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Detection of an FV3-like Ranavirus in Wood Frogs (*Lithobates sylvaticus*) and Green Frogs (*Lithobates clamitans*) in a Constructed Vernal Pool Network in Central New York State

Ranavirus, an emerging and often lethal pathogen of amphibians, reptiles, and fish, has been detected across the continental United States (Duffus et al. 2015). FV3-like ranaviruses have been reported at several locations in New York State in larval Spotted Salamanders (*Ambystoma maculatum*), Jefferson Salamanders (*Ambystoma jeffersonianum*), Wood Frogs (*Lithobates sylvaticus*), Green Frogs (*Lithobates clamitans*), Tiger Salamanders (*Ambystoma tigrinum*; Brunner et al. 2011; Titus and Green 2013; Crespi et al. 2015), and Eastern Box Turtles (*Terrapene carolina carolina*; Johnson et al. 2008). To determine baseline prevalence and patterns of a disease in the environment, systematic surveillance over multi-year periods is necessary. Here, we report ranavirus prevalence for two species across four years in a vernal pool array. We screened *L. sylvaticus* and *L. clamitans* for ranavirus in both natural and constructed woodland vernal pools to determine baseline prevalence estimates. *Lithobates sylvaticus* are commonly occurring at this study site and highly susceptible to ranavirus (Hoverman et al. 2011). *Lithobates clamitans*, however, are less susceptible to ranavirus infection, and exhibit lower mortality rates in a laboratory setting (Hoverman et al. 2011).

In 2010, the Upper Susquehanna Coalition in collaboration with the State University of New York College of Environmental Science and Forestry (SUNY-ESF) constructed 71 vernal pools in Svend O. Heiberg Memorial Forest, Onondaga County, New York State (42.77165°N, 76.08636°W; Fig. 1). These pools were designed in clusters of 1, 3, or 9 pools, contained within numbered landscape hexagons, with an additional cluster—the "microarray"—as shown in Fig. 1. From 2011–2014, all pools containing water were each sampled approximately every four weeks during wood frog tadpole development, for a total of three sampling events per pool, beginning from late May to early June, with the exception of 2013. Due to financial and logistical constraints, sampling in 2013 only occurred in one round in July. For all other years, earliest sampling began six to eight weeks after wood frog egg masses were observed in pools, allowing tadpoles to develop to at least Gosner stage 25 (Gosner 1960). A maximum of 42 pools contained either wood frog or green frog larvae in any given year.

Larval sampling at each interval described above was performed according to pipe sampling protocols as described in Werner et al. (2007), with samples spaced 1–2 m apart in pools greater than 5 m diameter. One sample per ca. 2 m^2 of surface area were taken in smaller pools. Captured tadpoles were stored in buckets containing water from their pool. In 2011 and 2012, buckets were combined and 30 tadpoles randomly sampled from the total sample population of each pool. In 2013 and 2014, an equal number of tadpoles were randomly sampled from each bucket until 10 (year 2013) or 30 (year 2014) individuals were reached. If less than the target number of individuals were sampled, all were used for further processing. All other individuals were immediately returned to their pool of origin, and those selected for further processing were humanely euthanized by immersion in 70% ethanol. Proper care was taken during sampling in all years to prevent contamination between sites by disinfecting all equipment with 10% bleach solution. Ponds were also visually monitored once weekly from May-August each year with the exception of 2013, in which ponds were visited once monthly.

Specimens were preserved in 95% ethanol and stored at 4°C for further processing. Samples from years 2011–2012 were stored together in jars per pool, as these were originally collected simply for Gosner staging as part of a separate study on population recovery at restored vernal pool sites (unpubl. data). Samples from 2013–2014 were stored individually. Ranavirus testing for 2011 was originally intended as preliminary screening, to justify further specimen collection and screening in subsequent years; thus, samples of only five individuals per pool were tested. In 2012–2013, ten individuals per pool where at least ten were captured were randomly selected for testing, to at minimum detect ranavirus outbreaks during these years (Gray et al. 2015). In 2014, all 30 individuals where at least 30 were captured were used. For 2011–2013 samples, liver tissue (up to 25 mg) was extracted, immersed in cell lysis buffer and digested with 10 µL Proteinase K. DNA was extracted and purified using a salt extraction method (Sambrook and Russell 2001). Ranavirus DNA was amplified by PCR using major capsid protein primers 4 and 5 in Mao et al. (1997), following thermocycling conditions for routine PCR described in the Quick-Load® Taq 2X Master Mix handbook (New England BioLabs® Inc.). Products were separated via 1% agarose gel electrophoresis and visualized with ethidium bromide. Negative and ambiguous results were re-amplified using PCR methods as described above.

For 2014 samples, ≤ 20 mg liver tissue was extracted, and DNA was purified using Qiagen DNeasy® Blood and Tissue kit according to manufacturer's protocols for animal tissue. Ranavirus DNA was amplified by conventional PCR using methods described above, and negative or ambiguous results were analyzed by quantitative PCR assay developed by Pallister et al. (2007). Twenty-three percent of conventional PCR negatives were declared positive by quantitative PCR. Quantitative PCR was conducted at Cornell University Animal Health Diagnostic Center using Applied Biosystems StepOne™ real-time PCR system and analyzed with StepOne software v2.3. DNA extractions and cPCR were completed at SUNY ESF. PCR products from four *L. sylvaticus* from 2011 were purified

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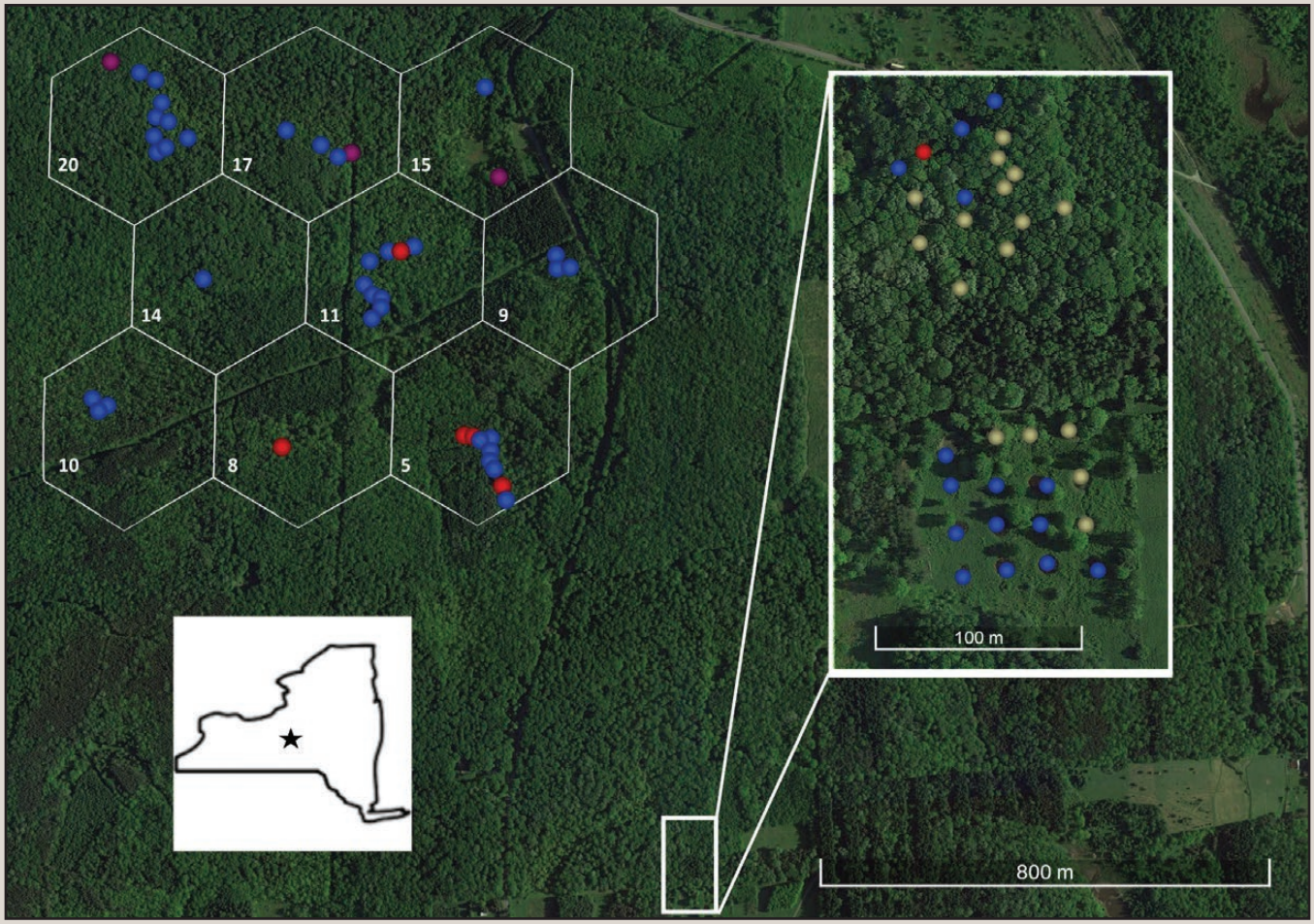

FIG. 1. Aerial view of Heiberg Forest, showing location within New York State (star, map inset). Landscape was divided into 9.3-ha hexagons, and clusters of either 1, 3, or 9 pools (blue dots) were constructed within selected hexagons. Microarray cluster (enlarged section) contained pools with hydroperiods shorter than one month post-spring thaw (beige dots); these pools remained dry during entire summer season each year and were not sampled. Purple dots indicate natural pool sites. Red dots indicate observed die-offs.

using Omega E.Z.N.A.® Cycle Pure Kit, and sequenced at Yale University DNA Analysis Facility. Samples were aligned using BioEdit v 7.2.5, and BLAST® analysis revealed 100% identity with Frog Virus 3 isolate D1 major capsid protein gene, GenBank accession #JQ771299.

Ranavirus prevalence, reported as total ranavirus-positive specimens/total specimens tested during each year, per species, ranged from 0.13–0.51 for *L. sylvaticus*, and 0.0–0.33 for *L. clamitans* (Table 1). Recurring die-offs (visual observation of dead tadpoles) of *L. sylvaticus* were observed during sampling in one hexagon 5 pool in June 2011, July 2013, and July 2014 (of eight total hexagon 5 pools containing water) (Fig. 1). A *L. sylvaticus* die-off occurred in a second hexagon 5 pool in July 2013. Other observed *L. sylvaticus* die-offs occurred in hexagon 11 in June 2011 (one pool of nine containing water), and the microarray in June 2012 (one pool of 16 containing water). Subcutaneous hemorrhaging was observed in *L. sylvaticus* tadpoles from one hexagon 9 pool in July 2014 (of three containing water). No die-offs were observed in hexagon 9 during weekly visits. Moribund *L. clamitans* (erratic swimming, lethargy, skin sloughing) and decomposing carcasses were seen in pool 5B in August 2014 (of eight hexagon 5 pools containing water). Die-offs of *L. clamitans* were observed in hexagon 8 (containing a single pool) in August 2012, and August 2013. In addition, three *A. maculatum* larvae captured during sampling in July 2014 from hexagon 5 exhibited subcutaneous hemorrhaging and extreme lethargy, and tested positive for ranavirus via qPCR.

Lithobates clamitans has been considered as a possible reservoir host for ranavirus (Hoverman et al. 2012), due to their lower rates of infection and mortality (Hoverman et al. 2011) and multi-year larval development periods. Many other species of pool-breeding amphibians are also present in Heiberg Forest (in order of highest to lowest observed abundance): Spring Peepers (*Pseudacris crucifer*), Spotted Salamanders (*A. maculatum*), American Toads (*Anaxyrus americanus*), Eastern Spotted Newts (*Notophthalmus viridescens*), and American Bullfrogs (*L. catesbeianus*). Each of these species is susceptible, in varying degrees, to ranavirus (Green et al. 2002; Hoverman et al. 2011; Forzán and Wood 2013; Richter et al. 2013), and further study of infection status in these species would provide more insight into transmission within the Heiberg system. However, other than *L. sylvaticus, L. clamitans,* and *A. maculatum*, no dead or moribund individuals of the Heiberg amphibian species were observed.

With baseline prevalence greater than zero throughout the spring and summer seasons, proper decontamination protocols should be adhered to by both researchers and recreational users of these forest properties. Our recommendations for continued

TABLE 1. Prevalence estimates for Wood Frog (*Lithobates sylvaticus*) and Green Frog (*L. clamitans*) populations by year. Sample sizes are indicated. *Sampling in 2011 did not include *L. clamitans*. **Sampling only occurred in July. †Values are as follows: (# ponds with observed die-offs of this species/# ponds with ≥ 1 ranavirus-positive specimen of this species/# ponds sampled containing this species). ‡Ponds were not sampled during these die-offs.

		Year			
		2011*	2012	2013**	2014
Lithobates sylvaticus	Prevalence	0.36 (N = 275)	0.13 (N = 436)	0.13 (N = 101)	0.51 (N = 817)
	Ponds†	2/20/34	1/9/22	2/7/11	1/29/32
Lithobates clamitans	Prevalence	NA	0.13 (N = 60)	0 (N = 12)	0.33 (N = 204)
	Ponds†	NA	1‡/2/14	1‡/0/3	1/15/18

surveillance include standardized screening methods and long-term population monitoring. Other studies have suggested that four years is not sufficient to determine population impacts of recurrent outbreaks (Petranka et al. 2003; Earl and Gray 2014). Adult amphibians should also be included in screening processes, as sub-lethally infected adults returning to breeding ponds could be sources of contamination (Brunner et al. 2004). *Lithobates sylvaticus* exhibits a source-sink metapopulation structure (Peterman et al. 2013) and may remain stable or decline at slower rates in hexagon clusters of 3 or 9 pools despite recurrent outbreaks; therefore examining ranavirus effects on populations in Heiberg forest could be useful for guiding future vernal pool construction efforts.

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Presence of *Ranavirus* in a Created Temporary Pool Complex in Southeastern New York, USA

Amphibian population declines have been attributed to habitat loss, fragmentation, and degradation prompting the protection and even creation of amphibian habitats, such as breeding pools (Lichko and Calhoun 2003; Calhoun et al. 2014). If well-constructed, these habitats should support the full suite of ecological interactions (Kolozsvary and Holgerson 2016), including parasitism. Viruses in the genus *Ranavirus* (family Iridoviridae), for instance, are common pathogens of pond-breeding amphibians, often leading to mass mortality events and sometimes long-term population declines (Green et al. 2002; Gray et al. 2009; Miller et al. 2011; Price et al. 2014; Duffus et al. 2015). Petranka et al. (2007) and Youker-Smith et al. (2016) have both reported that *Ranavirus* infections and die-offs occur in amphibian larvae from constructed ponds, especially in Wood Frogs (*Lithobates sylvaticus*) and Spotted Salamanders (*Ambystoma maculatum*), suggesting that amphibian populations in constructed ponds are at least epidemiologically similar to natural ponds. But such studies remain rare. Here we document *Ranavirus* infections in three amphibian species inhabiting a newly created temporary pool in southeastern New York, USA.

Dip-net surveys for amphibian larvae were conducted every other week in seven created temporary pools from early May through July (or until a pool was dry) in 2013 and 2014, as part of a larger study (pools created in 2006; see Kolozsvary and Holgerson 2016). The ponds were embedded in a mixed-deciduous forest intermixed with several large wetlands and other water bodies, adjacent to the Stewart International Airport in the towns of Newburgh and New Windsor, New York, USA (Fig. 1). On 19 June 2013, during regular sampling for water chemistry measurements, dozens of dead or moribund amphibian larvae were found scattered throughout created temporary pool B2 (41.494011°N, 74.122403°W; area = 275.19 m^2; maximum depth = 62 cm) in addition to dozens of actively swimming Wood Frog tadpoles. Pool B2 had substrate dominated by leaf litter, downed logs, with minimal vegetation—except for a small patch of submerged vegetation—and clear water, and thus visibility in the water column was high. Ten live Wood Frog larvae (Gosner stages 31–36) with varying degrees of petechial lesions and edema were collected with a dipnet. These and subsequent specimens were euthanized with tricaine methanesulfonate (MS-222), immediately placed on dry ice in the field, and transported to the laboratory where they were frozen. The specimens were then shipped to Washington State University on dry ice for virus screening.

On 22 June 2013, we returned to pool B2 to conduct the regular dip-net survey. We again observed dozens of Wood Frog larvae carcasses scattered throughout the pool, but saw substantially fewer Wood Frog larvae swimming in the pool than we had three days earlier. During the dip-net survey (20 person minutes), we captured only seven live Wood Frog larvae, all of which showed varying degrees of lesions and edema. We did not conduct a dip-net survey at the pool on 19 June, so we do not have a quantitative comparison of relative numbers of Wood Frog larvae between the two dates, but the difference was qualitatively striking. We did not intend to submit additional Wood Frog larvae for testing, so these seven larvae were released after the survey, but we did collect two Spotted Salamander (*Ambystoma maculatum*) larvae, one Spring Peeper (*Pseudacris crucifer*) larva, and an adult Red-spotted Newt (*Notophthalmus v. viridescens*). Petechial lesions were visible on the Spotted Salamander larvae, which were close to metamorphosis, but not on the Spring Peeper, which was a recent hatchling (Gosner stage < 27; note: the Spring Peeper was not tested for ranavirus.) The newt had red, swollen hind legs, especially proximally, where they join with the body, and thin, red, spidery veins visible on the ventral surface of its main body. An adult Red-spotted Newt had been seen consistently in the pool during previous sampling occasions, although it may or may not have been the newt we collected.

No other amphibian larvae were found in pool B2 during the next bi-weekly sampling event (5 July 2013), which was the last sampling event for this pool before the pool dried completely on 18 July 2013. No dead or visibly sick amphibians were observed in any of the other six created temporary pools in the complex throughout the sampling season.

In 2014, the same set of seven created temporary pools was sampled and no amphibian die-offs were observed in pool B2. However, on 25 May 2014, Wood Frog larvae with visible petechial lesions and edema were observed in pool C1 (41.496168°N, 74.1222207°W; area = 153.78 m^2; maximum depth = 37 cm). Visibility in the water of pool C1 was obscured by dark water color and areas of thick vegetation (primarily *Phragmites australis*), so the extent of the die-off was difficult to assess. Although five live Wood Frog larvae with visible signs of disease were collected, the larvae were not properly preserved on ice and

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