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Screening Natural History Collections for Historical Presence of *Batrachochytrium dendrobatidis* in Anurans from Oklahoma, USA

In North America, *Batrachochytrium dendrobatidis* (*Bd*) has been present for at least the last half-century (Ouellet et al. 2005); however, infectious amphibian diseases have remained poorly studied through most of the Great Plains of the United States (Steiner and Lehtinen 2008; Lannoo et al. 2011). In Oklahoma, *Bd* has been documented in wild populations only three times, with four counties surveyed (Steiner and Lehtinen 2008; Lannoo et al. 2011; Bd-maps 2015). It is imperative that a greater portion of the state be surveyed to develop a baseline understanding of the distribution and status of *Bd* statewide. A large-scale, statewide *Bd* survey project has been funded by the Oklahoma Department of Wildlife Conservation (ODWC) to determine the current distribution and prevalence of *Bd* in amphibians in Wildlife Management Areas (WMAs). To complement the project, we began sampling for the historical presence of *Bd* using museum specimens to provide a baseline for *Bd* presence in the state. We expected that *Bd* would be present and widespread throughout Oklahoma.

A total of 469 adult or juvenile (non-larval) anuran formalin-fixed specimens were swabbed using sterile, individually packaged, large rayon-tipped swabs (Puritan Medical Products), following methods described by Lannoo et al. (2011). After sampling, the swab tips were placed in sterile 1.5-mL microcentrifuge tubes with 2–3 drops of 70% ethanol and stored in a -20°C freezer. The swabs were left out overnight in a laboratory fume hood to remove ethanol prior to DNA extraction. Two DNA extraction protocols were used: PrepMan Ultra (4 samples) and Qiagen DNeasy Blood & Tissue Kit (384 samples). Both methods have been used successfully to test for *Bd* in museum specimens (Cheng et al. 2011). Prior to analysis, samples extracted following the PrepMan Ultra extraction protocol were diluted 1:10 to reduce potential inhibition during quantitative polymerase chain reaction (qPCR) analysis. Following the fast qPCR methods described by Kerby et al. (2013), we used 10µL reactions (3µL DNA extract plus 7µL cocktail) to determine if samples were positive (*Bd*+) or negative (*Bd*-). Additionally, each plate contained a negative control (nanopure water) and four sDNA standards. Samples were run on a StepOnePlus qPCR machine and the number of *Bd* gene copies was quantified with StepOne software v2.3 (Applied Biosystems). All samples were run in triplicate and considered positive if: 1) amplification occurred in at least two of the three wells and 2) the quantity was above 1.0. Samples were rerun if there were two wells with quantities near 1.0, or if sample values differed by an order of magnitude. All qPCR analyses were conducted at the Disease Testing Center at the University of South Dakota.

The anuran specimens used in this study were collected between 1924 and 2014, and were swabbed for *Bd* between

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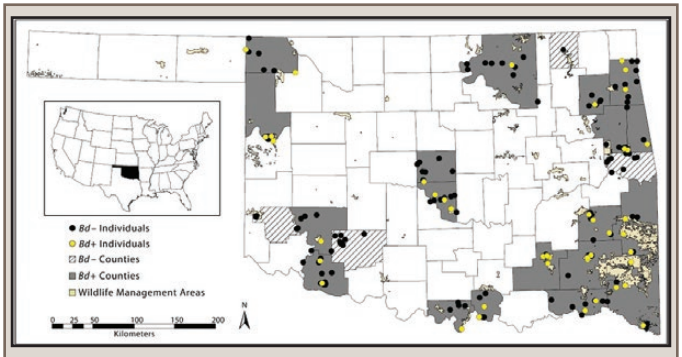

FIG. 1. Map of Oklahoma, USA showing *Batrachochytrium dendrobatidis* (*Bd*) sampled localities, counties, and Wildlife Management Areas (WMAs). Localities that yielded *Bd*+ (black dot) and *Bd*- (white dot) individuals, *Bd*+ counties (grey), *Bd*- counties (hatched), and WMAs (green) are all presented.

TABLE 1. List of amphibian species swabbed for *Batrachochytrium dendrobatidis* (*Bd*) by county (ecoregion) in Oklahoma, USA. Ecoregions are coded by the following: 1) Crosstimbers, 2) Mixed-grass, 3) Ouachita Mountains/Arkansas Valley/Western Gulf Coastal Plain, 4) Ozark, 5) Short-grass Prairie, 6) Tall-grass Prairie. Total sample size (N), number of Bd+ specimens (% prevalence), and mean *Bd* gene copies per sample (± 1 SD) are indicated.

County/Species (Ecoregion)	N	*Bd*+ (% Prevalence)	Mean *Bd* Gene Copies/Sample
Adair County (4)	14	7 (50%)	201.65 (± 267.39)
Acris blanchardi	2	1	
Hyla chrysoscelis/ versicolor	8	4	
Pseudacris crucifer	2	0	
Pseudacris fouquettei	2	2	
Atoka County (1,3)	31	14 (45%)	1374.00 (± 2603.63)
Acris blanchardi	7	3	
Hyla chrysoscelis/ versicolor	7	0	
Pseudacris fouquettei	9	8	
Pseudacris streckeri	1	1	
Gastrophryne olivacea	2	2	
Lithobates areolatus	2	0	
Lithobates sphenocephalus	3	0	
Cherokee County (4)	45	2 (4%)	112.25 (± 117.51)
Hyla cinerea	1	0	
Hyla chrysoscelis/ versicolor	15	0	
Pseudacris crucifer	23	2	
Gastrophryne carolinensis	1	0	
Gastrophryne olivacea	1	0	
Lithobates clamitans	3	0	
Lithobates sphenocephalus	1	0	
Choctaw County (1,3)	17	3 (18%)	8.66 (± 3.87)
Anaxyrus americanus	2	0	
Anaxyrus woodhousii	2	0	
Acris blanchardi	2	0	
Hyla cinerea	2	1	
Pseudacris streckeri	2	0	
Gastrophryne carolinensis	2	0	
Lithobates areolatus	2	2	
Lithobates catesbeianus	2	0	
Lithobates clamitans	1	0	
Cleveland County (1,2)	20	7 (35%)	55980.54 (± 144924.06)
Acris blanchardi	6	3	
Pseudacris streckeri	2	2	
Gastrophryne olivacea	2	0	
Lithobates blairi	2	0	
Lithobates catesbeianus	2	0	
Lithobates sphenocephalus	2	2	
Scaphiopus hurterii	2	0	
Spea bombifrons	2	0	
Comanche County (1,2)	27	0	0 (N/A)
Anaxyrus cognatus	1	0	
Anaxyrus punctatus	1	0	
Anaxyrus woodhousii	2	0	
Acris blanchardi	7	0	
Pseudacris clarkii	2	0	
Pseudacris streckeri	3	0	
Lithobates blairi	3	0	
Lithobates catesbeianus	3	0	
Lithobates sphenocephalus	3	0	
Scaphiopus couchii	2	0	

TABLE 1. Continued.

County/Species (Ecoregion)	N	*Bd*+ (% Prevalence)	Mean *Bd* Gene Copies/Sample
Delaware County (4,6)	21	4 (19%)	11.64 (± 27.41)
Anaxyrus americanus	2	0	
Acris blanchardi	2	0	
Hyla chrysoscelis/ versicolor	2	0	
Pseudacris fouquettei	2	0	
Gastrophryne carolinensis	2	1	
Gastrophryne olivacea	1	1	
Lithobates areolatus	2	0	
Lithobates catesbeianus	2	0	
Lithobates clamitans	2	0	
Lithobates palustris	2	1	
Lithobates sphenocephalus	2	1	
Ellis County (2,5)	35	5 (14%)	4300.63 (± 8687.79)
Anaxyrus woodhousii	4	0	
Acris blanchardi	7	4	
Gastrophryne olivacea	12	0	
Lithobates blairi	4	0	
Lithobates catesbeianus	4	0	
Spea bombifrons	4	1	
Greer County (2)	18	0	0 (N/A)
Anaxyrus debilis	6	0	
Anaxyrus punctatus	5	0	
Pseudacris clarkii	3	0	
Gastrophryne olivacea	2	0	
Lithobates blairi	1	0	
Spea bombifrons	1	0	
Harper County (2,5)	20	2 (10%)	115.91 (± 146.81)
Anaxyrus cognatus	4	1	
Anaxyrus woodhousii	4	0	
Acris blanchardi	2	0	
Lithobates blairi	6	1	
Lithobates catesbeianus	1	0	
Spea bombifrons	3	0	
Kiowa County (2)	18	4 (22%)	6522.19 (± 6154.54)
Anaxyrus cognatus	2	0	
Anaxyrus debilis	2	0	
Anaxyrus speciosus	2	0	
Anaxyrus woodhousii	2	0	
Acris blanchardi	3	1	
Lithobates blairi	3	3	
Lithobates catesbeianus	2	0	
Spea bombifrons	2	0	
Latimer County (3)	18	4 (22%)	65.46 (± 48.16)
Anaxyrus americanus	1	0	
Acris blanchardi	2	1	
Hyla cinerea	2	0	
Hyla chrysoscelis/ versicolor	2	1	
Gastrophryne carolinensis	2	0	
Gastrophryne olivacea	1	0	
Pseudacris fouquettei	3	0	
Lithobates catesbeianus	1	0	
Lithobates clamitans	1	0	
Lithobates palustris	2	1	
Lithobates sphenocephalus	1	1	

TABLE 1. Continued.

County/Species (Ecoregion)	N	*Bd*+ (% Prevalence)	Mean *Bd* Gene Copies/Sample
Le Flore County (3)	16	4 (25%)	272.08 (± 231.04)
Acris blanchardi	1	0	
Hyla chrysoscelis/ versicolor	4	2	
Pseudacris crucifer	1	1	
Gastrophryne carolinensis	1	0	
Lithobates areolatus	1	0	
Lithobates palustris	1	0	
Lithobates sphenocephalus	7	1	
Love County (1,2)	16	2 (13%)	341.79 (± 80.31)
Anaxyrus woodhousii	2	0	
Acris blanchardi	8	2	
Gastrophryne olivacea	2	0	
Lithobates catesbeianus	2	0	
Lithobates sphenocephalus	2	0	
Marshall County (1)	21	2 (10%)	97.76 (± 23.63)
Anaxyrus americanus	2	0	
Anaxyrus cognatus	2	0	
Anaxyrus woodhousii	2	0	
Hyla cinerea	4	1	
Pseudacris clarkii	2	1	
Pseudacris streckeri	2	0	
Gastrophryne olivacea	2	0	
Lithobates catesbeianus	1	0	
Lithobates sphenocephalus	2	0	
Spea bombifrons	2	0	
Mayes County (4,6)	14	1 (7%)	35.22 (N/A)
Anaxyrus americanus	2	0	
Anaxyrus woodhousii	1	0	
Acris blanchardi	2	0	
Hyla chrysoscelis/ versicolor	2	1	
Gastrophryne olivacea	2	0	
Lithobates catesbeianus	2	0	
Lithobates sphenocephalus	2	0	
Spea bombifrons	1	0	
McCurtain County (3)	20	9 (45%)	1820.14 (± 3213.97)
Anaxyrus americanus	5	2	
Pseudacris crucifer	2	0	
Pseudacris fouquettei	1	0	
Lithobates catesbeianus	1	0	
Lithobates palustris	1	1	
Lithobates sphenocephalus	10	6	
Nowata County (6)	1	0 (0%)	0 (N/A)
Acris blanchardi	1	0	
Oklahoma County (1,2)	21	1 (5%)	33.26 (N/A)
Anaxyrus americanus	3	0	
Anaxyrus cognatus	4	1	
Anaxyrus woodhousii	2	0	
Acris blanchardi	1	0	
Pseudacris clarkii	3	0	
Lithobates blairi	3	0	
Lithobates sphenocephalus	2	0	
Spea bombifrons	3	0	

TABLE 1. Continued.

County/Species (Ecoregion)	N	*Bd*+ (% Prevalence)	Mean *Bd* Gene Copies/Sample
Osage County (1,6)	17	1 (6%)	66.42 (N/A)
Anaxyrus cognatus	2	0	
Anaxyrus woodhousii	2	0	
Pseudacris clarkii	2	0	
Pseudacris streckeri	2	0	
Gastrophryne olivacea	2	0	
Lithobates blairi	1	0	
Lithobates catesbeianus	3	1	
Lithobates sphenocephalus	3	0	
Pushmataha County (1,3)	28	5 (18%)	6934.60 (± 9163.12)
Anaxyrus americanus	5	2	
Anaxyrus woodhousii	5	0	
Acris blanchardi	2	1	
Hyla cinerea	5	0	
Hyla chrysoscelis/ versicolor	4	0	
Pseudacris crucifer	1	1	
Gastrophryne carolinensis	4	0	
Lithobates clamitans	1	0	
Lithobates sphenocephalus	1	1	
Sequoyah County (3,4)	12	0 (0%)	0 (N/A)
Anaxyrus americanus	2	0	
Anaxyrus woodhousii	3	0	
Acris blanchardi	1	0	
Hyla cinerea	1	0	
Lithobates areolatus	1	0	
Lithobates catesbeianus	2	0	
Lithobates sphenocephalus	2	0	
Tillman County (2)	19	2 (11%)	77.93 (± 56.02)
Anaxyrus debilis	2	0	
Anaxyrus speciosus	7	0	
Anaxyrus woodhousii	4	1	
Acris blanchardi	2	1	
Gastrophryne olivacea	2	0	
Lithobates sphenocephalus	1	0	
Scaphiopus couchii	1	0	
TOTAL	477	79 (17%)	6506.99 (± 43277.97)

November 2014 and February 2015. Specimens from 22 of 31 documented Oklahoma species were swabbed from 23 of 77 counties in the state (Fig. 1). All specimens were from specific counties that have been or will be sampled for the ODWC project from 2015–2017. When possible, historic specimens collected within, or in close proximity to, WMAs in the state were chosen so that comparisons to with newly collected specimens could be made. Specimens examined came from Sam Noble Oklahoma Museum of Natural History (OMNH), Oklahoma State University Museum (OSU), and the Smithsonian National Museum of Natural History (USNM). Counties are represented by 12–45 swabbed individuals (Table 1), with the exception of Nowata County, which is represented by a single sample due to the paucity of vouchered specimens in museum collections.

The total number of amphibian specimens that tested positive for *Bd* was 79 (16.84% prevalence overall). Of the 23 sampled counties in Oklahoma, 19 counties had *Bd*+ specimens (Table 1). Counties with the highest prevalence rates (45–50%) were in the following ecoregions: a) Ouachita Mountains/Arkansas Valley/Western Gulf Coastal Plain, b) Crosstimbers, and c) Ozark (Oklahoma Department of Wildlife Conservation 2015; Table 1). The earliest *Bd*+ sample was from a specimen collected in 1926 (*Lithobates blairi*) and the most recent *Bd*+ sample was from an animal collected in 2014 (*Acris blanchardi*). The year with the highest prevalence (32.65%) was 2007. Of the 22 species swabbed, 18 had at least one individual that was *Bd*+ (Table 2). The four species of this study with no *Bd*+ specimens may be an artifact of small sample sizes (*Anaxyrus punctatus*, N = 6; *Lithobates clamitans*, N = 9; *Scaphiopus couchii*, N = 4; *S. hurterii*, N = 2; Table 2). The family Hylidae had the highest prevalence rates (24.18%) and the highest number of gene copies per sample (mean ± 1 SD: 10572.28 [± 57878.81]; Table 2).

TABLE 2. List of amphibian species swabbed for *Batrachochytrium dendrobatidis* (*Bd*) by family in Oklahoma. Total sample size (N), number of *Bd*+ specimens (% prevalence), and mean *Bd* gene copies per sample (± 1 SD) are indicated.

Family, Species	N	*Bd*+ (% Prevalence)	Mean *Bd* Gene Copies/Sample
Bufonidae	88	7 (8%)	140.39 (± 245.61)
Anaxyrus americanus	24	4 (17%)	172.86 (± 331.44)
Anaxyrus cognatus	15	2 (13%)	126.49 (± 131.85)
Anaxyrus debilis	10	0 (0%)	0 (N/A)
Anaxyrus punctatus	6	0 (0%)	0 (N/A)
Anaxyrus woodhousii	33	1 (3%)	38.32 (N/A)
Hylidae	187	44 (24%)	15072 (± 57878.812)
Acris blanchardi	58	17 (29%)	25774.07 (± 92654.75)
Hyla chrysoscelis/versicolor	44	7 (16%)	96.40 (± 193.78)
Hyla cinerea	15	2 (13%)	59.38 (± 77.91)
Pseudacris clarkii	12	1 (8%)	81.05 (N/A)
Pseudacris crucifer	29	4 (14%)	4363.01 (± 8508.89)
Pseudacris fouquettei	17	10 (59%)	758.63 (± 1266.99)
Pseudacris streckeri	12	3 (25%)	369.32 (± 389.91)
Microhylidae	43	4 (9%)	533.90 (± 1124.93)
Gastrophryne carolinensis	12	1 (8%)	17.82 (N/A)
Gastrophryne olivacea	31	3 (10%)	878.95 (± 1443.77)
Ranidae	120	22 (18%)	2054.55 (± 4206.33)
Lithobates areolatus	8	2 (25%)	10.84 (± 1.14)
Lithobates blairi	23	4 (17%)	2864.35 (± 3490.09)
Lithobates catesbeianus	31	1 (3%)	66.42 (N/A)
Lithobates clamitans	9	0 (0%)	0 (N/A)
Lithobates palustris	7	3 (43%)	357.04 (± 516.15)
Lithobates sphenocephalus	42	12 (29%)	2715.29 (± 5271.44)
Scaphiopodidae	24	1 (4%)	20.20 (N/A)
Scaphiopus couchii	4	0 (0%)	0 (N/A)
Scaphiopus hurterii	2	0 (0%)	0 (N/A)
Spea bombifrons	18	1 (6%)	20.20 (N/A)

Although these results are not exhaustive, these data indicate that as early as 1926, *Bd* was present in Oklahoma. Other recent studies of *Bd* in museum specimens have indicated that the pathogen has had a global presence much longer than most realized, but likely in a less virulent form (Ouellet et al. 2005; Shaw et al. 2013; Fong et al. 2015; Talley et al. 2015). With historical data collected from this study, we will be able to observe *Bd* prevalence within a temporal framework by comparing historic and modern specimens (or live individuals) found in the same localities. It is of particular concern that some of the highest prevalence rates were found in the Ozark and Ouachita Mountains/Arkansas Valley/Western Gulf Coastal Plain ecoregions, as these two areas are where the highest alpha diversity of amphibians in the state occurs. Additionally, these two ecoregions are home to nearly all of the amphibians currently listed as Species of Greatest Conservation Need in Oklahoma (Oklahoma Department of Wildlife Conservation 2015).

It is also important to further note that many of the *Bd*+ specimens had been stored in the same collection storage jar as other *Bd*+ specimens. It is possible that some of our results reflect a jar effect, where an infected specimen contaminated others in the same jar. At the Sam Noble Oklahoma Museum of Natural History, similar to many other natural history collections, specimens that are stored in the same jar are of the same species and were collected in the same county, but possibly at different times and/or localities. This may result in an overabundance of *Bd*+ individuals or a misrepresentation of date ranges; however, the oldest *Bd*+ specimen was alone in its jar, which lends confidence to the temporal documentation of *Bd* in the state. Other studies testing *Bd*+ historical museum specimens have also documented the potential of contamination within a single jar and taken various steps to avoid cross-contamination (Spitzen-Van der Sluijs et al. 2014; Fong et al. 2015; Talley et al. 2015). We reduced the possibility of cross-contamination at the time of swabbing, by sterilizing equipment and changing gloves between individuals. There were several jars that contained both *Bd*+ and *Bd*- specimens, from a variety of collection localities, so cross-contamination of individuals within a single jar is difficult to discern.

Studying the presence of *Bd* in museum specimens allows researchers to investigate if amphibian declines have been driven by the emergence of infectious diseases over time (Cheng et al. 2011). Museum collections are an important part of discovering origins of these diseases and comparing historic prevalence with that of recently collected specimens (Ouellet et al. 2005). This study adds evidence to our understanding of historical *Bd* distribution in the United States, provides insight on how and when this pathogen may have spread, and may help develop strategies to combat this disease (Talley et al. 2015). Contemporary surveys of amphibian infectious diseases are important

for establishing the current distribution of pathogens in a particular region, but to understand the complete story of *Bd*, it is necessary to couple modern efforts with the information from the past that historical specimens have to offer.

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Literature Cited

Bd-maps. 2015. Available at http://www.bd-maps.net (Accessed 14 October 2015).

Cheng, T. L., S. M. Rovito, D. B. Wake, and V. T. Vredenburg. 2011. Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. Proc. Nat. Acad. Sci. 108:9502–9507.

Fong, J. J., T. L. Cheng, A. Bataille, A. P. Pessier, B. Waldman, and V. T. Vredenburg. 2015. Early 1900s detection of *Batrachochytrium dendrobatidis* in Korean amphibians. PLOS ONE 10:e0115656.

Kerby, J. L., A. Schieffer, J. R. Brown, and S. Whitfield. 2013. Utilization of fast qPCR techniques to detect the amphibian chytrid fungus: a cheaper and more efficient alternative method. Methods Ecol. Evol. 4:162–166.

Lannoo, M. J., C. Petersen, R. E. Lovich, P. Nanjappa, C. Phillips, J. C. Mitchell, and I. Macallister. 2011. Do frogs get their kicks on Route 66? Continental U.S. transect reveals spatial and temporal patterns of *Batrachochytrium dendrobatidis* infection. PLOS ONE 6:e22211.

Oklahoma Department of Wildlife Conservation. 2015. Oklahoma Comprehensive Wildlife Conservation Strategy. Available at http://www.wildlifedepartment.com/CWCS.htm (Accessed 15 January 2016).

Ouellet, M., I. Mikaelian, B. D. Pauli, J. Rodrigue, and D. M. Green. 2005. Historical evidence of widespread chytrid infection in North American amphibian populations. Conserv. Biol. 19:1431–1440.

Shaw, S. D., L. F. Skerratt, A. Haigh, B. D. Bell, L. Daglish, P. J. Bishop, R. Summers, V. Moreno, S. Melzer, M. Ohmer, S. Herbert, D. Gleeson, L. Rowe, and R. Speare. 2013. The distribution and host range of *Batrachochytrium dendrobatidis* in New Zealand, 1930–2010. Ecology 94:2108–2111.

Steiner, S. L., and R. M. Lehtinen. 2008. Occurrence of the amphibian pathogen *Batrachochytrium dendrobatidis* in Blanchard's Cricket Frog (*Acris crepitans blanchardi*) in the U.S. Midwest. Herpetol. Rev. 39:193–196.

Talley, B. L., C. R. Muletz, V. T. Vredenburg, R. C. Fleischer, and K. R. Lips. 2015. A century of *Batrachochytrium dendrobatidis* in Illinois amphibians (1888–1989). Biol. Conserv. 182:254–261.