care guidelines and appropriate permits were obtained.

Literature Cited

Adams, M. J., S. Galvan, D. Reinitz, R. A. Cole, S. Pyare, M. Hahr, and P. Govindarajulu. 2007. Incidence of the fungus *Batrachochytrium dendrobatidis* in amphibian populations along the northwest coast of North America. Herpetol. Rev. 38:430–431.

Briggler, J. T., K. A. Larson, and K. J. Irwin. 2008. Presence of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) on hellbenders (*Cryptobranchus alleganiensis*) in the Ozark Highlands. Herpetol. Rev. 39:443–444.

———, and J. W. Prather. 2006. Seasonal use and selection of caves by plethodontid salamanders in a karst area of Arkansas. Am. Midl. Nat. 155:136–148.

Brodman, R., and J. T. Briggler. 2008. *Batrachochytrium dendrobatidis* in *Ambystoma jeffersonianum* larvae in southern Indiana. Herpetol. Rev. 39:320–321.

Elliott, W. R. 2005. Caves and karst. *In* P. W. Nelson, The Terrestrial Natural Communities of Missouri, pp. 475–491. The Missouri Natural Areas Committee, Jefferson City, Missouri.

———. 2007. Zoogeography and biodiversity of Missouri caves and karst. J. Cave Karst Stud. 69:135–162.

Johnson, T. R. 2000. The Amphibians and Reptiles of Missouri. 2nd ed. Missouri Department of Conservation, Jefferson City, Missouri. 400 pp.

Longcore, J. R., J. E. Longcore, A. P. Pessier, and W. A. Halteman. 2007. Chytridiomycosis widespread in anurans of northeastern United States. J. Wildl. Manage. 71:435–444.

Mittleman, M. B. 1950. Cavern-dwelling salamanders of the Ozark Plateau. Bull. Natl. Speleol. Soc. 12:12–15.

Ouellet, M., I. Mikaelian, B. D. Pauli, J. Rodrigue, and D. M. Green. 2005. Historical evidence of widespread chytrid infection in North American amphibian populations. Conserv. Biol. 19:1431–1440.

Pearl, C. A., E. L. Bull, D. E. Green, J. Bowerman, M. J. Adams, A. Hyatt, and W. H. Wente. 2007. Occurrence of the amphibian pathogen *Batrachochytrium dendrobatidis* in the Pacific Northwest. J. Herpetol. 41:145–149.

Petranka, J. W. 1998. Salamanders of the United States and Canada. Smithsonian Institution Press, Washington, D.C. 587 pp.

Piotrowski, J. S., S. L. Annis, and J.E. Longcore. 2004. Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. Mycologia 96:9–15.

Prather, J. W., and J. T. Briggler. 2001. Use of small caves by anurans during a drought period in the Arkansas Ozark. J. Herpetol. 35:675–678.

Reeves, M. K., and D. E. Green. 2006. *Rana sylvatica* (wood frog) chytridiomycosis. Herpetol. Rev. 37:450.

Resetarits, J. W., Jr. 1986. Ecology of cave use by the frog *Rana palustris*. Am. Midl. Nat. 116:256–266.

Sutton, M. R. 1993. Caves and cave wildlife in a mineral prospecting area, Oregon and Shannon counties, Missouri. J. Missouri Speleol. Soc. 33:1–138.

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The Presence of *Ranavirus* in Anuran Populations at Itasca State Park, Minnesota, USA

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Viruses in the genus *Ranavirus* are implicated as a possible contributing factor to global amphibian declines (Daszak et al. 1999). Previous studies have associated ranaviruses with mass amphibian die–offs throughout North America (Chinchar 2002 Greer et al. 2005; Williams et al. 2005) including Minnesota, USA (Green et al. 2002; Vandenlangenberg et al. 2003). However, it is unclear whether ranavirus infections exist in wild anuran populations without causing die-offs. This study investigates the presence of ranavirus in six anuran species at the headwaters of the Mississippi River in Itasca State Park, Minnesota, USA.

Methods.—We collected recently metamorphosed and adult frogs and toads by hand and with nets from twenty-six sites in Itasca State Park, Clearwater County, Minnesota (47.2397°N, 95.2075°W) in June and July 2009. Sites were chosen for maximum species diversity and for accessibility. There have been no reports of mass amphibian die-offs in the park, which has been the site of a University of Minnesota field station since 1909 (D. Biesboer, pers. comm., 30 July 2009; C. Handrick, pers. comm., 3 Aug 2009). Both field-based and lab-based courses are taught during summer months and there are few places in the state that have had such a consistent and thorough presence of biologists during peak periods of amphibian activity. This steady stream of students and faculty provides some level of confidence that amphibian die-offs will be observed and reported. That said, there is no guarantee that amphibian die-offs, particularly of aquatic larvae, went unobserved.

To minimize contamination among sites, nets and waders were rinsed with 5% bleach solution between collecting events. Captured frogs were kept individually in separate plastic bags. Individuals were classified as either young-of-the-year (YOY) or adults by comparing their snout-vent length to published sizes of adult frogs (Oldfield and Moriarty 1994; Wright and Wright 1995). Frogs were returned to the laboratory and humanely euthanized with MS-222 (tricaine methanesulfonate) (Simmons 2002). Liver samples were stored in RNAlater (Ambion, Austin, Texas, USA) or 99% ethanol at 4°C until processing. Vouchers were deposited in the Bell Museum of Natural History, University of Minnesota (JFBM).

We used disposable razor blades to subsample between 15–30 mg of liver tissues for DNA extraction using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, California, USA) follow-

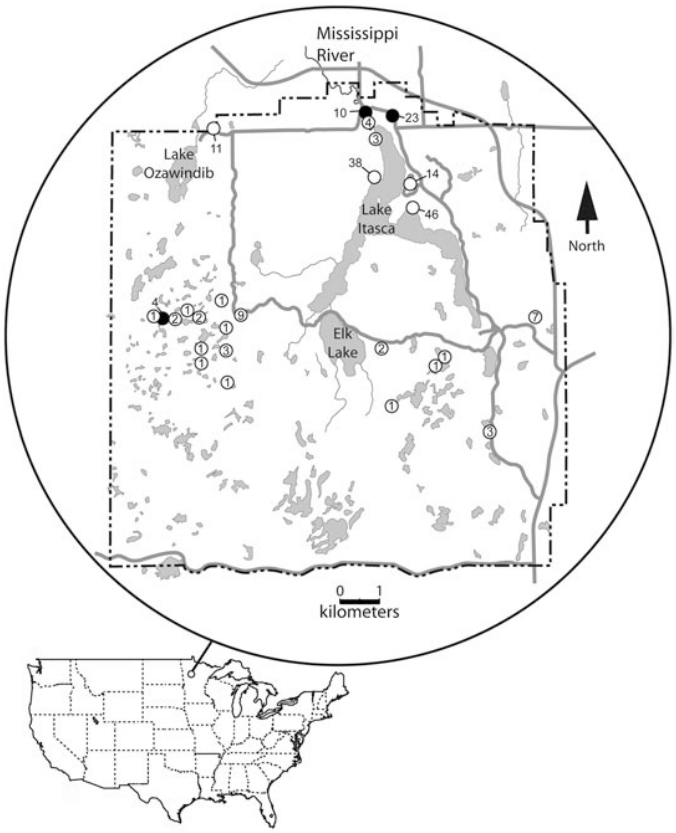

FIG. 1. Collecting locations (circles) of anurans screened for ranavirus at Itasca State Park, Minnesota, USA. Numbers reflect sample size at each site. Sites with ranavirus positive specimens indicated by filled circles, localities with no ranavirus positive specimens indicated by open circles. Park boundaries shown by a dashed line. Light gray areas are water.

ing the manufacturer's protocol. We screened DNA for the presence of ranavirus using polymerase chain reaction (PCR) to amplify a highly conserved ~500-bp fragment of the ranavirus major capsid protein (MCP) using the MCP4 and MCP5 primers (Mao et al. 1997). PCR was carried out in 12.5 μl volumes using conditions described by Greer et al. (2005) and the product visualized by gel electrophoresis on 1% agarose gels stained with ethidium bromide. We ran negative (water in place of genomic DNA) and positive controls (DNA extracted from a specimen verified to be ranavirus positive) with each PCR reaction.

All positive samples were verified by sequencing using Big Dye terminator cycle sequencing on an ABI 3730xl at the Advanced Genetic Analysis Center, University of Minnesota. Sequences were identified as ranavirus using BLAST (Altschul et al. 1990). We gel-purified the positive PCR product from *L. pipiens* prior to sequencing using 3% agarose due to the presence of some non-specific PCR products. Positive PCR products of *L. sylvatica* were purified using Exonuclease I and Shrimp Alkaline Phosphatase (Hanke and Wink 1994).

Results.—We collected and tested 191 YOY and adult frogs of six different species. Four individuals (2%) from two species tested positive for ranavirus (Table 1): three *Lithobates sylvatica* (JFBM 16793, 16923, and 17116) and one *Lithobates pipiens* (JFBM 16932). Four frog species, *Lithobates septentrionalis*, *Pseudacris crucifer, Hyla versicolor*, and *Anaxyrus americanus,* had no positive samples. We captured infected frogs from three different sites within Itasca State Park (Fig. 1); the infected *L. pipiens* shared a site with one of the infected *L. sylvatica* (JFBM 17116). One *L. sylvatica* (JFBM 17116) and the infected *L. pipiens* were adults while the two remaining *L. sylvatica* were recent YOY. Aside from one dead ranavirus-negative *L. pipiens* (cause of death undetermined), all specimens appeared healthy at the time of capture.

DNA sequences (Genbank accession # GQ856477) from the four infected individuals were identical to each other and to other Frog Virus 3 (FV3) sequences from infected North American amphibians (Genbank FJ459783, GQ144408, FJ601916, AY548484, and U36913) and the *Terrapene* Virus 3 (TV3) sequence (U82553) from a turtle (Mao et al. 1997).

Discussion.—FV3 was originally isolated from a Northern Leopard Frog (*L. pipiens*) collected from Minnesota or Wisconsin, and proved to be fairly benign in metamorphosed amphibians in laboratory experiments (Granoff et al. 1966; Tweedell and Granoff, 1968; see Williams et al. 2005 for historical review), but since then, amphibian ranaviruses have been primarily associated with mortality events, especially in larval amphibians (Gray et al. 2009, but see Gray et al. 2007). The presence of ranavirus in apparently healthy metamorphosed frogs at Lake Itasca State Park, an area with no reported amphibian die-offs, suggests that die-offs may not be an inevitable outcome of ranavirus infections and that ranavirus may be more common than previously thought.

There are several possible explanations for our observations of ranavirus in the absence of large-scale mortality events. These include: genetic resistance in these anuran populations (Green et al. 2002; Pearman et al. 2004; Teacher et al. 2009); a less virulent strain of FV3-like virus; the absence of some environmental conditions necessary to cause mass die-offs (Gahl and Calhoun 2008; Greer et al. 2005); or that die-offs occurred but went unnoticed or unreported, particularly since mortality in early, aquatic life history stages would be difficult to observe.

TABLE 1. Ranavirus infections in six frog species collected in Itasca State Park, Minnesota, USA, in 2009.

Species	Family	Adults positive / tested	YOY positive / tested	All individuals positive / tested	Prevalence in all individuals (95% CI)
Lithobates sylvatica	Ranidae	1/21	2/47	3/68	4.4 (2.01–12.69)
Lithobates pipiens	Ranidae	1/68	—	1/68	1.5 (0.01–8.63)
Lithobates septentrionalis	Ranidae	0/27	—	0/27	0 (0–10.85)
Pseudacris crucifer	Hylidae	0/3	0/2	0/5	0 (0–40.10)
Hyla versicolor	Hylidae	0/7	0/2	0/9	0 (0–26.93)
Anaxyrus americanus	Bufonidae	0/3	0/11	0/14	0 (0–19.08)

Other studies have confirmed that five of the frog species we examined are susceptible to ranavirus (Duffus et al. 2008; Green et al. 2002). Although the susceptibility of *A. americanus* to ranavirus remains unknown, ranavirus has been identified in other bufonids such as *Anaxyrus fowleri*, *Bufo bufo*, and *Rhinella marina* (Chinchar 2002; Cunningham et al. 2007; Zupanovic et al. 1998). Given our small sample sizes and the potential for false negatives (Greer and Collins 2007), we cannot eliminate the possibility that species we identified as negative were also infected at low levels.

It is important to document the baseline prevalence of ranavirus infections in amphibian populations in order to understand its epidemiology and transmission in wild populations. In much of the ranavirus literature, amphibians are sampled and tested only after or during die-offs. While this response-based sampling is valuable, it offers a skewed estimate of ranavirus prevalence and perhaps virulence in amphibian populations. Future studies should expand the limited documentation of baseline ranavirus levels in randomly-sampled wild populations and also investigate the factors that may cause sublethal infections to become virulent. Such research would allow scientists to better evaluate the causes and consequences of ranavirus infections in amphibian communities.

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Literature Cited

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403–410.

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

Cunningham, A. A., A. D. Hyatt, P. Russell, and P. M. Bennett. 2007. Experimental transmission of a ranavirus disease of common toads (*Bufo bufo*) to common frogs (*Rana temporaria*). Epidemiol. Infect. 135:1213–1216.

Daszak, P., L. Berger, A. A. Cunningham, A. D. Hyatt, D. E. Green, and R. Speare. 1999. Emerging infectious diseases and amphibian population declines. Emerg. Infect. Dis. 5:735–748.

Duffus, A. L. J., B. D. Pauli, K. Wozney, C. R. Brunetti, and M. Berrill. 2008. Frog Virus 3-like infections in aquatic amphibian communities. J.Wildl. Dis. 44:109–120.

Gahl, M. K., and A. J. K. Calhoun. 2008. Landscape setting and risk of *Ranavirus* mortality events. Biol. Cons. 141:2679–2689.

Granoff, A., P. E. Came, and D. C Breeze. 1966. Viruses and renal carcinoma of *Rana pipiens*: 1. The isolation and properties of virus from normal and tumor tissues. Virology 29:133–148.

Gray, M. J., D. L. Miller, J. T. Hoverman. 2009. Ecology and pathology of amphibian ranaviruses. Dis. Aquat. 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. Aquat. 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. Sc. 969:323–339.

Greer, A. L., M. Berrill, and P. J. Wilson. 2005. Five amphibian mortality events associated with ranavirus infection in south central Ontario, Canada. Dis. Aquat. Org. 67:9–14.

———, and J. P. Collins. 2007. Sensitivity of a diagnostic test for amphibian *Ranavirus* varies with sampling protocol. J. Wildl. Dis. 43:525–532.

Hanke, M., and M. Wink. 1994. Direct DNA-sequencing of PCR-amplified vector inserts following enzymatic degradation of primer and dNTPs. BioTechniques 17:858-860.

Mao, J., R. P. Hedrick, and V. G. Chinchar. 1997. Molecular characterization, sequence analysis, and taxonomic position of newly isolated fish Iridoviruses. Virology 229:212–220.

Oldfield, B., and J J. Moriarty. 1994. Amphibians and Reptiles Native to Minnesota. Univ. Minnesota Press, Minneapolis, Minnesota. 237 pp.

Pearman, P. B., T. W. J. Garner, M. Straub, and U. F. Greber. 2004. Response of the Italian agile frog (*Rana latastei*) to a *Ranavirus*, Frog Virus 3: A model for viral emergence in naïve populations. J. Wildl. Dis. 40:660–669.

Simmons, J. E. 2002. Herpetological Collecting and Collections Management, revised edition. SSAR, Shoreview, Minnesota. 153 pp.

Teacher, A. G. F., T. W. J. Garner, and R. A. Nichols. 2009. Evidence for directional selection at a novel major histocompatibility class I marker in wild common frogs (*Rana temporaria*) exposed to a viral pathogen (*Ranavirus*). PLoS ONE 4:e4616. doi:10.1371/journal.pone.0004616.

Tweedle, K., and A. Granoff. 1968. Viruses and renal carcinoma of *Rana pipiens*. V. Effect of frog virus 3 on developing frog embryos and larvae. J. Natl. Cancer Inst. 40:407–410.

Vandenlangenberg, S. M., J. T. Canfield, and J. A. Magner. 2003. A regional survey of malformed frogs in Minnesota (USA). Environ. Monit. Assess. 82:45–61.

Williams, T., V. Barbosa-Solomieu, and V. G. Chinchar. 2005. A decade of advances in Iridovirus research. Ad. Virus Res. 65:173–248.

Wright, A. H., and A. A. Wright. 1995. Handbook of Frogs and Toads of the United States and Canada. 3rd ed. Comstock Publishing Associates, Ithaca, New York.

Zupanovic, Z., C. Musso, G. Lopez, C. L. Louriero, A. D. Hyatt, S. Hengstberger, and A. J. Robinson. 1998. Isolation and characterization of iridoviruses from the giant toad *Bufo marinus* in Venezuela. Dis. Aquat. Org. 33:1–9.