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# PREVALENCE OF RANAVIRUS IN SPOTTED SALAMANDER (*AMBYSTOMA MACULATUM*) LARVAE FROM CREATED VERNAL POOLS IN WEST VIRGINIA, USA

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ABSTRACT: Ranavirosis is a disease of high concern for amphibians due to widespread documentation of its lethal and sublethal impacts and its high transmission potential across populations and species. We investigated whether spotted salamander (*Ambystoma maculatum*) ranavirus prevalence and viral load were associated with habitat characteristics, genetic diversity, corticosterone levels, and body size. In 2015 and 2016, we sampled 34 recently created vernal pools in the Monongahela National Forest, West Virginia, USA. We collected tail clippings from 1,128 spotted salamander larvae and waterborne hormone samples from 436 of those larvae, along with eight environmental characteristics of the pools. Over the 2-yr period, we detected ranavirus in 62% of pools, with prevalence ranging from 0% to 63% (mean, 7.68%). Spotted salamander size was positively correlated with ranavirus presence and viral load; however, we did not find associations between ranavirus prevalence or viral load and habitat characteristics, spotted salamander genetic diversity, relatedness, effective number of breeders, or corticosterone levels. The widespread occurrence of ranavirus in the vernal pools illustrates the potential for rapid natural introduction of the pathogen to created wetlands. Managers could consider monitoring local distributions of ranavirus before creation of new vernal pools to guide strategic placement of the wetlands to minimize occurrence and prevalence of this pathogen.

*Key words:* *Ambystoma maculatum*, amphibian, disease, pathogen, restoration, wetlands.

## INTRODUCTION

Ranaviruses are DNA viruses in the family Iridoviridae that cause infections that lead to hemorrhaging and necrosis of multiple organs, resulting in amphibian mortality (Chinchar 2002; Gray et al. 2009). Green et al. (2002) attributed most amphibian die-offs in the US from 1996 to 2001 to ranaviruses. The detection and distribution of ranavirosis are increasing. The mass die-offs and population declines caused by ranavirus make it a pathogen of concern for amphibian conservation (Earl and Gray 2014; Price et al. 2014; Duffus et al. 2015). Reptiles and fish are also susceptible to ranavirus and can function as reservoirs that are capable of transmitting the pathogen to amphibians (Brenes et al. 2014a, b). The infection can be transmitted to uninfected individuals through physical contact, by use of shared water or soil, or by consumption of infected tissue (Harp and Petranka 2006; Gray et al. 2009; Robert et al. 2011).

Susceptibility to ranavirus varies among amphibian species, with higher susceptibility associated with species inhabiting semipermanent pools and larvae with rapid development

(Hoverman et al. 2011). Amphibians in northeastern North America, such as wood frogs (*Rana sylvatica*) and spotted salamanders (*Ambystoma maculatum*), rely on vernal pools for breeding. Larvae in these fishless, ephemerally flooded wetlands are adapted to a short developmental period of 2–4 mo. Wood frogs and spotted salamanders have high and intermediate susceptibility to ranavirus, respectively (Hoverman et al. 2011), and die-offs are documented for both species (Green et al. 2002; Docherty et al. 2003).

Wetland characteristics can influence ranavirus infection and mortality rates (Gahl and Calhoun 2008, 2010). For example, Arizona tiger salamanders (*Ambystoma mavortium nebulosum*) in pools with less vegetative cover had higher infection rates of *Ambystoma tigrinum* virus, one of several ranaviruses, potentially due to the congregation of individuals around pool edges (Greer and Collins 2008). Ranavirus gene copy number and mortality rate of spotted salamander and wood frog larvae increased in high water temperatures (Brand et al. 2016; Hall et al. 2018); however, the association between temperature and mortality rates is strain and species dependent (Rojas et al. 2005; Echaubard et al. 2014). Susceptibility to ranavirus also varies by developmental stage. Mortality rates from ranavirus infection are higher in wood frog metamorphs than in earlier larval stages (Warne et al. 2011; Hall et al. 2018). Conversely, prevalence of the ranavirus Frog virus 3 decreased in American bullfrog (*Rana catesbeiana*) tadpoles as development progressed (Gray et al. 2007). Arizona tiger salamander larvae and metamorphs can sustain sublethal infection, thereby allowing the infection to persist in populations (Brunner et al. 2004; Greer et al. 2009).

Physiological condition and genetic diversity can also influence amphibian susceptibility to ranavirus (Daszak et al. 2000). Disease can elevate the concentration of stress hormones, particularly corticosterone, negatively impacting fitness of amphibian tadpoles (Warne et al. 2011; Gabor et al. 2013; Davis et al. 2020). Corticosterone is a glucocorticoid hormone that mobilizes resources for amphibians when additional energy is needed for proper immune system function, growth, and development and for response to environmental stressors (Romero 2004; Denver 2009; Crespi et al. 2013). In wood frogs, tadpoles infected with ranavirus had high corticosterone concentrations, fast developmental rates, and decreased body weight (Warne et al. 2011). Conversely, in northern leopard frog (*Rana pipiens*) tadpoles, sublethal infections of ranavirus reduced growth and development (Echaubard et al. 2010). Higher genetic diversity was correlated with reduced mortality from ranavirus infection in Italian agile frogs (*Rana latastei*; Pearman and Garner 2005).

Given that susceptibility to ranavirus infection is influenced by species, individual, and habitat factors, management actions have the potential to affect disease transmission and prevalence in amphibian populations (Wobeser 2002; Grant et al. 2018). Historically, loss and degradation of vernal pools have been high due to difficulties in mapping pools and limited protection for smaller pools that lack surface water connections to rivers (DiBello et al. 2016). This has resulted in conservation efforts to create new vernal pools to mitigate habitat loss (Calhoun et al. 2014). Presence of pool-breeding amphibians is a component of healthy vernal pool systems, and an important factor for producing ideal habitat conditions for amphibians is reducing the risk of spreading disease. Determining individual and habitat characteristics that influence host susceptibility to pathogens, pathogen occurrence and prevalence, and infection intensity would help inform managers in their efforts to create and maintain habitat (Grant et al. 2018).

Our objectives were to survey for ranavirus infection in larval spotted salamanders inhabiting recently created vernal pools and determine whether habitat characteristics, population genetics, or individual physiology was correlated with occurrence, prevalence, or viral load. We examined whether infection prevalence was related to pool age, water temperature, water pH, pool depth, pool diameter, vegetative cover and refuge within the pool, predator presence, number of pools

within 1 km, and average distance to other pools that were sampled. We tested for correlations between ranavirus occurrence, prevalence, or viral load and genetic diversity, effective number of breeders, or genetic relatedness. We tested for an association between average corticosterone level and ranavirus prevalence in a pool. We also compared individual corticosterone levels of larvae that were infected with ranavirus with those that were not and tested for an association with viral load. Finally, we tested whether larval body size was associated with occurrence or viral load.

## MATERIALS AND METHODS

We sampled vernal pools created by the US Forest Service (USFS) in the Monongahela National Forest, West Virginia, USA (Fig. 1). Pools were in east central West Virginia at elevations of 1,219–1,296 m. We refer to pools created in the same year and located geographically proximate to each other as regions (Barton Bench 2011, Lambert North 2013, and Lambert South 2014; Fig. 1). All pools were within 5 km of each other. Vernal pool creation was based on field conditions rather than predetermined designs as part of larger restoration projects (USFS 2014). The area was strip-mined for coal in the 1970s before USFS acquisition in the 1980s. Initial restoration efforts began in 2009 and included removal of nonnative Norway spruce (*Picea abies*) and red pine (*Pinus resinosa*) planted during mine reclamation; deep ripping to mitigate soil compaction; and planting aspen (*Populus* spp.), red spruce (*Picea rubens*), black cherry (*Prunus serotina*), wild raisin (*Viburnum nudum*), elderberry (*Sambucus nigra*), and service berry (*Amelanchier arborea*; Sandeno 2011; USFS 2014). Because of recent tree removal, most pools had an open canopy (Millikin et al. 2019). We did not locate any natural vernal pools in the area. Species using created vernal pools, in addition to spotted salamanders, included adult eastern newts (*Notophthalmus viridescens*) and adult and larval wood frogs, green frogs (*Rana clamitans*), American toads (*Anaxyrus americanus*), and Cope's gray tree frogs (*Hyla chrysoscelis*), with wood frog and green frog tadpoles being most common.

### Field sampling

In May and June 2015 and 2016, we caught spotted salamander larvae in vernal pools by using dipnets and seines. We captured 20.51±0.34 (mean±SEM; range, 14–30) spotted salamander larvae in 34 of the 46 pools sampled. In total, 22 pools were sampled in both years, 8 pools were sampled only in 2015, and 4 pools were sampled only in 2016 (Table 1). Upon capture, larvae were placed in individual high-density polyethylene specimen cups (Dynarex, Orangeburg, New York, USA) in 20 mL of distilled water premixed (to prevent osmotic shock) with R/O Right water conditioner (Kent Marine, Franklin, Wisconsin, USA; Millikin et al. 2019). We removed larvae from specimen cups after 1 h. We then measured the total larval length to the nearest millimeter. We collected a tail clip (<1 cm) that was immediately submerged in 100% ethanol (Pharmco, Lexington, Kentucky, USA) in a 1.5-mL microcentrifuge tube (Fisher Scientific, Pittsburgh, Pennsylvania, USA) for storage until DNA extraction for ranavirus screening and genetic analysis. We then released the spotted salamander at the capture site. To prevent cross-contamination, we sterilized all equipment (e.g., nets, waders, scissors, forceps) with 10% bleach (Clorox Bleach, Oakland, California, USA) between pools and between larvae.

In 2015, we collected tail clips from 616 spotted salamanders from 30 pools. We removed one pool containing 27 samples from the data set due to detection of ranavirus in an extraction of a negative control (water). The remaining samples included the following: 193 larvae (10 pools) in Barton Bench, 281 larvae (13 pools) in Lambert North, and 115 larvae (6 pools) in Lambert South. In 2016, we collected tail clips from 539 spotted salamanders (26 pools): 205 larvae (10 pools) in Barton Bench, 232 larvae (11 pools) in Lambert North, and 102 larvae (5 pools) in Lambert South.

### Molecular analysis

We extracted DNA from spotted salamander tail clips by using Wizard® SV 96 genomic DNA purification system kits (Promega, Madison, Wisconsin, USA) at West Virginia University. We standardized DNA concentration in water to 15 ng/μL and shipped it frozen to the Disease Testing Center, University of South Dakota, to test for ranavirus by using quantitative PCR (qPCR). We determined ranavirus prevalence and estimated the number of viral gene copies per larvae by following Forson and Storfer (2006). Each qPCR plate contained a negative control (no-template, water) and standardized dilution series of gBlocks containing the target sequence of DNA (major capsid protein) for a standard curve ($1e^2$–$1e^5$). We ran 2 μL of each sample in triplicate and considered it positive for ranavirus infection if at least two of the three replicates amplified and the gene copy number was >1.0 (Davis and Kerby 2016; Watters et al. 2018). Samples with one replicate positive were re-run using the same conditions to confirm positivity. We used StepOne

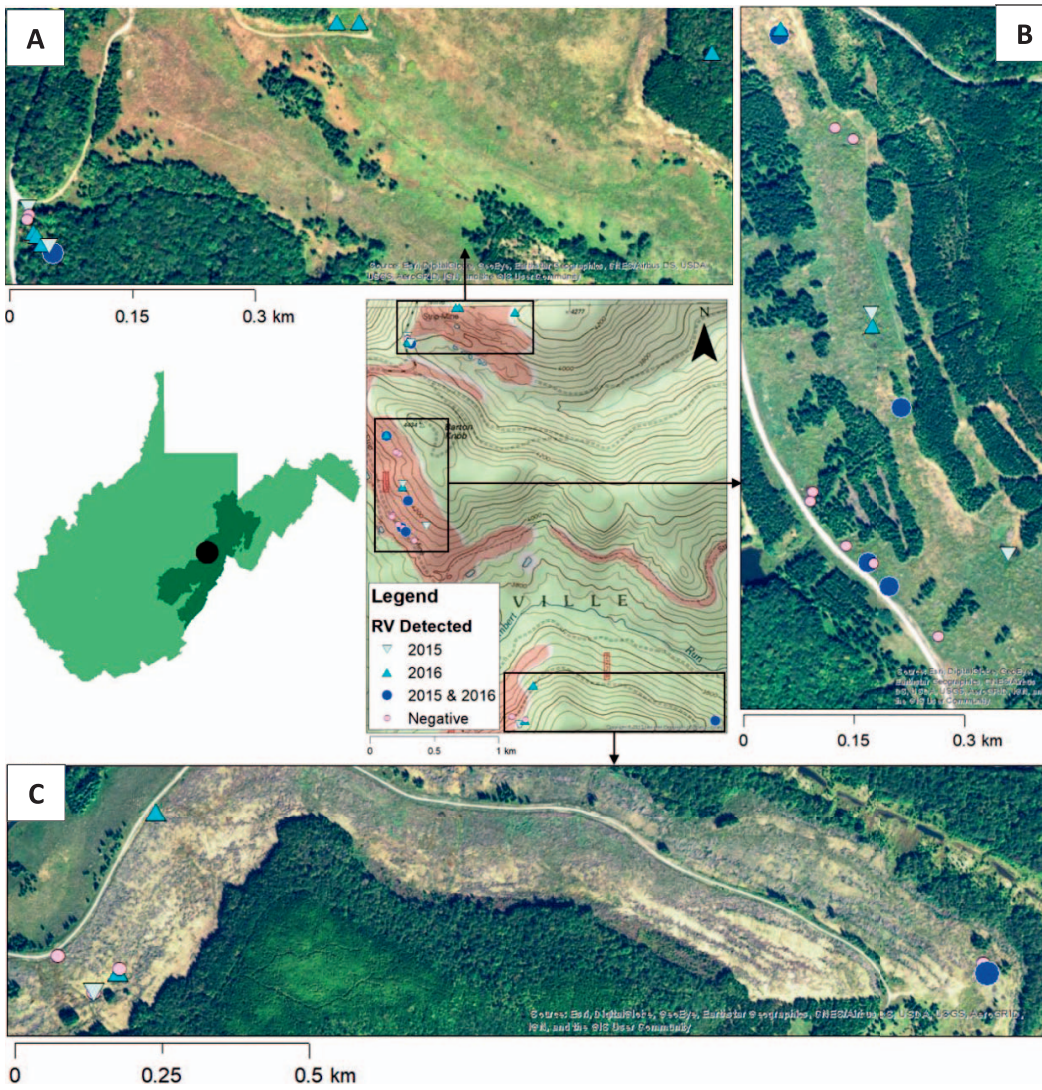


FIGURE 1. Map displaying study area in Randolph County, West Virginia, USA, in the Greenbrier District of the Monongahela National Forest. Triangles and circles represent locations of sampled created vernal pools. Regions are separated geographically from north to south: (A) Barton Bench, (B) Lambert North, and (C) Lambert South. State map displays Monongahela National Forest and the general area of sampling with a black circle. USA topo map accessed through ESRI © 2013 National Geographic Society, i-cubed; world imagery source: ESRI, DigitalGlobe, GeoEye, Earthstar Geographics, Centre National d'Etudes Spatiales/Airbus Defence and Space, US Department of Agriculture, US Geological Survey, AeroGRID, Instituto Geográfico Nacional, and the GIS User Community.

version 2.3 software (Applied Biosystems, 2010) to quantify viral gene copies based on average gene copies in the three replicates.

### Habitat variables

We recorded habitat characteristics at each pool to determine whether they influenced pathogen prevalence (Millikin et al. 2019). Pool diameter was based on an average of the longest distance across the pool and the perpendicular measurement. Pool depth was based on an average of three measurements along the longer transect of the pool. Using the line intercept method, we measured available cover (i.e., rocks, coarse woody debris, vegetation) within the pool

TABLE 1. Spotted salamander (*Ambystoma maculatum*) ranavirus prevalence and quantity of viral copies per 100 ng of DNA at 34 created vernal pools, Monongahela National Forest, West Virginia, USA, separated by sampling year (from 18 May to 10 June 2015 and from 23 May to 13 June 2016).

| | No.[b] | | Prevalence[c] (%) | | Viral copies (mean±SE) | |
|---|---|---|---|---|---|---|
| Pool[a] | 2015 | 2016 | 2015 | 2016 | 2015 | 2016 |
| B1 | 14 | —[d] | 21 | — | 43.00±28.33 | — |
| B11 | 20 | 20 | 10 | 5 | 35.96±24.75 | 14.37±14.37 |
| B2 | 20 | 20 | 0 | 0 | 0±0 | 0±0 |
| B4 | 20 | 20 | 0 | 0 | 0±0 | 0±0 |
| B5 | 20 | 23 | 0 | 4 | 0±0 | 17.52±17.52 |
| B7 | 20 | 21 | 0 | 10 | 0±0 | 13.70±10.90 |
| B8 | 15 | 21 | 0 | 10 | 0±0 | 5.72±3.94 |
| B9 | 20 | 21 | 5 | 0 | 15.67±15.67 | 0±0 |
| BB2 | 24 | 20 | 0 | 10 | 0±0 | 170.50±169.88 |
| BB8 | 20 | 20 | 0 | 30 | 0±0 | 442.62±197.32 |
| BB9 | – | 19 | — | 63 | — | 488,985.6±402,591.2 |
| LN10 | 25 | 20 | 28 | 10 | 148.02±60.25 | 84.83±76.43 |
| LN104 | 15 | 21 | 27 | 10 | 102.66±48.74 | 12.76±9.95 |
| LN105 | — | 20 | — | 5 | — | 21.98±21.98 |
| LN11 | 22 | 20 | 0 | 0 | 0±0 | 0±0 |
| LN18 | 25 | 20 | 16 | 10 | 56.27±28.29 | 18.23±12.66 |
| LN2 | 21 | 20 | 0 | 0 | 0±0 | 0±0 |
| LN24 | 25 | 20 | 0 | 0 | 0±0 | 0±0 |
| LN4 | 23 | 30 | 0 | 0 | 0±0 | 0±0 |
| LN5 | 20 | 21 | 0 | 0 | 0±0 | 0±0 |
| LN56 | 18 | — | 50 | — | 291.31±77.39 | — |
| LN62 | 22 | 20 | 5 | 5 | 13.20±13.20 | 8.88±8.88 |
| LN77 | — | 20 | — | 5 | — | 6.75±6.75 |
| LN78 | 22 | — | 55 | — | 679.05±250.85 | — |
| LN94 | 21 | — | 0 | — | 0±0 | — |
| LN96 | 22 | — | 0 | — | 0±0 | — |
| LS1 | — | 22 | — | 0 | — | 0±0 |
| LS119 | 20 | — | 0 | — | 0±0 | — |
| LS120 | 19 | 20 | 11 | 5 | 99.51±68.98 | 7.38±7.38 |
| LS125-34 | 22 | — | 5 | — | 20.63±20.63 | — |
| LS125-5 | 17 | 20 | 0 | 0 | 0±0 | 0±0 |
| LS2 | — | 20 | — | 5 | — | 2.18±2.18 |
| LS49 | 16 | — | 0 | — | 0±0 | — |
| LS69 | 21 | 20 | 0 | 5 | 0±0 | 3.05±3.05 |

[a] Pools include those from Barton Bench (B, BB), Lambert North (LN), and Lambert South (LS).

[b] Number of larvae tested per pool per sample year.

[c] Prevalence = percentage of larvae positive for ranavirus out of total larvae sampled at a pool.

[d] Dash indicates no data due to the site being dry or a lack of spotted salamanders.

along the two transects to the nearest centimeter (Egan and Paton 2004). We also measured the predominant vegetation: grass (Poacea), sedge (Cyperaceae), cattail (Typha spp.), and rush (Juncaceae) (GSCR). We calculated the proportion of pool cover and GSCR as the total length of cover intersecting transects divided by the cumulative length of both transects at the pool (Egan and Paton 2004). We determined number of pools within 1 km of each pool in ArcMap version 10.5.1 (Esri 2017) by using spatial join, and we calculated average distance to other pools by using point distance.

The presence of predators such as eastern newts, diving beetle (Dytiscidae) larvae, and dragonfly (Odonata) larvae was indexed as present

or absent (0/1) as determined by direct observation or capture while sampling for spotted salamanders. We ranked predator abundance at pools from 0, meaning that no predators were detected, to 3, meaning that all three taxa were observed (Millikin et al. 2019). We recorded pool age in years as the sample year minus the creation year. We measured water temperature and pH at the time of sampling by using a PCTestr™ 35 Oakton® waterproof multiparameter Testr™ (Cole-Parmer, Vernon Hills, Illinois, USA).

### Genetic diversity and corticosterone

We tested whether genetic and hormone data (Millikin et al. 2019) were correlated with ranavirus prevalence and viral load or were different between spotted salamander larvae that were or were not infected with ranavirus. We analyzed genetic data from larvae in 22 pools in 2015 and from larvae in 25 pools in 2016 (Millikin 2019). We examined whether occurrence, prevalence, or viral load was correlated with expected genetic heterozygosity, allelic richness, relatedness, and effective number of breeders (Millikin 2019). We calculated expected heterozygosity and allelic richness by using FSTAT, with 10,000 iterations based on a minimum sample size of 10 individuals (Goudet 1995). We measured relatedness with the mean within-population pairwise values by using the Queller and Goodnight (1989) estimator in GenAlEx (Peakall and Smouse 2006, 2012). We used COLONY to estimate effective number of breeders (Wang 2009; Wang et al. 2011; Millikin 2019). We sampled waterborne corticosterone levels from 436 spotted salamander larvae: 181 larvae across 27 pools in 2015 and 255 larvae across 26 pools in 2016 (Millikin et al. 2019). Waterborne hormone samples were filtered, purified, and concentrated using solid phase extraction, and corticosterone concentrations were measured in 96-well plates by using CORT ELISA kits (Cayman Chemicals Inc., Ann Arbor, Michigan, USA; Millikin et al. 2019). Waterborne corticosterone units account for body size by dividing by total length of the larvae and are based on corticosterone release rate per hour (pg/TL/h; Millikin et al. 2019).

### Statistical analysis

We treated pools as the sampling unit for analyses, except when looking at individual corticosterone levels and individual total length. Ranavirus infection prevalence represented the proportion of individual spotted salamander larvae with positive ranavirus detection out of the total number of larvae sampled at a pool. We standardized the mean quantity of ranaviruses to number of viral copies in 100 ng of DNA by taking the average quantity detected in a sample of 2 μL (containing 15 ng of DNA/μL, or 30 ng/2 μL sample) and multiplying it by (100/30).

We used beta regressions (betareg 3.1-2; Zeileis 2019), which are designed for analyzing response data restricted to defined intervals, such as rate and proportion data, to estimate relationships between environmental predictors and prevalence of ranavirus (Ferrari and Cribari-Neto 2004). The models use a beta distribution, thereby allowing for an asymmetric structure and do not require normalized rate and proportion response data (Cribari-Neto and Zeileis 2010). We compared models predicting prevalence at each pool by using the Akaike information criterion corrected for small sample size (AICc; Burnham and Anderson 2002) with AICcmodavg 2.1-0 (Mazerolle 2017). Model predictors included pool age, water temperature, water pH, predator presence, pool depth, pool diameter, pool cover, GSCR, pools within 1 km, average distance to other pools, average spotted salamander total length, average corticosterone level, expected heterozygosity, allelic richness, relatedness, and effective number of breeders.

Because our data lacked a normal distribution, we used a nonparametric Kruskal-Wallis test to compare prevalence and viral load among regions and sample years ($\alpha$=0.05). We used nonmetric multidimensional scaling (NMDS) ordination to visualize differences in pools with and without ranavirus present in relation to habitat characteristics (Zuur et al. 2007; Borcard et al. 2011). We used Bray-Curtis distance measurement, a random starting point, and two dimensions (Roberts 1986) using vegan 2.5-3 (Oksanen et al. 2018).

We used Kruskal-Wallis to test 1) whether pools with and without ranavirus differed in genetic expected heterozygosity, allelic richness, relatedness, and effective number of breeders; 2) individual levels of corticosterone between spotted salamander larvae infected with and those without ranavirus; and 3) differences in total length between spotted salamander larvae that were and were not infected with ranavirus. We used Spearman rank to test for a correlation between viral load and corticosterone level, and we also tested for correlations between viral load and total length of spotted salamander larvae. Total length data were missing for 10 of the larvae sampled for ranavirus. Analyses were conducted in R version 3.4.2 (R Core Team 2017).

## RESULTS

We detected ranavirus infection in 84 (7%) of 1,128 spotted salamander larvae from 21 (62%) of the 34 pools. At pools sampled both

TABLE 2. Spotted salamander (*Ambystoma maculatum*) ranavirus prevalence in larvae and created vernal pools, Monongahela National Forest, West Virginia, USA, by region (Barton Bench, Lambert North, Lambert South) and sample year (2015, 2016, 2015–16).

| | Larvae | | | | | | Pools | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2015 | | 2016 | | 2015–16 | | 2015 | | 2016 | |
| Region | $n$[a] | %[b] | $n$ | % | $n$ | % | $n$[c] | %[d] | $n$ | % |
| Barton Bench | 193 | 3 | 205 | 13 | 398 | 8 | 10 | 30 | 10 | 70 |
| Lambert North | 281 | 13 | 232 | 4 | 513 | 9 | 13 | 46 | 11 | 55 |
| Lambert South | 115 | 3 | 102 | 3 | 217 | 3 | 6 | 33 | 5 | 60 |

[a] $n$ = sample size of larvae/region.
[b] % = percentage of larvae infected with ranavirus.
[c] $n$ = sample size of pools/region.
[d] % = percentage of pools infected.

years, individuals in 13 of the 21 pools (62%) tested positive at least one of those years: 6 pools changed from negative to positive between years; 1 pool changed from positive to negative; and 6 pools were positive both years and prevalence at these 6 pools stayed the same or decreased between years (Table 1). We detected ranavirus at four of the eight pools that were only sampled in 2015. At three of the pools with positive detections during 2015, we caught 14–22 spotted salamander larvae in 2015 and either found no egg masses (two pools) or only caught 2 larvae (one pool) in 2016. Four of the five pools sampled only in 2016 had positive detections.

Ranavirus infection prevalence across sample years averaged <10% in all regions (Table 2). Prevalence was higher when examining percentage of pools infected compared with percentage of spotted salamander larvae. Prevalence of all larvae in a region was ≤13% in 2015 and 2016. Prevalence of pools infected was ≤46% in 2015, but as high as 70% in 2016 (Table 2). Within-pool prevalence ranged from 0% to 63% (mean±SE, 7.68±0.02%; Table 1). In 2015, prevalence ranged from 0% to 55%: 0–21% in Barton Bench, 0–55% in Lambert North, and 0–11% in Lambert South (Fig. 2). In 2016, prevalence ranged from 0% to 63%: 0–63% in Barton Bench, 0–10% in Lambert North, and 0–5% in Lambert South (Fig. 2).

We found no difference in prevalence or viral load among regions or between sample years ($P \geq 0.27$; Fig. 2). No environmental predictors were more supported than a null model for explaining prevalence of ranavirus infection (Supplementary Material Table S1). The NMDS ordination indicated almost

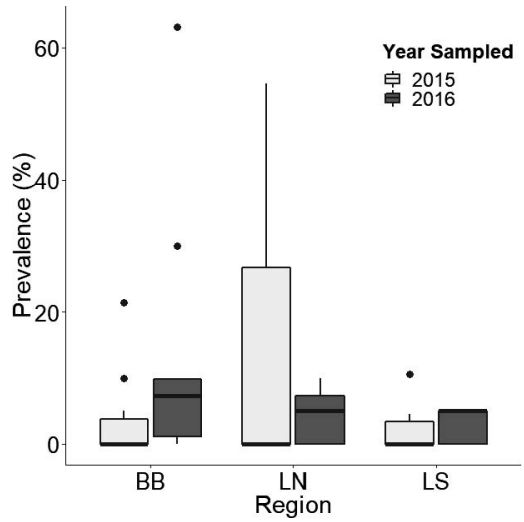


FIGURE 2. Boxplots displaying ranges of ranavirus prevalence (percentage of larvae with positive detections out of total sampled at a pool) in spotted salamander (*Ambystoma maculatum*) larvae within created vernal pools in the Monongahela National Forest, West Virginia, USA. Regions include Barton Bench (BB), Lambert North (LN), and Lambert South (LS). Sample years include 2015 ($n$=10, 13, and 6 pools for BB, LN, and LS, respectively) and 2016 ($n$=10, 11, and 5 pools for BB, LN, and LS, respectively).

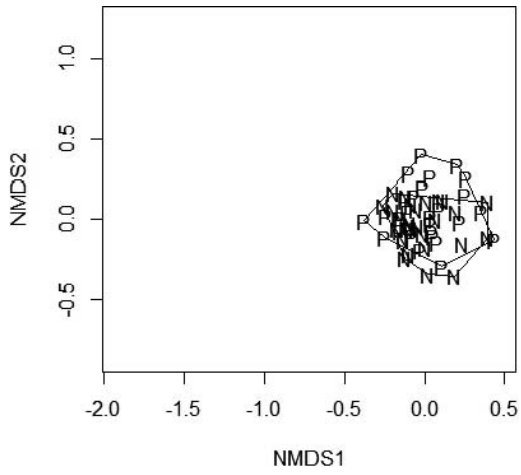


FIGURE 3. Nonmetric multidimensional scaling ordination of created vernal pools based on environmental characteristics, Monongahela National Forest, West Virginia, USA. Polygons demonstrate overlap of pools with positive (P) and negative (N) detections of ranavirus in spotted salamander (*Ambystoma maculatum*) larvae from sampling in 2015 and 2016: P detections in 28 pools and N detections in 27 pools.

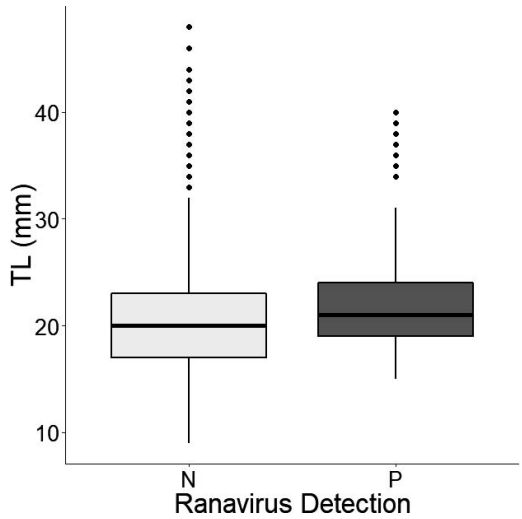


FIGURE 4. Boxplot displaying ranges in individual spotted salamander (*Ambystoma maculatum*) larval total length (TL in millimeters) separated by larvae with negative (N) and positive (P) detections for ranavirus: 1,034 larvae tested N with TL of 21.00±0.19 mm (mean±SEM), and 84 larvae tested P with TL of 22.52±0.64 mm, Monongahela National Forest, West Virginia, USA. Letters indicate significant difference (Kruskal-Wallis $\chi^2$=7.71; $P$=0.005).

complete overlap of pools with positive detections and pools without ranavirus (k=2, stress=0.25; Fig. 3).

In 2015, 8 (36%) of 22 pools with genetics data had positive detections for ranavirus. In 2016, 16 (64%) of 25 pools with genetics data had positive detections. There were no differences in any genetic parameters between pools with or without ranavirus ($P \geq 0.28$; Supplementary Material Fig. S1). There was no association between viral load and genetic diversity, effective number of breeders, or relatedness ($P \geq 0.08$).

We only detected ranavirus in 35 (8%) of 436 spotted salamander individuals sampled for corticosterone: 14 (8%) of 181 individuals sampled in 2015 and 21 (8%) of 255 individuals sampled in 2016. We found no difference in corticosterone levels between individual larvae infected with ranavirus (mean±SEM, 1.97±0.19 pg/TL/h) and those that were not (2.06±0.09 pg/TL/h; $P$=0.51; Supplementary Material Fig. S2). Corticosterone levels were also not correlated with viral load ($P$=0.53).

Spotted salamander larvae infected with ranavirus had greater total length, averaging 22.52±0.64 mm, than those that were not infected (21.00±0.19 mm; Kruskal-Wallis $\chi^2$=7.71; $P$=0.005; Fig. 4). Total length was also positively correlated with viral load (Spearman ρ=0.08; $P$=0.006; Fig. 5). Of these larvae, 1,034 tested negative and 84 tested positive for ranavirus.

## DISCUSSION

Our results indicate presence of ranavirus infection is widespread among recently created vernal pools in the Monongahela National Forest; however, most pools had low prevalence of ranavirus infection among spotted salamanders. Ranavirus infection was positively correlated with size of larvae, potentially due to either faster developmental rates or variation in susceptibility at different developmental stages. In wood frogs, lethal doses of ranavirus increased developmental rates, but decreased body weight (Warne et al. 2011).

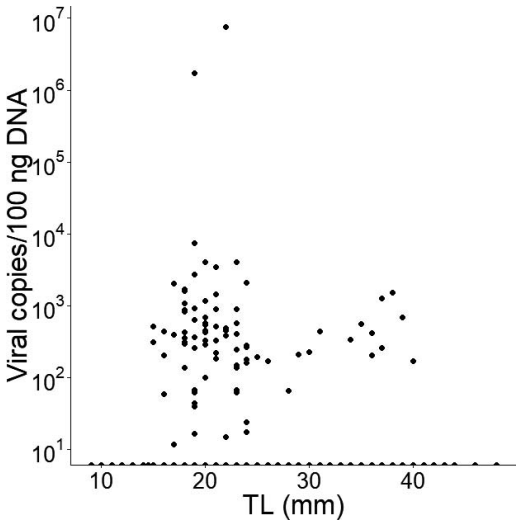


FIGURE 5. Biplot displaying correlation between spotted salamander (*Ambystoma maculatum*) larval total length and individual viral copies of ranavirus per 100 ng of DNA (Spearman $\rho$=0.08; $P$=0.006), Monongahela National Forest, West Virginia, USA. Note the log scale. Each dot represents 1 larva: 1,034 larvae that tested negative and 84 larvae that tested positive for ranavirus in corticosterone levels between the two groups.

Conversely, northern leopard frogs with sublethal ranavirus infections had reduced growth and development (Echaubard et al. 2010). Mortality due to ranavirus infection was higher in spotted salamander metamorphs than in larval stages for wood frogs, green frogs, and northern leopard frogs (Haislip et al. 2011; Hall et al. 2018). Observed die-offs often involve late-stage tadpoles and metamorphs (Green et al. 2002). This might indicate higher susceptibility later in larval development, particularly metamorphosis, which could reflect our findings of higher prevalence among larger larvae. However, this does not hold true for all species. Eastern spadefoot (*Scaphiopus holbrookii*) and Cope's gray treefrogs experimentally exposed to ranavirus in mesocosms had higher infection rate and mortality during the larval stages than during metamorphosis (Haislip et al. 2011). Our findings of larger spotted salamander larvae with higher infection loads could coincide with larval age, thereby making the association a product of increased time spent in the infected pool.

Disease risk is a major concern for habitat creation because resulting die-offs and spread of disease could negatively affect the population. Detection of ranavirus in more than half of the created pools exemplifies that risk. Ranavirus infection in both created and natural pools in North Carolina, USA, decreased amphibian juvenile output (Petranka et al. 2007). Ranavirus infection prevalence of eastern newts was 33% and 70% at two of five created vernal pools in Kentucky, USA, thus showing the potential for high infection rates in created habitat (Richter et al. 2013). In addition, ranavirus can persist in the population through sublethal infections of amphibian larvae, thereby allowing the larvae to complete metamorphosis and return or disperse to other pools to spread the infection (Brunner et al. 2004; Greer et al. 2009). Because we did not sample natural vernal pools, it is unknown whether the creation of vernal pools increased, decreased, or had no impact on the prevalence of ranavirus infection in the metapopulation; however, we suspect that the increased number of pools would help spotted salamander populations even if ranavirosis-driven mortality events occur.

At two pools sampled both years, we had positive detections for ranavirus infection the first year and found no larvae or egg masses the following year. One pool sampled both years changed from positive to negative for ranavirus infection, but it is difficult to know why this switch occurred. Six other pools changed from negative to positive, and six more pools were positive for both years of sampling, which could indicate sustained infections and spread of the disease at the created pools.

We did not find any associations between ranavirus infection prevalence and habitat characteristics. The lack of association between water temperature and prevalence reflects the uncertainty around this relationship, which varies by species and viral strain (Brand et al. 2016; Youker-Smith et al. 2018). Moreover, temporal water temperature readings may provide more insight than our point

measurement. Ranavirus infection prevalence was also not associated with vegetative cover. Arizona tiger salamanders in pools with less vegetative cover had higher infection prevalence, with observed larval clustering around the pool edge (Greer and Collins 2008). We found that water pH did not coincide with infection rates, which also held true for amphibians in California and Maine, USA (Gahl and Calhoun 2010; Tornabene et al. 2018). We found no association between ranavirus infection prevalence with pool size or depth. Conversely, prevalence of ranavirus in common frogs (*Rana temporaria*) increased with pond depth (North et al. 2015). For wood frogs and green frogs in created vernal pools, models predicted that deeper water would increase infection prevalence, potentially due to decreased likelihood of pool sediment freezing, thereby allowing infection reservoir species to persist in the pool (Youker-Smith et al. 2018). We also did not find an association between predator presence and ranavirus infection prevalence. Predators can function as disease reservoirs, alter behavior of prey, or act as a natural stressor with potential for immunosuppression, any of which might influence disease susceptibility. Others found predator cues did not affect mortality or infection of frog tadpoles (Haislip et al. 2012; Reeve et al. 2013). However, presence of newts increased ranavirus infection prevalence in common frogs (North et al. 2015).

Corticosterone levels were similar between infected and uninfected spotted salamander larvae, potentially because the viral load observed was not sufficient to activate the hypothalamus-pituitary-interrenal axis to elicit a corticosterone response, although corticosterone levels and larval size were positively correlated (Millikin et al. 2019). This relationship might also depend on progression of the disease. Wood frogs exposed to lethal doses of ranavirus exhibited elevated corticosterone levels (Warne et al. 2011). It is possible that ranavirus infection and ranavirosis do not activate the hypothalamus-pituitary-interrenal axis in spotted salamander larvae.

There were no correlations among genetic diversity parameters and disease prevalence. Higher genetic diversity may improve a population's ability to survive a disease outbreak (Daszak et al. 2000; Pearman and Garner 2005). Die-offs resulting from ranavirus infection might also result in reduced genetic diversity (Beebee 2012; Puschendorf et al. 2019). Our study was limited to observing infection load, rather than mortality or signs of disease. The observed infection loads at these created pools may be low enough to prevent detectable differences between pools infected and those that were not. Pools sampled were newly created, with the oldest created only 5 yr before sampling. Impacts to population genetics from disease introduction could take longer to observe.

In a concurrent study, we documented genetic connectivity and gene flow among created pools in this study system (Millikin 2019). Connectivity among pools is beneficial to ensure initial colonization and recolonization of pools after extinction events such as drought years. It also enables the spread of rare alleles enhancing genetic diversity. The connectivity we observed, which sustains metapopulations, might be facilitating the spread of ranavirus. We did not observe a trend in ranavirus prevalence across regions, spatial patterns, or associations with pool distance or density, indicating it was already widespread across the regions, reflecting the observed high connectivity.

Ranavirus is a pathogen of concern for amphibians because of its impact on individual fitness, ability to spread through sublethal infections, and resulting mortality (Green et al. 2002; Echaubard et al. 2010). Minimizing risk of disease introduction is an important consideration for habitat creation initiatives. This includes determining the distribution of the disease and which areas are at risk of exposure and monitoring ranavirus prevalence and its effect on the population. Although our study did not reveal important habitat characteristics to consider for vernal pool creation, we recommend additional studies to better understand spatiotemporal factors that influence the introduction and spread of ranavirus in newly created pool networks. In addition, continued monitoring of amphibian larvae

inhabiting created pools can provide insight to susceptibility among developmental stages (Haislip et al. 2011). Documenting and monitoring the occurrence of ranavirus is critical to creating vernal pools that do not serve as reservoirs of disease, while providing suitable breeding habitat.

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## SUPPLEMENTARY MATERIAL

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