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Authors: Harp, Elizabeth M., and Petranka, James W.

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RANAVIRUS IN WOOD FROGS (*RANA SYLVATICA*): POTENTIAL SOURCES OF TRANSMISSION WITHIN AND BETWEEN PONDS

Elizabeth M. Harp[1,2] **and James W. Petranka**[1,3]

[1] Department of Biology, University of North Carolina at Asheville, One University Heights, Asheville, North Carolina 28804, USA
[2] Current address: Department of Biology, Colorado State University, Ft. Collins, Colorado 80523, USA
[3] Corresponding author (email: petranka@unca.edu)

ABSTRACT: Members of the genus *Ranavirus* (family Iridoviridae) can cause catastrophic mortality of pond-breeding amphibians and are associated with an emerging infectious disease that may be contributing to amphibian declines. We conducted three experiments to examine factors that may affect transmission both within and between local breeding populations of the wood frog (*Rana sylvatica*). In a laboratory study, when exposed to moribund tadpoles collected during a local ranaviral die-off, uninfected tadpoles died as soon as 4 days after exposure. The onset of death was accelerated when tadpoles were allowed to scavenge on carcasses of infected tadpoles. In a mesocosm experiment that was conducted in outdoor wading pools, die-offs of tadpoles began approximately 19 days after infected tadpoles were added to pools containing uninfected tadpoles. Mass die-offs with greater than 98% mortality occurred in all pools, regardless of the initial tadpole density. In a second mesocosm experiment, the addition of water and bottom sediments that were collected from a pond during a ranaviral die-off did not result in lower tadpole survival or growth relative to controls. Only a small percentage of tadpoles appeared to be sick, and most tadpoles survived until the first individuals began metamorphosing within a pool. However, tests for ranavirus using polymerase chain reaction were positive for most pools that received contaminated sediment, suggesting that some infections were sublethal. Our results indicate that transmission within ponds is enhanced by scavenging and that spread between local ponds could occur via the transport of contaminated sediment by animals or humans.

Key words: Declining amphibians, North Carolina, *Ranavirus*, *Rana sylvatica*, scavenging, transmission.

INTRODUCTION

Amphibian populations have been declining worldwide since the 1990s (Alford et al., 2001), and possibly since the 1950s (Houlahan et al., 2000). Many factors have been implicated in these declines, including introduced predators (Kats and Ferrer, 2003), increased ultraviolet-B radiation (Blaustein et al., 2003), habitat destruction and degradation, chemical contaminants, and emerging diseases (Collins and Storfer, 2003). Although loss or degradation of habitat appears to be the most important cause of global amphibian declines, emerging infectious diseases have been implicated in the local extinction of several species and may be increasing in importance (Daszak et al., 2003).

Three pathogens may be contributing to amphibian declines: A water mold, *Saprolegnia ferax* (Kiesecker et al., 2001); a chytrid fungus, *Batrachochytrium dendrobatidis* (Berger et al., 1998); and a number of ranavirus species (Chinchar, 2002; Wang et al., 2003). The two pathogens most often cited as being involved in amphibian declines are the chytrid fungus and ranaviruses; direct evidence for declines is greatest for the chytrid fungus (Daszak et al., 2003). Little is currently known about the ecology and epidemiology of amphibian ranaviruses or their effects on host population demography.

The family Iridoviridae includes four genera: *Iridovirus*, *Chloriridovirus*, *Lymphocystivirus*, and *Ranavirus* (Williams et al., 1999). Of these, the first two infect invertebrates, primarily insects, whereas the last two infect amphibians, reptiles, and fish (Wang et al., 2003). In North America, ranavirus outbreaks have been associated with apparent short-term amphibian declines in Arizona (Jancovich et al., 1997), North Carolina (Petranka et al., 2003), and Saskatchewan (Bollinger et al., 1999); however, the long-term population

consequences are unclear. An analysis by Green et al. (2002) of 64 amphibian die-offs in the United States between 1996 and 2001 implicated ranaviruses in the majority of die-offs, with ranaviral infection occurring in 11 states, ranging from as far west as Utah to as far east as North Carolina and Maine. Ranaviruses are highly virulent, with reported mortality rates often greater than 90% (Green et al., 2002). Larval stages appear to be the most vulnerable, but adults of some species may be susceptible as well (Daszak et al., 1999). Symptoms of ranaviral infections are not always present, but they typically include lethargy, hemorrhaging, emaciation, and death (Daszak et al., 1999).

Ranaviral infections occur primarily in amphibians that breed in standing-water habitats, such as seasonal or semipermanent ponds. These tend to be patchily distributed across the landscape and often are clustered locally. As such, transmission may occur at different spatial scales and entail different mechanisms, depending on the proximity of local or regional populations. Examples include transmission within a single pond, between neighboring ponds within a local cluster, or between larger units, such as watersheds or geographic regions (Jancovich et al., 2005).

Laboratory experiments have demonstrated ranavirus transmission via water bath (Brunner et al., 2004; Pearman et al., 2004), cannibalism (Pearman et al., 2004), and close proximity of naïve individuals to sublethally infected individuals (Brunner et al., 2004). These mechanisms would likely operate within a local pond. Within a local cluster of ponds, the virus could be spread by animals that transport sediment or water from pond to pond, such as raccoons and mink, or by sublethally infected individuals that leave a pond as juveniles and return to a different pond to breed as adults (Brunner et al., 2004). In addition, field researchers could spread ranavirus by transporting sediment and water from pond to pond on fomites, such as nets and chest waders. Brunner et al. (2004) observed sublethally infected juvenile tiger salamanders (*Ambystoma tigrinum*) leaving their natal pond. Those authors also observed infected adult salamanders returning to a pond to breed, but they were unable to show vertical transmission. Thus, potential mechanisms of transmission between local ponds are not fully understood.

Another limitation in determining the biologic significance of mechanisms of transmission is that experimental studies of ranavirus transmission have been restricted to laboratory settings. Under these conditions, test animals may not be exposed to the normal array of environmental conditions (e.g., diel temperature fluctuations, exposure to ultraviolet-B radiation), microbial communities, or other environmental elements that could influence transmission. To the best of our knowledge, no experiments involving ranavirus transmission have been conducted in more realistic settings, such as outdoor mesocosms, using wild-caught amphibians and naturally obtained virus. Thus, the extent to which laboratory results can be applied with confidence to natural communities is poorly resolved.

We conducted experiments in the laboratory and in outdoor mesocosms with the wood frog (*Rana sylvatica*) to delineate potential mechanisms of transmission both within and between local ponds. We used wild-caught tadpoles and naturally contaminated water and sediment in outdoor mesocosm experiments to improve realism. Our primary goals were to determine: 1) whether direct contact and scavenging on wild-caught, infected tadpoles accelerates the development of infections; 2) whether exposure to infected tadpoles results in lethal infections under more natural conditions; 3) whether the initial density of uninfected tadpoles affects the severity or onset of outbreaks; and 4) whether exposure to small amounts of water or sediment from ponds undergoing active die-offs can trigger infections.

MATERIALS AND METHODS

Experiment 1: Test for transmission via exposure to infected tadpoles under laboratory conditions

We observed numerous cases of tadpoles scavenging on carcasses during natural ranaviral die-offs in ponds at the Tulula Wetlands (TW), Graham County, North Carolina, USA (35°16.432′N, 83°41.594′W) (Petranka et al., 2003). We subsequently conducted a 2×2 factorial experiment with five replications to determine whether healthy tadpoles would become infected when exposed to infected tadpoles and whether scavenging on conspecific carcasses would influence infection rates. The experimental treatments were, first, exposure to moribund or dead tadpoles that were either naturally infected or uninfected and, second, having access or no access to moribund or dead tadpoles. Infected tadpoles were collected from a pond at TW that was experiencing an active ranaviral die-off.

The experiment was conducted on a laboratory bench at ~25 C, and organisms were exposed to natural photoperiods from windows. Ranavirus has been documented only at one site in western North Carolina (the TW in Graham County). We have never observed symptomatic animals or die-offs of *R. sylvatica* in other local pond populations that we have observed in the region, suggesting that almost all populations are virus-free. We used test animals that were derived from egg masses from a pond near Mars Hill (MH), Madison County, North Carolina, USA (35°47.199′N, 82°33.682′W). During a 5-yr period that preceded the study, tadpoles in this population were observed nearly daily during the larval period of *R. sylvatica,* and symptomatic animals or ranavirus outbreaks were never observed (J.W. Petranka, pers. obs.). Negative controls used in polymerase chain reaction (PCR) analyses were from this site and consistently tested negative for ranavirus. Although test animals that were derived from eggs from the site were not screened for the virus, they were presumed to be virus-free.

We transferred egg masses to outdoor wading pools that held conditioned water, then grew the hatchlings and older tadpoles on commercial rabbit chow until they were ready for testing in the laboratory. Tadpoles were tested in 19×31×7-cm (4-l) plastic containers that were filled two-thirds full with aerated tap water. Municipal water that drains from crystalline bedrocks in the region has very low conductivity, so tap water was conditioned with approximately 100 mg/l of aquarium salts to reduce physiologic stress to tadpoles. We divided each container ($n=20$) in half across its width with nylon window screening (mesh diameter, 1.8 mm) that was secured to the sides and bottom with hot glue. For treatments that allowed access to carcasses ($n=10$), we cut a 4-cm^2 opening in the screens so that tadpoles could scavenge.

We added eight uninfected test animals from MH to each container on 7 May 2002, and did not feed tadpoles during the experiment to encourage scavenging. Tadpoles used for tests were approximately 2 mo posthatching and averaged 0.37±0.01 g (mean±SE). Gosner (1960) developmental stages varied from stage 33 to stage 37 and averaged stage 36.6±0.22 ($n=26$). The next day, we randomly assigned and added four dead or moribund tadpoles to one end of each container. Half of the treatments received infected tadpoles from a pond at TW that was experiencing an active ranaviral die-off. These tadpoles showed typical symptoms of ranaviral infections (Green et al., 2002), and most died within 1 or 2 days after they were added to containers.

One concern when designing the experiment was that infected tadpoles might develop secondary bacterial or fungal populations with the onset of death that could contribute to the mortality of test animals. This would confound determination of whether ranavirus was a significant source of mortality. To minimize this, the remaining containers ($n=10$) served as controls and received four uninfected, pithed tadpoles from MH that were added at the same time the infected tadpoles from TW were added. Control tadpoles were added to containers immediately after pithing, were briefly moribund, and died within 4 hr after being added to containers. As with infected tadpoles, test animals in control containers readily scavenged on the decomposing carcasses of pithed tadpoles.

We recorded the number of surviving test animals at approximately 24, 48, 72, 100, 111, 118, 135, and 163 hr after infected or pithed tadpoles were added to treatments. All test animals that died were preserved in 70% alcohol.

Common conditions of mesocosm experiments

We conducted two mesocosm experiments in 2003 in a partially shaded field in Asheville, Buncombe County, North Carolina, USA. We collected egg masses of *R. sylvatica* from Sandy Bottom (SB), Buncombe County, North Carolina (35°29.677′N, 82°35.457′W) on 6 March 2003 and incubated egg masses in outdoor wading pools to obtain hatchlings. *Rana sylvatica* tadpoles at this site have been

observed for 15 breeding seasons, and symptomatic animals or ranavirus outbreaks have never been observed (J.W. Petranka, pers. obs.). As with MH, test animals that were derived from eggs from the site were not screened for the virus and were presumed to be virus-free.

We conducted experiments in plastic wading pools (diameter, 0.9 m; depth when full, 20 cm) filled with aged tap water that was treated with aquarium salts. Pools were initially filled on 11 March 2003, and each received 50 southern red oak (*Quercus falcata*) leaves to provide cover and nutrients for the tadpoles. We added hatchlings from SB to pools on 17 March 2003 and, except where noted, fed tadpoles 1.5 g of commercial rabbit chow per pool weekly. To minimize the chance of cross-contamination, we draped plastic netting over the pools to exclude raccoons, and we restricted the use of each dip net to an individual pool. The pools were colonized by aquatic insects, such as midges, and also developed populations of zooplankton and algae that were introduced in pond water that was used to process and count hatchlings. Hatchling densities for all experimental treatments (range, 64–254 per m^2 of pool bottom) were within the range of hatchling densities in ponds at TW with documented ranavirus outbreaks between 1998 and 2001 (range, 26–790 per m^2; $n=37$) (Petranka et al., 2003).

All infected tadpoles and exposed sediment and water were collected from a complex of ponds at TW that had experienced ranavirus outbreaks annually since 1997 (Petranka et al., 2003). With respect to bottom sediment and water, we use the modifiers "exposed" to refer to samples collected from ponds during active ranaviral die-offs at TW and "unexposed" to refer to samples collected from a pond in Madison County with no history of ranavirus. All naïve animals in the mesocosm experiments were from SB and were presumed to be virus-free.

Experiment 2: Effect of tadpole density on ranavirus transmission

The primary goals of this experiment were to determine whether outbreaks could be induced under nonlaboratory conditions using wild-caught tadpoles and whether the time of onset and severity of ranavirus outbreaks are influenced by initial tadpole density. We used a randomized block design with six replicates to test whether initial hatchling density affects the time of onset or severity of die-offs following exposure to infected animals. We reasoned that crowding of tadpoles could affect the response variables by altering per-capita viral titers associated with scavenging, by increasing contact rates with infected conspecifics, or by weakening the immune system in association with intense competition for food resources.

We set out pools in six rows of three and randomly assigned low-, medium-, or high-density treatments to each pool. Densities were established by adding 100, 250, or 400 hatchlings, respectively, to each pool, which corresponds to densities of 64, 159, or 254 tadpoles/m^2, respectively. In similar mesocosm experiments with *R. sylvatica* (Holbrook and Petranka, 2004), this range of hatchling densities was sufficient to cause intraspecific competition that reduced tadpole growth.

Approximately 1 mo after adding hatchlings (20 April 2003), we added five moribund tadpoles to each pool within 4 hr of collection from a pond at TW that was undergoing an active ranaviral die-off (Petranka et al., 2003). Tadpoles were not fed after this date to increase the likelihood of scavenging on carcasses. Although our goal was to determine whether initial tadpole density affects the time to onset or the severity of disease, symptomatic animals were not observed after 13 days. To increase the likelihood of the disease manifesting, we added six additional moribund tadpoles from TW to each pool on 4 May (14 days after the initial addition of five infected tadpoles). We checked tadpoles at 1- to 2-day intervals for signs of ranaviral infection, such as lethargy, erratic swimming, emaciation, and hemorrhaging, as seen during die-offs at TW. We recorded the date when symptomatic animals were first observed in each pool. We removed all surviving tadpoles at the termination of the experiment on 19 May and preserved them in 70% ethanol.

Experiment 3: Test for transmission of ranavirus via contaminated sediments and water

We exposed tadpoles to pond sediment and water collected during an active ranavirus outbreak to test for transmission via sediment and water. Our primary goal was to determine whether the introduction of small quantities of contaminated water or sediment into pools, as might occur while researchers or animals move between sites, could cause ranaviral infections.

We conducted a 2×2 factorial experiment using a randomized block design, with replicates serving as blocks. We set out pools in six rows of four, with one each of the four treatments randomly assigned within each replicate. The two experimental treatments

were sediment type (exposed versus unexposed samples) and water type (exposed or unexposed samples).

We added 300 uninfected hatchlings to each pool on 17 March 2003 (density, 191 tadpoles/m^2). Approximately 1 mo later (20 April), we added approximately 0.3 kg wet weight of pond sediment (and associated bottom debris and organisms) and 2.0 l of water per pool to the appropriate treatments. We collected exposed sediment and water from a single pond at TW on 20 April during an active ranaviral die-off. At this time, many animals were dead or moribund with hemorrhagic tissues. We collected unexposed sediment and water from MH on 21 April. This site had no history of ranaviral die-offs. At each site, we used a posthole digger to remove the top 5 to 8 cm of mud and debris from pond bottoms. We collected sediment from throughout the ponds, and separately bagged samples that were added to individual pools. We collected water samples by filling 1-l bottles with water that was collected within 10 cm of the water surface in areas where sediment had not been disturbed by wading. Samples were transported from ponds to the field site in coolers and were added to pools within 4 hr of collection.

We checked pools every 1 to 2 days for signs of ranaviral infection, but we did not observe sick animals until 2 June (43 days after sediment and water were added), when metamorphosis began in nine of the pools. The initiation of metamorphosis had begun in all pools by 9 June, when the experiment was terminated. We preserved samples of tadpoles from pools for PCR analysis from 2 June through 9 June, and we collected the few tadpoles per pool (if any) that were lethargic or emaciated.

When the first metamorph was observed in a pool (as indicated by emergence of the front legs), we extensively dipnetted and then drained each pool to determine the number surviving to the initiation of metamorphosis. All surviving tadpoles were preserved in 70% ethanol to prevent potentially infected animals from escaping and infecting local populations. We terminated the experiment on 9 June, when metamorphosis began in the last pools, and used mean mass and survival to the initiation of metamorphosis as the response variables. We estimated mean mass at the initiation of metamorphosis by weighing a subsample of 20 tadpoles from each pool.

Identification of ranavirus in alcohol-preserved tadpoles

We used PCR to document infected animals in the mesocosm experiments conducted in 2003. The large number of hatchlings used in the experiments precluded analyzing all specimens. Instead, we analyzed pooled subsamples of preserved test animals to verify the presence of infected animals in specific treatments. Mass die-offs occurred in Experiment 2, and many tadpoles showed overt symptoms of ranaviral infections, such as hemorrhaging. We subsequently elected to assay only six pools to verify ranavirus in sick animals. We scrutinized the test animals in Experiment 3 more carefully (100% of pools were sampled), because most animals appeared to be healthy and infections may have been sublethal.

Genomic DNA was extracted from pooled samples of five whole tadpoles using a mixture of 50 μg/ml of proteinase K, 150 mM NaCl, 10 mM Tris, 25 mM ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulfate, and 152.5 μL/ml of ddH_2O per one to five tadpoles. Tadpoles were ground thoroughly with a mortar and pestle, incubated from 4 h to overnight at 65 C, and then centrifuged for 5 min at 1,000 × G. The aqueous layer was removed and subjected to a phenol-chloroform extraction followed by ethanol precipitation. Primers specific for the genus *Ranavirus* were the following: 5′-GTCTCTGGAGAAGAAGAA-3′ (MCP5), and 5′-ACTTGGCCACTTATGAC-3′ (MCP4) (Mao et al., 1996). A conserved region of the major capsid protein encoding region of ranavirus DNA (V.G. Chinchar, University of Mississippi Medical Center, Jackson, Mississippi, USA) was used as a positive control in all reactions. Tadpoles from MH were used as negative controls for each gel run and were consistently negative in all tests. For each PCR reaction (total volume, 25 μl), 18.25 μl of ddH_2O, 4 pmol of each primer, 1 μl of genomic DNA, 0.25 μl of dNTP mix (20 mM), 2.5 μl of buffer A, and 0.5 μl of *Taq* DNA polymerase were used. The PCR was performed under these conditions: 30 sec at 94 C, 30 sec at 54 C, and 5 min at 72 C, for a total of 35 cycles. Samples were then separated on a 1.5% agarose gel containing ethidium bromide.

Statistical analyses

We analyzed data using Statistical Analysis System (Ver 8.2, SAS Institute, Cary, North Carolina, USA). Block (replicate) effects were not significant for any of the experiments ($P>0.25$) and were eliminated from models to increase the error degrees of freedom and statistical power. We used angularly transformed percentage data for survival in all analyses. We analyzed survival over time in Experiment 1 using repeated-measures analy-

sis of variance (ANOVA). We also restricted the analysis to the last five sample intervals, because no mortality occurred during the first 72 hr. The assumption of equality of variances was violated, because survival in control treatments was 100%. However, preliminary analyses of main effects using ranked data (the nonparametric alternative) yielded nearly identical results compared with an analysis using nonranked data (infected animals: $P<0.0001$ in both cases; access treatment: $P=0.0009$ versus $P=0.0004$). We subsequently used nonranked data in the analyses to minimize bias in testing for interaction terms (see, e.g., Akritas et al., 1997). Because sphericity tests indicated that the assumption of equal variances was violated for samples across time, we used the Greenhouse-Geisser correction to adjust the model for a more conservative test.

In Experiment 2, we did not compare the time to the first observance of symptomatic animals, because the onset of the disease was rapid and highly synchronized across treatments (sick animals appeared in every pool within a 24-hr period). We compared the mean proportion surviving to 19 May using the Kruskal-Wallis k-sample test. In Experiment 3, we used ANOVA to test for the effects of sediment and water on survival and growth, and we used the mean value per pool to compare tadpole mass.

RESULTS

Experiment 1: Test for transmission via exposure to infected tadpoles under laboratory conditions

Healthy tadpoles scavenged nearly all infected and pithed carcasses within 24 hr and completely consumed carcasses within 111 hr. Mean survival to the termination of the experiment varied from 3% to 100% among treatments (Fig. 1). No mortality occurred in tadpoles exposed to uninfected (pithed) animals, regardless of whether test animals had access to and scavenged carcasses. In contrast, animals that had access to infected tadpoles began dying within 100 hr and exhibited symptoms of ranaviral disease (e.g., emaciation, lethargy, hemorrhaging on the skin). Survival to the termination of the experiment averaged 3.0±2.5% for tadpoles that had access to infected carcasses versus 7.5±2.0% for tadpoles with no access to infected carcasses. Repeated-measures ANOVA indicated that overall survival differed significantly for exposure to infected versus uninfected animals ($F_{1,16}=343$, $P<0.0001$) and for access versus no access to sick or pithed animals ($F_{1,16}=19.9$, $P=0.0004$). The interaction term also was significant ($P=0.0004$), because scavenging only lowered survival for the infected treatment. Within subjects, the time×infected and time×access interactions were significant ($P<0.0001$). These results reflect the facts that mortality only occurred in treatments receiving infected animals and that onset of death occurred sooner for tadpoles that scavenged on infected carcasses (Fig. 1). For example, after 111 hr, an average of 95% of tadpoles that had access to carcasses were dead, compared with 25% of tadpoles that were not allowed to scavenge (t-test: $t=4.33$; df=8; $P=0.003$).

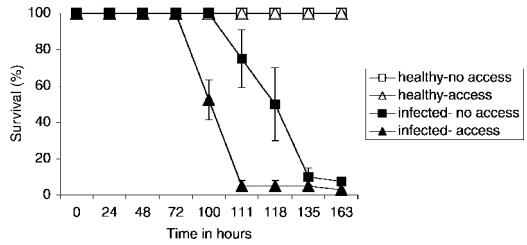

FIGURE 1. Survival of wood frog tadpoles over time after exposure to ranavirus-infected or uninfected dead tadpoles. Treatments were exposure to infected moribund tadpoles (versus controls with healthy tadpoles that were pithed) and access (versus no access) to tadpoles that allowed scavenging.

Experiment 2: Effect of tadpole density on ranavirus transmission

Most infected, moribund tadpoles died with 24 hr after they were added to pools. We observed that test tadpoles scavenged the tail fins of infected, moribund tadpoles within minutes after infected tadpoles were added to wading pools. Most carcasses of infected tadpoles were consumed within 2 days. Test tadpoles appeared to be healthy after 13 days (4 May), when a second allotment ($n=6$) of infected

TABLE 1. Results of polymerase chain reaction assays for ranavirus detection in surviving wood frog tadpoles from Experiment 3.[a]

Experimental treatment			
Sediment	Water	No. of samples (% positive)	Pools (% positive)
Exposed	Unexposed	11 (45%)	83%
Exposed	Exposed	9 (44%)	67%
Unexposed	Unexposed	6 (0%)	0%
Unexposed	Exposed	6 (0%)	0%

[a] Treatments reflect all combinations of exposed or unexposed sediment and water. Exposed samples were collected during an active ranavirus outbreak and unexposed samples from a pond without ranavirus. Data represent the number of samples analyzed, the percentage of samples that were positive (in parentheses), and the percentage of pools ($n=6$ per treatment) that were positive for ranavirus. Each sample reflects a pooled analysis of five tadpoles, and from one to three samples (5–15 tadpoles) were analyzed per pool.

tadpoles from TW was added to each pool. Moribund tadpoles with overt symptoms of ranaviral disease (e.g., emaciation, weak swimming, hemorrhaging tissues) were first observed 5 days after adding the second allotment of infected tadpoles. The onset of die-offs of test tadpoles was rapid. Sick animals were first observed on 9 May, at which point 38% of the pools were experiencing die-offs, and all density treatments had at least one pool undergoing a die-off. When observed the next day, all 18 pools had symptomatic animals and were undergoing die-offs. The onset of infection was so synchronized among pools that statistical tests were not conducted for the time to onset.

Mortality approached 100% for all pools by 19 May (10 days after the first observation of infected animals), when the experiment was terminated. The respective percentage survival (mean±SE) for low, medium, and high densities to 19 May was 1.16±1.2, 1.40±0.8, and 0.46±0.4%, respectively, and did not differ significantly among density treatments (Kruskal-Wallis k-sample test with chi-squared approximation: df=2, $P=0.61$). We assayed a single pooled sample (five tadpoles per pool) from six of the 18 pools (two pools per density treatment). Samples tested positive for ranavirus by PCR in four of six cases, and ranavirus was identified from each of the density treatments (two of the 100-tadpole pools and one each of the 250-tadpole and 400-tadpole pools). Because symptoms were identical in all the pools and ranavirus was detected in this initial screening, we did not conduct further testing and presumed that ranavirus was present in all pools.

Experiment 3: Test for transmission of ranavirus via contaminated sediments and water

Tadpoles in the four experimental treatments appeared to be healthy until 2 June, when the first metamorphosing tadpoles were observed in some pools. On this date, a small percentage of tadpoles in six pools appeared to be sick, and all contained exposed sediment. Analysis by PCR indicated ranaviral infections in tadpoles from nine of 12 pools containing exposed sediment. Samples from pools with unexposed sediment were consistently negative, regardless of whether they contained exposed or unexposed water (Table 1).

Mass die-offs, as seen in Experiment 2, did not occur in any of the pools through 9 June, when the experiment was terminated. Survival to the initiation of metamorphosis varied from 28% to 53% among treatments and was lowest in mesocosms that received a combination of exposed sediment and exposed water (Fig. 2). Despite PCR detection of ranavirus in tadpoles from most pools with exposed sediment treatments, the survival of tadpoles did not differ significantly for the

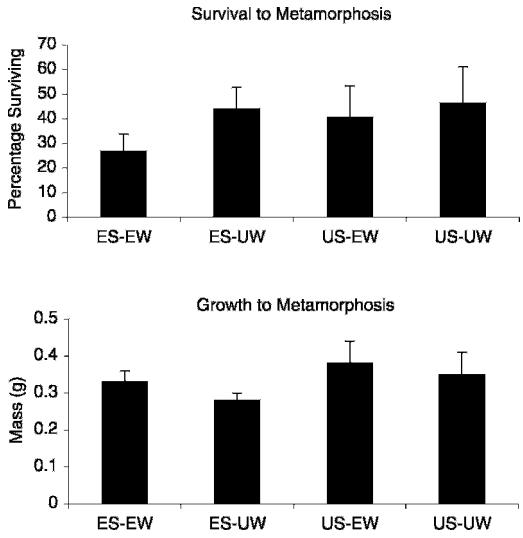

FIGURE 2. Growth and survival of wood frog tadpoles from hatching to the initiation of metamorphosis after exposure to different combinations of "exposed" (E) or "unexposed" (U) pond sediment (S) and water (W). Exposed samples were collected from a pond at Tulula Wetlands, Graham County, North Carolina, USA, during an active ranaviral die-off. Unexposed samples were from a pond at Mars Hill, Madison County, North Carolina, USA, with no history of ranavirus. Values are presented as the mean+SE.

main effect of addition of exposed versus unexposed sediment ($F_{1,20}=0.75$, $P=0.40$). Similarly, an orthogonal contrast of the exposed sediment and exposed water treatment (28% survival) with its control was not significant ($P=0.22$). These results suggest that most infections were sublethal. As expected based on PCR and the survival analysis, neither the main effect of addition of exposed versus unexposed water ($F_{1,20}=1.02$, $P=0.32$) or the interaction term ($F_{1,20}=0.20$, $P=0.66$) was significant.

Despite widespread detection of ranavirus by PCR in the exposed sediment treatment, little evidence suggested that infected animals were severely emaciated (Fig. 2). The mean mass of tadpoles at the initiation of metamorphosis varied from 0.28 g to 0.35 g among treatments and did not differ significantly for the main effects of the addition of exposed versus unexposed sediment ($F_{1,20}=1.60$, $P=0.22$), the addition of exposed versus unexposed water ($F_{1,20}=0.63$, $P=0.44$), or the interaction term ($F_{1,20}=0.03$, $P=0.86$).

DISCUSSION

Our results provide new insights regarding conditions that may facilitate transmission of ranaviral infections both within and between local ponds. They also provide evidence that results from laboratory studies appear to have application to more natural conditions. We did not attempt to identify the species of ranavirus or to quantify viral titers used in our experiments, and we encourage readers to interpret the results and conclusions within this context.

The laboratory scavenging experiment showed that ranavirus can be transmitted to healthy tadpoles via scavenging of infected carcasses or by exposure to water containing infected animals or carcasses. Animals that were denied direct contact with infected animals by fencing still developed the disease, albeit at a slower rate than for those that had access and scavenged. This demonstrates waterborne transmission (Jancovich et al., 2001), perhaps because viral particles were shed from moribund tadpoles. Our results are consistent with those of Pearman et al. (2004), who exposed a European anuran (*Rana latastei*) to frog virus 3 from North America. However, the laboratory results conflict with the results of Experiment 3, in which we were unable to induce infections by exposing test animals in mesocosms to pond water collected during an active outbreak of ranavirus. Because mass die-offs occurred in mesocosms in Experiment 2, these differences are best explained as dosage-dependent responses rather than abiotic conditions in mesocosms (e.g., lower temperatures) that prevented transmission of the disease.

At TW, where we have observed numerous die-offs, the onset of the disease

in a local pond is rapid, and complete die-offs of tadpoles may occur within 1 to 2 weeks after the first moribund tadpoles are observed. We observed similar patterns in wading pools in Experiment 2, suggesting that mesocosms may provide reasonable simulations of conditions that occur in natural ponds. In our density experiment, the addition of 11 moribund tadpoles per pool resulted in catastrophic mortality (>98%) similar to that seen in ponds at TW. Although we did not have control pools to verify that mass die-offs would not occur in the absence of ranavirus, we have conducted several mesocosm experiments with *R. sylvatica* to address community interactions and have never documented mass die-offs in experimental pools that lacked ranavirus. For example, a mesocosm experiment that was conducted in the same field in 2003 using comparable densities of *R. sylvatica* (Holbrook and Petranka, 2004) resulted in more than 50% of *R. sylvatica* surviving to metamorphosis. Similarly, we did not observe mass mortality in pools of Experiment 3, in which survival to metamorphosis in pools with unexposed sediment (no detectable ranavirus) averaged greater than 35%. These results, coupled with observations of numerous symptomatic animals in pools and PCR identification of ranavirus in preserved animals, lead us to confidently conclude that ranavirus was the cause of mass die-offs in pools.

Moribund tadpoles that were added to pools died within 1 to 2 days and were quickly consumed by scavenging animals. Although tadpole density could affect the per-capita consumption (dosage) of viral particles by scavenging tadpoles and contact rates with conspecifics, both survival and time to development of overt symptoms of the disease were independent of the density treatments. Parris and Beaudoin (2004) found a similar lack of correlation between density and disease in experiments with chytridiomycosis. The range of densities in our pools may have been insufficient to initiate a competition-mediated disease response. The onset of the disease was much faster in our laboratory experiment (4 versus 19 days), in which the ratio of infected to uninfected tadpoles (0.50) was much greater than in the density experiment (0.03–0.11). This difference may reflect dosage-dependent responses, as reported by Pearman et al. (2004), or perhaps differences in environmental conditions, such as water temperature or microbial communities.

Our second mesocosm experiment addressed whether ranavirus might be transmitted between ponds when small amounts of water or sediment are carried via animals (e.g., wading birds, raccoons), vehicles, or researchers. Cunningham et al. (2003) coined the term "pathogen pollution" to refer to the introduction of any pathogen into a new (or naïve) host species or population because of anthropogenic effects. Pathogens may be transmitted to a new species or population directly, or some environmental change may make it possible for a pathogen to cross geographic boundaries and reach areas that were previously inaccessible. Once ranavirus is present in a geographic region, it could be spread by fomites, such as nets or boats, or through artificial stocking of ponds for recreational fishing (Whittington et al., 1996) or use of bait salamanders (Jancovich et al., 2005).

Ranavirus outbreaks were not observed at TW in 1995 and 1996. A mass die-off was first observed in one of 18 seasonal or semipermanent ponds in 1997, and ranavirus appeared to spread quickly to seven additional seasonal ponds by the following year (Petranka et al., 2003). Our research teams regularly moved between ponds with wet field gear (chest waders and nets), which prompted concern that we were spreading the disease locally.

Our mesocosm study suggests that ranavirus can be transmitted by pond sediment; however, we found no evidence that exposure to small quantities of water or pond sediment from infected ponds can cause catastrophic die-offs, as seen in

Experiment 2 or in natural ponds. Because the density of hatchlings used in this experiment was near the middle range of those in the density experiment during which catastrophic mortality occurred, our results may, again, reflect a dosage-dependent response that produced sublethal infections (Pearman et al., 2004). Sick tadpoles appeared on the same day as the first metamorph in two of the eight mesocosms with sick animals. It is possible that if tadpoles had been allowed to develop longer, sick animals would have appeared after the first appearance of metamorphs. Because infections appeared to be sublethal, this experiment suggests that infected metamorphs might survive to breed in nearby ponds, where vertical transmission could potentially occur (Brunner et al., 2004).

An alternative hypothesis for the lack of catastrophic die-offs in Experiment 3 is the absence of predator-induced stress. In experimental tests, Van Buskirk and Relyea (1998) found that wood frog tadpoles had lower survival, growth, and development rates when in the presence of caged predators. Because mounting an immune response is costly (Carey, 1993), tadpoles exposed to predators may be more likely to succumb to ranaviral infection. Concordant with this hypothesis, Parris and Beaudoin (2004) found a significant interaction between predator and pathogen when larval gray treefrogs (*Hyla chrysoscelis*) were exposed to a chytrid fungus and reared in the presence of a predatory salamander, *Notophthalmus viridescens*.

This research provides the first empirical support for a mechanism of transmission between local ponds; however, much is still not understood about the host-pathogen dynamics of this system. Notably, it remains unknown to what extent hosts may evolve responses that minimize infection (Pfennig et al., 1991) or how ranavirus persists between outbreaks. Brunner et al. (2004) suggested that *A. tigrinum* virus may be maintained by being passed from parent to offspring, but to our knowledge, evidence for vertical transmission is lacking. The high rates of horizontal transmission (see Experiment 2) combined with vertical transmission would, theoretically, explain how such a virulent pathogen could persist (Lipsitch et al., 1995). However, given the high larval mortality rates associated with ranaviral infections (Green et al., 2002; Petranka et al., 2003) and low recruitment of infected metamorphs into terrestrial populations, the trade-off theory of virulence (for review, see Bull, 1994) predicts that vertical transmission, if it exists, would be relatively unimportant in this system.

Vertical transmission cannot be ruled out as a means of ranavirus persistence, but reservoir hosts likely maintain the virus between outbreaks. At TW, for example, 11 amphibian species share breeding ponds with *R. sylvatica,* and many could function as carriers that develop sublethal infections. Highly susceptible species, such as the wood frog, might act as amplifying hosts in the system. In laboratory transmission studies of several sympatric amphibian and fish species, Jancovich et al. (2001) were unable to produce a possible reservoir host for *A. tigrinum* virus. However, wild-caught individuals have not been tested for the presence of ranavirus. We suggest that surveys of natural amphibian communities for reservoir species are prudent.

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