – Jun 2012 | (-) |
| *Acris crepitans* | 8 (toe) | Mar – Jun 2012 | (3 +) |
| *Bufo houstonensis* | 6 (toe) | Mar – Jun 2012 | (-) |
| *Scaphiophus hurteri* | 82 (toe) | Mar – Jun 2012 | (-) |

Table 2: Samples obtained from Comal County in 2012 (N=122).

| Species | # of Samples | Date | qPCR (Bd) |
|---|---|---|---|
| *Bufo nebulifer* | 33 (toe) | Mar – May 2012 | (-) |
| *Acris crepitans* | 89 (toe) | Mar – May 2012 | (3 +) |

Table 3: Fifty six historical samples obtained from Tamaulipas State from 2004 - 2008.

| Species | # of Samples | Date | Elevation (m.) | qPCR (Bd) |
|---|---|---|---|---|
| *Rana catesbeiana* | 1 (toe) | 17-Oct-07 | 29.5 | (-) |
| *Rana berlandieri* | 1 (toe) | 11-Aug-07 | 75.6 | (-) |
| *Trachycephalus typhonius* | 1 (toe) | 28-May-05 | 89 | (-) |
| *Smilisca baudinii* | 1 (toe) | 24-May-05 | 102.3 | (-) |
| *Gastrophryne olivacea* | 3 (toes) | 18-Sep-06 | 127.3 | (-) |
| *Smilisca baudinii* | 1 (toe) | 18-Sep-06 | 127.3 | (-) |
| *Scinax staufferi* | 3 (toes) | 18-Sep-06 | 127.3 | (-) |
| *Smilisca baudinii* | 1 (toe) | 28-May-05 | 136 | (-) |
| *Bufo debilis* | 5 (toes) | 18-Sep-06 | 138 | (-) |
| *Scinax staufferi* | 1 (toe) | 30-May-05 | 242 | (-) |
| *Smilisca baudinii* | 1 (toe) | 30-May-05 | 242 | (-) |
| *Leptodactylus melanonotus* | 1 (toe) | 22-Sep-06 | 347 | (-) |
| *Smilisca baudinii* | 1 (swab) | 13-Jul-07 | 501 | (-) |
| *Rana berlandieri* | 1 (swab) | 11-Jul-07 | 510 | (-) |
| *Bufo nebulifer* | 1 (swab) | 10-Jul-07 | 588 | (-) |
| *Hypopachus variolosus* | 1 (swab) | 16-Oct-07 | 710 | (-) |
| *Syrrhophus cystignathoides* | 1 (swab) | 13-Jul-07 | 731 | (-) |
| *Syrrhophus cystignathoides* | 1 (toe) | 17-Sep-06 | 832 | (-) |
| *Bufo marina* | 1 (swab) | 16-Oct-07 | 832 | (-) |

Table 3, continued

| | | | | |
|---|---|---|---|---|
| *Eleutherodactylus augusti* | 1 (toe) | 17-Sep-06 | 849 | (-) |
| *Rana berlandieri* | 1 (swab) | 16-Oct-07 | 886 | (-) |
| *Bufo nebulifer* | 1 (swab) | 20-Aug-07 | 895 | (-) |
| *Smilisca baudinii* | 1 (swab) | 19-Aug-07 | 951 | (-) |
| *Syrrhophus sp.* | 2 (toes) | 21-Sep-06 | 952 | (-) |
| *Syrrhophus sp.* | 1 (swab) | 16-Oct-07 | 952 | (-) |
| *Syrrhophus cystignathoides* | 1 (swab) | 19-Aug-07 | 1004 | (-) |
| *Bufo nebulifer* | 1 (swab) | 6-Oct-08 | 1079 | (-) |
| *Bufo cognatus* | 2 (toes) | 12-Oct-07 | 1060-1064 | (-) |
| *Rana berlandieri* | 2 (swabs) | 6-Oct-08 | 1073-1089 | (-) |
| *Eleutherodactylus augusti* | 2 (toes) | 25-Sep-06 | 1203-1211 | (-) |
| *Syrrhophus sp.* | 1 (toe) | 25-Sep-06 | 1211 | (-) |
| *Smilisca baudinii* | 1 (toe) | 25-Sep-06 | 1211 | (-) |
| *Syrrhophus cystignathoides* | 1 (toe) | 26-May-05 | 1233 | (-) |
| *Bufo nebulifer* | 1 (toe) | 13-Oct-06 | 1245 | (-) |
| *Bufo marina* | 1 (toe) | 13-Oct-06 | 1245 | (-) |
| *Hyla miotympanum* | 2 (toes) | 26-May-05 | 1380 | (-) |
| *Syrrhophus cystignathoides* | 1 (toe) | 24-May-05 | 1327 | (-) |
| *Rana berlandieri* | 1 (toe) | 10-Jul-04 | 1544 | (-) |
| *Hyla eximia* | 4 (toes) | 27-May-05 | 2053 | (-) |
| *Spea multiplicata* | 1 (swab) | 18-Sep-07 | 2595 | (-) |

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