Herpetological Review, 2016, 47(1), 46–50.

First Detection of Ranavirus in Amphibians from Nebraska, USA

Ranaviruses are considered an emerging pathogen in amphibians due to recent mass-mortality events (Chinchar 2002; Green et al. 2002) even though they appear to have coevolved with local populations (Storfer et al. 2007). Amphibians infected with ranaviruses can display both physical (skin sloughing, erythema, lesions, swollen limbs) and behavioral signs (lack of equilibrium, erratic movements, lethargy) of infection, often culminating in organ necrosis and massive hemorrhaging (Gray et al. 2009; Miller et al. 2011). The importance of ranaviruses in amphibian epizootics is frequently dismissed because most ranavirus-associated mortality has occurred in common species (Gray et al. 2009); however, die-offs of uncommon species may occur more frequently than realized due to low encounter rates or small populations sizes (Green et al. 2002). While the trigger for the epizootic events is difficult to determine, it is believed to be the result of complex interactions between biotic and abiotic factors (Collins and Storfer 2003). In order to take steps to reduce the frequencies of epizootic events, baseline understanding of the distribution and knowledge of what species are affected by ranaviruses is needed.

While ranaviruses have been studied across the United States, there are still many regions where little is known about the presence of this pathogen, such as the Great Plains region. Specifically, ranaviruses have yet to be detected in amphibians from several states in the upper Midwest, including Iowa, Nebraska, and South Dakota, yet it is known from Minnesota (Green et al. 2002; Uyehara et al. 2010; Wolff et al. 2012) and North Dakota (Green et al. 2002). Here, we investigated the presence of ranavirus in nine species of amphibians from northeastern Nebraska: Western Tiger Salamander (*Ambystoma mavortium*), Woodhouse's Toad (*Anaxyrus woodhousii*), Blanchard's Cricket Frog (*Acris blanchardi*), Cope's Gray Treefrog (*Hyla chrysoscelis*), Boreal Chorus Frog (*Pseudacris maculata*), Plains Leopard Frog (*Lithobates blairi*), American Bullfrog (*L. catesbeianus*), Northern Leopard Frog (*L. pipiens*), and Plains Spadefoot (*Spea bombifrons*).

From 20 May–15 October 2013, we collected tissue samples from amphibians from 15 sites in Cedar and Dixon counties,

DREW R. DAVIS*
JACOB L. KERBY
Department of Biology, University of South Dakota,
414 East Clark Street, Vermillion, South Dakota 57069, USA

**Corresponding author; e-mail: drew.davis@usd.edu*

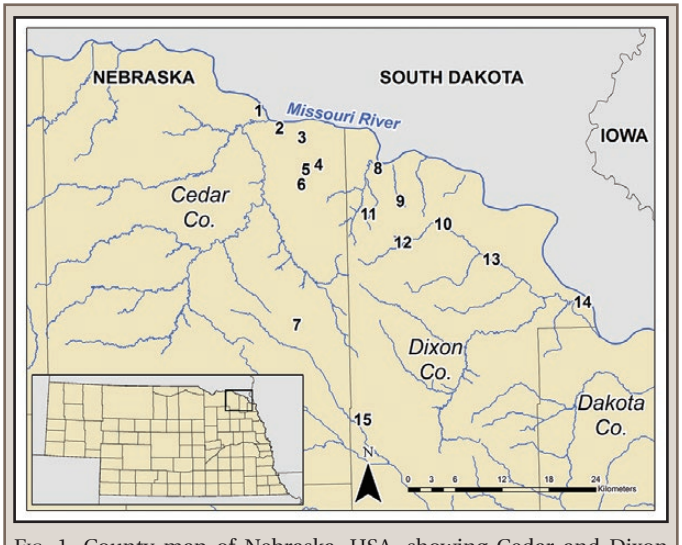

FIG. 1. County map of Nebraska, USA, showing Cedar and Dixon counties and sampling sites (numbers). Sampling site numbers correspond to sites in Table 1.

TABLE 1. Site information and amphibian species screened for ranavirus in northeast Nebraska, USA in 2013. County, geographic coordinates (datum: WGS 84), sampling dates, number of positive samples, and total sample size (N) are all indicated. L = larval, S = subadult, A = adult. Site numbers correspond to numbers in Fig. 1. Species abbreviations: ACBL = *Acris blanchardi*, AMMA = *Ambystoma mavortium*, ANWO = *Anaxyrus woodhousii*, HYCH = *Hyla chrysoscelis*, LIBL = *Lithobates blairi*, LICA = *Lithobates catesbeianus*, LIPI = *Lithobates pipiens*, PSMA = *Pseudacris maculata*, SPBO = *Spea bombifrons*.

Site No.	Site name / species	County	Lat / Long	Visit dates	Age class	RV+	N	RV prevalence
1	Bow Creek Recreational Area	Cedar	42.77963°N, 97.14584°W	23 May–13 Oct		26	38	0.68
	ANWO				S	6	9	0.67
	ACBL				L	9	9	1.0
	ACBL				S	3	5	0.6
	ACBL				A	1	3	0.33
	HYCH				L	3	3	1.0
	HYCH				S	0	1	0
	LIBL				L	2	3	0.67
	LIBL				S	2	2	1.0
	LICA				L	0	3	0
2	Cedar Co. OFW Property	Cedar	42.76166°N, 97.11384°W	8 June–25 July		18	22	0.82
	ANWO				A	1	1	1.0
	ACBL				A	4	4	1.0
	HYCH				L	2	2	1.0
	HYCH				S	1	1	1.0
	HYCH				A	1	1	1.0
	PSMA				S	1	1	1.0
	PSMA				A	0	1	0
	LIBL				L	1	1	1.0
	LIBL				S	0	1	0
	LIBL				A	0	1	0
	LICA				S	3	3	1.0
	LICA				A	3	4	0.75
	LIPI				A	1	1	1.0
3	Wiseman Monument	Cedar	42.74860°N, 97.08051°W	25 May–25 Sept		7	28	0.25
	ACBL				A	2	15	0.13
	HYCH				A	2	8	0.25
	PSMA				L	1	2	0.5
	PSMA				A	1	1	1.0
	LIBL				S	0	1	0
	LIPI				A	1	1	1.0
4	Stewart Pond	Cedar	42.71620°N, 97.05738°W	13 June, 24 July		17	44	0.39
	AMMA				L	13	35	0.37
	AMMA				S	1	3	0.33
	ACBL				A	1	1	1.0
	LIBL				L	1	1	1.0
	LIBL				S	1	4	0.25
5	Driver Pond	Cedar	42.70952°N, 97.07526°W	2 June		2	5	0.4
	ACBL				A	2	4	0.5
	LIBL				A	0	1	0
6	Rolfes Farm Pond	Cedar	42.69722°N, 97.07945°W	27 May–24 July		32	40	0.8
	AMMA				L	4	9	0.44
	SPBO				L	28	29	0.97
	SPBO				A	0	2	0
7	Dead Creek	Cedar	42.53375°N, 97.09383°W	8 June		1	1	1.0
	SPBO				A	1	1	1.0
8	Mulberry Bend	Dixon	42.72101°N, 97.96245°W	20 May, 5 July		2	2	1.0
	ANWO				S	1	1	1.0
	ANWO				A	1	1	1.0
9	Dixon Co. OFW Property	Dixon	42.67293°N, 97.92782°W	9, 11 Sept		8	13	0.62
	AMMA				S	0	2	0
	ANWO				A	1	1	1.0
	ACBL				A	3	4	0.75
	LIBL				A	2	2	1.0
	SPBO				S	2	4	0.5

Nebraska, USA (Fig. 1; Table 1). In general, northeastern Nebraska has been poorly sampled for amphibians (see Davis et al. 2014). Individuals were euthanized via submersion in MS-222, and liver samples were collected and stored in 95% ethanol. Genetic tissue samples and vouchered, whole-body specimens were collected at each sampling location; however, at sites with high population densities, vouchered collections were supplemented with additional, non-destructive tissue sampling

TABLE 1. Continued.

Site No.	Site name / species	County	Lat / Long	Visit dates	Age class	RV+	N	RV prevalence
10	North Aowa Creek	Dixon	42.64455°N, 96.86473°W	29 May, 22 Aug		1	3	0.33
	ANWO				S	1	1	1.0
	LIBL				A	0	1	0
	SPBO				A	0	1	0
11	East Creek	Dixon	42.65847°N, 96.97946°W	26 May		2	3	0.67
	ANWO				A	1	1	1.0
	SPBO				A	1	2	0.5
12	Buckskin Hills WMA	Dixon	42.62562°N, 96.92729°W	24–26 Sept		5	10	0.5
	ACBL				S	1	2	0.5
	ACBL				A	0	1	0
	LIBL				A	1	1	1.0
	LICA				L	3	3	1.0
	LICA				S	0	2	0
	LICA				A	0	1	0
13	South Aowa Creek	Dixon	42.60251°N, 96.79097°W	29 May		1	2	0.5
	HYCH				A	0	1	0
	SPBO				A	1	1	1.0
14	Ponca, Nebraska	Dixon	42.55847°N, 96.64724°W	16 Aug	0	2	0	
	LIBL				S	0	2	0
15	Dixon, Nebraska	Dixon	42.42207°N, 96.99834°W	8 June		1	2	0.5
	ANWO				A	1	2	0.5

TABLE 2. Amphibian species sampled in 2013 from northeast Nebraska, USA in 2013. Family, number of positive samples, total sample size (N), pooled RV prevalence (number RV+ individuals/total number sampled across sites), and viral gene copies of RV+ individuals (presented as mean over range).

Species	Family	RV+	N	RV Prevalence	Viral Gene Copies
Ambystoma mavortium	Ambystomatidae	18	49	0.37	2.6×10^5 ($318.5–2.5e^6$)
Anaxyrus woodhousii	Bufonidae	13	17	0.76	1.2×10^8 ($66.0–1.5e^9$)
Acris blanchardi	Hylidae	26	49	0.53	2.7×10^4 ($69.3–2.1e^5$)
Hyla chrysoscelis	Hylidae	9	17	0.53	6.1×10^3 ($404.5–1.8e^4$)
Pseudacris maculata	Hylidae	3	5	0.60	3.5×10^8 ($151.5–1.0e^9$)
Lithobates blairi	Ranidae	10	20	0.50	3.2×10^4 ($74.9–2.5e^5$)
Lithobates catesbeianus	Ranidae	9	16	0.56	2.2×10^6 ($412.2–1.8e^7$)
Lithobates pipiens	Ranidae	2	2	1.0	1.6×10^8 ($503.7–3.2e^8$)
Spea bombifrons	Scaphiopodidae	33	40	0.83	1.0×10^{10} ($85.6–1.6e^{11}$)

PHOTOS BY DREW R. DAVIS

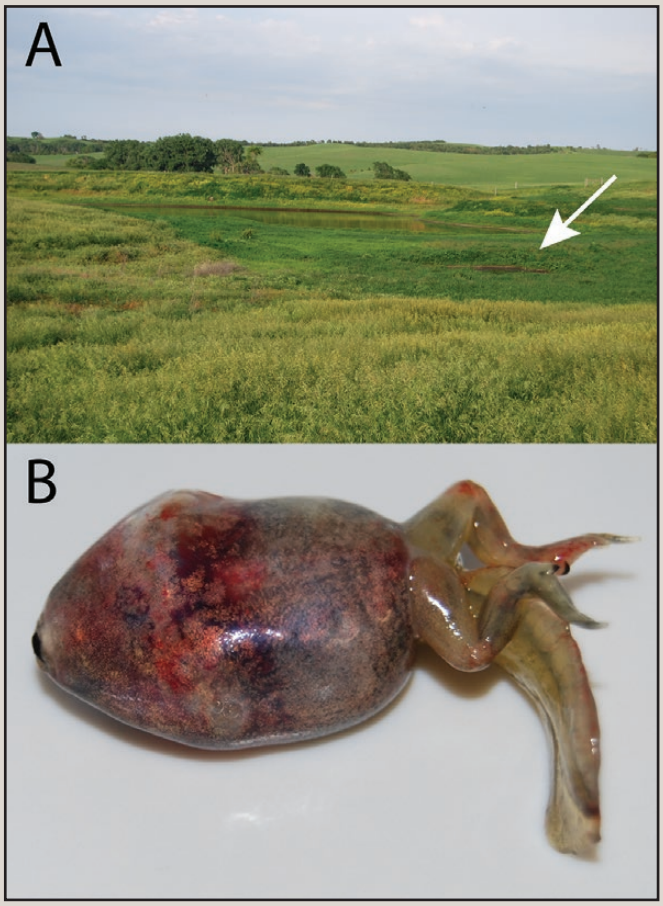

FIG. 2. A) Rolfes Farm Pond in Cedar Co., Nebraska, USA, indicating a shallow side pool (white arrows) where a mass mortality event of Plains Spadefoot (*Spea bombifrons*) tadpoles was observed on 13 June 2013. B) Ventral view of a moribund Plains Spadefoot tadpole collected from this site showing clinical signs of ranavirus infection (erythema, swelling).

(tail or toe clips), followed by specimen release. All individuals were collected via dip net, seine, or by hand. All equipment was sterilized between individuals with a 10% bleach solution to eliminate contamination risk.

Ranavirus presence was detected following a modified protocol outlined by Kerby et al. (2011), and DNA was extracted from samples using Qiagen DNeasy Blood and Tissue Kits. All samples were run in triplicate and ranavirus detection was determined via qPCR (StepOnePlus, Applied Biosystems). Each plate contained a negative control and a standardized dilution series of gBlocks (IDT) that contained the target sequence of DNA to use as a standard curve. This method provides an estimate of the number of gene copies present in a sample. Samples were only considered positive (RV+) if two of three replicates were positive. Viral gene copies were determined by averaging values from each sample. All analyses were conducted at the Disease Testing Center at the University of South Dakota.

A total of 215 samples were collected from this region in 2013, and we detected ranavirus in 123 (57.2%) of these samples (Tables 1, 2). Ranavirus was detected in samples from all nine species and at 14 of the 15 (93.3%) sites sampled. Detection in these nine species includes the first reported detection of ranavirus in the Woodhouse's Toad, Boreal Chorus Frog, and Plains Spadefoot (Miller et al., 2011). We detected ranavirus infection in at least half of all individuals sampled from every species except Western Tiger Salamanders (Table 2). While the prevalence of infection in some species may be artificially inflated due to limited sample sizes, the overall high prevalence may be indicative that ranavirus infection is common and widespread. In addition to detecting presence of ranavirus infection, we were also able to quantify viral gene copies within each sample. Average viral gene copies ranged across several orders of magnitude (10^3–10^{10}); observed average values were lowest in Cope's Gray Treefrog tissues (6.1×10^3) and highest in Plains Spadefoot tissue (1.0×10^{10}; Table 2).

In addition to detecting ranavirus in all species of amphibians, we detected a die-off of Plains Spadefoot tadpoles in Dixon Co. on 13 June 2013. In this instance, 28 moribund tadpoles (all infected with ranavirus) were collected in a small, turbid, side pool along Rolfes Farm Pond (Fig. 2A). Most tadpoles from this site were moribund and showed clinical signs of ranavirus infection (erythema, swelling, erratic movement; Fig. 2B). Highly turbid water prevented an accurate assessment of the number of dead tadpoles. Other than this group of Plains Spadefoot tadpoles, all other sampled amphibians did not show any clinical signs of ranavirus infection. The lack of clinical signs of infection is not unexpected because ranaviruses are believed to have existed with local amphibian hosts, yet have increased virulence when introduced to amphibians outside their original range (Storfer et al. 2007). While the commercial use and movement of amphibians may contribute to pathogen pollution, and thus the introduction of strains outside their native region (Picco and Collins 2008), it is unlikely that this is the cause of this particular die-off as this is an area of low human population, it is not along a major interstate corridor, and is not a site where fishing and where the potential release of bait amphibians occurs. Alternatively, other natural or anthropogenic factors such as high density, agricultural contaminants, and direct access by cattle may be responsible (Forson and Storfer 2006; Kerby et al. 2011). Rolfes Farm Pond is a site where cattle are present and have direct access to this wetland, and previous work has suggested that the prevalence of ranavirus in amphibians is greater in wetlands where cattle have access, which may increase individual stress and decrease water quality (Gray et al. 2007). Plains Spadefoot adults are explosive breeders and reproduce in ephemeral ponds, as larval development is extremely rapid (as fast as 13 days; King 1960). At this site, water levels were shallow, resulting in a high density of individuals, and when combined with the presence of cattle, may have contributed to the observed die-off. Many wetlands in this region share similar characteristics (direct cattle-access, ephemeral) to our site where this die-off was detected and while we were unable to detect them, die-offs may be occurring at these sites as well.

Our survey resulted in the first confirmed detection of ranavirus in Nebraska amphibians and helps fill in information gaps regarding this pathogen across the Great Plains. In order to more fully understand potential effects on individuals, populations, and species, knowledge of the distribution of this pathogen across space, time, and hosts is critical (Ostfeld et al. 2005). Overall, high prevalence of ranavirus infection was detected across both sites and species sampled. These results are especially important when considered in relation to pathogen pollution resulting from commercial movement of amphibians. The commercial trade of bait amphibians may continue to spread ranavirus around North America, introducing novel strains of ranavirus to regions and amphibians where virulence is expected to be greater. Large numbers of bait amphibians are being exported from much of the upper Midwest, including Nebraska and being

transported to multiple states in the southwestern United States (Picco and Collins 2008). Though a single die-off was observed, all other individuals infected with ranavirus showed no clinical signs of disease. Land managers should consider the role of external stressors on triggering mortality events and work to minimize and eliminate anthropogenic stressors, such as eliminating cattle access to wetlands, which may affect amphibian populations in order to curb regional amphibian declines.

Acknowledgments.—We thank J. Farkas, D. Quist, C. Siler, J. Vlcek, and members of the Kerby Lab for field and lab assistance, and landowners for permission to sample on private property. Helpful comments on this manuscript were provided by the GrEBE Discussion Group at the University of South Dakota. Specimens and tissue samples were collected under Nebraska Game and Parks Commission Scientific and Educational Collecting Permits (#293, #294) issued to DRD and under an approved University of South Dakota IACUC protocol (#15-02-13-16C). Funding for this study was provided by a grant awarded to DRD from the Nebraska Herpetological Society.

Literature Cited

Chinchar, V. G. 2002. Ranaviruses (family Iridoviridae): emerging cold-blooded killers. Arch. Virol. 147:447–470.

Collins, J. P., and A. Storfer. 2003. Global amphibian declines: sorting the hypotheses. Divers. Distrib. 9:89–98.

Davis, D. R., S. R. Siddons, and J. L. Kerby. 2014. New amphibian and reptile county records from eastern Nebraska, USA. Herpetol. Rev. 45:99–100.

Forson, D. D., and A. Storfer. 2006. Atrazine increases ranavirus susceptibility in the tiger salamander, *Ambystoma tigrinum*. Ecol. Appl. 16:2325–2332.

Gray, M. J., D. L. Miller, and J. T. Hoverman. 2009. Ecology and pathology of amphibian ranaviruses. Dis. Aqua. Org. 87:243–266.

———, ———, A. C. Schmutzer, and C. A. Baldwin. 2007. *Frog virus 3* prevalence in tadpole populations inhabiting cattle-access and non-access wetlands in Tennessee, USA. Dis. Aqua. Org. 77:97–103.

Green, D. E., K. A. Converse, and A. K. Schrader. 2002. Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996–2001. Ann. New York Acad. Sci. 969:323–339.

Kerby, J. L., A. J. Hart, and A. Storfer. 2011. Combined effects of virus, pesticide, and predator cue on the larval tiger salamander (*Ambystoma tigrinum*). EcoHealth 8:46–54.

King, O. M. 1960. Observations on Oklahoma toads. Southwest. Nat. 5:102–103.

Miller, D., M. Gray, A. Storfer. 2011. Ecopathology of ranaviruses infecting amphibians. Viruses 3:2351–2373.

Ostfeld, R. S., G. E. Glass, and F. Keesing. 2005. Spatial epidemiology: an emerging (or re-emerging) discipline. Trends Ecol. Evol. 20:328–336.

Picco, A. M., and J. P. Collins. 2008. Amphibian commerce as a likely source of pathogen pollution. Conserv. Biol. 22:1582–1589.

Storfer, A., M. E. Alfaro, B. J. Ridenhour, J. K. Jancovich, S. G. Mech, M. J. Parris, and J. P. Collins. 2007. Phylogenetic concordance analysis shows an emerging pathogen is novel and endemic. Ecol. Lett. 10:1075–1083.

Uyehara, I. K., T. Gamble, and S. Cotner. 2010. The presence of *Ranavirus* in anuran populations in Itasca State Park, Minnesota, USA. Herpetol. Rev. 41:177–179.

Wolff, B. G., S. M. Conway, and C. J. Dabney, III. 2012. *Batrachochytrium dendrobatidis* and ranavirus in anurans inhabiting decorative koi ponds near Minneapolis, Minnesota, USA. Herpetol. Rev. 43:427–429.