Journal of Aquatic Animal Health 30:291–301, 2018

ISSN: 0899-7659 print / 1548-8667 online
DOI: 10.1002/aah.10041

COMMUNICATION

Concurrent Infection of *Batrachochytrium dendrobatidis* and Ranavirus among Native Amphibians from Northeastern Oklahoma, USA

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Abstract

Global amphibian decline continues to be a great concern despite our increased understanding of the causes behind the observed patterns of the decline, such as habitat modification and infectious diseases. Although there is a large body of literature on the topic of amphibian infectious diseases, pathogen prevalence and distribution among entire communities of species in many regions remain poorly understood. In addition to these geographic gaps in our understanding, past work has focused largely on individual pathogens, either *Batrachochytrium dendrobatidis* (*Bd*) or ranavirus (RV), rather than dual infection rates among host species. We sampled for prevalence and infection load of both pathogens in 514 amphibians across 16 total sites in northeastern Oklahoma. Amphibians were caught by hand, net, or seine; they were swabbed to screen for *Bd*; and liver tissue samples were collected to screen for RV. Overall results of quantitative PCR assays showed that 7% of screened individuals were infected with RV only, 37% were infected with *Bd* only, and 9% were infected with both pathogens simultaneously. We also documented disease presence in several rare amphibian species that are currently being monitored as species of concern due to their small population sizes in Oklahoma. This study synthesizes a growing body of research regarding infectious diseases among amphibian communities in the central United States.

Amphibian populations worldwide are undergoing a rapid decline due to a number of factors, including climate change, habitat loss, the pet trade, pollution, and emerging infectious diseases, such as chytridiomycosis and ranaviruses (RVs; Daszak et al. 1999; Berger et al. 2016; Rollins-Smith 2017; Scheele et al. 2017). The disease chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), has been a primary focus of disease-related studies on global amphibian declines for nearly three decades (Jancovich et al. 2005; Lips et al. 2006; Vredenburg et al. 2010; Olson et al. 2013; Scheele

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Received November 21, 2017; accepted July 22, 2018

et al. 2017). Causing loss of skin function and ion imbalance, the fungal infection can lead to mortality via cardiac arrest in most infected amphibians (Voyles et al. 2009). In contrast, despite their discovery in the 1960s (Granoff et al. 1966), RVs were given far less attention by wildlife and conservation biologists until recently (Duffus 2009; Chinchar and Waltzek 2014). Collectively a group of viruses in the family Iridoviridae, RVs cause lethargy, hemorrhaging, and organ necrosis, eventually leading to death (Gray and Chinchar 2015; Chinchar et al. 2017). However, because physical signs associated with RV infection are not always present, it was not until diagnostic approaches improved that researchers became more acutely aware of the devastating effects these systemic pathogens were having on wildlife, particularly amphibians (Green et al. 2002; Gray et al. 2009b; Chinchar and Waltzek 2014; Isidoro-Ayza et al. 2017).

Today, genetic testing of amphibian tissue samples is necessary to determine whether an individual is infected with RV (St-Amour and Lesbarrères 2007). Ranavirus-infected amphibians tend to have high mortality, often greater than 90% (Green et al. 2002), and lethal strains have also been linked to declines in a number of amphibian species in North America, from the western tiger salamander *Ambystoma mavortium* (Jancovich et al. 1997; Bollinger et al. 1999; Green et al. 2002; Collins et al. 2004) to the boreal toad *Anaxyrus boreas* (Chinchar and Waltzek 2014). Furthermore, a recent study of 247 wild anuran mortality events that were documented in North America between 2009 and 2015 found RV to be the cause of 92 events (37%), whereas 50 mortality events (20%) were linked to *Bd* (Isidoro-Ayza et al. 2017).

Both pathogens can be spread from direct contact between animals as well as transmitted in water, with increased transmission linked to human-mediated pathogen dispersal through aquatic recreational activities and the movement of infected individuals for food, sport, or the pet trade (Jancovich et al. 2005; Harp and Petranka 2006; Picco and Collins 2008; Schloegel et al. 2009). For example, chytridiomycosis has been known to spread at a rate of 688 m/year in a southern California metapopulation of mountain yellow-legged frogs *Rana muscosa* and Sierra Nevada yellow-legged frogs *R. sierrae*, which generated a change in infection rate from 0% to 98% in the span of a single year (Vredenburg et al. 2010). Furthermore, numerous other factors, such as invasive species, environmental contaminants, and climatic variation, are known to exacerbate infection levels by allowing pathogens like *Bd* to spread beyond historical geographic limits or by exerting a detrimental impact on host immunity (Forson and Storfer 2006; Puschendorf et al. 2006; Collins 2010; Rollins-Smith et al. 2011; Rollins-Smith 2017). Climate change specifically is expected to alter host–pathogen interactions dramatically in the future, increasing the global risk for amphibian mass die-offs (Pounds et al. 2006; Rohr and Raffel 2010; Raffel et al. 2013, 2015; Rollins-Smith 2017).

Currently, intensive and repeated sample collection at both broad and fine scales continues to be critical for monitoring regional infection rates among amphibian communities over time (Gray et al. 2017). Perhaps more importantly, a paucity of information exists on the impact of concurrent infections with fungal and viral pathogens on frog and salamander populations. Research into concurrent amphibian infections is relatively new; therefore, it remains unclear how amphibian populations respond to the presence of more than one pathogen, particularly in terms of mortality level (Souza et al. 2012). In the last decade, a few studies have shown lower prevalence of concurrent *Bd*/RV infections in wild populations (i.e., 5%: Souza et al. 2012, Whitfield et al. 2013; 20–30%: Warne et al. 2016) compared with captive populations (i.e., 100%: Miller et al. 2008, Kik et al. 2012; 49%: Warne et al. 2016). Because most existing studies on concurrent infections have focused on a small taxonomic group and/or sample sizes, it is difficult to draw broader inferences from reported prevalence rates (i.e., four individuals, one species: Fox et al. 2006; 12 individuals, three species: Kik et al. 2012; 97 individuals, one species: Souza et al. 2012).

Recently, research focused on amphibians in Oklahoma has shown *Bd* prevalence rates from 30% to 100% at several localities in southeastern Oklahoma (Marhanka et al. 2017), whereas no studies of RV prevalence rates in this area have yet been published. Despite a marked increase in research across the state, little remains known about the long-term impacts of infectious diseases on native species, and no studies have investigated large-scale prevalence of concurrent infections among amphibian communities. Herein, we report on the first documented cases of *Bd* and RV in northeastern Oklahoma and describe the rates of concurrent *Bd*/RV infection.

METHODS

Field data collection.— Fieldwork was conducted in April and September–October 2015 and March–June 2016 in northeastern Oklahoma, with surveys conducted in the following counties: Adair, Cherokee, Delaware, Mayes, Muskogee, Nowata, Osage, and Sequoyah (Figure 1). Survey efforts focused on ponds, streams, and wetlands in Oklahoma Department of Wildlife Conservation (ODWC) Wildlife Management Areas (WMAs), National Wildlife Refuges, or Oklahoma State Park lands. Overall, 16 distinct sites were sampled, with 3–133 individuals sampled per site (total $N = 514$ individuals). Each site was sampled for a total of 24–48 h, with amphibians captured by hand,

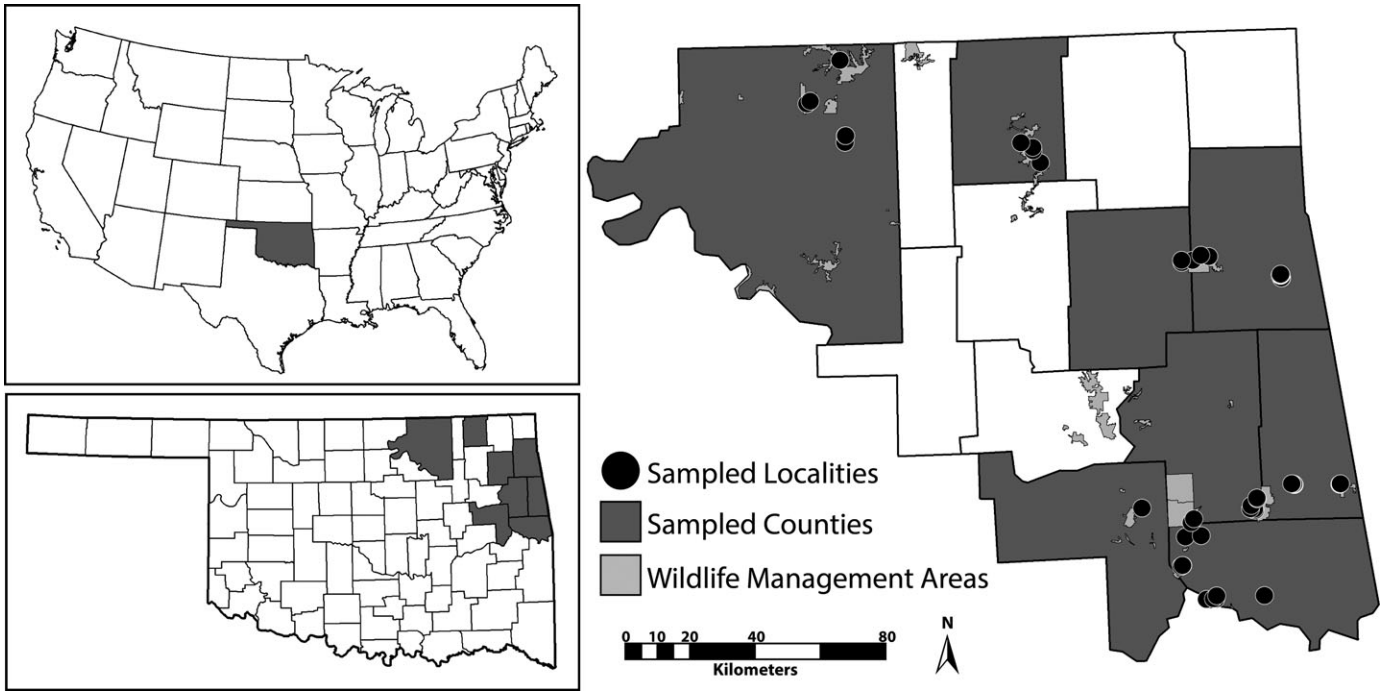

FIGURE 1. Map of the United States (upper left), showing the location of the state of Oklahoma. Map of Oklahoma (bottom left) shows the focal counties of this study in dark gray. Close-up map of northeastern Oklahoma (right) depicts the sites (black circles) and counties (dark gray) that were sampled. Polygons indicating Oklahoma Department of Wildlife Conservation Wildlife Management Areas (light gray) are also shown for reference.

aquatic trap, dip net, or seine and then kept in individual plastic bags until they were swabbed and euthanized. All reusable equipment was sterilized between sample collections using 10% bleach, as recommended by Gray et al. (2017). Amphibians were transported back to the Sam Noble Oklahoma Museum of Natural History (SNOMNH) prior to disease sample collection.

Amphibian skin was swabbed for fungal spores on the ventral, lateral, and dorsal portions of the trunk; on the hind limbs (five swipes per region); and on the webbing between hind limb toes, where the highest concentration of *Bd* zoospores is often found (Lannoo et al. 2011). Animals were then euthanized via submersion in a chlorobutanol solution prior to the dissection of genetic material from the liver for RV screening, as recommended by St-Amour and Lesbarrères (2007) and Gray et al. (2012). Vouchered specimens were fixed in 10% buffered formalin and transferred to 70% ethanol within 1 week after preservation, in accordance with herpetological museum best practices (Simmons 2015). All vouchered material was deposited at the SNOMNH: preserved specimens were deposited in the Herpetology Collection, and tissue samples were deposited in the Oklahoma Collection of Genomic Resources. All samples were stored in a −20°C freezer from the time of sample collection up to DNA extraction; swab storage varied from 5 to 6 months, and tissue storage varied from 6 to 8 months. Each swab was stored dry in a 1.5-mL microcentrifuge tube, and tissues were stored in 95% ethanol in a 2-mL cryovial. Throughout swabbing and tissue collection, sterile techniques were employed to prevent cross-contamination (Gray et al. 2017). All animals were collected under ODWC Scientific Collecting Permits accorded to C.D.S. (2015: Permit 6147; 2016: Permit 6666) and J.L.W. (2015: Permit 6148; 2016: Permit 6503). Research was approved by the Institutional Animal Care and Use Committee (Protocol Number R14-026) at the University of Oklahoma.

Extraction of DNA and disease screening.—The DNA from *Bd* swabs was extracted with the PrepMan Ultra (Life Technologies) reagent and protocol (Cheng et al. 2011); DNA was also extracted from liver tissue samples via a high salt extraction method (Esselstyn et al. 2008). Both types of DNA extract from each amphibian were then stored at −20°C until used for pathogen screening. Quantitative PCR (qPCR) techniques were utilized to determine the presence/absence of *Bd* or RV in collected samples and to estimate the number of gene copies (infection load) per sample (Kerby et al. 2013; Davis and Kerby 2016). Prior to qPCR analysis, DNA extracts were diluted 1:10 for *Bd* swab samples and 1:2 for RV tissue samples to reduce the effects of potential inhibitors, following standardized published protocols (Hyatt et al.

2007; Watters et al. 2016; Marhanka et al. 2017). Primers used for the *Bd* assay target the internal transcribed spacer (ITS-1) ribosomal RNA gene (forward primer: ITS1-3 Chytr; reverse primer: 5.8S) as described by Boyle et al. (2004), while those used for RV target the major capsid protein (MCP) gene as described by Forson and Storfer (2006).

The qPCR assays of samples were run in triplicate (three reactions per sample) with standard dilutions of known pathogen gene copy number (DNA quantities) based on customized double-stranded DNA fragments (gBlocks; Integrated DNA Technologies) from ITS-1 (*Bd*) and MCP (RV). The 750-base-pair gBlocks contained sequences of the target PCR fragment for each pathogen and were run with the samples to create a standard curve, which allowed us to quantify the gene copy number in samples. Four standards were created for each pathogen gBlock by serially diluting the gBlock stock of known DNA quantities into concentrations ranging from 1×10^1 to 1×10^4. Standards of 1×10^1 to 1×10^3 were run in triplicate, with a 1×10^4 standard run in duplicate next to a single negative control sample (molecular grade water), per plate. Disease loads for 2015 samples were quantified using a StepOnePlus Real-Time PCR System and StepOnePlus software version 2.3 (Applied Biosystems, Inc. [ABI]) at the University of South Dakota; for 2016 samples, QuantStudio version 3.0 (ABI) was used at the SNOMNH Genomics Core Facility. The qPCR protocol followed Kerby et al. (2013) for *Bd* and Davis and Kerby (2016) for RV, with 3 μL of DNA added to a 7-μL cocktail solution (TaqMan Fast Advanced Master Mix, ABI; forward primer; reverse primer; TaqMan probe, ABI; and molecular grade water). Both *Bd* and RV were run on qPCR systems for 50 cycles. A sample was considered positive for a pathogen if (1) at least two of the three wells were amplified and (2) the resulting mean gene copy number was above 1.0 (Davis and Kerby 2016; Watters et al. 2016; Marhanka et al. 2017). To determine final infection load per sample, *Bd* and RV gene copy numbers were multiplied by the dilution factor (10 for *Bd*; 2 for RV) and then were multiplied again by a value in order to reach the total original extraction volume for each sample. Differences in disease prevalence between amphibian orders (Anura versus Caudata) were analyzed with a chi-square test of independence (χ^2). For those species with sample sizes greater than 20 and a nonzero value for each category (not infected; infected with RV only [RV+]; infected with *Bd* only [*Bd*+]; and infected with both RV and *Bd* [RV+/*Bd*+]), additional chi-square tests of independence were completed. For mean gene copy number per sample (i.e., infection load), results were analyzed using nonparametric Kruskal–Wallis tests for all species pooled and for those species with greater than five individuals testing positive per disease.

RESULTS

Out of 514 individuals screened for the two focal pathogens, 46.30% showed no infection, 7.20% were infected with RV only (RV+), 37.35% were infected with *Bd* only (*Bd*+), and 9.14% were infected with both RV and *Bd* (RV+/*Bd*+; Table 1). When comparing Anura (frogs and toads) to Caudata (salamanders and newts), anurans had both the highest *Bd*+ prevalence (39.93% versus 33.65%) and the highest RV+/*Bd*+ prevalence (11.22% versus 6.16%; Table 1). However, higher RV+ prevalence was observed in Caudata (9.00%) than in Anura (5.94%; Table 1). These observed differences in infection patterns between Caudata and Anura were statistically significant ($\chi^2 = 8.40$, df = 3, $P = 0.04$; Table 2).

Among the species with greater sample sizes available for analysis ($N > 20$), the *Bd*+ rate was highest for eastern newts *Notopthalmus viridescens* (81.48%) followed by pickerel frogs *R. palustris* (76.92%; Table 1). For RV, the highest prevalence was 19.35% in western slimy salamanders *Plethodon albagula*, followed closely by cave salamanders *Eurycea lucifuga* at 19.05% (Table 1). Of the 22 species screened in this study, 11 showed evidence of dual infections. For those individuals infected with both pathogens, American bullfrogs *R. catesbeiana* had the highest prevalence at 34.33% (Table 1). Additional chi-square tests of independence were performed on those species with sufficient sample sizes within Anura (Blanchard's cricket frog *Acris blanchardi*, Cope's gray tree frog *Hyla chrysoscelis*/gray tree frog *H. versicolor*, American bullfrog, and southern leopard frog *R. sphenocephala*) and Caudata (long-tailed salamander *E. longicauda*, Oklahoma salamander *E. tynerensis*, and western slimy salamander). Statistically distinct infection patterns were visible for several pairs of the anuran species, but for Caudata, the only significant difference was that between the long-tailed salamander and the Oklahoma salamander (Table 3).

Due to the high variation in infection load for both pathogens (ranging from 10^3 to 10^7) and small sample sizes, all amphibian species were pooled for analysis. Infection load was significantly higher for *Bd* than for RV, regardless of single or dual infection ($H = 40.422$, $P < 0.001$; Figure 2; Table 1). When completing a further comparison of infection load for each pathogen in single versus dual infections, we found that when species were combined, the *Bd* infection load was significantly higher in the individuals infected with *Bd* only ($H = 4.720$, $P = 0.029$), but we observed no difference among RV+ individuals ($H = 2.396$, $P = 0.122$; Figure 2). However, in American bullfrogs and southern leopard frogs, for which we had sufficient sample sizes per category ($n > 5$), we found the opposite pattern in that infection loads were highest in *Bd*+ individuals that were co-infected as compared to individuals infected by a single pathogen only

TABLE 1. List of amphibian species screened for infectious disease in northeastern Oklahoma, with summaries by total sample size (*N*) and infection prevalence values (% by number observed). Additionally, the mean infection load (MIL; ±SD) is represented for each individual category (RV+ = ranavirus positive; *Bd*+ = *Batrachochytrium dendrobatidis* positive); for dual infection (RV+/*Bd*+), the MIL is provided for each pathogen (RV and then *Bd*).

Classification	*N*	Not infected	RV+ only MIL	*Bd*+ only MIL	RV+/*Bd*+ RV MIL, *Bd* MIL
Anura (total)	303	42.90% (130)	5.94% (18) 27,306.78 ± 46,995.58	39.93% (121) 2,008,944.97 ± 15,488,936.13	11.22% (34) 43,104.89 ± 97,799.52 483,028.57 ± 953,367.78
Bufonidae					
American toad *Anaxyrus americanus*	16	62.50% (10)	12.50% (2) 3,936.25 ± 395.99	25.00% (4) 544,719.67 ± 775,816.47	0.00% (0) N/A
Woodhouse's toad *A. woodhousii*	3	66.67% (2)	0.00% (0) N/A	33.33% (1) 9,128.45 ± N/A	0.00% (0) N/A
Hylidae					
Blanchard's cricket frog *Acris blanchardi*	76	47.38% (36)	1.32% (1) 1,911.51 ± N/A	50.00% (38) 5,729,073.51 ± 27,014,740.87	1.32% (1) 3,321.15 ± N/A 189,620.65 ± N/A
Cope's gray tree frog *Hyla chrysoscelis*/ gray tree frog *H. versicolor*	41	75.61% (31)	2.44% (1) 16,409.01 ± N/A	19.51% (8) 26,983.42 ± 75,120.29	2.44% (1) 252.83 ± N/A 36,042.21 ± N/A
Spring peeper *Pseudacris crucifer*	11	27.27% (3)	0.00% (0) N/A	72.72% (8) 433,788.45 ± 891,931.18	0.00% (0) N/A
Boreal chorus frog *P. maculata*	7	14.29% (1)	0.00% (0) N/A	85.71% (6) 66,431.07 ± 125,765.93	0.00% (0) N/A
Microhylidae					
Eastern narrow-mouthed toad *Gastrophryne carolinensis*	1	0.00% (0)	0.00% (0) N/A	100.00% (1) 296.67 ± N/A	0.00% (0) N/A
Ranidae					
American bullfrog *Rana catesbeiana*	67	31.31% (21)	13.43% (9) 29,689.77 ± 35,172.17	20.896% (14) 548,767.44 ± 1,759,536.66	34.33% (23) 31,197.05 ± 113,127.47 554,434.86 ± 1,075,454.77
Green frog *R. clamitans*	20	50.00% (10)	0.00% (0) N/A	50.00% (10) 208,829.41 ± 707,258.9548	0.00% (0) N/A
Pickerel frog *R. palustris*	13	15.39% (2)	0.00% (0) N/A	76.92% (10) 209,094.08 ± 492,323.46	7.69% (1) 737,648.29 ± N/A 450.50 ± N/A
Southern leopard frog *R. sphenocephala*	48	29.17% (14)	10.42% (5) 39,624.21 ± 79,030.66	43.75% (21) 208,829.41 ± 707,258.95	16.67% (8) 851.46 ± 1,526.82 430,607.04 ± 531,813.02
Caudata (total)	211	51.18% (108)	9.00% (19) 743.15 ± 528.12	33.65% (71) 93,461.51 ± 235,282.46	6.16% (13) 73,487.07 ± 195,969.197 77,654.16 ± 139,403.73

TABLE 1. Continued.

Classification	*N*	Not infected	RV+ only MIL	*Bd*+ only MIL	RV+/*Bd*+ RV MIL, *Bd* MIL
Ambystomatidae					
Ringed salamander *Ambystoma annulatum*	4	0.00% (0)	50.00% (2) 205.02 ± 92.54	0.00% (0) N/A	50.00% (2) 391.47 ± 142.37 231,035.16 ± 324,729.22
Spotted salamander *A. maculatum*	7	100.00% (7)	0.00% (0) N/A	0.00% (0) N/A	0.00% (0) N/A
Small-mouthed salamander *A. texanum*	2	50.00% (1)	0.00% (0) N/A	0.00% (0) N/A	50.00% (1) 208.26 ± N/A 1,435.6 ± N/A
Plethodontidae					
Long-tailed salamander *Eurycea longicauda*	37	43.24% (16)	13.51% (5) 1,129.27 ± 482.43	37.84% (14) 142,883.72 ± 29,115.50	5.41% (2) 1,474.48 ± 261.35 2,976.66 ± 4,163.35
Cave salamander *E. lucifuga*	21	66.67% (14)	19.05% (4) 828.44 ± 652.80	14.29% (3) 48,439.65 ± 83,055.61	0.00% (0) N/A
Many-ribbed salamander *E. multiplicata*	4	50.00% (2)	25.00% (1) 837.08 ± N/A	25.00% (1) 8,448.49 ± N/A	0.00% (0) N/A
Grotto salamander *E. spelaea*	1	100.00% (1)	0.00% (0) N/A	0.00% (0) N/A	0.00% (0) N/A
Oklahoma salamander *E. tynerensis*	38	78.95% (30)	2.63% (1) 340.46 ± N/A	13.16% (5) 2,471.09 ± 2,845.90	5.26% (2) 192.90 ± 130.13 3,507.52 ± 3,709.29
Western slimy salamander *Plethodon albagula*	31	67.74% (21)	19.35% (6) 595.35 ± 391.27	9.68% (3) 7,009.78 ± 11,277.54	3.23% (1) 183.40 ± N/A 241,933.20 ± N/A
Ozark zigzag salamander *P. angusticlavius*	12	91.67% (11)	0.00% (0) N/A	8.33% (1) 52.47 ± N/A	0.00% (0) N/A
Salamandridae					
Eastern newt *Notopthalmus viridescens*	54	9.26% (5)	0.00% (0) N/A	81.48% (44) 100,977.16 ± 266,607.48	9.26% (5) 190,164.51 ± 267,452.20 58,219.31 ± 67,951.95
All amphibians	514	46.30% (238)	7.20% (37) 13,665.99 ± 34,989.22	37.35% (192) 1,300,615.15 ± 12,682,081.86	9.14% (47) 51,555.55 ± 130,999.10 370,903.73 ± 839,574.58

(American bullfrog: $H = 13.592$, $P = 0.004$; southern leopard frog: $H = 13.086$, $P < 0.001$; Table 1).

DISCUSSION

Numerous factors are contributing to global patterns of amphibian declines, including habitat loss, pollution inputs to freshwater ecosystems, climate change, and infectious diseases; collectively, they play interconnected roles in exacerbating stress on species' survival (Daszak et al. 1999; Berger et al. 2016; Rollins-Smith 2017; Scheele et al. 2017). Without a robust threat assessment analysis for amphibian species in Oklahoma, it is difficult to determine which of the many environmental stressors are major drivers in the spread and prevalence of *Bd* and RV regionally. Furthermore, the severity of the problem is poorly understood, both in relation to the degree of state and federal habitat monitoring and the impact that popular recreational activities (i.e., hunting and fishing) have on the spread of infectious diseases. For example, the State of

TABLE 2. Results of chi-square (χ^2) tests of independence employed to identify distinct patterns of infectious status (RV+ = ranavirus positive; *Bd*+ = *Batrachochytrium dendrobatidis* positive; RV+/*Bd*+ = dual infection) between two amphibian orders, Anura and Caudata. Asterisks indicate significant differences, with a significance level of 0.05.

Infection status comparison	χ^2	*P*
Not infected versus RV+ only	3.09	0.50
Not infected versus *Bd*+ only	5.05	0.08
Not infected versus RV+/*Bd*+	2.69	0.03*
RV+ only versus *Bd*+ only	4.93	0.10
RV+ only versus RV+/*Bd*+	1.44	0.03*
Bd+ only versus RV+/*Bd*+	3.09	0.23

TABLE 3. Results of the pairwise comparisons of infectious status by amphibian species, as evaluated by chi-square (χ^2) tests of independence and grouped by order. Asterisks indicate significant differences, with a significance level of 0.05.

Species comparison	χ^2	*P*
Anura		
Blanchard's cricket frog versus Cope's gray tree frog/gray tree frog	10.399	0.01*
Blanchard's cricket frog versus American bullfrog	41.188	<0.01*
Blanchard's cricket frog versus southern leopard frog	17.246	<0.01*
Cope's gray tree frog/gray tree frog versus American bullfrog	25.335	<0.01*
Cope's gray tree frog/gray tree frog versus southern leopard frog	19.934	<0.01*
American bullfrog versus southern leopard frog	8.288	0.04*
Caudata		
Long-tailed salamander versus Oklahoma salamander	11.179	0.01*
Long-tailed salamander versus western slimy salamander	7.748	0.052
Oklahoma salamander versus western slimy salamander	5.338	0.149

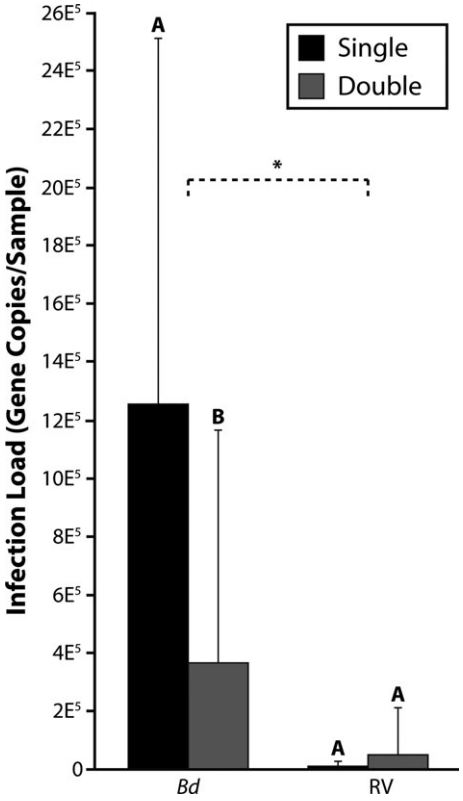

FIGURE 2. Infection load (+SD) by pathogen (as calculated by mean gene copies per sample) for both single (black) and dual (gray) infections (*Bd* = *Batrachochytrium dendrobatidis*; RV = ranavirus) in northeastern Oklahoma amphibians. The asterisk represents a significant difference in infection load between pathogen types when individuals with both single and dual pathogens were pooled. Nonoverlapping letters above the bars in each category represent significant differences in infection load within each pathogen type (single versus dual).

Oklahoma currently has 73 land holdings designated as WMAs; with a few exceptions (i.e., U.S. Fish and Wildlife Service refuges and several military bases), these WMAs represent some of the more pristine and undisturbed natural habitats throughout the state. Many of these areas are used extensively by the public, both from a recreational standpoint and for research and educational endeavors, which can contribute to the spread of infectious disease by direct transmission of *Bd* spores and RV virions on recreational equipment and the movement of larval salamanders as bait (Jancovich et al. 2005; Picco and Collins 2008; Schloegel et al. 2009; Gray et al. 2017).

In addition, the spread of amphibian pathogens in both the United States and abroad has now been linked to trade activities associated with the pet and food industries (Schlaepfer et al. 2005). Ranavirus strains in China share close phylogenetic similarity with a frog virus 3 (FV3) strain associated with North American populations of the northern leopard frog *R. pipiens* (Zhang et al. 2001; Schloegel et al. 2009). Farmed amphibians are known to be carriers of both focal pathogens (Zhang et al. 2001; Mazzoni et al. 2003), and a nascent body of literature highlights the role played by the American bullfrog as a potential vector for disease spread (Daszak et al. 2004; Garner et al. 2006; Schloegel et al. 2009; Gervaisi et al. 2011). Unfortunately, with all state-listed amphibian species of greatest conservation concern documented within or in close proximity to WMA habitats, such wildlife trade and bait distribution pose an even greater risk than is likely appreciated. Despite this remaining uncertainty,

the results of our study are clear in showing significant infection levels for *Bd* present in northeastern Oklahoma (42%), similar to high levels of infection observed recently across the southeastern portion of the state (68%; Marhanka et al. 2017). Such high prevalence of *Bd* among amphibian species, particularly the eastern newt (81%), has now been observed across much of Oklahoma and even throughout the United States (Rothermel et al. 2008; Groner and Relyea 2010; Marhanka et al. 2017).

In contrast, the observed RV infection levels by species were unexpectedly low in comparison with other studies describing RV throughout the USA. For example, Davis and Kerby (2016) found much higher levels of RV prevalence in hylids (Blanchard's cricket frog: 56%; Cope's gray tree frog: 53%) and ranids (American bullfrog: 56%) from northeastern Nebraska than we found in the present study. We observed a high prevalence of RV infection in plethodontid salamanders (western slimy salamander: 19%; cave salamander: 19%). Higher infection levels of RV within the genus *Plethodon* are particularly interesting, as members of this genus are fully terrestrial (and often fossorial), and it is unlikely that they were infected via direct or indirect contact with an infected amphibian in a water body. In comparison, *Eurycea* spp. have an aquatic component to their life cycle and therefore may show an increased susceptibility to infection at early stages of development (Gray et al. 2009a). Moreover, Muletz et al. (2014) found no incidence of concurrent *Bd* and RV infection in the plethodontid taxa they sampled (genera *Desmognathus*, *Eurycea*, and *Plethodon*), even in areas where *Bd* infection rates were high in other amphibians.

The observed patterns of concurrent infection in this study are higher than expected relative to other published research on wild-caught amphibians in North America (Souza et al. 2012; Whitfield et al. 2013; Muletz et al. 2014). This could be the result of a much larger sample size or larger geographic area of disease screening, or perhaps the incidence of simultaneous infections is more common than we are aware (Warne et al. 2016). Both *Bd* and RV are recognized to cause significant physiological stress to amphibian populations (Rollins-Smith 2017); thus, infection by one or both pathogens likely depresses the host amphibian's immune system, possibly making it difficult to fight off other infections (Rollins-Smith et al. 2011; Rollins-Smith 2017). We did not observe any mortality events throughout this study, despite recording significant rates of dual infection. This lack of visible mortality could be due to several distinct possibilities: (1) amphibians with higher infection loads for both pathogens succumbed to one or both diseases prior to our survey efforts; (2) our 24–48-h sampling period may have been too limited to observe mortality in the field (Gray et al. 2009b; Woodhams et al. 2011); and (3) a large number of documented mortality events in North America occur among amphibian larvae rather than adults (Cunningham et al. 1996; Green et al. 2002), but since our survey efforts focused largely on adults, we may have missed signs of larval die-offs.

The observed pattern in infection load for *Bd* in single versus dual infections (when all species were pooled) is of particular interest, as it may indicate disease competition within the host or a heightened immune system response due to the presence of both pathogens. However, recent laboratory research has shown that *Bd* infections normally suppress immune systems through lymphocyte inhibition in the Panamanian golden frog *Atelopus zeteki* (Ellison et al. 2014) and the African clawed frog *Xenopus laevis* (Fites et al. 2014). In addition, research on RV-infected African clawed frogs showed an increase in the production of FV3 antibodies in response to a second RV infection as compared to their first infection (Maniero et al. 2006). In contrast, when statistical analyses were completed for the American bullfrog and the southern leopard frog individually, our results indicated that *Bd*+ infection loads were statistically higher in dual infections rather than single infections for both species. Documented tolerance to both pathogens (Garner et al. 2006; Gervaisi et al. 2011) and the fact that ranid species with long larval development periods are less susceptible to RV infections (Miller et al. 2008) may account for the higher infection loads observed. Varying immune responses, combined with additional potential environmental stressors, make it difficult to determine why we have observed these differing infection load patterns. As neither immune responses nor long-term survivorship were components of our study's survey efforts, we are now presented with several interesting avenues for future research.

Six amphibian Species of Greatest Conservation Need (SGCN) are found in northeast Oklahoma: the ringed salamander, many-ribbed salamander, grotto salamander, Oklahoma salamander, Ozark zigzag salamander, and crawfish frog *R. areolatus* (ODWC 2015). Of these species, all but the crawfish frog were observed and collected at our sampling sites and screened for disease. Although sample sizes were small for the focal SGCN, we observed RV+ or *Bd*+ individuals from all species except the grotto salamander. We also observed instances of co-infection in both ringed salamanders (50%) and Oklahoma salamanders (5.26%). With continued interest in the population health of these six species among state wildlife managers and conservation management programs, efforts should be made to regularly monitor disease prevalence in and population health of these taxa on an annual basis. The importance of repeated sampling and monitoring cannot be overemphasized, since several recent, long-term studies of amphibian communities have confirmed significant population declines associated with infectious disease (Teacher et al. 2010; Price et al. 2014). In addition to repeat

monitoring of amphibian communities, increased attention must be paid to the health of amphibian larvae. Less information is available on pathogen prevalence at these earlier stages of development, and amphibian larvae experience increased susceptibility to infection, particularly by RV (Hoverman et al. 2010; Bayley et al. 2013; Landsberg et al. 2013; Reeve et al. 2013; Grayfer et al. 2014; Koubourli et al. 2017).

Our study joins a growing body of research on amphibian infectious diseases in Oklahoma; together, these studies have improved our understanding of infection patterns observed across the state. In addition, the results of the present study have provided the first large-scale assessment of two emergent pathogens among entire communities of amphibians in Oklahoma, generating a baseline for future research into the organismal- and community-level impacts of concurrent infection on native species of vertebrates.

ACKNOWLEDGMENTS

Funding for this research was provided by an ODWC grant (Number F14F01225 [T-80-1]) and an Oklahoma City Zoo and Botanical Gardens Conservation Action Now grant to C.D.S. and J.L.W. Work was further supported in part by a grant from the Research Council of the University of Oklahoma Norman Campus to C.D.S. We appreciate the members of the Siler Laboratory for assistance in fieldwork, DNA extractions, and pathogen screening. We also thank the two anonymous reviewers for their critical feedback and helpful insights. There is no conflict of interest declared in this article.

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