First Report of *Batrachochytrium dendrobatidis* (Chytridiomycota: Rhizophydiales) from American Bullfrog, *Rana catesbeiana* (Anura: Ranidae) from Western Arkansas

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Abstract: A single American bullfrog, *Rana catesbeiana*, collected in May 2023 from Polk County, Arkansas, harbored the skin fungus, *Batrachochytrium dendrobatidis* (*Bd*). However, the host showed no obvious pathological signs of infection. Three larval salamanders and six additional anurans collected from the same general locality were negative for *Bd* as well as other amphibian pathogens, including Ranavirus (specifically FV3) and *Batrachochytrium salamandrivorans* (*Bs*). This is the first time *R. catesbeiana* from Arkansas has been reported with *Bd* as well as being the fourth species of amphibian from the state harboring the fungus.

Introduction

Chytrid fungus causes the disease chytridiomycosis, originally produced by *Batrachochytrium dendrobatidis* (*Bd*). It was initially discovered in 1989 and is an amphibian chytrid that causes a skin infection, with an affinity to occur in anurans (frogs and toads) worldwide (Lips et al. 1999). Unfortunately, the disease has caused a serious decline in, and in some instances, extinction of more than 200 species of amphibians worldwide and poses the greatest threat to biodiversity of any known disease (Stuart 2004; Skerratt et al. 2007; Pounds et al. 2016; Collins 2020).

Arkansas supports a great diversity of 56 species and subspecies of amphibians (Trauth et al. 2004). However, to date, only three (5%) species from the state have been reported to be infected with the fungus. Federally endangered Ozark hellbenders, *Cryptobranchus alleganiensis bishopi* Grobman collected from the Eleven Point River in Randolph County

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were reported to harbor *Bd* (Briggler et al. 2008; Hardman et al. 2020). In another study, *Bd* was found in Blanchard's cricket frogs, *Acris blanchardi* Harper from the Wapanocca National Wildlife Refuge in Crittenden County (Hanlon et al. 2014). Interestingly, no specimens exhibited clinical signs of a *Bd* infection (e.g., lethargy, sloughing of skin), perhaps indicating that *Bd* in this population may persist without yielding to *Bd*-induced mortality. In a third study, frogs collected from the Bald Knob National Wildlife Refuge (White County) and Felsenthal National Wildlife Refuge (Union County), including postmetamorphic *A. blanchardi* from the former and tadpoles of Coastal Plains leopard frog, *Rana sphenocephala utricularia* (Cope) from the latter site, harbored *Bd* (Rothermel et al. 2008). Obviously, much more work needs to be done in the state to survey additional populations of amphibians for *Bd* and other amphibian pathogens. Here we attempt to help fill that void with examination of four species of amphibians from Arkansas for amphibian pathogens, specifically *Bd*.

Methods

Host collection and processing

On 31 July and 1 June 2023, the following 10 amphibians from Polk County were collected by hand and examined for *Bd* as well as for other amphibian pathogens, including Ranavirus (specifically FV3) and *Batrachochytrium salamandrivorans* (*Bs*): three juvenile Ouachita dusky salamanders, *Desmognathus brimleyorum* Stejneger from Grace Fountain Spring at Big Fork (34°29′07.1232″N, -93°58′05.16″W), and adults of four green frogs, *Rana clamitans* (Latreille), two American green treefrogs, *Dryophytes* (=*Hyla cinerea*) *cinereus* (Schneider), and a single juvenile American bullfrog, *Rana catesbeiana* (Shaw) from the Ouachita Mountains Biological Station (OMBS) pond (34°27'43.4484''N, -93°59'54.3264''W). We followed the protocols of Livo (2004) and Standish et al. (2018) by swabbing the ventral and dorsal skin of each amphibian (including the hind-toe webbing of anurans) 15 to 20× with 15.24 cm sterile cotton-tipped applicators (Medline, Northland, Illinois) and preserving the swabs in individual sterile vials containing 95% DNA grade ethanol. Several preventative steps were taken to avoid cross-contamination by keeping individuals separate from the time of collection, using a new pair of gloves to handle each specimen, and swabbing individuals before taking their snout-vent length (SVL) measurements. Following processing, all amphibians were photographed and released unharmed at their site of collection. We follow the use of the genus *Rana* versus *Lithobates* for North American ranid frogs following Yuan et al. (2016).

Molecular techniques

To examine swab DNA, ethanol was decanted, and swabs were air-dried. Sample DNA was extracted using 150 µL of the PrepMan™ Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, Massachusetts) following the manufacturer's instructions. Extracted DNA was analyzed using a multiplex quantitative PCR (qPCR) targeting *B. dendrobatidis* (*Bd*), *B. salamandrivorans* (*Bsal*) and frog virus 3-like Ranavirus as described by Standish et al. (2018). A synthetic gBlock® containing all three amplicons was used as a positive control and standard curve of 10× dilutions ranging from 10^7 to one copy per reaction was used to quantify copies per µL (Standish et al. 2018). Sample concentration (copies per ng total DNA) was calculated using a Qubit™ 3.0 Fluorometer (Thermo Fisher Scientific) and the Qubit™ DNA High Sensitivity Kit (Thermo Fisher Scientific) following the manufacturer's instructions.

Results

Ranavirus and *B. salamandrivorans* were not detected. One of 10 (10%) of the amphibians sampled harbored *Bd*. The host was a juvenile *R. catesbeianus* collected from the OMBS pond. The specimen appeared otherwise healthy and exhibited no overt symptoms of the infection. All other amphibians sampled were negative for the presence of pathogens.

No significant inhibition was observed in and qPCR reactions and amplification was only observed in one sample of this host. Samples

were initially screened (not quantified) then archived at -80º as we did not recognize the significance of this positive result. After two weeks samples, extracts were quantified and copy number determined. An average Cq value of 30.99 was observed across triplicate reactions which contained an average of 2382 copies of the *Bd* target amplicon or 9102 *Bd* copies per ng of total DNA.

Discussion

Interestingly, American bullfrogs have been reported to be seemingly resistant (asymptomatic) to the disease and may act as vectors or reservoir hosts (Daszak et al. 2004; Hanselmann et al. 2004). Although the factors are not fully understood, one characteristic that appears to protect *R. catesbeiana* from *Bd* involve the production of immunoprotective peptides (Eskew et al. 2018). This resistance has been thought to allow *R. catesbeiana* to spread *Bd* to various parts of North America and even other continents (Jenkinson et al. 2016; Yap et al. 2018). In addition, Garner et al. (2006) detected *Bd* infections in introduced *R. catesbeiana* from six countries, including Brazil, Canada (British Columbia), France, Italy, United Kingdom, and Uruguay; they also found *Bd* in introduced American bullfrogs from Arizona, USA. Standish et al. (2018) identified *Bd* and FV3 from 33.3% and 16.7%, respectively, of adult *R. catesbeiana* sampled in Wisconsin with none of the bullfrog tadpoles sampled testing positive for either pathogen. Rothermel et al. (2008) reported five of 229 (2%) individuals of *R. catesbeiana* from Georgia, North Carolina, and Virginia harbored *Bd* but specimens from Florida, Louisiana, Mississippi, and South Carolina were not infected; no *R. catesbeiana* from Arkansas were sampled. However, in adjacent Oklahoma, several studies have reported *Bd* in *R. catesbeiana* from the state either rarely or in high prevalence (Watters et al. 2016, 2018, 2019, 2021; Marhanka et al. 2017; Nichols et al. 2022).

We suggest additional amphibians from the state be surveyed for *Bd* as well as other amphibian pathogens. Undoubtedly, more individuals will be discovered to harbor this fungus, including the possibility, more importantly, of finding it in Arkansas endemic and protected species.

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References

Briggler JT, Larson KA, Irwin KJ. 2008. Presence of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) on hellbenders (*Cryptobranchus alleganiensis*) in the Ozark highlands. Herpetol Rev 39:443–444.

Collins JP. 2010. Amphibian decline and extinction: what we know and what we need to learn. Dis Aquat Organ 92:93–99.

Daszak P, Streiby A, Cunningham AA, Longcore JE, Brown CC, Porter D. 2004. Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. Herpetol J 14:201–207.

Eskew EA, Shock BC, LaDouceur EEB, Keel K, Miller MR, Foley JE, Todd BD. 2018. Gene expression differs insusceptible and resistant amphibians exposed to *Batrachochytrium dendrobatidis*. Royal Soc Open Sci 5:170910. http://dx.doi.org/10.1098/rsos.170910

Garner TWJ, Perkins M, Govindarajulu P, Seglie D, Walker SJ, Cunningham AA, Fisher MC. 2006. The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*. Biol Lett 2:455–459.

Hanlon S., Smith S, Gerby JL, Berg E, Peterson W, Parris MJ, Moore JE. 2014. Occurrence of *Batrachochytrium dendrobatidis* in Wapanocca National Wildlife Refuge, Arkansas, USA. Herpetol Rev 45:31–32.

Hanselmann R, Rodriguez A, Lampo M, Fajardo-Ramos L, Aguirre AA, Kilpatrick AM, Rodriguez JP, Daszak P. 2004. Presence of an emerging pathogen of amphibians in introduced bullfrogs (*Rana catesbeiana*) in Venezuela. Biol Cons 120:115–119.

Hardman RH, Irwin KJ, Sutton WB, Miller DL. 2020. Evaluation of severity and factors contributing to foot lesions in endangered Ozark hellbenders, *Cryptobranchus alleghaniensis bishopi*. Front Vet Sci 7:34. doi: 10.3389/fvets.2020.00034

Jenkinson TS, Betancourt RCM, Lambertini C, Valencia-Aguilar A, Rodriguez D, Nunes-de-Almeida CHL, Ruggeri J, Belasen AM, da Silva Leite D, Zamudio KR, Longcore JE, Toledo LF, James TY. 2016. Amphibian-killing chytrid in Brazil comprises both locally endemic and globally expanding populations. Mol Ecol 25:2978–2996.

Lips KR, Brem F, Brenes R, Reeve JD, Alford RA, Voyles J, Carey C, Livo L, Longcore JE, Pessier AP, Nichols DK. 1999. *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia 91:219–227.

Livo LJ. 2004. [online]. Methods for obtaining *Batrachochytrium dendrobatidis* (*Bd*) samples for PCR testing. Colorado Division of Wildlife, Denver, Colorado. Available from: http://wildlife.state. co.us/NR/rdonlyres/710BBC95-2DCF-4CF9-8443-D4561DBC3B69/0/PCRsampling2004.pdf. (Accessed 30 July 2023)

Marhanka EC, Watters JL, Huron NA, McMillin SL, Winfrey CC, Curtis DJ, Davis DR, Farkas JK, Kerby JL, Siler CD. 2017. Detection of high prevalence of *Batrachochytrium dendrobatidis* in amphibians from southern Oklahoma, USA. Herpetol Rev 48:70–74.

Nichols MH, Smith SN, Watters JL, Siler CD. 2022. Implementation of a citizen science program to assess chytridiomycosis (*Batrachochytrium dendrobatidis*) prevalence in amphibians across Oklahoma, USA. Proc Okla Acad Sci 102:1–18.

Pounds JA, Bustamante MR, Coloma LA, Consuegra JA, et al. 2006. Widespread amphibian extinctions from epidemic disease driven by global warming. Nature 439:161–167.

Rothermel BB, Walls SC, Mitchell JC, Dodd CK Jr, Irwin LJ, Green DE, Vazquez V, Petranka JW, Stevenson DJ. 2008. Widespread occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in the southeastern USA. Dis Aquat Organ 82:3–18.

Skerratt LF, Berger L, Speare R, Cashins S, McDonald KR, Phillott A, Hines H, Kenyon N. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth 4:125–134.

Standish I, Leis E, Schmitz N, Credico J, Erickson S, Bailey J, Kerby J, Phillips K, Lewis T. 2018. Optimizing, validating, and field testing a multiplex qPCR for the detection of amphibian pathogens. Diseases of Aquatic Organisms 129:1–13.

Stuart SN. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783–1786.

Trauth SE, Robison HW, Plummer MV. 2004. The amphibians and reptiles of Arkansas. Fayetteville (AR): University of Arkansas Press. 421 p.

Watters JL, Davis DR, Yuri T, Siler CD. 2018. Concurrent infection of *Batrachochytrium dendrobatidis* and ranavirus among native amphibians from northeastern Oklahoma, USA. J Aquat Anim Health 30:291–301.

Watters JL, Flanagan RL, Davis DR, Farkas JK, Kerby JL, Labonte MJ, Penrod ML, Siler CD. 2016. Screening natural history collections for historical presence of *Batrachochytrium dendrobatidis* from anurans in Oklahoma, USA. Herpetol Rev 42:214–220.

Watters JL, Hall SE, Siler CD. 2021. Human impacts on the prevalence of the amphibian infectious diseases, *Batrachochytrium dendrobatidis* and ranavirus, in Oklahoma, USA. Proc Okla Acad Sci 101:1–13.

Watters JL, McMillin SL, Marhanka EC, Davis DR, Farkas JK, Kerby JL, Siler CD. 2019. Seasonality in *Batrachochytrium dendrobatidis* detection in amphibians in central Oklahoma, USA. J Zoo Wildl Med 50:492–497.

Yap TA, Koo MS, Ambrose RF, Vredenburg VT. 2018. Introduced bullfrog facilitates pathogen invasion in the western United States. PLoS One 13 (4):e0188384. doi: 10.1371/journal.pone.0188384.

Yuan, Z-Y, Zhou W-W, Chen X, Poyarkov NA Jr, Chen H-M, Jang-Liaw N-H, Chou W-H, Matzke NJ, Iizuka K, Min M-S, Kuzmin SL, Zhang Y-P, Cannatella DC, Hillis DM, Che J. 2016. Spatiotemporal diversification of the true frogs (genus *Rana*): A historical framework for a widely studied group of model organisms. Syst Biol 65:824–842.

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