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SEASONALITY IN *BATRACHOCHYTRIUM DENDROBATIDIS* DETECTION IN AMPHIBIANS IN CENTRAL OKLAHOMA, USA

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Abstract: Chytridiomycosis, an infectious disease caused by the fungus *Batrachochytrium dendrobatidis* (chytrid or *Bd*), has not been well studied in Oklahoma. This is of particular concern regarding the connection between seasonality and chytrid infection. To further investigate this connection, chytrid prevalence and infection load were quantified within amphibians in central Oklahoma from March to October, across two sites in Oklahoma Co. and two sites in Cleveland Co. The results show a trend between seasonality and chytrid, with spring and fall showing higher prevalence and summer showing lower prevalence, which coincides closely with the preferred chytrid growth temperatures. Additionally, periods of high rainfall in May 2015 are linked to increased chytrid prevalence, as has been suggested by other research. Additionally, species exhibiting high chytrid prevalence follow the results of previous studies: Blanchard's cricket frog (*Acris blanchardi*), American bullfrog (*Rana catesbeiana*), and southern leopard frog (*Rana sphenocephala*).

Key words: Amphibian decline, *Batrachochytrium dendrobatidis*, Chytrid, Chytridiomycosis, Infectious disease, Weather.

BRIEF COMMUNICATION

Chytridiomycosis, an infectious disease caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), has been implicated in the decline of amphibian species across the globe.[21,26] This is of particular concern because symptoms are not often visible in an individual (i.e., lethargy, skin sloughing), mortality events are not well documented, and spread of the disease can be human mitigated.[3,7] Additionally, disease susceptibility can vary greatly by species and developmental stage, even in the same habitat.[6,20,21,25] Numerous laboratory and experimental studies have been conducted to better understand how temperature impacts *Bd* prevalence in wild populations, with data indicating that *Bd* may do well at cooler temperatures and can even withstand freezing, whereas maximum growth occurs between 17°C and 20°C.[10,17] The pathogen's ability to infect the host amphibian decreases at temperatures above 23°C.[1] Not only does the pathogen appear to die at temperatures exceeding 26–30°C,[9,24] but also, exposing infected host amphibians to temperatures exceeding 30°C has been recommended as a possible course of treatment.[28] Studies have also shown that infection prevalence decreases seasonally in regions experiencing higher temperatures for part of the year.[9]

Considering the fact that *Bd* can have variable effects on different amphibian populations, additional studies have been conducted to observe amphibian immune responses in regard to varying temperature.[1,9,17,28] These studies have shown that amphibian immune defenses are weakened in cooler temperatures.[19,20] Therefore, one would expect that amphibians might be more susceptible to infection in regions with cooler temperatures or in regions that experience stronger seasonal fluctuations in temperature.[10] Unlike natural studies, laboratory experiments are useful in predicting the correlation between *Bd* prevalence and temperature due to the ability to monitor individuals throughout time and have noninfected controls; however, they cannot truly predict how amphibians will respond in natural conditions. Therefore, repeated observations of wild populations are necessary to better understand how the fungus interacts with amphibian hosts in natural environments. This study examined seasonal variation in *Bd* prevalence within central Oklahoma, building on recent work on amphibian infectious disease in the state.[13,27] Our a priori

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expectations were that *Bd* prevalence would be greatest during seasons within the optimal temperature range for the pathogen and when the immune systems of amphibian host species may be suppressed, such as spring or fall.

To determine whether *Bd* prevalence in amphibians in central Oklahoma is correlated with spring–fall seasonal variation in weather, anuran communities were repeatedly sampled during their primary active periods, during 18 sampling trips in Cleveland and Oklahoma counties. In total, 246 amphibians were sampled during spring (March and May; $N = 92$), summer (June and August; $N = 90$), and fall (October; $N = 64$) in 2015. The Oklahoma Co. sites were along the shore of Northeast Lake near the Oklahoma City Zoo (site 1; 35.51705, –97.47129) and a wetland located within a residential intersection at NE 50th Street and N. Bartell Road (site 2; 35.52229, –97.43267); both areas have high human impact levels. In Cleveland Co., one sampling area was located at Sutton Urban Wilderness Park and adjacent residential ponds (site 3; 35.24266, –97.42689), with moderate human impact levels. The second sampling area for Cleveland Co. was the Lexington Wildlife Management Area (WMA; site 4; 35.04437, –97.24004), which exhibited low human impact levels. Amphibians from each site were screened once per sample month, with the exception of NE 50th Street and N. Bartell Road, which was only sampled in spring (May only) and summer (June only), due to low water levels resulting in an inability to find amphibians during the sampling timeframe.

During each survey, amphibians were caught after dark by hand, dipnet, or seine in streams, ponds, and wetlands. A total of 10 anuran species were collected, composed of 202 adults, 19 juveniles/metamorphs, and 22 tadpoles (Table 1). Sterile techniques were used to prevent cross-contamination between individuals.[7] Potential *Bd* samples were collected using established protocols by rubbing a rayon-tipped swabs along the amphibian skin to dislodge fungal spores (ventral, lateral, and dorsal portions of the trunk, hindlimbs, and toe webbing).[11] The swab was then placed into sterile, individually labeled vials with no preservative and stored at –20°C until DNA extractions were performed.

Extractions were completed from each swab using published methodologies[3] and the extraction buffer PrepMan Ultra (Life Technology, Thermo Fisher Scientific, Foster City, CA, USA 94404). Extracts were diluted 1:10 with 0.25× TE buffer (ProMega Corp, Fitchburg, WI 53711, USA) before being shipped for analysis to the Disease Testing and Sequencing Facility at the University of South Dakota. Quantitative polymerase chain reaction (qPCR) techniques were used to determine the presence/absence of *Bd* genetic material and to estimate the number of gene copies per sample, or *Bd* load,[8] using StepOne software v2.3 (Applied Biosystems, Thermo Fisher Scientific). The *Bd* assay target primers followed published protocols, using the *ITS-1* rRNA gene.[2] All samples were run in triplicate, with positive controls of known *Bd* gene copies (gBlock DNA quantities $1e^1$–$1e^4$) and a negative control (distilled water). Samples were considered positive for *Bd* (*Bd*+) if amplification occurred in at least two of the three wells and if the mean *Bd* gene copy number was greater than 1.0,[8] with samples rerun as needed. Descriptive statistics were conducted on our results with groups pooled by site, species, or season, due to small sample sizes.

The results of qPCR analysis were examined for patterns of seasonal variation in *Bd* prevalence and load. Of the 246 amphibians screened, a total of 103 (41.87%) were *Bd*+; however, none of our sampled individuals showed visible signs of infection. Of the 10 species collected, only 4 had sufficient sample sizes for interpretation ($N \geq 10$; sampled in all three seasons); all show relatively high *Bd* prevalence: *Hyla chrysoscelis/versicolor* (53%), *Acris blanchardi* (47%), *Rana catesbeiana* (30%), and *R. sphenocephala* (33%; Table 1). There were no discernable patterns in *Bd* load within species, as those with the highest loads (i.e., *Anaxyrus woodhousii*, *Gastophryne olivacea*, *Pseudacris fouquettei*) were also among the lowest sample sizes (Table 1). Amphibians collected in the fall had the highest *Bd* prevalence (73%), followed by spring (51%) and summer (10%; Table 1). For those species sampled across all three seasons, the same seasonal pattern can be identified within each species (Table 1). Additionally, there was a trend of higher *Bd* loads in the spring months, with both summer and fall comparably one order of magnitude lower (Table 1). Last, the percent of *Bd*+ individuals across sites was similar (Table 2). An examination of *Bd* load found that both Oklahoma Co. sites (sites 1 and 2) had much higher infection loads than in Cleveland Co. (sites 3 and 4; Table 2). No temperature or rainfall amounts were directly recorded as part of this study; weather station temperature and rainfall amounts for Oklahoma and Cleveland counties are provided (Table 3).[15]

Table 1. Comparison of *Bd* screening by species, including sample size (*N*), prevalence of *Bd*+ individuals (*Bd* %), and average *Bd* load (±1 SD).

	Spring			Summer			Fall			Total		
Species	*N*	*Bd* %	*Bd* Load Avg. (±SD)	*N*	*Bd* %	*Bd* Load Avg. (±SD)	*N*	*Bd* %	*Bd* Load Avg. (±SD)	*N*	*Bd* %	*Bd* Load Avg. (±SD)
Bufonidae	8	75.00	283,277.39 (±550,735.32)	2	0.00	N/A	1	100.00	2,455.97 (N/A)	11	63.64	284,064.62 (±550,264.38)
Anaxyrus americanus	3	66.67	72,790.99 (±95,002.47)	0	N/A	N/A	1	100.00	2,455.97 (N/A)	4	75.00	49,345.98 (±78,496.75)
Anaxyrus woodhousii	5	80.00	4,601,003.60 (±710,67.78)	2	0.00	N/A	0	N/A	N/A	7	57.14	4,601,003.60 (±710,676.78)
Hylidae	44	61.36	5,055,514.83 (±21,465,642.86)	41	7.32	303,260.14 (±431,738.85)	37	75.68	209,101.11 (±513,866.94)	122	47.54	2,470,060.55 (±14,704,889.20)
Acris blanchardi	29	55.17	687,818.45 (±2,187,688.29)	33	9.09	303,260.14 (±431,738.85)	36	75.00	216,235.43 (±522,240.74)	98	46.94	385,939.83 (±1,345,856.24)
Hyla chrysoscelis/versicolor	11	72.73	930,109.09 (±2,523,724.96)	5	0.00	N/A	1	100.00	16,474.41 (N/A)	17	52.94	828,594.13 (±2,380,291.32)
Hyla cinerea	0	N/A	N/A	3	0.00	N/A	0	N/A	N/A	3	0.00	N/A
Pseudacris clarkii	3	66.67	56,629,537.70 (±78,118,737.50)	0	N/A	N/A	0	N/A	N/A	3	66.67	56,629,537.70 (±78,118,737.50)
Pseudacris fouquettei	1	100.00	4,793,857.08 (N/A)	0	N/A	N/A	0	N/A	N/A	1	100.00	4,793,857.08 (N/A)
Microhylidae	6	83.33	7,169,662.47 (±15,880,028.20)	2	0.00	N/A	0	N/A	N/A	8	62.50	7,169,662.47 (±15,880,028.20)
Gastrophryne olivacea	6	83.33	7,169,662.47 (±15,880,028.20)	2	0.00	N/A	0	N/A	N/A	8	62.50	7,169,662.47 (±15,880,028.20)
Ranidae	34	26.47	23,630.75 (±48,853.73)	45	13.33	11,502.30 (±11,646.19)	26	69.23	33,327.73 (±58,870.29)	105	31.43	26,714.84 (± 50,296.01)
Rana catesbeiana	32	25.00	24,832.83 (±52,084.35)	11	18.18	17,599.07 (±21,210.82)	11	54.55	64,543.87 (±90,669.30)	54	29.63	38,820.25 (±66,823.18)
Rana sphenocephala	2	50.00	14,014.13 (N/A)	34	11.76	8,453.92 (±6,238.55)	15	80.00	17,719.66 (±28,671.26)	51	33.33	15,321.51 (±24,262.35)
Total	92	51.09	3,713,742.25 (±16,997,506.45)	90	10.00	108,754.92 (±260,701.05)	64	73.44	137,386.94 (±405,013.00)	246	41.87	1,766,814.24 (±11,557,984.10)

Table 2. Comparison of *Bd* screening by sample site, including sample size (*N*), prevalence of *Bd*+ individuals (*Bd* %), and average *Bd* load (±1 SD).

Site	N	*Bd* %	*Bd* Load Avg. (±SD)
Oklahoma Co.			
Oklahoma City Zoo (1)	59	57.62	1,477,820.27 (±6,219,816.95)
50th and Bartell (2)	37	62.16	5,568,914.14 (±23,237,913.49)
Cleveland Co.			
Sutton Urban Wilderness (3)	103	27.18	117,894.07 (±321,354.06)
Lexington WMA (4)	47	38.30	19,439.89 (±27,500.96)
Total	246	41.87	1,766,814.24 (±11557984.10)

Amphibian host species are known to be highly linked to both *Bd* susceptibility and infection load.[6,20,21] The results of this study indicated that for those species with larger sample sizes, *A. blanchardi* was among the highest prevalence. Its ubiquitous presence at nearly every aquatic water body throughout its Oklahoma range suggests that it is found in high densities, which likely contributes to its well-documented history of *Bd* infection.[4,13,23,27] Likewise, its range overlaps with all 16 at-risk Oklahoma amphibian species,[16] and as such, it could be used as a focal study organism for understanding temporal and spatial dynamics of amphibian infectious disease risk throughout much of the state. Additionally, both *R. catesbeiana* and *R. sphenocephala* exhibited high prevalence and lower infection loads. Previous studies of *R. catesbeiana* have shown them to be a tolerant to *Bd* infections, which allows them to act as a global vector for the disease.[5,6,22] Other research has suggested that *R. sphenocephala*, and its close relative *R. pipiens*, may have similar *Bd*-resistant properties as *R. catesbeiana* and can therefore be localized carriers of the disease within the United States.[12,29]

These findings indicate a strong pattern of seasonality associated with the presence and prevalence of *Bd*. Disease prevalence and infection load increased during the cooler months (March, May, October) and decreased during the warmer months (June, August). Climatologic data from within the sampling period show that temperatures in 2015 were comparable to typical Oklahoma seasons.[14,15] Previous research regarding *Bd* exhibits maximum growth[10,17] coincides with the observed temperatures of the spring and fall 2015 sampling seasons (Table 3), resulting in the two highest prevalence levels observed. Summer 2015 resulted in the lowest *Bd* prevalence levels, as would be expected as the lethal temperature is approached,[9,28] which coincides with the optimum amphibian immune response temperature.[19,20] Other than the extremely high rainfall in May 2015 (14.33–23.39 inches; Table 3), rainfall amounts during our sampling period are typical for Oklahoma.[14,15] Previous research has indicated that extreme variation in precipitation can lead to increased prevalence in *Bd* and other amphibian infectious diseases.[18] These conclusions join other bodies of research stating that the highest rates of *Bd* prevalence are correlated with the onset of heavy rainfall,[10,25] in addition to a number of studies demonstrating a correlation between *Bd* prevalence in wild amphibians and seasonality across the United States.[4,11]

It is important to recognize that all four sampling sites across Oklahoma and Cleveland counties are located within the Cross Timbers ecological region in central Oklahoma, an area that is undergoing rapid urban development.[16] Because of this similarity in locality, climate, and urbanization, one would expect each site to

Table 3. Weather station monthly summaries for 2015 for Oklahoma County, North Station (35.55556, –97.51056; 360 m elevation) and Cleveland County (35.23583, –97.46472; 357 m elevation).[15]

	Oklahoma Co.		Cleveland Co.	
Month	Mean temperature (°C)	Mean rainfall (in.)	Mean temperature (°C)	Mean rainfall (in.)
March	11.5	2.78	11.28	2.42
May	18.89	14.33	18.89	23.39
June	26.5	4.94	26.17	2.95
August	26.61	1.05	26.39	1.74
October	17.78	3.50	17.5	2.87

experience similar seasonal infection patterns throughout the year. Similar patterns of *Bd* prevalence were observed between the sites, as was expected based on these climatic similarities; however, the higher *Bd* infection load observed in the two Oklahoma Co. sites is likely linked to the increased human impacts in the area.[7] For future studies, it would be beneficial to test for seasonal disease prevalence in other ecological regions across the state that may have different climates, exhibit greater levels of annual seasonal variation, or have fewer anthropogenic impacts. Additionally, longer temporal studies with increased taxonomic and seasonal sampling are needed to better assess amphibian infection patterns through time to make statewide management strategies to protect vulnerable Oklahoma amphibians. We recommend that Oklahoma residents undergoing aquatic recreational activities, particularly in the spring and fall months, fully disinfect their equipment (i.e., boats, waders) to help further prevent disease spread during these particularly active chytrid seasons.[7]

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