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Investigating Ranavirus Prevalence in Central Oklahoma, USA, Amphibians

Ranaviruses are viral pathogens that cause systemic problems in the liver, digestive tract, and kidneys of amphibians (Daszak et al. 1999) and are among the infectious diseases implicated in the global decline of amphibian populations (Kiesecker 2002; Blaustein et al. 2003; Harp and Pentranka 2006; Lips et al. 2006; Raffel et al. 2006). To date, little is known about ranavirus (RV) prevalence among wild amphibian populations in the central United States (but see Duffus et al. 2015; Kirschman et al. 2017). Although the historical and recent patterns of pathogen prevalence of another amphibian pathogen, *Batrachochytrium dendrobatidis* (*Bd*), have been documented throughout Oklahoma, USA (Steiner and Lehtinen 2008; Lannoo et al. 2011; Watters et al. 2016, 2018; Marhanka et al. 2017), few studies have focused on RV in the state. In this study, we provide the first assessment of RV infection among wild amphibian populations in central Oklahoma.

We conducted repeated sampling trips to survey and screen amphibians across a five-month period from four sites in Cleveland and Oklahoma counties, in central Oklahoma (Fig. 1). Sampling occurred during Spring (March and May), Summer (June and August), and Fall (October) in 2015. At each site, all amphibian species encountered were captured and sampled, regardless of life stage. Ten anuran species (Tables 1, 2) were collected for a total of 198 adults, 16 juveniles, 2 metamorphs, and 23 tadpoles. We categorized adults as full-grown anurans; juveniles as metamorphosed, but smaller, individuals with four limbs and no tail; metamorphs as individuals with four limbs and a reduced tail; and tadpoles as individuals with full tails and fewer than four limbs. In Oklahoma County, we surveyed a site adjacent to a stream outflow of Northeast Lake near the Oklahoma City Zoo (Fig. 1: Site 1), and a wetland site located within a residential neighborhood at NE 50th St. and N. Bartell Rd. (Fig. 1; Site 2). We sampled two sites in Cleveland County: Sutton Urban Wilderness Park (Fig. 1: Site 3) and Lexington Wildlife Management Area (WMA; Fig. 1: Site 4). The surveys were conducted on either the second or fourth week of each focal month. When different sites were surveyed in the same month, site assessments were conducted within ten days of each other; however, Sutton Urban Wilderness Park (Site 3) was the only site surveyed twice in one month. In addition, Sutton Urban Wilderness Park (Site 3) was not sampled in May and the wetland in the residential neighborhood (Site 2) was not sampled in March, August, or October, due to low water levels and the inability to find amphibians. Between ponds, all equipment, waders, and boots were decontaminated using a 10% bleach solution to ensure pathogens would not be spread among sample sites (Gray et al. 2017).

Animals were collected in the field at night, by hand, dip net, or seine. Adult, juvenile, and metamorph anurans were placed in individual clean collection bags. If multiple tadpoles of the same species were collected at a site, they were held in one collection bag for up to 24 h before being euthanized and processed as a tadpole lot, with 5–10 individuals subsampled for RV from each group. Tadpoles that were not sampled for RV were vouchered as a group representing a developmental series for an unrelated study. We did not encounter or sample any individuals that were

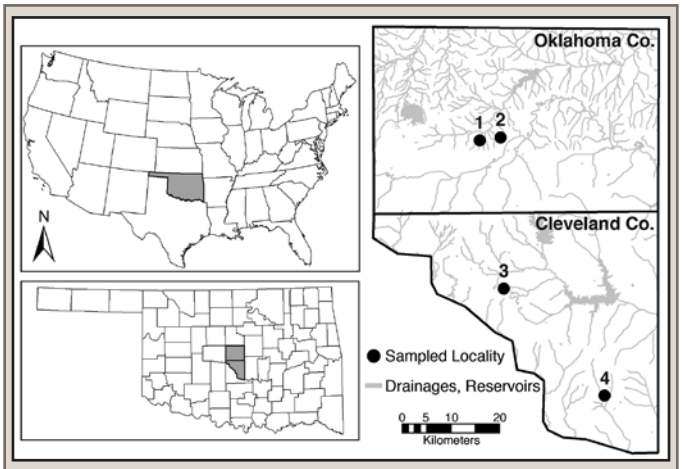

FIG. 1. Map of samples sites for amphibians tested for ranavirus in central Oklahoma, USA. Black circles represent the sampled sites: near the Oklahoma City Zoo (Site 1: 35.51705°N, 97.47129°W); intersection of NE 50th St. and N. Bartell Rd. in Oklahoma City (Site 2: 35.52229°N, 97.43267°W); Sutton Urban Wilderness Park (Site 3: 35.24266°N, 97.42689°W); Lexington Wildlife Management Area (Site 4: 35.04437°N, 97.24004°W).

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TABLE 1. Prevalence of ranavirus (RV) in amphibian populations in central Oklahoma, USA, summarized per site across sampling dates, including species, life stage, number of individuals who tested positive out of the total number sampled (RV+/total), Mean Viral Load, and Infection Load Standard Deviation (Std. Dev.). We report high RV prevalence among *Lithobates catesbeianus* tadpoles collected from Site 1 on 21 March 2015; however, because multiple tadpoles of the same species were held in one bag after collection, we removed these individuals from all statistical summaries because of the potential for false positives (Brunner et al. 2007; Gray et al. 2018).

Site	Date (2015)	Species	Life stage	Tissue type	Number RV+/total	Mean viral load	Infection load Std. Dev.
Oklahoma City Zoo (Site 1)	21-Mar	*Lithobates catesbeianus*	Tadpole	Tail	14/19	412	240
	12-May	*Acris blanchardi*	Adult	Liver	0/6	N/A	N/A
	24-Jun	*Acris blanchardi*	Adult	Liver	0/4	N/A	N/A
		Anaxyrus woodhousii	Adult	Liver	1/2	2,221	N/A
		Lithobates catesbeianus	Adult	Liver	0/1	N/A	N/A
		Lithobates sphenocephalus	Adult	Liver	0/3	N/A	N/A
	22-Aug	*Acris blanchardi*	Adult	Toe	0/1	N/A	N/A
		Lithobates catesbeianus	Adult	Toe	0/3	N/A	N/A
			Tadpole	Tail	0/1	N/A	N/A
	29-Oct	*Acris blanchardi*	Adult	Toe	2/18	151	42
		Lithobates catesbeianus	Adult	Toe	0/2	N/A	N/A
50th & Bartell, Oklahoma City (Site 2)	12-May	*Acris blanchardi*	Adult	Liver	0/3	N/A	N/A
		Anaxyrus americanus	Adult	Liver	0/1	N/A	N/A
		Anaxyrus woodhousii	Adult	Liver	0/6	N/A	N/A
		Gastrophryne olivacea	Adult	Liver	1/3	1,264	N/A
		Hyla chrysoscelis/versicolor	Adult	Liver	0/10	N/A	N/A
		Lithobates catesbeianus	Juvenile	Liver	0/1	N/A	N/A
		Pseudacris clarkii	Adult	Liver	0/2	N/A	N/A
		Pseudacris fouquettei	Adult	Liver	0/1	N/A	N/A
	24-June	*Hyla cinerea*	Adult	Liver	1/2	753	N/A
		Lithobates catesbeianus	Adult	Liver	1/3	295,920	N/A
			Metamorph	Tail	0/1	N/A	N/A
		Lithobates sphenocephalus	Tadpole	Tail	1/1	1,060	N/A
Sutton Urban Wilderness Park (Site 3)	12-Mar	*Acris blanchardi*	Adult	Liver	1/7	283	N/A
		Lithobates catesbeianus	Tadpole	Tail	1/1	927	N/A
	23-Mar	*Acris blanchardi*	Adult	Liver	0/6	N/A	N/A
		Lithobates catesbeianus	Adult	Liver	0/13	N/A	N/A
				Toe	0/1	N/A	N/A
	23-June	*Acris blanchardi*	Adult	Liver	0/9	N/A	N/A
		Lithobates sphenocephalus	Adult	Liver	0/1	N/A	N/A
			Juvenile	Liver	0/10	N/A	N/A
			Tadpole	Tail	0/1	N/A	N/A
			Metamorph	Tail	1/1	245	N/A
	21-Aug	*Acris blanchardi*	Adult	Toe	5/8	851	894
		Gastrophryne olivacea	Adult	Liver	0/1	N/A	N/A
		Hyla chrysoscelis/versicolor	Adult	Toe	1/2	238	N/A
		Lithobates sphenocephalus	Adult	Toe	1/12	210	N/A
	15-Oct	*Acris blanchardi*	Adult	Toe	2/6	626	13
		Lithobates catesbeianus	Adult	Toe	0/6	N/A	N/A
			Juvenile	Toe	0/3	N/A	N/A
		Lithobates sphenocephalus	Adult	Toe	2/11	322	141
Lexington WMA (Site 4)	11-Mar	*Acris blanchardi*	Adult	Toe	2/6	634	162
		Lithobates sphenocephalus	Adult	Toe	0/1	N/A	N/A
	10-May	*Acris blanchardi*	Adult	Liver	1/2	109	N/A
		Gastrophryne olivacea	Adult	Liver	0/1	N/A	N/A
		Hyla chrysoscelis/versicolor	Adult	Liver	0/1	N/A	N/A
		Lithobates sphenocephalus	Adult	Liver	0/1	N/A	N/A
	22-June	*Acris blanchardi*	Adult	Liver	2/10	925	969
		Hyla chrysoscelis/versicolor	Adult	Liver	0/3	N/A	N/A
		Lithobates sphenocephalus	Juvenile	Liver	1/1	634	N/A
	20-Aug	*Lithobates catesbeianus*	Adult	Liver	0/1	N/A	N/A
		Lithobates sphenocephalus	Adult	Liver	1/2	910	N/A
	19-Oct	*Acris blanchardi*	Adult	Toe	1/12	349	N/A
		Anaxyrus americanus	Adult	Liver	0/1	N/A	N/A
		Hyla chrysoscelis/versicolor	Juvenile	Liver	0/1	N/A	N/A
		Lithobates sphenocephalus	Adult	Liver	1/3	342	N/A

TABLE 2. Comparison of ranavirus (RV) prevalence by host amphibian species from Oklahoma, USA, including sample size (N), number of individuals who tested negative (RV-), number of individuals who tested positive (RV+), Mean Viral Load and Infection Load Standard Deviation (Std. Dev.). We report the abnormally high infection load for a liver sample collected from a single *Lithobates catesbeianus*; however, this sample was not included in any Mean Viral Load calculations to avoid potential biases. Additionally, the 19 *L. catesbeianus* tadpoles collected from Site 1 in March were removed from the total calculations reported here because of the potential for false positives, see text for full explanation.

Species	N	Tissue type	RV-	RV+	RV%	Mean viral load	Infection load Std. Dev.
Bufonidae							
Anaxyrus americanus	2	Liver	2	0	0%	N/A	N/A
Anaxyrus woodhousii	8	Liver	7	1	12.5%	2,221	N/A
Hylidae							
Acris blanchardi	47	Liver	43	4	8.5%	560	704
	51	Toe	39	12	23.5%	619	603
Hyla chrysoscelis/versicolor	15	Liver	15	0	0%	N/A	N/A
	2	Toe	1	1	50%	238	N/A
Hyla cinerea	2	Liver	1	1	50%	753	N/A
Pseudacris clarkii	2	Liver	2	0	0%	N/A	N/A
Pseudacris fouquettei	1	Liver	1	0	0%	N/A	N/A
Microhylidae							
Gastrophryne olivacea	5	Liver	4	1	20%	1,264	N/A
Ranidae							
Lithobates catesbeianus	19	Liver	18	1	5.3%	295,920	N/A
	3	Tail	2	1	33.3%	927	N/A
	15	Toe	15	0	0%	N/A	N/A
Lithobates sphenocephalus	21	Liver	18	3	14.3%	628	284
	3	Tail	1	2	66.7%	653	577
	24	Toe	21	3	12.5%	285	119
TOTAL/MEAN	220		190	30	13.6%	815	245

dead or showed outward signs of RV infection. After collection, specimens were either: (1) euthanized in aqueous chloretone (Simmons 2015) and a piece of liver tissue was collected from the individual, or; (2) non-destructively sampled, with a toe or tail clip taken immediately after capture (only for common species sampled during the Fall). To minimize cross-contamination, dissection equipment was cleaned and sterilized, using 95% ethanol, and gloves were changed between each individual (Gray et al. 2017). Tissue samples (liver or toe) were preserved in 95% ethanol (St-Amour and Lesbarrères 2007). DNA from tissue subsamples was extracted via a high salt extraction method (Esselstyn et al. 2008), with extracts stored at -20°C until used for pathogen screening.

Prior to quantitative PCR (qPCR) analysis at the Disease Testing and Sequencing Facility at the University of South Dakota, DNA extracts were diluted 1:2 with a 0.25 TE buffer solution to reduce the effects of potential inhibitors, following standardized published protocols (Watters et al. 2018). Primers used for the RV assay target a ~70bp fragment of the Major Capsid Protein (MCP) gene as described by Forson and Storfer (2006). Hall et al. (2016) demonstrated that this specific qPCR primer set is likely to detect all ranaviruses known to circulate in North American amphibian populations. All qPCR assays of samples were run in triplicate (three reactions per sample) for 50 cycles, with standard dilutions of known pathogen gene copy number (DNA quantities) based on customized, double-stranded DNA fragments (qBlocks; Integrated DNA Technologies) from the MCP gene (Watters et al. 2018). The 750-base-pair gBlocks contain known copy numbers of the PCR fragment target for RV, which allowed us to quantify the gene copy number amplified in each sample. Four standards were created for the RV pathogen gBlock by serially diluting the gBlock stock of known DNA quantities into concentrations ranging from $1e^1$–$1e^4$. As with study samples, each gBlock standard was run in triplicate on each qPCR assay plate, with the exception of the $1e^4$ standard which was run in duplicate next to a single negative control sample (sterile, molecular grade water). Infection loads were quantified using a StepOnePlus Real-Time PCR System and StepOnePlus software version 2.3 (Applied Biosystems, Inc. [ABI]), following the protocols of Davis and Kerby (2016) and Watters et al. (2018), 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). Samples were considered positive for ranavirus (RV+) if (1) at least two of the three (triplicate) wells were amplified and (2) the resulting mean gene copy number was above 1.0 (Davis and Kerby 2016; Watters et al. 2016, 2018; Marhanka et al. 2017). To determine final infection load per sample, RV gene copy numbers were multiplied by the dilution factor (two for RV) and then were multiplied again by a value in order to reach the total original extraction volume for each sample (Davis and Kerby 2016; Watters et al. 2018).

Out of the 220 amphibians screened, 30 (13.64%) tested positive for RV infection across all sites (Table 2). The highest prevalence of RV+ amphibians was observed during the month of August (25.81%), followed by June (15.09%). The months of March, May, and October had lower prevalence of RV (8.82%,

5.26%, and 12.7%, respectively). The high prevalence of RV infection in August was driven by *Acris blanchardi* individuals from Site 3 (Table 1). We report high RV prevalence among *Lithobates catesbeianus* tadpoles collected near the Oklahoma City Zoo in March (73.68%; Table 1); however, because multiple tadpoles of the same species were held in one bag after collection, we removed these individuals from statistical summaries to account for potential false positives as a result of infected animals shedding virus into the water in which groups of tadpoles were held (Brunner et al. 2007; Gray et al. 2018). When we summarized RV prevalence by season, Summer (19%) and Fall (13%) had the highest number of RV+ individuals followed by Spring (8.22%). Looking at the summaries by site, we found the highest percentage of RV+ individuals at Site 4 (19.57%), followed by Site 3 (14.14%). Sites 1 and 2 had lower percentages of RV prevalence (7.32% and 11.76%, respectively). Additionally, the average RV viral infection load differed between months. Specimens collected in June had the highest viral infection load (966.23 mean gene copies/sample), followed by August (701.76 mean gene copies/sample). March, May, and October had lower viral infection loads (619.59, 686.71, and 361.25 mean gene copies/sample, respectively). The average viral infection load for the month of June does not include the abnormally high liver sample collected from a single *L. catesbeianus* individual from Site 2 (Table 1). A previous study that utilized identical field and molecular techniques found higher average RV viral infection loads in northeastern Oklahoma (Watters et al. 2018), while another found very similar infection loads in southeastern Oklahoma (Davis et al. 2019).

We found the highest number of infected individuals came from the family Hylidae (*A. blanchardi*, 16.33%; *Hyla chrysoscelis/versicolor*, 5.88%) followed closely by Ranidae (*L. catesbeianus*, 5.41%; *L. sphenocephalus*, 16.27%). Comparing the different tissue types and developmental stages sampled, we observed higher prevalence of RV infection in the tail tissues obtained from *L. sphenocephalus* tadpoles (Tables 1, 2). Additionally, our study screened individuals from species that are not often reported in RV manuscripts (Table 2). Despite smaller sample sizes for *Anaxyrus woodhousii* and *Hyla cinerea*, we found a low level of RV infection among these species (Table 2). Both species of *Pseudacris* tested were negative for RV; however, our sample sizes do not allow for definitive reporting on whether these species are negative for RV in central Oklahoma, especially considering *P. clarkii* has been reported as RV+ in other studies (Torrence et al. 2010). Additionally, this is one of the first reports of RV infection in the Microhylidae species *Gastrophryne olivacea* (Table 2; Davis et al. 2019). Although RV is fairly well documented in the United States, Oklahoma remains largely understudied (but see Watters et al. 2018; Davis et al. 2019). More robust surveys are needed to determine the threat of this pathogen to amphibian communities in the state.

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