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Two Ranavirus-Associated Mass-Mortality Events among Larval Amphibians in Illinois, USA

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Growing evidence suggests that ranaviruses are globally distributed pathogens that infect a diversity of ectothermic vertebrates, and can cause sudden mass-mortality events, especially in larval amphibians (Brunner et al. 2004, 2015; Gray et al. 2009; Warne et al. 2016). Ranavirus epizootics in larval amphibians can also amplify viral titers in the environment (Hall et al. 2016) and potentially export sublethally infected metamorphs to the surrounding ecosystem, creating a possible transmission route to sympatric hosts (Brunner et al. 2007). Despite a growing awareness of disease threats to amphibians, ranavirus epizootics are likely undetected and underreported because they are often rapid and cryptic (Brunner et al. 2015). Given the broad range of hosts threatened by ranaviruses (Duffus

et al. 2015), documenting the geographic range, spread, and effects of the disease, particularly mass die-offs is important for conservation and management efforts.

Here, we report observations consistent with ranavirus-induced mass-mortality events in larvae of two amphibian species over two years (2015 and 2016) in southern Illinois, USA. These are the first reported ranavirus-related mass-mortality events among amphibians in the region, although ranavirus was previously detected on skin swabs of amphibians from Illinois and Wisconsin (Sekowska et al. 2014). We also report findings of water samples examined for ranavirus DNA collected from four water bodies in Illinois, including at the site of one of the observed mortality events, supporting the occurrence of ranavirus in the region prior to the die-off events.

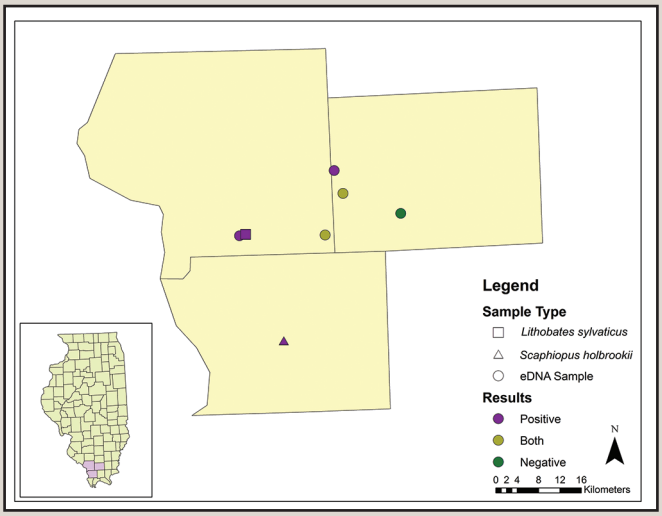

FIG. 1. A map of ranavirus-related mass mortality events and ranavirus eDNA samples from Jackson, Union, and Williamson counties, Illinois. The square represents a Wood Frog (*Lithobates sylvaticus*) die-off, the triangle an Eastern Spadefoot (*Scaphiopus holbrookii)* die-off, and circles are eDNA samples. For all symbols, purple indicates the presence of ranavirus as detected by qPCR, green represents no detection of ranavirus, and yellow represents sites where ranavirus was detected only in some samples.

We collected water samples in four water bodies in southern Illinois to sample for ranavirus environmental DNA (eDNA) in May and June of 2014 (Table 1; Fig. 1). Water was collected in sterile 500 mL containers and filtered through 0.22 µm micropore PVDF membrane filters (PVD023050, Sterlitech, Kent, Washington, USA) via vacuum filtration to collect eDNA (Hall et al. 2016).

We also collected amphibian larvae from two die-offs. The first occurred among Eastern Spadefoot (*Scaphiopus holbrookii*) larvae in a natural sinkhole pond in Jonesboro, Union County, Illinois (Table 1; Fig 1). We first observed lethargic and moribund larvae with swollen hind limbs and erythema on 28 July 2015. We collected four recently-deceased larvae by dip net and stored them in 70% ethanol. Subsequently, we found hundreds of dead and dying metamorphs at the water's edge on 29 and 30 July 2015. These metamorphosing larvae displayed gross lesions consistent with ranavirus, including erythema and ecchymosis on the ventral surface of the abdomen and hind limbs (Miller et al. 2015).

The second die-off occurred among Wood Frog (*Lithobates sylvaticus* [*Rana sylvatica*]) larvae in a wetland in the Cave Creek Valley of Jackson County, Illinois (Table 1; Fig. 1). On 17 April 2016, we observed some moribund larvae on the water's surface, displaying no visible lesions. We collected seven of the moribund larvae by dip net and stored them in 70% ethanol. The moribund Wood Frog larvae were present alongside hundreds of apparently healthy conspecifics, as well as larvae of four other taxa: Spring Peepers (*Pseudacris crucifer*), Trilling Chorus Frogs (*Pseudacris triseriata* complex), Spotted Salamanders (*Ambystoma maculatum*), and Marbled Salamanders (*Ambystoma opacum*), none of which exhibited signs of disease. Upon return to the site less than 36 h later to conduct a broader survey for a potential epizootic event, we did not observe or capture a single larvae or carcass of any species despite extensive searching and core (enclosure) sampling. All larvae in the pond were in early

TABLE 1. Average ranavirus titer in plaque forming unit equivalents (PFU) per sample and location of amphibian die-offs.

Species or eDNA sample	County	GPS coordinates	Sample date	Number positive / tested	Log10 Mean Viral Load ± SD
Scaphiopus holbrookii	Union	37.459350°N 89.268647°W	28 July 2015	4 / 4	7.80 ± 0.22
Lithobates sylvaticus [*Rana sylvatica*]	Jackson	37.641145°N 89.340451°W	17 April 2016	7 / 7	7.37 ± 0.26
eDNA Sample (500 mL)	Jackson	37.639368°N 89.353640°W	16 May 2014 14 June 2014	1 / 1 1 / 1	2.22 2.03
eDNA Sample (500 mL)	Jackson	37.63488°N 89.172319°W	16 May 2014 14 June 2014	0 / 2 1 / 2	 1.61 ± 1.13
eDNA Sample (500 mL)	Williamson	37.703788°N 89.130864°W	16 May 2014 14 June 2014	1 / 1 0 / 1	2.19
eDNA Sample (500 mL)	Williamson	37.665870°N 89.010335°W	16 May 2014 14 June 2014	0 / 1 0 / 1	
eDNA Sample (500 mL)	Williamson	37.742933°N 89.147505°W	16 May 2014 14 June 2014	1 / 1 1 / 1	1.77 2.13

developmental stages (less than Gosner Stage 35) such that metamorphosis over 36 h would not be possible. Furthermore, this pond was small (68.3 ± 25.0 m^2 when filled), with sparse aquatic vegetation, and thus easily sampled. Indeed, larvae were captured by core sampling in previous years (2013–2014) at densities as low as 0.34 larvae • m^{-2}.

We used a quantitative PCR assay (qPCR) to test for the presence of ranavirus DNA in the collected larvae and eDNA samples. Liver samples were dissected from larvae with equipment that was disinfected between individuals with 10% bleach for approximately 15 minutes and then flame sterilized for 20 seconds with a butane torch. Uninfected, lab-raised Wood Frog larvae were dissected alongside the sample larvae with the same equipment to serve as extraction controls for cross contamination. DNA was extracted from liver tissues or membrane filters (eDNA) using Mini-Spin columns (PureLink DNA extraction kit, ThermoFisher Inc., Waltham, Massachusetts, USA). Samples were standardized to 20 ng/µL and DNA copies quantified by qPCR using TaqMan primers and probes targeting a 70-bp region within the major capsid protein (MCP) sequence of ranaviruses in a StepOnePlus™ System (ThermoFisher Inc., Waltham, Massachusetts, USA) using the methods of Picco et al. (2007). We included extraction controls for liver samples, molecular grade distilled water as a negative control for the eDNA samples, and molecular grade distilled water as a no-template control in each assay. A liver from a Wood Frog larva infected with a 100% lethal dose of ranavirus was included as a positive control. Viral load was estimated relative to serial dilution of ranavirus standards extracted from cultured epithelioma papulosum cyprini (EPC) cells at a known concentration (10^8 PFU/ml). All field-collected larvae tested positive for ranavirus (Table 1) with viral titers that ranged from 7.11–8.11 ($\log_{10}$ PFU). There was no amplification of the template, the extraction controls, or the negative controls, therefore cross-contamination was unlikely.

These are the first reported ranavirus-associated die-offs in southern Illinois, despite long-term surveillance at both sites. At the Jonesboro location, Eastern Spadefoot breeding activity has been monitored since April 1996 (Palis 2012, 2016). Subsequent to each breeding event, larval development is monitored every 1–3 days through metamorphosis or larval mortality resulting from premature pond drying. The only previously observed mass die-offs of larvae were associated with premature drying of the pond. To our knowledge this is only the second report of ranavirus-related mortality in Eastern Spadefoots (Miller et al. 2011; Duffus et al. 2015). The Cave Creek site was part of a larger study of the surrounding wetlands. It was one of eight sites that were drift fenced from January 2013 to March 2015. The sites were monitored daily from spring fill until cessation of amphibian activity in autumn and thereafter monitored weekly in the winter, but no other die-offs were observed. These patterns of rarely observed disease-associated die-offs could suggest that ranavirus epizootics in Illinois (and more generally) are either a new threat driven by the spread of emerging virulent ranavirus strains, or that such die-offs are cryptic and challenging to detect. Speculatively, the latter explanation is likely, as ranavirus associated die-offs are sporadic (Hall et al. 2016), and when a mass mortality event occurs there is a very narrow window in which to observe and collect samples. Indeed, the die-offs we observed at both sites appeared to be dramatic. The rapid disappearance of animals and carcasses (< 36 h), particularly at the Cave Creek site, suggests that identifying a mass mortality event associated with ranavirus is restricted to a narrow time window of just a few days to perhaps a week (see also Wheelwright et al. 2014).

The reservoirs for ranavirus and the ecological factors driving outbreaks are generally poorly understood. In the Eastern Spadefoot case, the ranavirus outbreak may have been related to near-complete drying of the sinkhole by 2100 h on 19 July 2015 when thousands of larvae were confined to a 1.3-cm deep, 50 cm × 60 cm pool, followed by refilling during a thunderstorm starting at ca. 0100 h on 20 July 2015. By contrast, we detected no obvious environmental factors that could explain the second outbreak and putative die-off at the Cave Creek site. Ranavirus eDNA was detected in a nearby pond in this wetland matrix two years prior to the observed die-off, suggesting the virus was present in the area, but again, no die-offs were observed.

Wood Frogs are also highly susceptible to infection and ranavirus-induced mortality and may act as amplification hosts for ranaviruses (Brunner et al. 2015). Indeed, they have been shown to increase community level mortality when they are the first species exposed to ranavirus (Brenes 2013). These findings suggest that community composition and dynamics, potentially in conjunction with abiotic factors (e.g., thermal stress, hydroperiod, pollution), may interact to influence the spread and likelihood of ranavirus epizootics (St-Amour et al. 2008; Echaubard et al. 2010).

The work of Hall et al. (2016) and our eDNA analysis suggest that surveillance using water sampling can potentially increase the probability of detecting ranavirus and potential epizootics. We detected ranavirus eDNA from water at multiple sites, including a pond at the Cave Creek site two years prior to the observed mortality event (Table 1, Fig. 1). Although these eDNA data do not provide insight into the reservoirs or factors that could lead to a potential outbreak, they do suggest that ranavirus may be present throughout southern Illinois. Three of our sites showed eDNA viral titers above 100 PFU, a level which is near the median lethal dose for Wood Frogs (Warne et al. 2011) and associated with ranavirus die-offs in Connecticut, USA (Hall et al. 2016). Furthermore, these results suggest repeated eDNA surveillance may provide a means to more efficiently identify the prevalence and potential threat of ranavirus across time and space (Johnson and Brunner 2014; Hall et al. 2016). At a minimum, these data suggest more intensive surveillance is necessary to ascertain the prevalence of the pathogen and severity of the threat to amphibians and other ectothermic vertebrates.

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Literature Cited

Brenes, R. M. 2013. Mechanisms Contributing to the Emergence of Ranavirus in Ectothermic Vertebrate Communities. Ph.D. Dissertation, University of Tennessee, Knoxville, Tennesseee.

Brunner J. L., D. M. Schock, and J. P. Collins. 2007. Transmission dynamics of the amphibian ranavirus *Ambystoma tigrinum* virus. Dis. Aquat. Organ. 77:87–95.

———, ———, E. W. Davidson, and J. P. Collins. 2004. Intraspecific reservoirs: complex life history and the persistence of a lethal ranavirus. Ecology 85:560–566.

———, A. Storfer, M. J. Gray, and J. T. Hoverman. 2015. Ranavirus ecology and evolution: from epidemiology to extinction. *In* M. J. Gray

and V. G. Chinchar (eds.), Ranaviruses: Lethal Pathogens of Ectothermic Vertebrates, pp. 71–104. Springer International Publishing, Cham, Switzerland.

Duffus A. L., T. B. Waltzek, A. C. Stohr, M. C. Allender, M. Gotesman, P. H. Whittington, M. K. Hines, and Colleagues. 2015. Distribution and host range of ranaviruses. *In* M. J. Gray and V. G. Chinchar (eds.), Ranaviruses: Lethal Pathogens of Ectothermic Vertebrates, pp. 9–57. Springer International Publishing, Cham, Switzerland.

Echaubard P., K. Little, B. Pauli, and D. Lesbarrères. 2010. Context-dependent effects of ranaviral infection on northern leopard frog life history traits. PLoS ONE 5: e13723.

Gray M. J., D. L. Miller, and J. T. Hoverman. 2009. Ecology and pathology of amphibian ranaviruses. Dis. Aquat. Organ. 87:243–66.

Hall E. M., E. J. Crespi, C. S. Goldberg, and J. L. Brunner. 2016. Evaluating environmental DNA-based quantification of ranavirus infection in wood frog populations. Mol. Ecol. Resour. 16:423–433.

Johnson A. F., and J. L. Brunner. 2014. Persistence of an amphibian ranavirus in aquatic communities. Dis. Aquat. Organ. 111:129–138.

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

———, A. P. Pessier, and R. J. Whittington. 2015. Comparative pathology of ranaviruses and diagnostic techniques. *In* M. J. Gray and V. G. Chinchar (eds.), Ranaviruses: Lethal Pathogens of Ectothermic Vertebrates, pp. 171–208. Springer International Publishing, Cham, Switzerland.

Palis, J. G. 2012. Breeding frequency and success of eastern spadefoots, *Scaphiopus holbrookii*, in southern Illinois. Proc. Indiana Acad. Sci. 121:158–162.

———. 2016. The eastern spadefoot, Illinois' storm frog. Illinois Audubon 19–21.

Picco, A. M., J. L. Brunner, and J. P. Collins. 2007. Susceptibility of the endangered California tiger salamander, *Ambystoma californiense*, to ranavirus infection. J. Wildl. Dis. 43:286–290.

Sekowska, J. M., M. C. Allender, and C. A. Phillips. 2014. Prevalence and spatial distribution of frog virus 3 in free-ranging populations of northern cricket frogs (*Acris crepitans*), American bullfrogs (*Lithobates catesbeianus*), and northern leopard frogs (*Lithobates pipiens*) in western Illinois and Wisconsin, U.S.A. Herpetol. Rev. 45:245–247.

St-Amour, V., W. M. Wong, T. W. J. Garner, and D. Lesbarrères. 2008. Anthropogenic influence on prevalence of 2 amphibian pathogens. Emerg. Infect. Dis. 14:1175–1176.

Warne, R. W., E. J. Crespi, and J. L. Brunner. 2011. Escape from the pond: stress and developmental responses to ranavirus infection in wood frog tadpoles. Funct. Ecol. 25:139–146.

———, B. La Bumbard, S. La Grange, V. T. Vredenburg, and A. Catenazzi. 2016. Co-infection by chytrid fungus and ranaviruses in wild and harvested frogs in the tropical andes. PLoS ONE 11:1–15.

Wheelwright, N. T., M. J. Gray, R. D. Hill, and D. L. Miller. 2014. Sudden mass die-off of a large population of wood frog (*Lithobates sylvaticus*) tadpoles in Maine, USA, likely due to ranavirus. Herpetol. Rev. 45:240–242.