Herpetological Review, 2013, 44(4), 615–618.

Detection of Ranavirus in Ohio, USA

Of the many proximal factors that potentially contribute alone or in combination to the global concern about amphibian population declines (e.g., Stuart et al. 2004), habitat conversion (e.g., Homan et al. 2004) and infectious diseases (e.g., Daszak et al. 2003; Miller et al. 2011) are dominant concerns. To examine the differential patterns of habitat use for adult amphibians, the amphibian community of Taylor-Ochs Pond, Ohio, USA (40.0538°N, 82.3105°W) has been extensively studied since Spring 2005 (Homan et al. 2008, 2010). This 0.1-ha temporary pond supports breeding populations of *Ambystoma maculatum* (Spotted Salamanders), *Lithobates sylvaticus* (Wood Frogs), and *Anaxyrus americanus* (American Toads). As part of our long-term study, we have surveyed the pond by dip-netting weekly each summer since 2005. Prior to the summer of 2012, the only mass mortality of larval amphibians witnessed was clearly associated with early drying of the pond. However, in late May 2012, a time in which the pond had a suitably high water level, routine dip-netting of Taylor-Ochs Pond revealed evidence of mass mortality among larval amphibians. Larval individuals of four species, *A. maculatum*, *L. sylvaticus*, *A. americanus*, and, uncharacteristically, *Lithobates clamitans* (Green Frogs), were present in the pond at that time. Although we did not attempt to formally

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FIG. 1. Ponds sampled for larval amphibians in Granville, Ohio, USA. No larval amphibians were found in either Gurvis Pond or Wood Duck Pond; a recent metamorph was sampled from Wood Duck Pond. Griesse Pond is upstream of Taylor-Ochs Pond, while Gurvis, Olde Minnow, Spring Peeper, and Wood Duck Ponds are downstream.

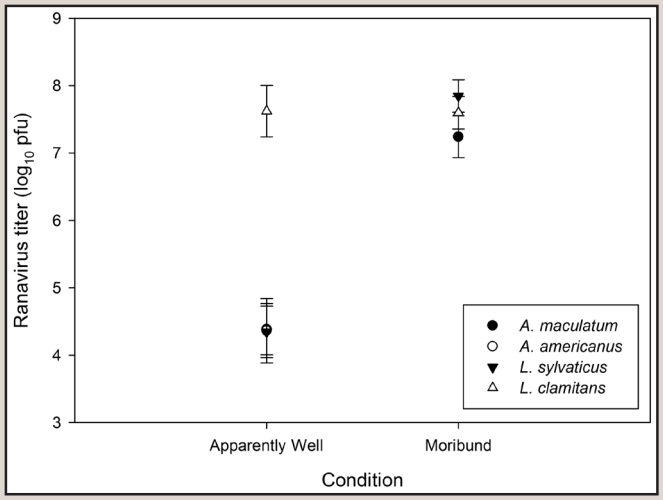

FIG. 2. Log_{10} virus titer (mean ± SE) of apparently well and moribund larval samples collected from Taylor-Ochs Pond, Ohio, USA. For apparently well *Ambystoma maculatum* (N = 2), *Anaxyrus americanus* (N = 5), *Lithobates clamitans* (N = 2), *L. sylvaticus* (N = 2). For moribund *A. maculatum* (N = 3), *A. americanus* (N = 0), *L. clamitans* (N = 5), *L. sylvaticus* (N = 5).

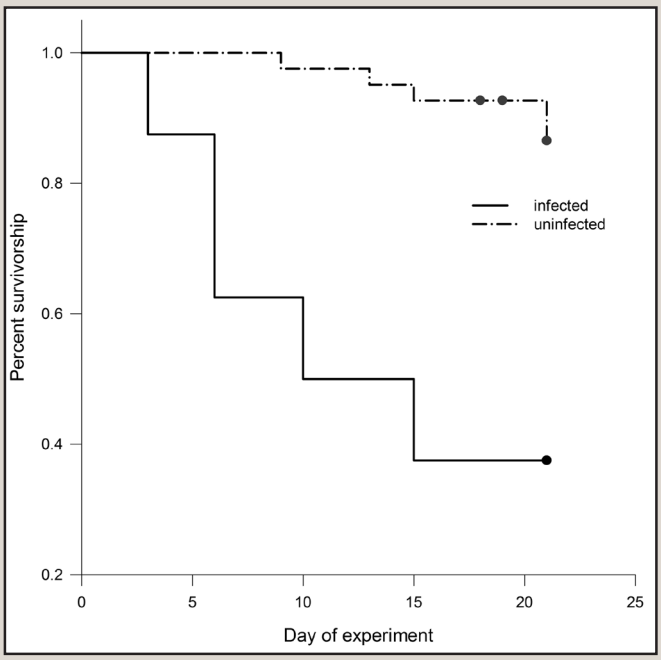

FIG. 3. Kaplan-Meier survival curves for infected (N = 8) and uninfected (N = 41) *Anaxyrus americanus* larvae from Taylor-Ochs Pond, Ohio, USA. Dots represent individuals that were removed during or at the end of the experiment, but were alive at the time of removal.

quantify the magnitude of mortality in the pond, we estimate that hundreds of *A. maculatum*, *L. clamitans*, and *L. sylvaticus* showed signs of illness in the field; only *A. americanus* seemed unaffected.

We suspected ranavirus might have been associated with and contributed to the high mortality based on the signs of individuals, which included hemorrhaging, swelling, erythema, and lethargy (Miller et al. 2011). To determine whether ranavirus was present, on 24 May 2012 we haphazardly collected five each of *A. maculatum* and *A. americanus* larvae, and seven each of *L. clamitans* and *L. sylvaticus* larvae from Taylor-Ochs Pond using dip-nets. Each animal was classified as either moribund (presence of any gross signs consistent with ranavirus infection, as described above) or apparently well (no signs present) at the time of capture. Each animal was bagged individually at the pond, euthanized using MS-222 (2.5g/L buffered to a pH of 7), frozen individually at –80°C, and screened for ranavirus as described below.

Additionally, on 29 and 30 May 2012, we haphazardly collected 49 apparently healthy *A. americanus* larvae from Taylor-Ochs Pond with dip-nets and brought them back to the laboratory. Given their apparent health in the field, we were interested in carefully monitoring individuals over time to document whether they experienced mortality and whether or not that mortality was associated with ranavirus infection. Larvae were housed individually in 500-mL plastic cups with approximately 300 mL of water. Water, either aged tap water or a blend of aged tap and Taylor-Ochs Pond water, was changed every three days. We varied the water source because we originally intended to look for effects of continued exposure to pond water on the mortality and infection status of the larvae; however, the infection rates were the same among the treatments, so we pooled the data to look only at infection and survival. Larvae were fed Tetra fish food every three days *ad libitum*, and were kept in the laboratory at room temperature (21–24°C) in a natural light:dark cycle. Larvae were kept for a period of 21 days. If an animal metamorphosed before the end of that period, it was euthanized and frozen as described above. If a pre-metamorphic animal died before the end of the study, it was bagged individually and frozen at –80°C. All surviving animals at day 21 were euthanized and frozen as above. At the completion of the study, all animals were tested for ranavirus infection, as described below.

In late June, after the confirmation of ranavirus in Taylor-Ochs Pond (see below), we sampled one pond directly upstream of Taylor-Ochs (Griesse Pond) and four ponds downstream of Taylor-Ochs Pond (Gurvis, Olde Minnow, Spring Peeper, and Wood Duck Ponds, Fig. 1). Griesse Pond is a permanent pond that directly feeds Taylor-Ochs Pond via a single stream channel. It has a surface area of ~1.5 ha, and is 200 m upstream of Taylor-Ochs Pond. Gurvis Pond is a permanent pond with an area of ~1.3 ha and is 487 m downstream of Taylor-Ochs Pond; Olde Minnow Pond is a permanent pond with an area of ~0.1 ha and is 684 m downstream of Taylor-Ochs Pond; Spring Peeper Pond is a temporary pond with an area of ~0.1 ha and is 783 m

TABLE 1. Summary of ranavirus infection in *Ambystoma maculatum*, *Lithobates catesbeianus*, *Hyla versicolor*, *L. clamitans*, and *Anaxyrus americanus* in ponds upstream (Griesse) and downstream (Olde Minnow, Spring Peeper and Wood Duck) of Taylor-Ochs Pond, in central Ohio, USA. The 95% confidence interval in the proportion infected is calculated using the method of Wilson (1927) with a correction for continuity (Newcombe 1988).

Site	Date	Coordinates	Species	Infected/Tested	95% CI
Griesse Pond	21 June 2012	40.0546°N, 82.3102°W	*A. maculatum*	0/4	0–0.604
Olde Minnow Pond	21 & 25 June 2012	40.0506°N, 82.3057°W	*L. catesbeianus*	3/3	0.310–1
Spring Peeper Pond	21 & 25 June 2012	40.0501°N, 82.3055°W	*A. maculatum*	1/1	0.055–1
			H. versicolor	0/4	0–0.604
			L. clamitans	1/2	0.027–0.973
Wood Duck Pond	21 June 2012	40.0500°N, 82.3057°W	*A. americanus*	0/1	0.055–1

downstream; Wood Duck Pond is a semi-permanent pond with an area of ~0.2 ha and is 806 m downstream of Taylor-Ochs pond (Fig. 1). Surface areas of ponds and distances between ponds were determined using ImageJ software and satellite photos (Schneider et al. 2012). To avoid potential cross-contamination among ponds, equipment was disinfected with a 10% bleach solution between ponds. In all cases, larvae in these ponds were sampled haphazardly (see Table 1 for sample sizes). We could not find any amphibian larvae in either Gurvis Pond or Wood Duck Pond, but one recent *A. americanus* metamorph was collected from the shore of Wood Duck Pond. All animals were euthanized and frozen as described above and tested for ranavirus. Individuals were kept isolated from one another throughout all procedures.

To screen for ranavirus, we extracted DNA from a combination of tissues from each animal (liver, interrenal glands, and the upper intestine, dissected with sterilized forceps and scissors) using the Qiagen DNeasy Blood and Tissue kit following the manufacturer's instructions (QIAGEN Inc, Valencia, California, USA). We ground up the entirety of very small individuals in 2-mL screw cap tubes filled with a small volume of 1.0-mm diameter silicon-carbide sharp particles (BioSpec Products, Bartlesville, Oklahoma, USA), and then topped up with tissue lysis buffer. These individuals were then homogenized for 45 s using a Mini-BeadBeater-16 (BioSpec Products, Bartlesville, Oklahoma) and DNA was extracted from a 200-µL aliquot of the homogenate. The concentration of extracted DNA was measured using a NanoDrop-2000 (Thermo-Scientific) and, if necessary, diluted to approximately 20ng DNA/µL with elution buffer.

Extracted DNA from each sample was screened for ranavirus in triplicate 20-µL reactions on 96-well plates on a StepOnePlus (Applied Biosystems, Foster City, California, USA) with 5 µL of DNA template (~100ng) using a Taqman realtime polymerase chain reaction (qPCR) with primers and probe that amplify a 70-bp region within the major capsid protein of all known ranaviruses (Brunner and Collins 2009). The unknown samples were quantified by comparing against DNA extracted from a frog virus 3-like ranavirus grown in *Epithilium papilloma cyprinia* cells and serially diluted from 10^2 to 10^7 plaque-forming units (pfu). Each plate also had two no-template controls. Samples with amplification in two or three wells within 35 cycles were scored as positive. Those without amplification in any of the wells were scored as negative. Ambiguous samples were re-run and if at least one well showed amplification the sample was scored as positive. Viral quantities for positive samples are reported as the mean of the $\log_{10}$ (pfu) across all wells of the sample.

In addition, virus isolation was attempted from two moribund, ranavirus-positive animals, one *L. sylvaticus* and one *L. clamitans*. For each animal, the sample was homogenized in 2% fetal bovine serum in minimum essential medium with Hanks salts (HMEM; HyClone, Ottawa, Ontario, Canada) with the Mini-Beadbeater-16 as described above. The homogenates were then filtered through a 0.45-µm filter and 1 mL inoculated onto 90% confluence monolayers of fathead minnow (FHM) cells in 6-well plates for 1 h before being overlayed with 3 mL of 5% FBS-HMEM growth media. Samples were passed two to three times and observed for signs of cytopathic effects using an inverted light microscope. Isolation of ranavirus was verified using conventional PCR with primers 4 & 5 for the major capsid protein (MCP) gene (Mao et al. 1996). The ~500 pb product was sequenced with an ABI 3730 (Applied Biosystems, Foster City, California, USA) and compared with published ranavirus sequences on GenBank using a BLAST search.

We found that all 24 specimens initially collected from Taylor-Ochs Pond were infected with ranavirus (Fig. 2). We successfully isolated a *Ranavirus* from two moribund frogs. The sequence of the MCP gene of these viruses was >99% identical to the type ranavirus frog virus 3 (FV3). To examine the patterns of $\log_{10}$ viral quantities among the four amphibian species in Taylor-Ochs Pond, we ran a two-factor ANOVA to look for effects of species, condition, and any interaction between the two (JMP 10.0, 2012). Excluding *A. americanus*, none of which were moribund, moribund tadpoles had a much higher titer than those that were apparently well ($F_{1,17} = 17.5$, $P < 0.001$). Although sample sizes were small, *L. clamitans* had a significantly higher mean virus titer than *A. maculatum*, *A. americanus*, and *L. sylvaticus* among apparently well individuals ($F_{3,7} = 13.5$, $P = 0.003$; Tukey HSD, $P < 0.05$), whereas there was no significant difference in virus titers among moribund individuals of different species ($F_{2,10} = 1.8$, $P = 0.22$, Fig. 2).

Among the 49 *A. americanus* larvae that were maintained in the laboratory, ranavirus DNA was detected in eight. We generated Kaplan-Meier Survival Curves and used log-rank tests (JMP 10.0, 2012) to look for differences in survivorship between infected and uninfected animals, as well as ANOVA to look for differences in the $\log_{10}$ viral titers among those that died or survived. Infected individuals had significantly lower survivorship than uninfected individuals ($\chi^2_1 = 15.2$, $P < 0.0001$; Fig. 3). Three of the

eight infected larvae survived to the end of the 21-day experiment; two of these survivors metamorphosed during the study. Among the larvae that were infected, there was no difference in virus titer between those that died during the study (mean ± SD = 3.67 ± 3.45 $\log_{10}$ pfu) and those that survived (mean ± SD = 0.90 ± 0.53 $\log_{10}$ pfu; $t_6 = -1.76$, $P = 0.22$), nor was there a difference in virus titer between the surviving metamorphs (mean ± SD = 1.0 ± 0.66 $\log_{10}$ pfu) and the others (mean ± SD = 3.16 ± 3.34 $\log_{10}$ pfu; $t_6 = -0.85$, $P = 0.43$). However, our statistical power was low for both tests (Power = 0.21 and 0.11, respectively).

In our upstream and downstream sampling, we did not detect ranavirus in the four *A. maculatum* larvae found in Griesse Pond, upstream of Taylor-Ochs, but we did find ranavirus downstream, in *L. catesbeianus* from Olde Minnow Pond and in *A. maculatum* and *L. clamitans*, but not *Hyla versicolor*, from Spring Peeper Pond (Table 1). Given our small sample sizes it is not possible to exclude ranavirus occurrence from any site or make meaningful comparisons in prevalence among ponds or species.

In the summer of 2012, Taylor-Ochs Pond in Granville, Ohio experienced a mass die-off event concurrent with the confirmed presence of an FV3-like ranavirus. To our knowledge, this is the first published report of ranavirus in Ohio although ranaviruses have been confirmed in other nearby states, including Illinois and Wisconsin (J. Sekowska, pers. comm.), New York (Brunner et al. 2011), Pennsylvania (Glenney et al. 2010), and Tennessee (Gray et al. 2009; Green et al. 2002). While we cannot be sure that ranavirus caused the observed mortality event, the prevalence of infection among *A. maculatum*, *L. sylvaticus*, and *L. clamitans*, each of which experienced high levels of mortality, as well as the positive relationship between infection and mortality in the laboratory-housed *A. americanus*, suggest that ranavirus likely played a role. In addition to finding ranavirus in our focal pond, we also confirmed its presence in two downstream ponds. Across these three ponds, we identified ranavirus infection among *A. maculatum*, *L. catesbeianus*, *L. clamitans*, *L. sylvaticus*, and *A. americanus*, all of which have had documented ranavirus infections previously (as reviewed by Miller et al. 2011). Whether *H. versicolor* was infected and we simply did not have the sample size to detect it remains unknown, although ranavirus infection in this species has been documented elsewhere (Duffus et al. 2008).

Among the larvae initially sampled from Taylor-Ochs Pond, ranavirus titers were higher in moribund animals than in apparently healthy animals, which is consistent with the finding of Brunner et al. (2005) that higher viral doses are associated with more rapid expression of symptoms. Interestingly, apparently well *L. clamitans*, a species not found in Taylor-Ochs in the preceding seven years, had viral titers that were higher than those of the other species. We hypothesize that a large spring rain event that occurred in 2012 brought the *L. clamitans* larvae to Taylor-Ochs from the upstream Griesse Pond. Although we cannot confirm that *L. clamitans* larvae were the source of the Taylor-Ochs infection, their unusual presence in the community as well as their relatively high viral loads are consistent with this hypothesis. We plan to continue monitoring Taylor-Ochs Pond, as well as the upstream and downstream ponds to document further occurrence and potential impacts of continuing ranavirus presence. Understanding the role of potential co-infection of *Batrachochytrium dendrobatidis*, or another pathogen, in larval health is a secondary objective for future monitoring.

Acknowledgments.—We thank Mr. and Mrs. Griesse and Mrs. Gurvis for allowing us to interview them and sample the ponds on their respective properties. Funding of this project was possible through the Anderson and Bowen Endowments. Animal handling procedures were approved by Denison University's Institutional Animal Care and Use Committee (IACUC) and by a Wild Animal Permit (14-113) provided to RNH by the Ohio Division of Wildlife.

Literature Cited

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

———, and J. P. Collins. 2009. Testing assumptions of the trade-off theory of the evolution of parasite virulence. Evol. Ecol. Res. 11:1169–1188.

———, K. Richards, and J. P. Collins. 2005. Dose and host characteristics influence virulence of ranavirus infections. Oecologia 144:399–406.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2003. Infectious disease and amphibian population declines. Diversity and Distributions 9:141–150.

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.

Glenney, G. W., J. T. Julian, and W. M. Quartz. 2010. Preliminary amphibian health survey in the Delaware Water Gap National Recreation Area. J. Aquat. Anim. Health 22:102–114.

Gray, M. J., D. L. Miller, and J. T. Hoverman. 2009. First report of ranavirus infecting lungless salamanders. Herpetol. Rev. 40:316–319.

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.

Homan, R. N., M. A. Atwood, A. J. Dunkle, and S. B. Karr. 2010. Movement orientation by adult and juvenile wood frogs (*Rana sylvatica*) and American toads (*Bufo americanus*) over multiple years. Herpetol. Conserv. Biol. 5:64–72.

———, B. S. Windmiller, and J. M. Reed. 2004. Critical thresholds associated with habitat loss for two vernal pool-breeding amphibians. Ecol. Appl. 14:1547–1553.

———, C. D. Wright, G. L. White, L. F. Michael, B. S. Slaby, and S. A. Edwards. 2008. Multiyear study of the migration orientation of *Ambystoma maculatum* (spotted salamanders) among varying terrestrial habitats. J. Herpetol. 42:600–607.

Mao, J., T. N. Tham, G. A. Gentry, A. Aubertin, and V. G. Chinchar. 1996. Cloning, sequence analysis, and expression of the major capsid protein of the iridovirus Frog Virus 3. Virology 216:431–436.

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

Newcombe, R. G. 1998. Two-sided confidence intervals for the single proportion: Comparison of seven methods. Statistics in Medicine 17:857–872.

Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9:671–675.

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 306:1783–1786.

Wilson, E. B. 1927. Probable inference, the Law of Succession, and statistical inference. J. Amer. Stat. Assoc. 22:209–212.