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Ranavirus is Common in Wood Frog (*Lithobates sylvaticus*) Tadpoles throughout Connecticut, USA

Ranavirus is responsible for 40–60% of amphibian mass mortality events in the United States (Gray et al. 2009; Green et al. 2002). In 2011, a private citizen in Lebanon, Connecticut shipped two live and five dead Wood Frog (*Lithobates sylvaticus*) larvae and one dead Spotted Salamander (*Ambystoma maculatum*) larva to the USGS National Wildlife Health Center in Madison, Wisconsin, after finding moribund and dead animals. Histological abnormalities from the two live frog larvae and one dead ambystomatid were typical of *Ranavirus* infection (Gray et al. 2009). Polymerase chain reaction (PCR) assays for the major capsid protein confirmed that isolated viruses were *Ranavirus*. Following this event, we learned of several instances throughout northeastern Connecticut where moribund or dead tadpoles were observed, but with no further action taken. We therefore undertook a statewide survey for the presence of *Ranavirus* in the summers of 2012 and 2013. Our specific objectives were to survey for the presence of *Ranavirus* in wetlands across the state of Connecticut and quantify the proportion of infected larvae within each wetland.

We focused on surveying larval Wood Frogs due to their high susceptibility to *Ranavirus* infections and widespread distribution across the northeastern USA (Hoverman et al. 2012), however, larvae of some other species were opportunistically collected and tested for *Ranavirus*. In 2012, we sampled eight wetlands in northeastern Connecticut, near where previous die-offs had been reported: four within the Yale-Myers (YM) Research Forest, and four within surrounding public lands in the town of Lebanon, Connecticut. Wetlands on public properties were identified via frog-call surveys in late March and early April. In 2013, we randomly selected state-owned properties distributed across Connecticut, with the condition that two properties were a minimum of 15 km apart to ensure samples were from presumed separate populations. Within each state property, we located three individual wetlands by performing a series of nighttime frog-call surveys during the Wood Frog breeding season in late March and early April. We then revisited wetlands beginning in early to mid-June to collect Wood Frog larvae. All sampling occurred within a time period of two weeks, and thus sampled larvae were at a similar stage of development (average Gosner stage ~ 35).

In both years we conducted dip net sweeps until at least 30 frog larvae were captured, a sample size sufficient to have 95% confidence that the prevalence of infection is < 10% if all samples tested negative (Gray et al. 2015). If Wood Frogs were absent from a site, we did not collect larvae of other species. These wetlands contained Gray Tree Frogs and other species not commonly found with Wood Frogs, suggesting habitat conditions were not ideal for Wood Frog breeding activity. We recorded any gross signs of disease or *Ranavirus* infection (e.g., lethargy, emaciation, areas of hemorrhaging). We rinsed tadpoles with sterile water and then euthanized tadpoles individually in a solution of MS-222 (Argent Laboratories, Richmond, Washington, USA). Once individuals were unresponsive to tactile stimuli, we placed individuals in separate whirl-pak bags (Nasco, Fort Atkinson, Wisconsin, USA) containing 90% ethanol. Between sites, we disinfected all field equipment with a 10% bleach solution, scrubbing any mud from the treads on our boots, and allowing equipment to dry fully (adapted from Phillott et al. 2010).

Liver samples of collected Wood Frog larvae, as well as larval Green Frogs and Spotted Salamanders that were collected opportunistically at two wetlands, were screened for *Ranavirus* DNA with a quantitative real time polymerase chain reaction (qPCR)

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TABLE 1. Average *Ranavirus* titer in plaque-forming unit equivalents (Avg. PFU) and number of *Lithobates sylvaticus* individuals infected out of number of individuals sampled from 29 wetlands at 16 properties across the state of Connecticut, USA. All individuals were collected at the larval stage (~ Gosner stage 35) during the month of June. Wetlands were given a four-letter code for identification (ID). Results are presented in chronological order.

Wetland ID	Property name	GPS coordinates	Year sampled	Avg. PFU	No. infected
ALTW	Aldo Leopold WMA	41.47816N, -73.28854W	2013	5.04E+08	2/3
CMMM	Cromwell Meadows WMA	41.59010N, -72.66185W	2013	2.41E+04	8/10
CMMO	Cromwell Meadows WMA	41.58778N, -72.66057W	2013	1.29	1/10
COCS	Cockaponset SF	41.40500N, -72.52228W	2013	32.1	1/10
COGL	Cockaponset SF	41.42698N, -72.53826W	2013	465.35	9/10
DHGG	Devil's Hopyard SP	41.48024N, -72.34840W	2013	0	0/10
DHRG	Devil's Hopyard SP	41.48039N, -72.34548W	2013	3.81	5/10
DHSH	Devil's Hopyard SP	41.48172N, -72.34744W	2013	81.78	8/10
HOSP	Housatonic SF	41.89636N, -73.39604W	2013	0	0/10
HOTW	Housatonic SF	41.89072N, -73.39882W	2013	0.8	1/10
NATB	Naugatuck SF	41.44533N, -73.08320W	2013	69.81	2/6
NHFP	Nathan Hale SF	41.76262N, -72.35644W	2013	9.51E+03	8/10
NHND	Nathan Hale SF	41.76258N, -72.34759W	2013	1.36E+07	10/10
NHTT	Nathan Hale SF	41.75644N, -72.34168W	2013	8.45E+07	3/3
PAMT	Paugusset SF	41.40675N, -73.20113W	2013	1.99	1/10
PEBH	People's SF	41.94928N, -72.99620W	2013	14.7	7/10
PEKI	People's SF	41.95935N, -73.00730W	2013	1.39	1/10
PESS	People's SF	41.94405N, -72.99606W	2013	10.7	7/10
RODT	Roraback WMA	41.73442N, -73.06288W	2013	0	0/4
ROSY	Roraback WMA	41.73449N, -73.05755W	2013	3.15	1/10
SGRC	Sleeping Giant SP	41.43345N, -72.88143W	2013	0	0/10
LEBB*	Bartlett Brook WMA	41.59752N, -72.26767W	2012, 2013	159, 1.66E+05	20/22, 10/10
LEPO*	Pomeroy State Park	41.69548N, -72.22221W	2012, 2013	1.77E+04, 1,465	20/23, 10/10
LEBR*	Lebanon Private Property	Private residence, NA	2012, 2013	3.84E+07, 15.5	22/22, 10/10
LEGR	Lebanon Airline Trail	41.614320N, -72.367532W	2012	2.95E+06	7/9
YMAT*	Yale Myers Forest	41.96678N, -72.15372W	2012, 2013	183.2, 115.3	8/10, 10/10
YMWP*	Yale Myers Forest	41.95498N, -72.12379W	2012, 2013	1.67E+07, 1.01E+08	18/18, 10/10
YME8*	Yale Myers Forest	41.96442N, -72.14852W	2012, 2013	48, 4.58E+03	6/6, 7/10
YMLP*	Yale Myers Forest	41.96683N, -72.15061W	2012, 2013	770.9, 1.53E+06	9/10, 10/10

in the Amphibian Disease Diagnostic Laboratory at Washington State University. DNA was extracted with Qiagen DNEasy Blood and Tissue kit following the manufacturer's instructions (QIAGEN Inc, Valencia, California, USA) and then ca. 5 µL of DNA template was included in triplicate 20 µL reactions on 96-well plates qPCR reaction with primers and probe that amplify a 70-bp region within the major capsid protein of all known amphibian ranaviruses in North America (Picco and Collins 2008). A 10-fold serial dilution of DNA extracted from a Frog Virus 3-like *Ranavirus* grown and titered in *Epithilium papilloma cyprinia* cells was used as a standard against which unknown samples were quantified. Samples were scored positive if ≥ 2 of the three wells had clear amplification and negative of zero of three wells had amplification. If there was amplification in just one of the three wells, the sample was re-run and considered positive if ≥ 1 well was positive and negative if not. *Ranavirus* titers are reported as $\log_{10}$ (average plaque-forming units; PFU) of all replicates of the sample.

In 2012, most Wood Frog larvae (110 of 120; 91.7%) tested from the eight wetlands were positive for *Ranavirus* (Table 1), and *Ranavirus* was found in all eight wetlands (Fig. 1). In 2013, we collected a total of 727 larval Wood Frogs from 35 wetlands across the state of Connecticut. For logistical and financial reasons we screened a subset of these samples for *Ranavirus*: 256 Wood Frogs from 28 of 35 wetlands sampled in 2013 (Table 1, Fig. 1). We submitted up to ten individual Wood Frogs per wetland for qPCR testing. We detected *Ranavirus* in 24 of 28 wetlands tested, at 13 of 14 properties (Table 1). Of the 256 larval Wood Frogs tested, 142 (53.6%) were positive for *Ranavirus*. The prevalence of *Ranavirus* was ≥ 50% in 16 of 28 wetlands and 100% in seven wetlands (Table 1). We also detected *Ranavirus* in all five *Lithobates clamitans* larvae from the one site they were collected (ALTW; see Table 1 for site name acronyms), but in none of the *A. maculatum* larvae from two sites (N = 4, NATB; N = 6, RODT). We cannot be certain that the pathogen was absent in the four wetlands in which *Ranavirus* was not detected, only that the prevalence was probably ≤ 28% (based on Wilson confidence interval on a proportion, Newcombe 1998). Average titers were generally similar among tadpoles within the same wetland, but varied by orders of magnitude among wetlands in both years (Table 1), from low of 1.29 PFU in CMMO to a high of 5.04×10^8 PFU in ALTW.

The widespread occurrence of *Ranavirus* among our sampled wetlands aligns with other recent surveys (Glenney et al.

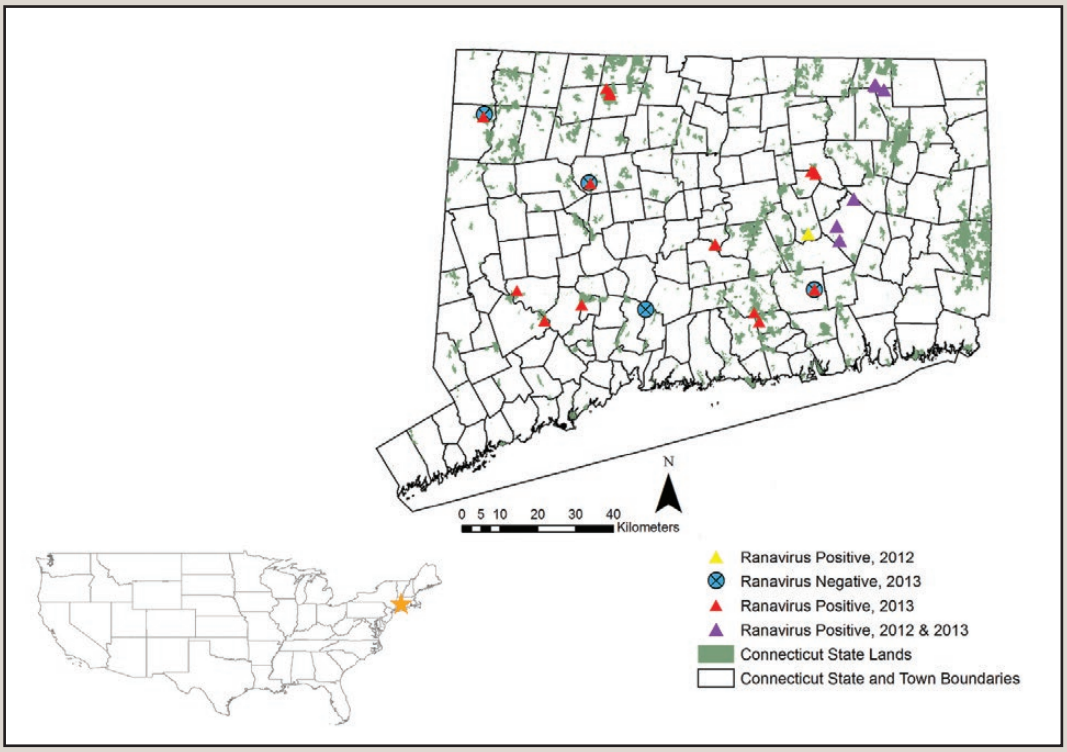

FIG. 1. Distribution of 29 wetlands on 16 properties across Connecticut, USA surveyed for *Ranavirus* in 2012 and 2013. Symbols denote the presence or absence of *Ranavirus*-infected Wood Frog (*Lithobates sylvaticus*) tadpoles by year sampled. Connecticut's location relative to the extent of the USA is indicated with a star on the inset panel.

2010; Brunner et al. 2011; Hoverman et al. 2012; Rothermel et al. 2013; Crespi et al. 2015). We observed signs of a mass mortality event at only one wetland in one year (YMLP in Table 1). Here on 4 June 2013 we observed ≥ 100 frog larvae floating near the surface of water with little ability to right themselves or control their direction of movement. These larvae showed little to no response to our movements when we walked through the wetland; we also found individuals that already appeared to be deceased. When in the hand, these larvae had noticeably swollen hind limbs, as well as petechial hemorrhaging across the ventral surface and on the hind limbs. Titers were high in this wetland (1.53×10^6 PFU on average, Table 1), but were not the highest observed in this sampling season. No larval amphibians collected or observed at any other wetland showed gross signs of infection. However, *Ranavirus*-related mortality events can progress rapidly within a wetland (e.g., Wheelwright et al. 2014) and the majority of our sites were visited only once during the larval period, so even though carcasses of Wood Frog larvae may remain detectable for a week or more during die-off events (E. M. Hall, pers. comm.) our odds of detecting a die-off in any given wetland likely was low. Still, our results demonstrate that reported mortality events severely underestimate the distribution of *Ranavirus* across a landscape, especially when surveys sacrifice temporal replication (i.e., many repeat visits to a single site) to improve spatial coverage of a landscape (i.e., few visits to many sites). More intensive monitoring within a wetland is needed to elucidate the dynamics and outcomes of *Ranavirus* epidemics.

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