trade in amphibians and introduced amphibian species. Fungal Biol. Rev. 2:2–9.

Garland, S., A. Baker, A. D. Phillott, and L. F. Skerratt. 2009. BSA reduces inhibition in a TaqMan assay for the detection of *Batrachochytrium dendrobatidis*. Dis. Aquat. Org. 92:113–116.

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.

Hyatt, A. D., D. G. Boyle, V. Olsen, D. B. Boyle, L. Berger, D. Obendorf, A. Dalton, K. Kriger, M. Hero, H. Hines, R. Phillot, R. Cambell, G. Marantelli, F. Gleason, and A. Colling. 2007. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. Dis. Aquat. Org. 73:175–192.

IUCN SSC Amphibian Specialist Group. 2011. *Atelopus patazensis*. *In* IUCN 2011. IUCN Red List of Threatened Species. Version 2011.1. <www.iucnredlist.org>. Accessed 27 October 2011.

Kriger, K. M., and J.-M. Hero. 2007. The chytrid fungus *Batrachochytrium dendrobatidis* is non-randomly distributed across amphibian breeding habitats. Diversity and Distributions 13:781–788.

———, and ———. 2008. Altitudinal distribution of chytrid (*Batrachochytrium dendrobatidis*) infection in subtropical Australian frogs. Austral Ecol. 33:1022–1032.

———, ———, and K. J. Ashton. 2006. Cost efficiency in the detection of chytridiomycosis using PCR assay. Dis. Aquat. Org. 71:149–154.

La Marca, E., K. R. Lips, S. Lötters, R. Puschendorf, R. Ibáñez, J. V. Rueda-Almonacid, R. Schulte, C. Marty, F. Castro, J. Manzanilla-Puppo, J. E. García-Pérez, F. Bolaños, G. Chaves, J. A. Pounds, E. Toral, and B. E. Young. 2005. Catastrophic population declines and extinctions in Neotropical harlequin frogs (Bufonidae: *Atelopus*). Biotropica 37:190–201.

Lips, K. R., J. Diffendorfer, J. R. Mendelson III, and M. W. Sears. 2008. Riding the wave: Reconciling the roles of disease and climate change in amphibian declines. PLoS Biol. 6:441–454.

———, J. K. Reaser, B. E. Young, and R. Ibáñez. 2001. Amphibian Monitoring in Latin America: A Protocol Manual. Society for the Study of Amphibians and Reptiles, Salt Lake City, Utah. 115 pp.

———, Reeve, J. D., and L. R. Witters. 2003. Ecological traits predicting amphibian population declines in Central America. Conserv. Biol. 17:1078–1088.

Lötters, S., R. Schulte, J. H. Cordova, and M. Veith. 2005. Conservation priorities for harlequin frogs (*Atelopus* spp.) of Peru. Oryx 39:343–346.

Sánchez, D., A. Chacón-Ortiza, F. León, B. A. Han, and M. Lampo. 2008. Widespread occurrence of an emerging pathogen in amphibian communities of the Venezuelan Andes. Biol. Conserv. 141:2898–2905.

Seimon, T. A., G. Hoernig, P. Sowell, S. Halloy, and A. Seimon. 2005. Identification of chytridiomycosis in *Telmatobius marmoratus* at 4450m in the Cordillera Vilcanota of southern Peru. *In* Studies on the Andean frogs of the genera *Telmatobius* and *Batrachophrynus*. Monografias de Herpetologia 7:275–283.

———, A. Seimon, P. Daszak, S. R. P. Halloys, L. M. Schloegel, C. A. Aguilar, P. Sowell, A. D. Hyatt, B. Konecky, and J. E. Simmons. 2007. Upward range extension of Andean anurans and chytridiomycosis to extreme elevations in response to tropical deglaciation. Global Change Biol. 13:288–299.

Stuart, S. M., J. S. Chanson, N. A. Cox, B. E. Young, A. S. L. Rodriques, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783–1786.

Venegas, P. J., A. Catenazzi, K. Siu-Ting, and J. Carrillo. 2008. Two new harlequin frogs (Anura: *Atelopus*) from the Andes of northern Peru. Salamandra 44:163–176.

Vié, J. -C., C. Hilton-Taylor, and S. N. Stuart. 2009. Wildlife in a Changing World—An Analysis of the 2008 IUCN Red List of Threatened Species. UCN, Gland, Switzerland. 180 pp.

von May, R., A. Catenazzi, A. Angulo, J. L. Brown, J. Carrillo, G. Chávez, J. H. Córdova, A. Curo, A. Delgado, M. A. Enciso, R. Gutiérrez, E. Lehr, J. L. Martínez, M. Medina-Müller, A. Miranda, D. R. Neira, J. A. Ochoa, A. J. Quiroz, D. A. Rodríguez, L. O. Rodríguez, A. W. Salas, T. Seimon, A. Seimon, K. Siu-Ting, J. Suárez, J. Torres, and E. Twomey. 2008. Current state of conservation knowledge of threatened amphibian species in Peru. Trop. Conserv. Sci. 1:376–396.

Walker, S. F., J. Bosch, V. Gomez, T. W. J. Garner, A. A. Cunningham, D. S. Schmeller, M. Ninyerola, D. A. Henk, C. Ginestet, C. -P. Arthur, and M. C. Fisher. 2010. Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. Ecol. Lett. 13:372–382.

Herpetological Review, 2012, 43(2), 293–295.

Further Presence of Ranavirus Infection in Amphibian Populations of Tennessee, USA

An estimated 43% of amphibian species across the globe are in decline (Stuart et al. 2004). These declines have been attributed to various diseases and anthropogenic impacts such as habitat destruction (Daszak et al. 1999; Price et al. 2006). Of particular interest to this study are ranaviruses, which are known to be a causal factor in amphibian die-offs worldwide (Cunningham et al. 1996; Fox et al. 2006; Green et al. 2002). Gray et al. (2009a) suggested that ranaviruses can have detrimental effects on localized populations, potentially leading to species declines.

In the eastern United States, ranavirus infections have been documented in 33 amphibian species (Miller et al. 2011). In Tennessee, ranavirus has been found in plethodontid salamanders in the Southern Appalachian Mountains (Gray et al. 2009b) and in various species of anurans in farm ponds (Gray et al. 2007; Hoverman et al. 2011a). To date, ranavirus infection in amphibians has not been reported in western Tennessee. Our objective

CHRISTOPHER J. O'BRYAN*
Austin Peay State University, Center of Excellence for Field Biology, P.O. Box 4718, Clarksville, Tennessee 37044, USA; Clemson University, School of Agriculture, Forest, and Environmental Sciences, 261 Lehotsky Hall, Clemson, South Carolina 29673, USA
MATTHEW J. GRAY
274 Ellington Plant Sciences Building, Center for Wildlife Health, Department of Forestry Wildlife and Fisheries, Institute of Agriculture, University of Tennessee, Knoxville, Tennessee 37996, USA; e-mail: mgray11@utk.edu
CHAD S. BROOKS
Austin Peay State University, Center of Excellence for Field Biology, P.O. Box 4718, Clarksville, Tennessee 37044, USA; e-mail: brooksc@apsu.edu
**Corresponding author; e-mail: obryan2@clemson.edu*

TABLE 1. Prevalence and 95% confidence intervals of ranavirus infection in larval amphibians inhabiting four ponds located at the Milan Army Ammunition Plant, Milan, Tennessee, USA, April 2010. Pond coordinates are withheld as these locations are on U.S. Department of Defense property.

	Pond Prevalence and 95% Confidence Interval			
Species	Pond NC1	Pond NC3	Pond NC6	Pond NC9
Ambystoma maculatum		0.33 0.06–0.79	1.0 0.21–1.0	0.5 0.33–0.67
A. opacum		0 0–0.66		
A. talpoideum		0.1 0.02–0.40		
Hyla chrysoscelis / *H. versicolor* complex		0.06 0.01–0.26	0.04 0.01–0.18	0.43 0.16–0.75
Lithobates clamitans			0 0–0.43	
L. sphenocephalus	0.18 0.08–0.34			

was to test for the occurrence of ranavirus in larval amphibian populations at one site in western Tennessee.

We conducted our study at the Milan Army Ammunition Plant (MLAAP) in Milan, Tennessee, USA (35.911977°N, 88.702903°W). The MLAAP was established in 1941 with over 9124 hectares in Carroll and Gibson counties (Brew and Markol 2001). Although herpetological research has been conducted at the MLAAP, none has focused on amphibian pathogens.

We opportunistically captured amphibian larvae from four fishless ponds on the MLAAP during April 2010 using dip nets. Upon capture, larvae were rinsed with aged tap water, placed in separate containers, and transported to Austin Peay State University (APSU). In total, we captured 136 larvae of the following species: *Ambystoma maculatum* (N = 32), *A. opacum* (N = 2), *A. talpoideum* (N = 10), *Hyla chrysoscelis/H. versicolor* complex (N = 53), *Lithobates clamitans* (N = 5), and *L. sphenocephalus* (N = 34). Before moving between ponds, all field personnel disinfected boots, waders, and field collection equipment with a 5% sodium hypochlorite (bleach) solution (Bryan et al. 2009). At APSU, amphibian larvae were euthanized by complete immersion in 80% ethanol after sedation in a 5% ethanol bath. Approximately 50% of the liver was collected for ranavirus testing. The liver is a known site of ranavirus infection in North American amphibians (St-Amour and Lesbarrères 2007), and commonly used in surveillance studies (e.g., Hoverman et al. 2011a). Sterilized instruments and different gloves were used between each animal to prevent cross contamination.

We isolated genomic DNA using standard phenol-chloroform techniques. Liver samples were initially incubated in 100 µl of 1 mg/ml collagenase (Sigma-Aldrich Chemical Company, St. Louis, Missouri, USA) in phosphate buffered saline for 4 hours at 37°C followed by an additional incubation using 100 µl of proteinase K for 16 hours at 37°C. After incubation, the digested tissue samples were triturated to disrupt cellular matrixes, and an equal volume of a 1:1 ratio of phenol:chloroform was added. The sample was vortexed thoroughly for 10 seconds and set at room temperature for approximately 5 minutes. The sample was then centrifuged at 10,000 x g for 5 minutes, and the top aqueous layer was moved to a new tube. Approximately 5 volumes of 100% ethanol and 100 µl of 3 M sodium acetate were added to the sample for DNA precipitation. Samples were placed in a -80°C for at least 15 minutes then centrifuged at 10,000 x g for 10 minutes. The resulting DNA pellet was washed once in 80% ethanol and subsequently centrifuged at 10,000 x g for 5 minutes. The DNA pellet was vacuum-dried, resuspended in 20 µl of molecular grade water, and stored frozen for future ranavirus testing.

We tested for the presence of *Ranavirus* DNA in duplicate for each sample using polymerase chain reaction (PCR) with primers specific for the major capsid protein of the virus (Mao et al. 1997). We used the PCR primers for *frog virus 3* (FV3) published in Mao et al. (1997), which is the type species of *Ranavirus*. These primers have been shown to be reliable for detecting FV3-like ranaviruses in Tennessee amphibians (Gray et al. 2007). The PCR amplified products were visualized on a 0.84% agarose electrophoresis gel, which was stained with ethidium bromide. DNA sequence analysis of PCR samples amplified using the aforementioned primers revealed a greater than 98% DNA sequence identity to NCBI's genomic database for the FV3 major capsid protein gene with an accession number of JQ771299. The PCR amplified products were purified using a Promega Wizard PCR clean-up kit and sent to Vanderbilt University for genomic sequencing.

The major capsid protein of *Ranavirus* was detected in 29 specimens, including a first detection in a wild population for *A. talpoideum*. Percent positives ranged from 0–43% (Table 1), with the highest percentage of infection in *Hyla chrysoscelis/versicolor* complex. Infection was not detected in *A. opacum* and *L. clamitans*.

We detected FV3-like ranaviruses in four amphibian species, with a first species detection in *A. talpoideum* (Miller et al. 2011). Interestingly, Hoverman et al. (2011b) were unable to cause infection in this species when challenged in the laboratory with two strains of FV3-like ranaviruses. One of the highest percent infections was in *A. maculatum*, which are frequently associated with ranavirus die-offs in eastern North America (Brunner et al.

2011; Gahl and Calhoun 2010; Green et al. 2002; Petranka et al. 2003; Todd-Thompson 2010). In the laboratory, Hoverman et al. (2011b) reported that *A. maculatum* larvae had relatively low susceptibility to ranaviral disease. We also documented infection in larval *H. chrysoscelis/H. versicolor* complex and *L. sphenocephalus*, which has been reported previously (Miller et al. 2011). Our findings support previous surveillance and laboratory studies demonstrating that ranaviruses can infect multiple species in an amphibian community (Brunner et al. 2011; Duffus et al. 2008; Hoverman et al. 2010; Schock et al. 2008). Given that ranaviruses are common pathogens in North America that can result in disease emergence in amphibian, reptile, and fish populations (Gray et al. 2009a), state and federal natural resource agencies should consider establishing ranavirus surveillance programs.

Acknowledgments.—Funding for this project was provided by the Presidential Research Scholarship fund through APSU Department of Undergraduate Research and the Tennessee Herpetological Society (Chad Lewis Memorial Grant). We thank the Center of Excellence for Field Biology for the generous use of their equipment and facilities. We are grateful to Floyd Scott for his review of this manuscript and for field assistance provided by Nathan Parker and Michael Fulbright. All sampling was approved by the Tennessee Wildlife Resources Agency (Scientific Collection Permit #1990) and followed APSU Institutional Animal Care and Use Committee protocol #10-002R.

Literature Cited

Brew, P., and J. Markol. 2009. Installation Action Plan for Milan Army Ammunition Plant; c2000–2009 [cited October 27, 2009]. Available at: http://www.globalsecurity.org/military/library/report/enviro/MLAAP_IAP.pdf

Brunner, J. L., K. E. Barnett, C. J. Gosier, S. A. McNulty, M. J. Rubbo, and M. B. Kolozsvary. 2011. Ranavirus infection in die-offs of vernal pool amphibians in New York, USA. Herpetol. Rev. 42(1):76–79.

Bryan, L. K., C. A. Baldwin, M. J. Gray, and D. L. Miller. 2009. Efficacy of select disinfectants at inactivating *Ranavirus*. Dis. Aquat. Organ. 84(2):89–94.

Cunningham, A. A., T. E. S. Langton, P. M. Bennett, J. F. Lewin, S. E. N. Drury, R. E. Gough, and S. K. MacGregor. 1996. Pathological and microbiological findings from incidents of unusual mortality of the common frog (*Rana temporaria*). Philos. Trans. R. Soc. Lond. B. 351(1347):1539–1557.

Daszak, P., L. Burger, A. A. Cunningham, A. D. Hyatt, D. E. Green, and R. Speare. 1999. Emerging infectious diseases and amphibian population declines. Emerg. Infect. Dis. 5(6):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(1):109–120.

Fox, S. F., A. L. Greer, R. Torres-Cervantes, and J. P. Collins. 2006. First case of ranavirus-associated morbidity and mortality in natural populations of the South American frog *Atelognathus patagonicus*. Dis. Aquat. Org. 72:87–92.

Gahl, M. K., and A. J. K. Calhoun. 2010. The role of multiple stressors in ranavirus-caused amphibian mortalities in Acadia National Park wetlands. Can. J. Zool. 88(1):108–121.

Gray, M. J., D. L. Miller, and J. T. Hoverman. 2009a. Ecology and pathology of amphibian ranaviruses. Dis. Aquat. Org. 87(3):243–266.

———, ———, and ———. 2009b. First report of ranavirus infecting lungless salamanders. Herpetol. Rev. 40(3):316–319.

———, ———, 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. Organ. 77(2):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.

Hoverman, J. T., M. J. Gray, N. A. Haislip, and D. L. Miller. 2011a. Widespread occurrence of ranavirus in pond-breeding amphibian populations. EcoHealth DOI: 10.1007/s10393-011-0731-9.

———, ———, ———, and ———. 2011b. Phylogeny, life history, and ecology contribute to differences in amphibian susceptibility to ranaviruses. EcoHealth 10.1007/s10393-011-0717-7.

———, ———, and D. L. Miller. 2010. Anuran susceptibilities to ranaviruses: role of species identity, exposure route, and novel virus isolate. Dis. Aquat. Org. 89(2):97–107.

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

Miller, D. L., M. J. Gray, and A. Storfer. 2011. Ecopathology of ranaviruses infecting amphibians. Viruses 34:2351–2373.

Petranka, J. W., S. S. Murray, and C. A. Kennedy. 2003. Responses of amphibians to restoration of a southern Appalachian wetland: Perturbations confound postrestoration assessment. Wetlands 23(2):278–290.

Price, S. J., M. E. Dorcas, A. L. Gallant, R. W. Klaver, and J. D. Willson. 2006. Three decades of urbanization: Estimating the impact of land-cover change on stream salamander populations. Biol. Conserv. 133(4):436–441.

Schock, D. M., T. K. Bollinger, V. G. Chinchar, J. K. Jancovich, and J. P. Collins. 2008. Experimental evidence that amphibian ranaviruses are multi-host pathogens. Copeia 2008(1):133–143.

St-Amour, V., and D. Lesbarreres. 2007. Genetic evidence of *Ranavirus* in toe clips: an alternative to lethal sampling methods. Conserv. Genet. 8(5):1247–1250.

Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. L. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306(5702):1783–1786.

Todd-Thompson, M. 2010. Seasonality, variation in species prevalence, and localized disease for Ranavirus in Cades Cove (Great Smoky Mountains National Park) amphibians. Master's thesis, University of Tennessee. http://trace.tennessee.edu/utk_gradthes/665/