Batrachochytrium dendrobatidis (Chytridiomycota: Rhizophydiales) from Blanchard's Cricket Frog, *Acris blanchardi* (Anura: Hylidae) and American Bullfrog, *Rana catesbeiana* (Ranidae) from Arkansas

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Abstract: Between August 2023 and August 2024, 42 amphibians including, 14 many-ribbed salamanders, *Eurycea multiplicata*, three Blanchard's cricket frogs, *Acris blanchardi*, seven spring peepers, *Pseudacris crucifer*, four American bullfrogs, *Rana catesbeiana*, and four green frogs, *Rana clamitans* from Arkansas, as well as nine Woodhouse' toads, *Anaxyrus woodhousii*, and one southern leopard frog, *Rana sphenocephala utricularia* from Texas were examined for the skin fungi, *Batra-*
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chochytrium dendrobatidis (*Bd*), *B. salamandrivorans* (*Bsal*), and frog virus 3-like Ranavirus (FV3). Swabs from one of four (25%) *R. catesbeiana* and one of three (33%) *A. blanchardi* from Arkansas tested positive for *Bd*: the other amphibians sampled were negative for all three pathogens. Neither host showed any obvious pathological signs of infection. This is the second time *A. blanchardi* and *R. catesbeiana* from Arkansas has been reported with *Bd*.

Introduction

Chytrids belong to the Kingdom Fungi, Phylum Chytridiomycota, Class Chytridiomycetes, and Order Rhizophydiales. The fungus causes the disease chytridiomycosis, originally generated by *Batrachochytrium dendrobatidis* Longcore, Pessier, and Nichols (1999) (*Bd*), an amphibian chytrid (Lips et al. 1999; Longcore et al. 1999). The skin infection has a predilection to anurans (frogs and toads) and has been reported on every amphibian inhabitant of the continent. Unfortunately, the disease has resulted in a serious decline and extinction of more than 200 species of amphibians worldwide and poses the greatest threat to their biodiversity of any known amphibian disease (Stuart 2004; Skerratt et al. 2007; Pounds et al 2016; Collins 2020).

In Arkansas, 10 to 20% of endangered Ozark hellbenders, *Cryptobranchus alleganiensis bishopi* Grobman sampled from the Eleven Point River watershed in Randolph County were reported to harbor *Bd* (Briggler et al. 2008; Hardman et al. 2020). The fungus was also found in Blanchard's cricket frogs, *Acris blanchardi* Harper from Wapanocca National Wildlife Refuge in Turrell (Crittenden County) (Hanlon et al. 2014). However, no frog exhibited pathological signs of a *Bd* infection (e.g., lethargy, skin sloughing), indicating that *Bd* in this population of *A. blanchardi* may endure in populations without dying from *Bd*-induced mortality. In another study, *A. blanchardi* collected from the Bald Knob National Wildlife Refuge in White County and southern leopard frog, *Rana sphenocephala utricularia* (Cope) tadpoles from Felsenthal National Wildlife Refuge in Union County harbored *Bd* (Rothermel et al. 2008). A more recent study by McAllister et al. (2023) reported *Bd* in a single *R. catesbeiana* from Polk County.

A similar species, *B. salamandrivorans* Martel, Blooi, Bossuyt, and Pasmans, 2013 *(Bsal)* also causes chytridiomycosis but primarily occurs in caudate amphibians (salamanders) (Martel et al. 2013). It was initially discovered in captive and native fire salamanders, *Salamandra salamandra* (L.) in Belgium and the Netherlands, where it led to significant declines in salamander populations. Since then, *Bsal* has also been noticed in captive salamanders in the United Kingdom and Germany. To date, it has not been reported from any salamander from North America.

Another emerging disease include members of the genus *Ranavirus* (an iridovirus), capable of infecting a wide variety of poikilothermic vertebrate hosts including fish, amphibians, and reptiles (Whittington et al. 2010; Marschang 2011; Miller et al. 2011). It is known to have caused amphibian die-offs on five continents (Gray et al. 2009; Miller et al. 2011) and is capable of infecting amphibians from at least 14 families and over 72 individual species, with the majority of cases in members of the anuran family Ranidae (Miller et al. 2011; see their Table 1). While ranavirus infections have been documented in numerous areas across the USA (Allender et al. 2011; Gray et al. 2012; Goodman et al. 2013; Duffus et al. 2015), infections have been rarely detected in herps in Arkansas. Hardman et al. (2020) discovered ranavirus in 6% of *C. a. bishopi* in the state. However, Hanlon et al. (2016) surveyed the prevalence of ranaviruses in five species of turtles of the Wapanocca Wildlife Refuge and none were infected. In neighboring Oklahoma, ranavirus was found in 12 species of amphibians, some in eastern counties bordering the western border of Arkansas (Davis et al. 2019).

The two-fold purpose of the present study is: (1) survey several amphibians from Arkansas and Texas for these emerging amphibian pathogens, and (2) to document additional reports of *Bd* in two common anurans of the state. We

hope to spur others in examining additional amphibians of Arkansas for pathogens, particularly populations that are listed as endangered, threatened, or are species of special concern or greatest conservation need (see: https://www.agfc.com/education/arkansas-wildlife-action-plan/).

Methods

Host collection and processing

Between August 2023 and August 2024, 14 many-ribbed salamanders, *Eurycea multiplicata* (Cope) from Montgomery, three *A. blanchardi* from Fulton, seven spring peepers, *Pseudacris crucifer* (Wied-Neuwied) from Polk, four *R. catesbeiana* from Fulton (n = 2) and Nevada (n = 2), and four green frogs, *Rana clamitans* (Latrielle) from Columbia (n = 1) and Polk (n = 3) counties, Arkansas, were collected by hand or dipnet. In addition, a *R. s. utricularia* from Lamar, and nine juvenile Woodhouse' toads, *Anaxyrus woodhousii* (Girard) from Morris counties, Texas, were also collected in a similar manner. We followed the sampling protocols of Livo (2004) and Standish et al. (2018) for collecting samples from potential hosts for *Bd*, *Bsal*, and 3-like Ranavirus. After processing, some specimens were photographed and released unharmed at their collection site while others were deposited as vouchers in the Northeast Texas Community College Vertebrate Collection, Mt. Pleasant, Texas (NTCCVC). We prefer the use of the genus *Rana* versus *Lithobates* for North American ranid frogs following Yuan et al. (2016).

Molecular Techniques

Swab DNA was extracted using 150 µL of the PrepMan™ Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, Massachusetts) following the manufacturer's instructions. Multiplex quantitative PCR (qPCR) was performed, targeting FV3, *Bd*, and *Bsal* as detailed by Standish et al. (2018). A synthetic gBlock® containing all three target amplicons was used as a positive control and standard curve. Serial dilutions (10×) ranging from 10^7 to one copy per reaction were used to quantify copies per µL (Standish et al. 2018). Sample concentration (copies/ng total DNA) was determined 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

Batrachochytrium salamandrivorans and FV3 were not detected in any samples. Swabs from one of two (50%) *R. catesbeianus* (urostyle length [UL] = 140 mm) collected in March 2024 from Town Creek at Salem, Fulton County (36°06′4.3848″N, -91°05′7.9224″W), and one of three (33%) *A. blanchardi* (UL = 22 mm) collected in March 2024 from Big Creek S of Mammoth Spring off co. rd. 81, Fulton County (36°26′22.46″N, -91°29′21.85″W), tested positive for *Bd*. Both host voucher specimens were deposited in the NTCCVC. The other amphibians sampled from four other Arkansas and two Texas counties were negative for *Bd*. Across duplicate reactions, swabs contained an average of 4.5 *Bd* copies/ng and 297 *Bd* copies/ng of total DNA for *R. catesbeiana* and *A. blanchardi*, respectively. The ITS copy in zoospores can vary significantly from 10 to 144 copies/zoospore, so we estimated based on CT values, resulting copy number, and lack of disease signs as these detections are subclinical infections representing <10 zoospores (Longo et al. 2013).

Discussion

In our survey, both anurans found to be infected with *Bd* have the widest geographical distribution within the state (Trauth et al. 2004). Both species have also been previously reported to harbor *Bd* in Arkansas (Rothermel et al. 2008; Hanlon et al. 2014; McAllister et al. 2023). The fungus has now been reported in amphibians from six of 75 (8%) counties of the state (Fig. 1). The apparent gaps in *Bd* distribution among amphibians in other counties (especially in the Ozarks of Northwest Arkansas) may be biased due to the lack of surveys conducted on them. In addition, as far we know no anuran from the state has been reported with FV3 although the endangered cau-

date *C. a. bishopi* from the Ozark highlands was found to be infected (Hardman et al. 2020).

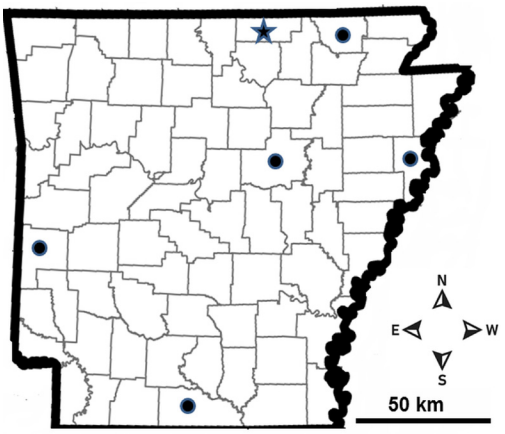

Figure 1. Counties in Arkansas where anuran hosts have been reported with Bd. Dots = previous records; star = new record.

Currently, *Bsal* is not known to occur in North America, but entry for its introduction apparently exists. Although we did not find either *Bd* or FV3 in our small sample of frogs (n = 10) from Texas, studies in the state have confirmed the presence of *Bd* in several amphibians (Gaertner et al. 2009a, 2009b, 2010, 2012; Gascon et al. 2009; Saenz et al. 2010; Marshall et al. 2019).

We (McAllister et al. 2023) noted previously that American bullfrogs appear to be asymptomatic (resistant) to *Bd* and may act as vectors or reservoir hosts (Daszak et al. 2004; Hanselmann et al. 2004). Garner et al. (2006) detected *Bd* infections in introduced *R. catesbeiana* from six countries, including those introduced into Arizona. In addition, Standish et al. (2018) sampled adult *R. catesbeiana* in Wisconsin and identified *Bd* and FV3 with a prevalence of 33% and 17%, respectively; however, none of the American bullfrog tadpoles sampled tested positive for either pathogen. Rothermel et al. (2008) reported from a large sample of *R. catesbeiana* (n = 229), five (2%) individuals from Georgia, North Carolina, and Virginia harbored *Bd* although specimens from Florida, Louisiana, Mississippi, and South Carolina were not infected. In Oklahoma, *Bd* in *R. catesbeiana* was reported either rarely or in high prevalence in several surveys (Watters et al. 2016, 2018, 2019, 2021; Marhanka et al. 2017; Nichols et al. 2022).

As far as our other host species (*A. blanchardi*) is concerned, this small frog has been reported to be the host of *Bd* in several studies, including those in Arkansas (Rothermel et al. 2008; Hanlon et al. 2014), Oklahoma (Watters et al. 2016; 2018, 2019, 2021; Marhanka et al. 2017; Nichols et al. 2022), and Texas (Gaertner et al. 2009, 2012). Interestingly, none of these studies reported that specimens appeared moribund or lethargic with obvious skin lesions, so this frog may also share some vector or reservoir host attributes for *Bd* with *R. catesbeiana*.

Additional amphibians from the state should be surveyed for *Bd* as well as for other amphibian pathogens. Gaertner et al. (2009a) noted that because central Texas has a significant number of endangered and endemic species of amphibians (see Dixon 2013), an increase of positive environmental factors, specifically permanent water sources, could subsequently result in an increase of the abundance and transmission of *Bd*, and thus might have a negative effect from *Bd* on the amphibian populations in the region. Therefore, we predict that with additional surveys in Arkansas, more individuals will be found to harbor this fungus, including the possibility of finding any of these pathogens in Arkansas' protected amphibians.

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