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A *Ranavirus*-related Mortality Event and the First Report of *Ranavirus* in New Jersey

Members of the genus *Ranavirus* in the family Iridoviridae have been documented on all continents with native amphibians except for Africa, though not always in amphibians (Gray et al. 2009). These viral pathogens can infect amphibians, fish, and reptiles. Infected animals typically exhibit skin lesions, organ and tissue swelling, and eventually die of organ failure (Gray et al. 2009 and references within). Ranavirus typically kills amphibians within a few days to a week of infection (Harp and Patrenka 2006), and can be transmitted via the environment, direct contact with an infected individual, or consumption of an infected individual (Pearman et al., 2004).

Although ranavirus has been detected throughout North America, including New York State (Johnson et al. 2008; Brunner et al. 2011) and the Delaware Water Gap of Pennsylvania (Glenney et al. 2010), there are currently no reports of ranavirus in New Jersey. Here we document the occurrence of ranavirus in New Jersey.

In May 2011 we were alerted to a *Lithobates clamitans* tadpole die off in Ocean County, NJ (Robert Zappalorti, pers. comm.). At the site we observed tadpoles that displayed signs consistent with ranaviral disease including lethargy, swelling, and red skin lesions. Additionally, we observed *Anaxyrus fowleri* tadpoles in the same area consuming the carcasses of dead *L. clamitans* tadpoles. Approximately one week after the initial *L. clamitans* die off, there was mass *A. fowleri* tadpole mortality. To assess whether ranavirus was the cause of this tadpole die off, we screened living and dead *L. clamitans* and *A. fowleri* tadpoles as well as other opportunistically sampled animals for the presence of *Ranavirus* DNA using Polymerase Chain Reaction (PCR) and quantitative PCR (q-PCR) at this and several nearby ponds (Table 1, Fig. 1).

Animals were sampled from five ponds located in Ocean County, New Jersey (USA), within an area that is being managed for the benefit of Pine Snake (*Pituophis melanoleucus*) populations (Fig. 1). The first site, Mitigation Pond (MP), is a retention pond immediately adjacent to a capped landfill. One side of the basin is lined and retains water year-round. The remainder of the basin is unlined, and the water level rises and falls with the water table seasonally. The second and third sites (MF Ponds) are located in close proximity to one another at the edge of a field that was artificially cleared for pine snake management (Management Field 2). Management Field 2 Breeding Pond (MF2BP2) is a small artificially constructed, unlined pond. MF2 Vernal Pool (MF2VP) is a small temporary pool covering a section of an unused unpaved road. The fourth site, Hay Pond (HP), is a large, heavily vegetated natural permanent pond. The final site, Costco Pond (CP), is a lined irrigation pond at the edge of the property near the roadside, across the road from a major shopping center. Sampling occurred on 17 May, 26 May, and 16 June 2011. Two other large permanent ponds (Beach Pond (BP) and Spotted Pond (STP)) were only sampled on 26 May.

We retrieved dead and dying *L. clamitans* tadpoles and symptomatic *A. fowleri* tadpoles by net, stored them on ice for approximately three hours, and then froze them at -20° C for 24

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TABLE 1. Sample collection information and ranavirus detection results. Data presented as number testing positive (+) out of number tested (N) by traditional PCR (first column of numbers) and q-PCR (second column). Samples negative by traditional PCR and positive with q-PCR are bold.

Site	Species	Life Stage	First Sample(5/17/11)			Second Sample (5/26/11)			Third Sample (6/16/11)		
			Symptomatic?	+/N	+/N	Symptomatic?	+/N	+/N	Symptomatic?	+/N	+/N
BP (Beach Pond)	*L. clamitans*	Adult	—	—		No	0/2	0/2	—	—	
HP (Hay Pond)	*Hyla* species	Tadpole	—	—		—	—		No	0/1	0/1
	L. clamitans	Tadpole	No	0/2	0/2	—	—		—	—	
	L. clamitans	Adult	No	0/2	0/2	No	0/4	0/4	No	0/7	0/7
	L. sphenocephala	Adult	No	0/2	0/2	No	0/1	0/1	—	—	
CP (Costco Pond)	*A. fowleri*	Tadpole	No	0/4*	2/4*	No	0/5	0/5	No	0/4	0/4
MF2BP2	*A. fowleri*	Tadpole	No	0/3*	1/3*	No	0/6	0/6	—	—	
(Management Field 2 Breeding Pond)	*A. fowleri*	Metamorph	—	—		—	—		No	0/5	0/5
	L. clamitans	Tadpole	Yes	3/3	3/3	—	—		—	—	
MF2VP	*A. fowleri*	Tadpole	No	0/2*	0/2*	No	0/7	0/7	No	0/1	0/1
(Management Field 2 Vernal Pool)	*L. clamitans*	Adult	No	0/1	0/1	—	—		—	—	
Mitigation Pond (MP)	*A. fowleri*	Tadpole	No	0/5*	5/5*	Yes	3/6	3/6	—	—	
	A. fowleri	Metamorph	—	—		—	—		No	0/6	0/6
	Hyla species	Tadpole	—	—		—	—		Dead	0/10	0/10
	L. clamitans	Tadpole	Yes	16/18	16/18	Yes	1/2	1/2	—	—	
	L. sphenocephala	Adult	—	—		Dead	1/1	1/1	—	—	
STP (Spotted Pond)	*L. clamitans*	Tadpole	—	—		No	0/1	0/1	—	—	
	L. clamitans	Adult	—	—		No	0/1	0/1	—	—	

* Water in which the tadpole was held was screened for ranavirus, not a tissue sample.

h before DNA extraction. DNA has been shown to be present in the environment even when an organism is no longer present (Ficetola et al. 2008) so we attempted to determine if water containing asymptomatic *A. fowleri* tadpoles could test positive for *Ranavirus* DNA. We placed all apparently healthy (asymptomatic) *A. fowleri* tadpoles in 1.5 ml eppendorf tubes and allowed them to "swim" in the tube for approximately 30 seconds. Healthy tadpoles were released back to the pond and the water in the eppendorf tubes they had been in (approx. 100 ml) was frozen until DNA extraction.

We caught adult animals by net or hand and removed one toe for DNA extraction using scissors sterilized in 95% EtOH. Toes were stored at room temperature in 1.5 mL eppendorf tubes filled with Drierite desiccant to preserve DNA (Chase and Hills 1991). All adult frogs, with the exception of one dead *L. sphenocephala* (Table 1), appeared to be healthy and were released at the point of capture immediately after tissue removal. Additionally, we removed a small piece of tail tissue that included skin and muscle but no bone from a dead common Snapping Turtle (*Chelydra serpentina*) and collected a shed skin from a northern water snake (*Nerodia sipedon*) found at STP during the second sampling session.

We extracted DNA from tissue and the water that had held asymptomatic *A. fowleri* tadpoles using the Qiagen QIAamp DNA Mini Kit following the manufacturer's instructions. We digested the entire body of *A. fowleri* tadpoles and equivalent amounts of tail tissue from the *L. clamitans* tadpoles. (Since the dead tadpoles displayed varying degrees of decay, harvesting of internal organs was not possible.) Tail tissue was removed using scissors sterilized with 10% bleach. We extracted DNA from a rear leg of the *A. fowleri* metamorphs, and a toe for adult frogs. Additionally, we extracted DNA from the dead *C. serpentina* tail tissue and the shed skin from *N. sipedon* (Table 1).

We used *Ranavirus*-specific primers MCP4 and MCP5 designed by Mao et al. (1997) to amplify an approximately 530 bp fragment of the *Ranavirus* major capsid protein gene using both traditional and q-PCR. For traditional PCR we used 2 µl of DNA in 25 µl reactions that included the following components: 0.4 µM forward and reverse primers, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.1 U/µl taq polymerase. We ran PCR reactions under the following conditions: 1 cycle of 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, followed by a final 7-min extension at 72°C. All reactions were run in a GeneAmp 9700 Thermocycler (Applied Biosystems). Appropriate negative controls using water in place of DNA were included with each PCR run. PCR products were checked for the presence of the *Ranavirus*-specific fragment on 2% 1XTAE gels by SYBR Safe (Invitrogen) staining and UV illumination. Samples were considered positive if there was a DNA fragment present at approximately 530 bp.

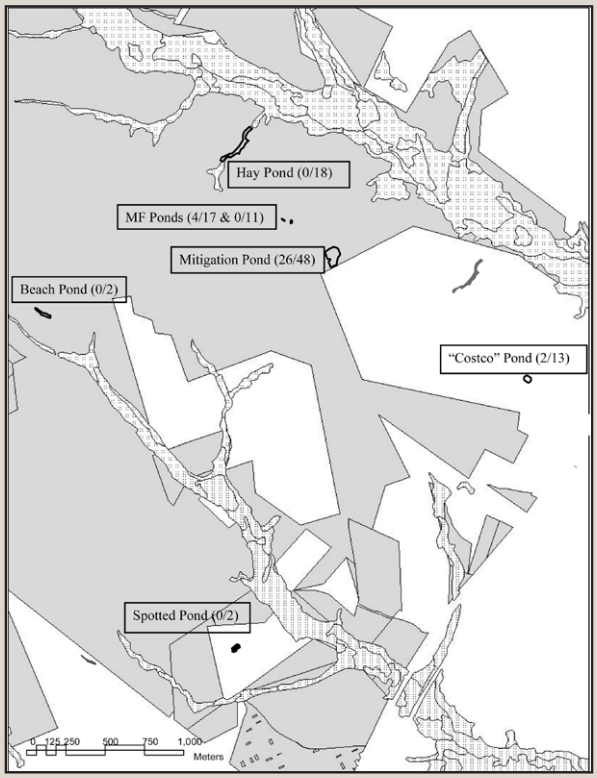

FIG. 1. Map of sampling locations in Ocean County, New Jersey. Numbers in parentheses represent number of samples testing positive for *Ranavirus* DNA / number tested.

PCR product for six putative positive samples was sequenced on an ABI 3130 Genetic Analyzer and compared to known ranavirus major capsid protein DNA sequence to confirm the presence of ranavirus DNA. All six samples were identical to each other and shared 99% similarity to an isolate of Frog Virus 3, confirming their identity as a ranavirus.

We used q-PCR to re-screen all samples. We used 4.6 µl of DNA in 10-µl q-PCR reactions that included the following components: 0.4 µM forward and reverse primers, 5 µl of Brilliant II SYBR Green q-PCR Master Mix (Agilent Technologies), and H_2O to a final volume of 10 µl. We ran positive control reactions using 2 µl of *L. clamitans* tadpole DNA that tested positive for ranavirus using traditional PCR and negative control reactions using water in place of DNA under the same conditions. PCR reactions were subjected to 1 cycle of 95°C for 10 min followed by 40 cycles of 95°C for 45 sec, 50°C for 30 sec, and 72°C for 30 sec. All q-PCR reactions were run on a StepOne Plus Real Time PCR System (Life Technologies). A sample was considered positive if the fluorescence was at least as high as the positive control.

Of the 114 animals sampled, 24 tested positive for the presence of *Ranavirus* DNA with traditional PCR and an additional eight (total of 32) tested positive with q-PCR (Table 1). The water from all 14 of the asymptomatic *A. fowleri* tadpoles from the first sampling session tested negative for ranavirus using traditional PCR, but eight tested positive with q-PCR suggesting q-PCR may be more appropriate for sampling when pathogen concentration is low. Ranaviral DNA was detected in three of the seven ponds tested, although low sample sizes prevent us from excluding its presence from the others (Fig. 1). No ranavirus was detected in the dead snapping turtle or skin shed of the water snake.

To our knowledge, this is the first published account of the presence of *Ranavirus* in the state of New Jersey. Our research demonstrates the need to sample during multiple time periods when a ranavirus outbreak is suspected of occurring. Our initial screening of *A. fowleri* tadpoles during the first sampling session, combined with their healthy physical form, gave the appearance they were not infected with *Ranavirus*. We only detected the presence of *Ranavirus* in multiple tadpoles of this species with traditional PCR after 10 days of exposure to known infected *L. clamitans* individuals in the same pond. Although we detected *Ranavirus* DNA using a non-lethal technique in asymptomatic *A. fowleri* tadpoles, further comparative tests between our non-lethal sampling (tadpole water) and tissue sampling will be necessary before we can determine if this non-lethal method is sensitive enough to consistently detect the presence of ranavirus. Future work at this site should focus on continued monitoring and screening for ranavirus over multiple seasons, and on increasing sampling effort, since for some sites where we had no positive results we did not have sufficient sample size to conclusively say those areas are actually disease free.

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