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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 (e.g., Bradley et al. 2002; Schlaepfer et al. 2007; Woodhams et al. 2008; Pearl et al. 2009; Chatfield et al. 2012), which can be devastating when a species is susceptible to the disease caused by *Bd*, chytridiomycosis (Rachowicz et al. 2006; Murray et al. 2009; Pilliod et al. 2010). 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 catesbeianus*) are often positive for *Bd*, although some species usually do not exhibit clinical signs of the disease (Garner et al. 2006; Steiner and Lehtinen 2008; Pearl et al. 2009). *Bd*-reservoir species, in this regard, may lead to patterns of higher *Bd* occurrences at sites with increasing species richness (Olson et al. 2013), 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 treefrogs, the Green Treefrog (*Hyla cinerea*) and Gray Treefrog (*H. 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 hylids appears to be relatively low, although *Bd* has been detected in several species. For example, Rizkalla (2010) found no *Bd* among three *Hyla* species in Florida, and studies conducted in Wisconsin, Minnesota, East Texas, Louisiana, and Georgia found that all *Hyla* samples were negative for *Bd* (Timpe et al. 2008; Rodriguez et al. 2009; Sadinski et al. 2010; Saenz et al. 2010; Brannelly et al. 2012). However, *Bd*-positive individuals have been detected in the Canyon Treefrrog (*H. arenicolor*), Gray Treefrog, Cope's Gray Treefrog (*H. chrysoscelis*), and Pacific Treefrog (*P. regilla*) (Bradley et al. 2002; Fellers et al. 2011; Muelleman and Montgomery 2013). 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

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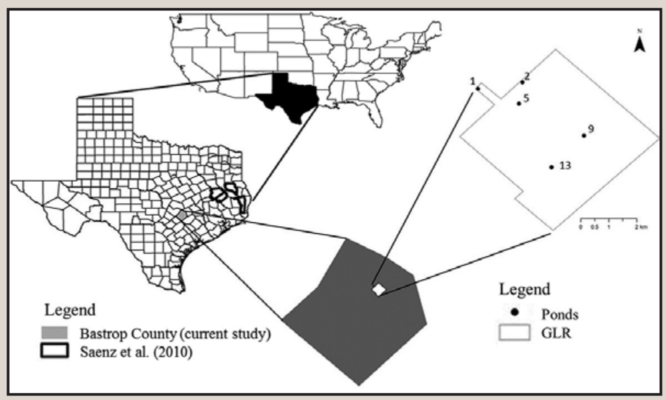

Fig. 1. Map showing USA with Texas highlighted in black (top) and more specifically the map of Texas with outlined counties (bottom left). Current study was conducted at Griffith League Ranch (GLR) in Bastrop County (highlighted in gray) and four additional counties outlined in bold represent counties surveyed by Saenz et al. (2010), the only other study that assessed *Batrachochytrium dendrobatidis* (*Bd*) in treefrogs in Texas. Out of 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.

Table 1. Green and Gray Treefrogs (*Hyla cinerea* and *H. 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 2014	2015	Prevalence (%)
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

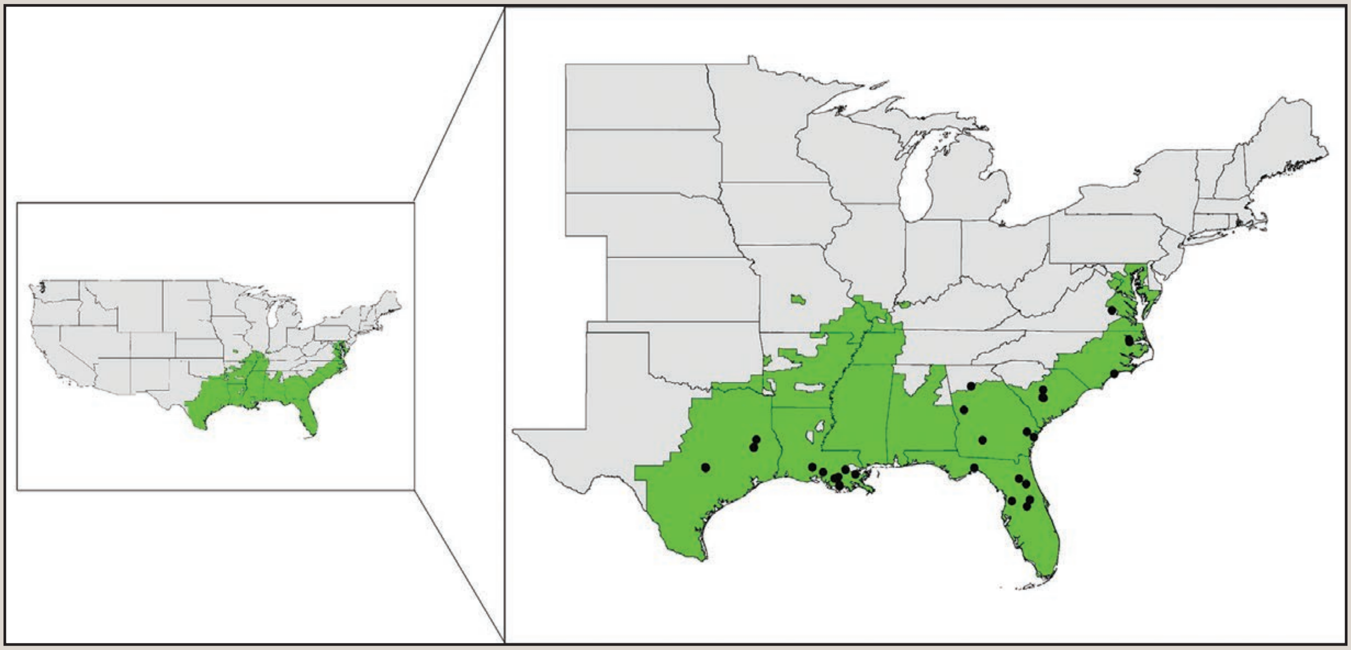

FIG. 2. United States map outlining the Green Treefrog (*Hyla cinerea*) distribution (green). Circles represent the regions where treefrogs were surveyed for *Batrachochytrium dendrobatidis* (*Bd*) and found to be *Bd*-negative. To date, no Green Treefrogs have been found to be *Bd*-positive.

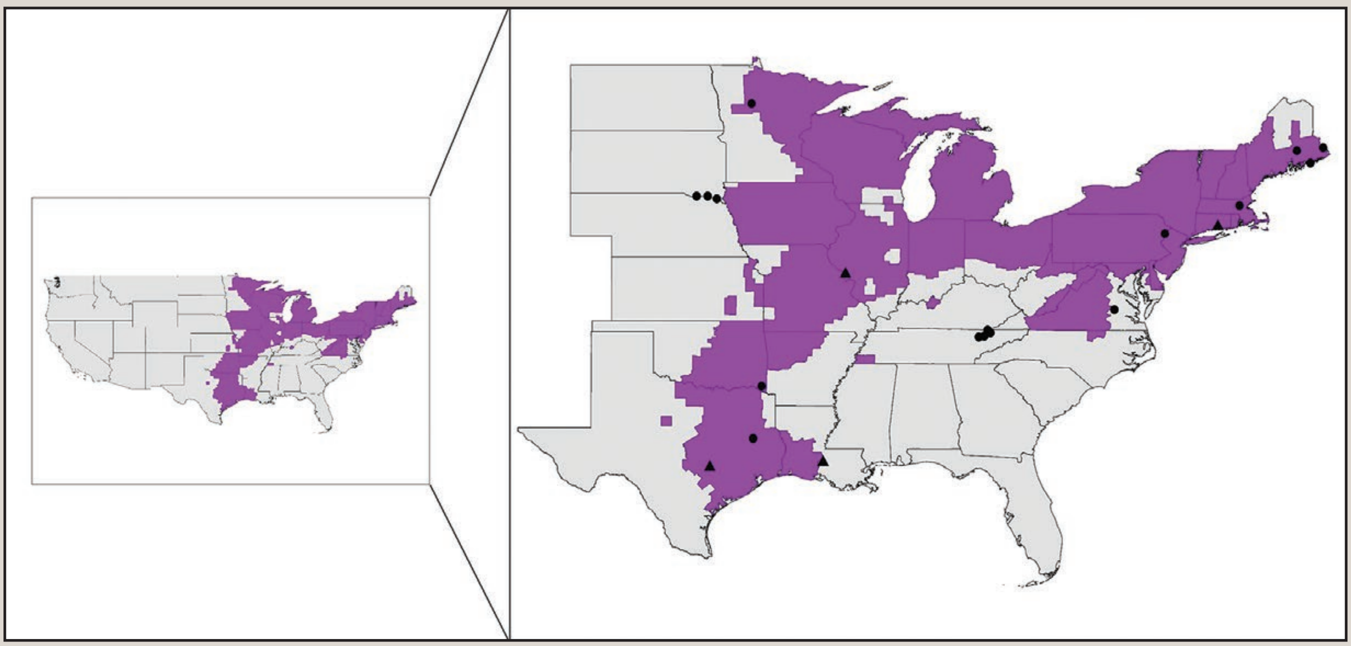

FIG. 3. United States map outlining the Gray Treefrogs (*Hyla versicolor*) distribution (purple). Circles represent the regions where treefrogs were surveyed for *Batrachochytrium dendrobatidis* (*Bd*) and found to be *Bd*-negative. Triangles represent the regions in which the frogs were found to be *Bd*-positive.

small, ranging from 1 to 42 (mean = ~18), with the exception of Brannelly et al. (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 *Bd* occurrence has not been widely studied, with the exception of studies in 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% *Bd* occurrence whereas Villamizar-Gomez (2013) tested the Houston Toad, Gulf Coast Toad, Blanchard's Cricket

Table 2. Studies that assessed *Batrachochytrium dendrobatidis* (*Bd*) prevalence in Green and Gray Treefrogs (*Hyla cinerea* and *H. 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.net (accessed 25 May, 2016); Rothermel et al. (2008)
South Carolina	*H. cinerea*	-	Daszak et al. (2005); www.Bd-maps.net (accessed 25 May, 2016); 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.net (accessed 25 May, 2016)
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)

Frog, and Hurter's Spadefoot (*Scaphiopus hurteri*) in the same region, and found 3% overall *Bd* 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 traditional 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 five standards 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 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 1). Positive samples were found in ponds 1, 2, and 5 (Fig. 1). Our literature review (Table 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), but was detected in 4 of 12 states (Fig. 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, temperatures, and precipitation can have a great influence on *Bd* occurrence among Anurans (Retallick et al. 2004; Pullen et al. 2010; Sapsford et al. 2013; Xie et al. 2016). 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. 2005). 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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