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# *Ranavirus* Infection in Die-offs of Vernal Pool Amphibians in New York, USA

Amphibian ranaviruses have an apparently wide distribution in North America. The type virus in the genus *Ranavirus*, frog virus 3 (FV3), was isolated from aclinically infected leopard frogs (*Lithobates pipiens*) collected in Wisconsin and Minnesota in 1962 (Granoff et al. 1965). Since then, FV3-like viruses have been identified in California (Mao et al. 1999), Saskatchewan (Schock et al. 2008), Tennessee (Gray et al. 2007), North Carolina (Petranka et al. 2003), Alabama and Arkansas (Wolf et al. 1969), Ontario (Greer et al. 2005), Vermont (Clark et al. 1968), and Maine (Bank et al. 2007). To date, however, ranaviruses have not been reported from amphibians in New York State, although they have been isolated from Eastern Box Turtles (*Terrapene carolina carolina*) on Long Island, New York (Johnson et al. 2008). Here we report that FV3-like ranaviruses are widespread in New York State and associated with recurrent die-offs of larval Spotted and Jefferson Salamanders (*Ambystoma maculatum* and *A. jeffersonianum*) and Wood Frog tadpoles (*Lithobates sylvaticus*) in vernal pools.

During routine monitoring of vernal pools for amphibian breeding from May through early July in 2008 and 2009, we noted die-offs in several vernal pools in four locations in New York: the Albany Pine Bush, the Huntington Wildlife Forest in the Adirondack Mountains, the Teatown Lake Reservation in the lower Hudson Valley, and the Mohonk Preserve in the Shawangunk Mountains.

In each case, live larvae or tadpoles were haphazardly collected by hand or with a dip net, sent to the laboratory of JLB in pond water on ice, and frozen at -80°C until they could be screened for ranavirus infection. Samples were collected opportunistically and so sample sizes varied and were generally small. Samples from the Albany Pine Bush and Teatown Lake Reservation were bagged by pond rather than individually such that their independence cannot be guaranteed. Care was taken,

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however, to prevent contamination between ponds by disinfecting sampling equipment between ponds with a 10% bleach solution.

In early to mid-May 2008, KEB and CG visited a pond and vernal pool (ca. 150 m apart) located in the Albany Pine Bush where essentially every tadpole (apparently all *L. sylvaticus*) visible from shore or caught with a dip net was moribund or dead. The tadpoles had edema, diffuse petechial lesions along the ventral surface, especially near the cloaca and base of the tailfin, and about a third had a torn peritoneum, apparently because their abdomens were severely bloated. Similar mortality events were not observed in these two ponds during the two or three visits per breeding season in the previous several years. In 2009, both ponds dried by mid June, earlier than usual, and no apparent disease-associated mortality was noted.

In mid-June 2008, SAM observed dying *L. sylvaticus* tadpoles in three of eight vernal pools that have been regularly monitored for amphibian breeding in the Huntington Wildlife Forest (HWF), Essex County, New York, in the Adirondack Mountains. Moribund (listless, occasionally edematous, with petechial lesions) and apparently healthy *A. maculatum* larvae as well as *L. sylvaticus* tadpoles were collected from four vernal pools, one of which had obvious signs of mortality (pond VP65-2; named according to a grid at the HWF) and three of which did not (VP43-2, VP73-3, and VP70-1). In VP65-2, an apparently healthy adult, female Green Frog (*L. clamitans*) was captured by hand and a toe-clip was taken with a sterile razor blade. On 10 June 2009, moribund and dead *L. sylvaticus* tadpoles were found in two ponds that were sampled the previous year (VP43-2 and VP65-2) and one that had not (VP42-1). Five live tadpoles were collected across all three ponds.

On 19 June 2008, MJR noticed dead wood frog tadpoles in a vernal pool (HV2) at the Teatown Lake Reservation, in Westchester County, New York in the lower Hudson Valley; many more dead tadpoles were observed 26 June. On 30 June 2008 one to two live individuals per species were collected from each of five ponds: three (HV2, HV3, and HV4) where dead and dying *Ambystoma* larvae, probably *A. maculatum*, were noted, along with apparently healthy Grey Tree Frog tadpoles (*Hyla versicolor*), and two (CL1, and DH2) where dead *L. sylvaticus* tadpoles were found. One other vernal pool observed on that day did not have signs of mortality and no samples were collected from it. Five of seven vernal pools at Teatown Lake Reservation had die-offs of amphibian larvae in early July 2009 as well, but samples were not collected.

On 15 June 2009, MBK found a die-off in the James Stokes vernal pool in the Mohonk Preserve, Ulster County. Many dead *L. sylvaticus* tadpoles were found as well as apparently healthy and moribund *A. maculatum* and *A. jeffersonianum* larvae. Two

Table 1. *Ranavirus* infection status, no. positive samples/no. tested, of amphibian samples from New York, USA examined using both PCR of the MCP gene and virus isolation with cell culture. Parentheses represent animals that were transported in the same container; cross contamination cannot be ruled out. # indicates MCP gene was sequenced. * indicates lateral halves of the same individuals were tested by PCR.

| Site | Date | Pond | Coordinates | Species | PCR | Cell culture |
|---|---|---|---|---|---|---|
| Albany Pine Bush | | | | | | |
| | 11 May 2008 | Pond1 | 42.7016°N, 73.8615°W | *Lithobates sylvaticus* | (9/10)# | — |
| | | Pond2 | 42.7012°N, 73.8683°W | *L. sylvaticus* | (1/10)# & (0/4) | (6/6) |
| Huntington Wildlife Forest | | | | | | |
| | 24 June – 3 July 2008 | VP43-2 | 43.9838°N, 74.2048°W | *Ambystoma maculatum* | 0/1 | — |
| | | | | *L. sylvaticus* | 0/5 | — |
| | | VP65-2 | 43.9825°N, 74.2330°W | *A. maculatum* | 3/3# | — |
| | | | | *L. sylvaticus* | 0/5 | — |
| | | | | *L. clamitans* adult | 1/1 | — |
| | | VP73-3 | 44.0091°N, 74.2226°W | *L. sylvaticus* | 0/1 | — |
| | | VP70-1 | 43.9842°N, 74.2230°W | *A. maculatum* | 1/3# | — |
| | 10 June 2009 | VP42-1 | 43.9831°N, 74.2070°W | *L. sylvaticus* | — | 2/2 |
| | | VP43-2 | 43.9838°N, 74.2048°W | *L. sylvaticus* | — | 0/1 |
| | | VP65-2 | 43.9825°N, 74.2330°W | *L. sylvaticus* | — | 2/2 |
| Teatown Lake Reservation | | | | | | |
| | 30 June 2008 | CL1 | 41.2076°N, 73.8562°W | *L. sylvaticus* | (0/2) | (2/2)* |
| | | HV2 | 41.2148°N, 73.8220°W | *L. sylvaticus* | 0/1 | — |
| | | HV3 | 41.2154°N, 73.8218°W | *Ambystoma* spp. | (2/2)# | (2/2)* |
| | | HV4 | 41.2144°N, 73.8217°W | *Ambystoma* spp. | (2/2)# | (1/1)* |
| | | | | *H. versicolor* | (0/2) | — |
| | | DH2 | 41.2024°N, 73.8263°W | *Ambystoma* spp. | (1/2)# | — |
| Mohonk Preserve | | | | | | |
| | 17 June 2008 | James Stokes | 41.8038°N, 74.1120°W | *A. maculatum* | — | 2/2 |
| | | | | *A. jeffersonianum* | — | 2/2 |

of each ambystomatid species were collected alive, all with lesions and two with slight edema by the following day. Apparently healthy *H. versicolor* tadpoles and later metamorphs, and *L. clamitans* tadpoles were observed in this pond during periodic sampling at James Stokes vernal pool throughout the rest of the summer. No morbidity or mortality was noted during a visit two weeks earlier, or at another study site, North Mud pond. These two ponds had been studied extensively in the previous two years, but this was the first time a die-off was observed.

The carcasses or, for larger animals, one lateral half of a carcass was ground in cell lysis buffer with ca. 1 ml silicone-carbide sharp particles (1 mm diameter) using a Bead Beater 96 (Biospec Products, Bartlesville, Oklahoma, USA) and digested with 10 µl Proteinase K. DNA was then extracted using a standard salt extraction protocol (Sambrook and Russell 2001). In order to minimize surface contamination, the samples from Teatown and the Albany Pine Bush, which were bagged and transported in groups, were surface sterilized with a 10% solution of commercial bleach in water and then rinsed with distilled water before being placed in an individual tube and extracted. It is impossible, however, to guarantee independence among these samples. Animals from the Adirondacks and Mohonk Preserve were individually bagged.

Samples were screened with PCR using primers MCP4 and MCP5 (Mao et al. 1997), which target a ca. 500 bp region of the 5' end of the major capsid protein (MCP) gene. PCR products were separated by gel electrophoresis on 1.5% agarose gels and visualized with ethidium bromide staining. Samples were tested at least twice, but up to four times to resolve ambiguous or contradictory results, and scored by majority rules.

PCR products from two *L. sylvaticus* from the Albany Pine Bush, two *A. maculatum* from the Huntington Wildlife Forest, and three *Ambystoma* sp. from the Teatown Lake Reservation (Table 1) were purified using the QIAEX II gel extraction kit (Qiagen, Valencia, California, USA) and submitted to the Nevada Genomics Laboratory for sequencing in the forward direction. Sequences were aligned using Geneious 4.0.2 (Biomatters Ltd.) resulting in a common 471 bp of sequence.

Virus isolation was attempted in the laboratory of JLB on six dead *L. sylvaticus* tadpoles from the Albany Pine Bush; five moribund *L. sylvaticus* tadpoles from the Huntington Wildlife Forest; one moribund and one dead *A. maculatum* and two moribund *A. jeffersonianum* larvae from the Mohonk Preserve; and the un-extracted lateral halves of two *L. sylvaticus* tadpoles and three *Ambystoma* larvae from the Teatown Lake Reservation (Table 1). Samples were homogenized in 5% fetal bovine serum in minimum essential medium with Hanks salts (HyClone, Ottawa, ON, Canada) in tubes filled with silicone-carbide sharp particles (Biospec Products) using a Mini-Beadbeater-96 (Biospec Products). The homogenates were then filtered

through a 0.45-µm filter and inoculated onto *epithelioma papulosum cyprini* (EPC) cells. Samples were passed twice, except for the sample from VP43-2 in which bacterial contamination prevented a second pass, and observed for signs of cytopathic effects using an inverted light microscope at 100× magnification. Isolation of ranavirus was verified using PCR, as above.

Twenty-two of 57 animals tested positive for ranavirus with PCR and ranavirus was isolated from 19 of 20 animals, including the lateral halves of two tadpoles that were negative by PCR (Table 1). Animals from all three regions tested positive, including *Ambystoma* larvae and *L. sylvaticus* tadpoles, as well as the toe of one adult *L. clamitans*, which consistently produced weakly visible bands. Given our small samples sizes, imperfect diagnostic tests (Greer and Collins 2007), and because these samples were collected opportunistically from die-offs, which can occur very rapidly and last for only two or three weeks (Gray et al. 2009), it seems likely that our data underestimate the prevalence, geographic range, and host range of these ranaviruses. Indeed, ranaviruses have been identified in all of the species we tested (Duffus et al. 2008; Gray et al. 2009), even though we never detected ranavirus in *H. versicolor*.

The major capsid protein (MCP) gene sequences of all eight sequenced samples were identical to each other and to the published sequence of Frog Virus 3 (GenBank accession # AY548484) across all 471 bases. It is not clear whether these virus isolates are in fact a single, widespread virus. The MCP is highly conserved among ranavirus species, and so sequence data from the MCP gene alone is not sufficient to differentiate species (Chinchar et al. 2009). Moreover, Schock et al. (2008) have shown that ranavirus isolates with identical MCP sequences can have distinct restriction profiles and perhaps different ecology interactions. Isolates from *L. sylvaticus* and *A. maculatum* collected from two vernal pools in the Teatown Lake Reservation, however, were statistically indistinguishable in terms of the rate and timing of mortality that they caused in an $LD_{50}$ study in *L. sylvaticus* (Warne et al. 2010) and *A. maculatum* (JLB, unpubl. data). Further study is required to determine whether there is one widespread or many, somewhat distinct ranaviruses in New York State.

We must emphasize that while ranaviruses are known to cause catastrophic mortality in larval amphibians (Gray et al. 2009) and several of the virus isolates we collected are highly lethal (Warne et al. 2010) we did not screen for other agents or complete a post mortem exam on these larvae and so we cannot conclude that ranavirus was the cause of these vernal pool die-offs, but merely that amphibians were infected with ranavirus during these die-offs. Whatever the cause, these were not the first die-offs noted in the Huntington Wildlife Forest, which has been systematically monitored for breeding activity every year since the mid 1990s. There was a report of "red-leg," which can be associated with ranaviral disease (Green et al. 2002), in *L. sylvaticus* tadpoles during mortality events in 1999 and 2000 (Quail 2001). Similarly, a die-off of *L. sylvaticus* tadpoles and *A. maculatum* larvae was observed in 2006, with moribund animals showing similar signs of infection as seen in the 2008 and 2009 die-offs. It is worth noting that there is no obvious sign of declines in breeding output in the vernal pools of the Huntington Wildlife Forest (SAM, unpubl. data). Moreover, die-offs may not be completely catastrophic to recruitment. Pond VP65-2, which had a die-off in mid-June of 2008, produced *L. sylvaticus* metamorphs by mid-July.

In summary, FV3-like ranaviruses were identified in live and dead *Ambystoma* larvae and *L. sylvaticus* tadpoles during mortality events in vernal pools across New York State. These epidemics and the occurrence of ranavirus recurred in the same ponds over two years, as has been observed elsewhere (e.g., Petranka et al. 2007). It remains to be determined whether these virus isolates represent a single widespread virus or whether there are several closely related strains of ranavirus with different ecologies. It is clear, however, that these virus isolates are capable of infecting multiple hosts. Whether they were the cause of the die-offs we observed or have long-term impacts on their host populations is not known, but the potential for ranaviruses to cause catastrophic mortality has been established (Gray et al. 2009; Green et al. 2002; Teacher et al. 2010). We urge amateur and professional biologists working in vernal pools in New York State to take basic steps to prevent the spread of these highly lethal viruses including washing and disinfecting boots and equipment between sites and taking care not to move water, sediment, or organic materials between ponds (Bryan et al. 2009; Green et al. 2009).

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