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Short Communication

Widespread Occurrence of Ranavirus in Pond-Breeding Amphibian Populations

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Abstract: Ranaviruses are an emerging threat for many amphibian populations, yet their distribution in amphibian communities and the association of infection with possible stressors and species is not fully understood due to historically sparse surveillance. Agricultural practices that reduce the water quality of amphibian breeding habitats (e.g., cattle access to wetlands) and environmental stressors (e.g., lower temperatures) may contribute to ranavirus emergence. We tested larval amphibians for ranavirus infection across four seasons in farm ponds (n = 40) located in Tennessee, USA. Cattle at various densities were allowed access to half of the sampled ponds. Ranavirus infections were detected in nine species and in 33 of the sampled ponds (83%), illustrating widespread occurrence of the pathogen. Species within the family Ranidae were the most frequently infected. In 13 of the ponds containing infected individuals, prevalence exceeded 40% during at least one season. Infections were detected in multiple seasons in 20 of the sampled ponds containing infections, suggesting that ranaviruses are relatively persistent in these systems. Cattle had negative effects on water quality (turbidity and ammonia) and there was a positive association between cattle abundance and ranavirus prevalence in the summer. Counter to previous field studies in North America, we found a significant positive association between water temperature and ranavirus prevalence in the fall sampling events. Despite these findings, the influences of cattle and temperature on ranavirus prevalence were not consistent across seasons. As such, the mechanisms driving high ranavirus prevalence across the landscape and over time remain unclear. Given the widespread occurrence of ranaviruses in wild amphibians, we encourage the implementation of surveillance programs to help identify potential drivers of emergence. Sites with high ranavirus prevalence should be monitored annually for outbreaks, and the long-term effects on population size determined.

Keywords: conservation, cattle, emerging infectious disease, frog virus 3, Iridoviridae, stressors, water quality, host range, amphibian larvae, farm ponds

Introduction

A group of viruses belonging to the genus *Ranavirus* (Family Iridoviridae) is responsible for amphibian mass mortality events in adults and larvae from the Americas,

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Europe, and Asia (Miller et al. 2011). These widespread die-offs have sparked a diversity of research programs addressing the molecular biology, ecology, and evolution of ranaviruses. To date, however, the majority of data regarding the distribution of ranaviruses in wild amphibian populations has come from sites with disease outbreaks. For example, over 20 species across five families have been reported in die-off events in North America (Jancovich et al. 1997; Bollinger et al. 1999; Green et al. 2002; Carey et al. 2003; Docherty et al. 2003; Petranka et al. 2003; Greer et al. 2005; Schock and Bollinger 2005; Harp and Petranka 2006; Petranka et al. 2007; Duffus et al. 2008; Gahl and Calhoun 2008; Torrence et al. 2010). Unfortunately, these die-off reports offer little insight into the prevalence of the pathogen within and among amphibian species in the absence of epizootics (Greer et al. 2009). Given that ranaviruses are known to infect a diversity of host species (Schock et al. 2008; Gray et al. 2009; Hoverman et al. 2011), there is a great need for field surveillance studies to obtain unbiased estimates of pathogen prevalence within and among host species, identify potential drivers of emergence, and assess their threat to wild amphibian populations.

Based on recent experimental studies, infection prevalence is likely to differ among amphibian species from wild populations (Schock et al. 2008; Hoverman et al. 2010, 2012). For instance, an experimental exposure study involving 19 amphibian species from seven amphibian families demonstrated that species within the family Ranidae tended to have relatively high susceptibility to infection by FV3-like ranaviruses compared with the other families (Hoverman et al. 2011). These findings corroborate field patterns of disease occurrence; species representing the Ranidae are the most frequently reported taxa in mortality events (e.g., Green et al. 2002). While the mechanisms underlying species differences in susceptibility to ranavirus infection remain to be tested, these studies suggest that ranid species may be the most commonly infected group in amphibian communities (Hoverman et al. 2011).

The host's environment can play an important role in disease emergence. Any environmental change or ecological interaction that compromises immune function has the potential to affect susceptibility to pathogens (Lloyd 1995). It has been shown that anthropogenic stressors reduce immune function in hosts and increase disease prevalence (Carey et al. 1999; Daszak et al. 1999, 2001; Bruno et al. 2003; Rohr et al. 2008). For amphibians, cattle grazing near aquatic habitats has been suspected as a significant anthropogenic stressor resulting in disease emergence (Johnson and Lunde 2005; Gray et al. 2007). Recent studies have documented greater ranavirus prevalence in cattle-access wetlands compared with non-access wetlands for green frogs (*Lithobates clamitans*) and tiger salamanders (*Ambystoma tigrinum*) but not American bullfrogs (*L. catesbeianus*; Gray et al. 2007; Greer and Collins 2008). Greater prevalence was associated with elevated levels of nitrogenous compounds and reduced availability of emergent vegetation, which may stress amphibians and lead to greater contact rates between individuals, respectively (Gray et al. 2007; Greer and Collins 2008). Together, these studies suggest that cattle grazing around wetlands can influence ranavirus disease dynamics and may be an important factor in disease emergence in amphibians.

In addition to anthropogenic stressors, natural stressors have been hypothesized as factors in disease emergence. For larval amphibians, water temperature may be an important factor affecting ranavirus prevalence. Rojas et al. (2005) reported an increase in *Ambystoma tigrinum* virus (ATV) virulence at temperatures below 18°C. In another study, American bullfrog tadpoles were eight times more likely to be infected with ranavirus in winter than in summer, and green frog tadpoles were five times more likely to be infected in fall than in summer (Gray et al. 2007). Although the replication rate of ranaviruses tends to be slower at lower temperature, these authors and others have hypothesized that increased pathogen infectivity at lower temperatures could be related to a corresponding decrease in amphibian host immune function (Maniero and Carey 1997; Carey et al. 1999; Forbes et al. 2004; Rojas et al. 2005; Raffel et al. 2006). Thus, seasonal changes in water temperature may be an important driver in ranavirus emergence and contribute to temporal variation in prevalence.

To enhance our understanding of the distribution of ranaviruses, we conducted field surveillance for the pathogen in populations of pond-breeding amphibian species located across four counties of eastern Tennessee, USA (Fig. 1). Given the diversity of pond-breeding amphibian species in our study area (Redmond and Scott 1996), our study represents the most extensive surveillance for ranaviruses in amphibians to date. To assess the possible roles of anthropogenic and natural stressors on ranavirus prevalence within amphibian populations, we conducted our study across four seasons in farm ponds that had and did not have cattle access. Based on previous findings, our hypotheses were that prevalence of ranavirus would be (1) greater in cattle-access ponds and (2) greatest during the lower temperature seasons (i.e., fall and winter). We also hypothesized that infection prevalence would differ among

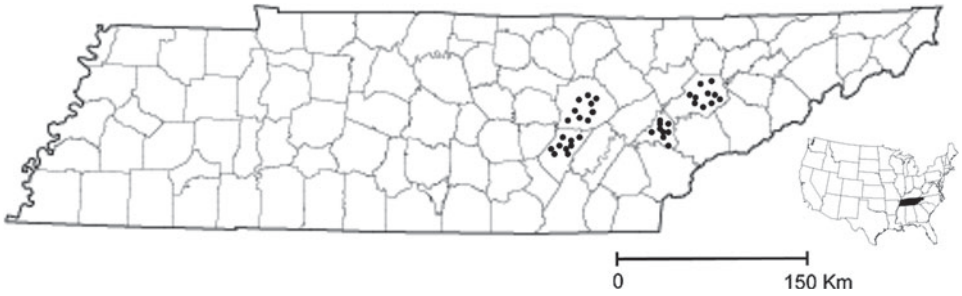

Fig. 1. Map showing the location of the 40 sampled farm ponds for ranavirus surveillance in eastern Tennessee. The ponds were divided equally among four counties in the Cumberland Plateau (Cumberland and Bledsoe counties) and Tennessee River Ridge and Valley (Knox and Loudon counties) physiographic regions. *Inset* shows the United States with the state of Tennessee *highlighted.*

sympatric amphibian species with ranids representing the most frequently infected group.

Methods

Study Sites

Our study was conducted in the Cumberland Plateau (CP) (Cumberland and Bledsoe Counties) and the Tennessee River Ridge and Valley (TRRV) (Knox and Loudon Counties) physiographic regions of Tennessee, USA, during 2008 and 2009 (Fig. 1). We sampled 40 ponds (20 ponds per year) equally distributed between the two regions. We chose this sampling scheme because our main interest was in seasonal dynamics within a pond rather than yearly dynamics and because we were not able to logistically sample 40 ponds each year. Ponds were identified in each region using U.S. Fish and Wildlife Service National Wetlands Inventory maps (http://www.fws.gov/wetlands/). For each county, we randomly selected one cardinal quadrant and constructed a list of potential ponds for the study. Landowners were contacted to gain access privilege and inquire about pond history. Suitable ponds for the study were identified based on the following criteria: (1) the pond did not dry completely during the summer 2007 drought (National_Climatic_Data_Center 2008), (2) the pond was >5 years old and had emergent vegetation, (3) there were no streams flowing into the pond that could contain agricultural point source pollution, (4) the pond was not adjacent to major roads, and (5) there was no artificial oxygenation of the pond. We also classified each pond as cattle access or non-access. For cattle-access ponds, the number of cattle that had access to ponds was recorded based on information provided by the landowner. From this list of ponds, we randomly selected 10 cattle-access and non-access ponds for each region and year (Table 4 in Appendix).

Pond Sampling

Amphibians (larvae for all species except red-spotted newts) and fish were sampled in January (winter), April (spring), July (summer), and October (fall) of 2008 and 2009 using seine and dip net protocols. We divided each pond into four cardinal quadrants. We randomly selected one quadrant and its opposing quadrant to conduct the seine net sampling. For each seine haul, we positioned the seine 2 m from the shore and pulled the seine for 10 m parallel to the shore (Schmutzer et al. 2008). Dip net sampling occurred for 15 min in each quadrant. Within each quadrant, we sampled all available amphibian habitats, which included submersed vegetation, detritus, and mud extending from the shoreline to 1 m in water depth. At the conclusion of dip net sampling, we recorded the total number of sweeps, which we used to estimate catch-per-unit-effort (CPUE) in each pond. For both the seine and dip net samples, we counted and identified all captured amphibian larvae and fish to species. We identified fish because they are an amphibian predator and could affect densities (Wellborn et al. 1996). Although fish may be a reservoir for ranaviruses (Picco et al. 2010), they were not sampled for ranavirus infections. There were two cases when we were unable to identify the amphibian larvae to species. We encountered larvae of the plethodontid genus *Desmognathus* ($n = 2$) and small hatchlings of the genus *Lithobates* (most likely *L. catesbeianus* or *L. clamitans*; $n = 11$). Thus, we recorded *Desmognathus* sp. and *Lithobates* hatchling, respectively. For each amphibian species collected, we randomly selected up to five individuals for ranavirus testing. Each selected individual was rinsed with sterile water and placed into a glass jar containing 500 ml of sterile water for transport to the University of Tennessee. In fall 2008, we did not sample one pond (R31) because it had dried. Thus, we excluded this pond from the analysis for that sample date.

We measured water quality at each pond on each sample date. The water quality variables included dissolved oxygen (mg l^{-1}), pH, temperature (°C), turbidity (FTU), and ammonia (ppm), which are known to be impacted by cattle and potentially cause stress in larval amphibians (Schmutzer et al. 2008). We used handheld YSI meters (Yellow Springs Instruments, Yellow Springs, OH, USA) to measure dissolved oxygen, pH, and temperature in the field. All measurements were taken 2 m from the south end of the shoreline in each pond. Given that we sampled multiple ponds on the same day, we could not account for diurnal changes in dissolved oxygen and temperature. Based on our previous work, we anticipated that seasonal and cattle-access effects would account for the majority of variation in these two variables (Gray et al. 2007; Schmutzer et al. 2008). We collected a 500-ml water sample from each pond to measure the remaining water quality variables using a LaMotte Smart2 colorimeter (LaMotte Company, Chestertown, MD, USA).

Necropsy and Ranavirus Testing

All collected individuals were transported to the University of Tennessee Joe Johnson Animal Research and Teaching Unit where they were euthanized by immersion in benzocaine hydrochloride. We removed a section of the liver and kidney for virus testing and froze the tissues at −80°C. The liver and kidney samples for each individual were pooled into a single microcentrifuge tube. We collected the liver and kidney because these organs are known sites of virus infection (Tweedell and Granoff 1968; Gantress et al. 2003; Converse and Green 2005; Miller et al. 2007). Moreover, based on experimental challenges in the laboratory for 19 amphibian species, we documented that ranavirus infection can occur in the majority of the species that we collected from the field and the liver and kidney are reliable organs for testing ranavirus infection (Hoverman et al. 2011). New gloves were used when handling each individual, and all equipment was disinfected with 0.75% Nolvasan® (2% chlorhexidine diacetate; Fort Dodge Animal Health, Fort Dodge, Iowa, USA; Bryan et al. 2009) after use with each individual.

For ranavirus testing, we extracted DNA from the pooled liver and kidney sample for each individual using a DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA). The extracted DNA was concentrated to 50 µl using a Savant DNA120 SpeedVac Concentrater (Thermo Fisher Scientific, Pittsburgh, PA). Conventional PCR targeting an approximately 450-bp region of the major capsid protein (MCP) gene was then used, following the protocol and primer sets reported by Mao et al. (1996, 1997; primers MCP4 and MCP5). The PCR products were resolved via electrophoresis on a 1.0% agarose gel. Two negative controls (water and gDNA extracted from a ranavirus-negative tadpole) and two positive controls (cultured ranavirus and gDNA extracted from an experimentally infected and confirmed ranavirus-positive tadpole) served as controls for the PCR runs. The PCR reactions were repeated to confirm results. We further verified the PCR products as ranavirus DNA by using real-time quantitative PCR (*Taq*Man qPCR) following Picco et al. (2007), and as described by Hoverman et al. (2010). A sample was declared positive only if both conventional PCR and qPCR results were positive. In laboratory experiments, we had considerable success with detecting ranavirus DNA using *Taq*Man qPCR with high correlation ($R^2 = 0.978$) between positive infection and mortality from ranaviral disease across 19 amphibian species (Hoverman et al. 2011). However, we acknowledge that low-grade infections may not be detected by PCR (Green et al. 2009), thus our infection prevalence estimates may be lower than true levels.

Statistical Analyses

The data set consisted of the abiotic and biotic characteristics of each pond and the infection prevalence of collected individuals within each pond across seasons. The biotic response variables were CPUE for fish and amphibians and amphibian species richness. Our infection data set consisted of the percent of infected individuals in the sample of collected amphibians. We pooled all species for analyses to reduce variation in the response variables due to low species-specific captures per pond, and because we were interested in community-level responses to ranavirus. We used repeated-measures analysis-of-variance (rm-ANOVA) to test the effects of sample year, cattle access, and physiographic region on the response variables among seasons. Univariate within-subjects tests were conducted using the Huynh–Feldt degrees of freedom correction factor when the assumption of sphericity was violated. We conducted mean comparisons using Fisher's LSD test if a response variable was significant.

Although community-level responses to ranaviruses were our major interest, we used multiple regression analysis to examine whether the presence of particular species that were infected at sites explained significant variation in ranavirus prevalence. For this analysis, we used only sites ($n = 61$) and species ($n = 9$) for which ranaviruses were detected. We also used stepwise multiple regression analysis

to examine the relative importance of pond characteristics in explaining ranavirus prevalence. Explanatory variables included pH, dissolved oxygen, temperature, ammonia, turbidity, cattle abundance, CPUE for fish and amphibians, and amphibian species richness. Given that each pond was repeatedly sampled (i.e., seasons were not independent) and ranavirus was not detected across all seasons for a particular pond, we conducted a regression analysis separately for each season. Data were combined across years for each regression because ponds were different replicates between years.

Results

Ranavirus Infection

We detected ranavirus infections in 83% ($n = 33$) of the ponds (Table 5 in Appendix). Prevalence varied from 4 to 90% across seasons within ponds with ranavirus infections. For ponds with infected individuals, 39% ($n = 13$) had $\geq$40% ranavirus prevalence and 6% ($n = 2$) had $\geq$85% ranavirus prevalence during at least one sampling event. Ranavirus infections were found during multiple seasons in 61% ($n = 20$) of the ponds containing infected individuals. In addition, ranavirus was detected in consecutive seasons in 42% ($n = 14$) of the ponds. Of the 13 species collected during our sampling (Table 1), infections were detected in nine species (69%, Fig. 2a). The percent of infected individuals varied from 3 to 75% for the nine species in which infections were detected. Of these nine species, the eastern tiger salamander had the greatest percent infection (Fisher's exact test $P \leq 0.007$), although only eight individuals were tested. Infections were detected in all four ranid species encountered during our sampling and infection prevalence averaged 16%. Green frogs had the greatest number of infected individuals followed by American bullfrogs and pickerel frogs. At the pond-level, green frogs were the

Table 1. List of amphibian species detected during field surveillance for ranavirus within farm ponds in eastern Tennessee, USA

Family	Scientific name	Common name
Ranidae	*Lithobates sphenocephalus*	Southern leopard frog
Ranidae	*Lithobates palustris*	Pickerel frog
Ranidae	*Lithobates clamitans*	Green frog
Ranidae	*Lithobates catesbeianus*	American bullfrog
Hylidae	*Hyla chrysoscelis*	Cope's gray tree frog
Hylidae	*Pseudacris feriarum*	Southeastern chorus frog
Hylidae	*Acris crepitans*	Northern cricket frog
Bufonidae	*Anaxryus americanus*	American toad
Microhylidae	*Gastrophryne carolinensis*	Eastern narrow-mouthed toad
Plethodontidae	*Desmognathus* sp.	Lungless salamander
Ambystomatidae	*Ambystoma tigrinum*	Eastern tiger salamander
Ambystomatidae	*Ambystoma talpoideum*	Mole salamander
Salamandridae	*Notophthalmus viridescens*	Red-spotted newt

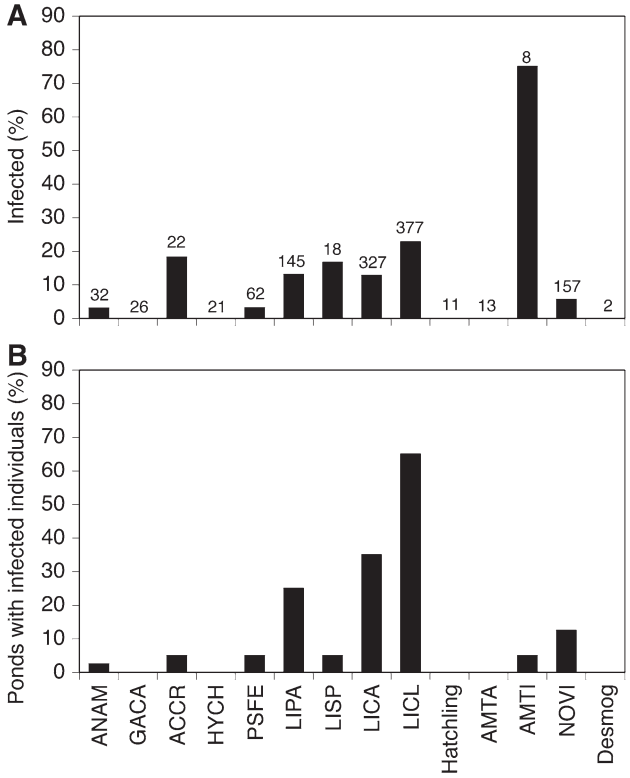

Fig. 2. Percentage of ranavirus-infected individuals (**a**; sample size indicated above *bars*) and the percentage of ponds in which infected individuals were detected (**b**) for species collected during the surveillance of ponds in eastern Tennessee. Species designations are ANAM, *Anaxyrus americanus*; GACA, *Gastrophryne carolinensis*; ACCR, *Acris crepitans*; HYCH, *Hyla chrysoscelis*; PSFE, *Pseudacris feriarum*; LIPA, *Litobates palustris*; LISP, *L. sphenocephala*; LICA, *L. catesbeianus*; LICL, *L. clamitans*; Hatchling, *Lithobates* hatchling; AMTA, *Ambystoma talpoideum*; AMTI, *A. tigrinum*; NOVI, *Notophthalmus viridescens*; Desmog, *Desmognathus* sp.

mostly commonly infected species followed by American bullfrogs and pickerel frogs (Fig. 2b). The percentage of species infected at a given pond within a season averaged 63%. There were no significant effects of season, cattle access, or region on infection prevalence (Table 2; Fig. 3a). However, infection prevalence was positively associated with the presence of infected green frogs and bullfrogs ($F_{2,58} = 8.6$, $P = 0.001$, adjusted $R^2 = 0.201$, Beta for bullfrogs = 0.364, Beta for green frogs = 0.359).

Observed Die-Off Event

In October 2008, we encountered mass mortality (>20 individuals) of American bullfrog and green frog tadpoles during our sampling of one non-access pond located in Knox County (Pond R68; Table 4 in Appendix). Dead tadpoles were observed at the surface and while dipnetting. Both species had classic gross signs of ranaviral disease including edema (swelling) and erythema (skin reddening from capillary congestion) of the body and hindlimbs. Of the sampled larvae, 100 and 80% of American bullfrogs and green frogs, respectively, tested positive for ranavirus infections. We revisited the pond 10 days later and observed additional dead tadpoles (100% of 20 individuals were ranavirus positive) suggesting that the mortality event may have lasted several weeks.

Biotic and Abiotic Characteristics

Amphibian species richness was 76% greater in non-access ponds compared with cattle-access ponds (Table 2). In addition, species richness was greatest in the spring and summer, intermediate in the fall, and lowest in the winter ($P \leq 0.013$; Fig. 3b). Species richness did not differ between the spring and summer ($P = 1.0$). The CPUE for amphibians was seven times greater in the TRRV compared with the CP (Table 2). The CPUE for fish was not affected by season, cattle access, or region (Table 2).

For ammonia and turbidity, there was a significant effect of cattle access (Table 3). Ammonia and turbidity were 2.4 times and 3.4 times greater, respectively, in cattle-access ponds compared with non-access ponds. There were significant effects of season on dissolved oxygen and pH (Table 3; Fig. 4a, b). Dissolved oxygen was greatest in the winter, intermediate in the spring and fall, and lowest in the summer. From winter through fall, pond pH tended to increase. There were significant effects of season and the season × region interaction for pond temperature (Table 3;

Table 2. Results of repeated-measures ANOVAs examining the effects of sample year, cattle access, and region on the catch-per-unit effort (CPUE) for fish and amphibians, amphibian species richness, and pond-level ranavirus prevalence (percent infected) across seasons in Tennessee, USA

Within subjects	CPUE—fish		CPUE—amphibians		Amphibian species richness		Ranavirus prevalence	
	$F_{2,61}$	P	$F_{2,72}$	P	$F_{3,108}$	P	$F_{3,43}$	P
Season	2.9	0.070	2.1	0.132	13.0	**0.000**	2.6	0.067
Season × access	2.2	0.127	0.5	0.625	1.8	0.160	0.9	0.440
Season × region	1.5	0.243	2.0	0.149	1.9	0.142	0.2	0.844
Season × access × region	0.9	0.397	0.4	0.701	0.4	0.757	1.5	0.231
Between subjects	**CPUE—fish**		**CPUE—amphibians**		**Amphibian species richness**		**Ranavirus prevalence**	
	$F_{1,32}$	P	$F_{1,36}$	P	$F_{1,36}$	P	$F_{1,16}$	P
Access	1.6	0.217	1.8	0.193	13.7	**0.001**	0.3	0.567
Region	0.1	0.794	4.7	**0.037**	0.0	0.960	0.6	0.465
Access × region	0.0	0.313	0.8	0.373	0.7	0.394	0.3	0.600

Note Univariate within-subjects tests were conducted using the Huynh–Feldt degrees of freedom correction factor because the assumption of sphericity was violated.

Bold values indicate $P \leq 0.05$.

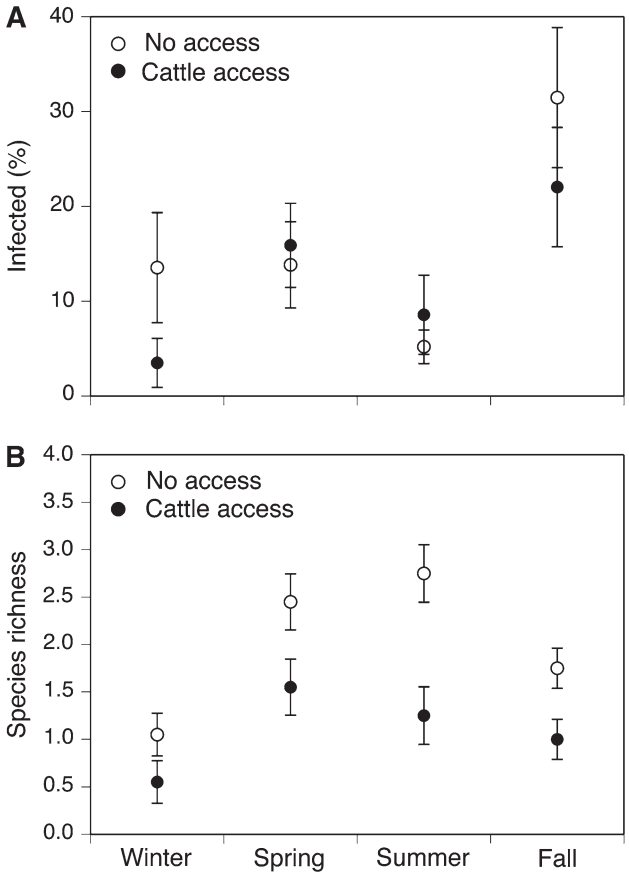

Fig. 3. Prevalence of ranavirus in collected amphibians (**a**) and amphibian species richness (**b**) in cattle-access (*closed circles*) and non-access (*open circles*) ponds across seasons for ponds sampled in eastern Tennessee. Data are least-squares means ± 1 SE.

Fig. 4c). Within both regions, pond temperature was lowest in the winter, intermediate in the spring and fall, and highest in the summer ($P < 0.001$). The season × region interaction was driven by higher pond temperature in the TRRV compared with the CP in the summer and fall ($P \leq 0.016$) but not in the winter and spring ($P \geq 0.051$).

Multiple Regression Analysis

In the summer, the most significant predictor of ranavirus prevalence was the model composed solely of cattle abundance ($F_{1,11} = 5.1$, $P = 0.048$, adjusted $R^2 = 0.271$, Beta = 0.580); greater prevalence was associated with greater cattle abundance. In the fall, the most significant predictor of ranavirus prevalence was the model composed solely of temperature ($F_{1,21} = 5.3$, $P = 0.032$, adjusted $R^2 = 0.169$, Beta = 0.457); greater prevalence was associated with higher pond temperatures. There was not a significant predictor of ranavirus prevalence in the winter or spring.

Discussion

We found that ranavirus infections were widespread in ponds located in eastern Tennessee; ranavirus infections were detected in nine species and in 33 of the sampled ponds (83%). In 13 of ponds containing infected individuals (39%), prevalence exceeded 40%. These findings suggest that ranaviruses may be common in the breeding habitats of pond-breeding amphibians and that a large proportion of the amphibian community can be infected at a site. Also, these results suggest that reliance on mortality events may greatly underestimate the geographic extent of ranaviruses in amphibian communities.

We detected ranavirus infections in multiple seasons within a large fraction ($n = 20$; 61%) of the ponds, which suggests that ranaviruses are relatively persistent in permanent aquatic environments. Previous research has shown that pond drying may inactivate ranaviruses, which would eliminate them from temporary habitats each year (Brunner et al. 2007). However, the permanent hydroperiod of our ponds coupled with the frequent occurrence of hosts such as American bullfrog and green frog tadpoles and adult eastern newts throughout the year may provide the opportunity for long-term persistence of ranaviruses. Indeed, infected American bullfrogs, green frogs, and eastern newts were found in 35, 65, and 13% of the sampled ponds, respectively. Moreover, there was a significant association between the presence of infected American bullfrogs and green frogs and infection prevalence in the ponds. Thus, these species may be important reservoirs that facilitate the transmission of ranaviruses throughout the year (Gray et al. 2009).

Despite the high prevalence and persistence of ranavirus in the sampled ponds, there was only one die-off event observed during our sampling. There is the possibility that mortality events occurred in the three months between each of the sampling events. Given that mortality rates closely track infection rates (Brunner et al. 2007; Cunningham et al. 2007; Hoverman et al. 2011), additional mortality events likely occurred during our study but were undetected. For example, our finding of two ponds with prevalence levels ≥85% suggests that die-offs may have been imminent but missed due to a single visit in that season. Our results underscore the need for intensive monitoring over time to assess the impacts of ranaviruses on amphibian populations.

Of the 13 amphibian species encountered during sampling, nine of the species collected tested positive for ranavirus infection. These species represent five amphibian

Table 3. Results of repeated-measures ANOVAs examining the effects of sample year, cattle access, and region on the abiotic characteristics (ammonia, turbidity, dissolved oxygen, pH and pond temperature) of the sampled ponds across seasons in Tennessee, USA

Within subjects	Ammonia		Turbidity		DO		pH		Temperature	
	$F_{2,83}$	P	$F_{3,89}$	P	$F_{3,105}$	P	$F_{3,100}$	P	$F_{3,105}$	P
Season	0.3	0.757	1.5	0.229	30.8	**0.001**	7.7	**0.001**	606.8	**0.001**
Season × access	1.0	0.382	0.9	0.451	1.6	0.190	2.2	0.095	1.1	0.340
Season × region	1.6	0.210	0.7	0.556	0.6	0.642	1.4	0.262	8.2	**0.001**
Season × access × region	1.1	0.362	0.6	0.583	0.1	0.963	0.8	0.505	0.7	0.549
Between subjects	**Ammonia**		**Turbidity**		**DO**		**pH**		**Temperature**	
	$F_{1,35}$	P	$F_{1,35}$	P	$F_{1,35}$	P	$F_{1,35}$	P	$F_{1,35}$	P
Access	5.3	**0.027**	11.0	**0.002**	2.5	0.120	3.6	0.065	1.1	0.309
Region	2.4	0.128	0.2	0.650	0.9	0.362	1.7	0.204	1.9	0.175
Access × region	2.1	0.161	0.0	0.923	1.9	0.173	0.1	0.745	0.0	0.951
					31.5	**0.001**				

Note Within-subjects tests were conducted using the Huynh–Feldt degrees of freedom correction factor if the assumption of sphericity was violated for a variable.

Bold values indicate $P \leq 0.05$.

families (Ranidae, Bufonidae, Hylidae, Ambystomatidae, Salamandridae) that are common inhabitants of ponds and wetlands across North America. While our findings demonstrate that ranaviruses have a broad host range, species within the family Ranidae, which comprised >70% of our sampled individuals, were the main contributors to patterns of infection. We detected infections in all four ranid species that were sampled at our sites and they were the most commonly infected group. This suggests that ranids are highly susceptible to ranavirus infections, which is supported by previous laboratory research (Hoverman et al. 2011). Additionally, we detected infections in 63% of the species, on average, at a site suggesting that not all members of the amphibian community may be simultaneously infected. To date, few studies have examined the role of transmission of ranaviruses within amphibian communities. For example, Duffus et al. (2008) proposed a conceptual model of interspecific transmission based on the observation of FV3-like infections in multiple co-occurring amphibian species within ponds. A fundamental assumption of this model is that species could be combined into a single group of susceptible individuals. Although this assumption adds simplicity to the model, variation in species susceptibility to infection and disease progression will likely require a more complex model that includes species-specific parameters to understand ranavirus transmission. Future experimental studies that quantify within- and between-species transmission will help inform model development and advance our understanding of ranavirus dynamics within amphibian communities.

We documented substantial differences in the water quality and the amphibian community between cattle-access and non-access ponds. Cattle-access ponds had higher turbidity and ammonia concentrations than non-access ponds. Importantly, the mean ammonia concentration exceeded levels (>0.6 mg l^{-1}) known to have lethal and sublethal effects on larvae of some amphibian species (Jofre and Karasov 1999). We captured 76% fewer species in cattle-access ponds compared with non-access ponds. Importantly, there were no differences in the abundance of predatory fish between the two pond types suggesting that fish were not driving the difference in amphibian richness and abundance. Schmutzer et al. (2008) reported lower water quality, species richness, and abundance of amphibian larvae in cattle-access ponds compared with non-access ponds. Burton et al. (2009) also reported that cattle reduce shoreline vegetation, which functions as breeding habitat for many amphibian species. Less shoreline vegetation in cattle-access ponds also may cause tadpoles to congregate in the remaining cover and increase ranavirus transmission rates (Greer and Collins 2008). Together, these data suggest that allowing cattle access in wetlands can negatively impact amphibian populations.

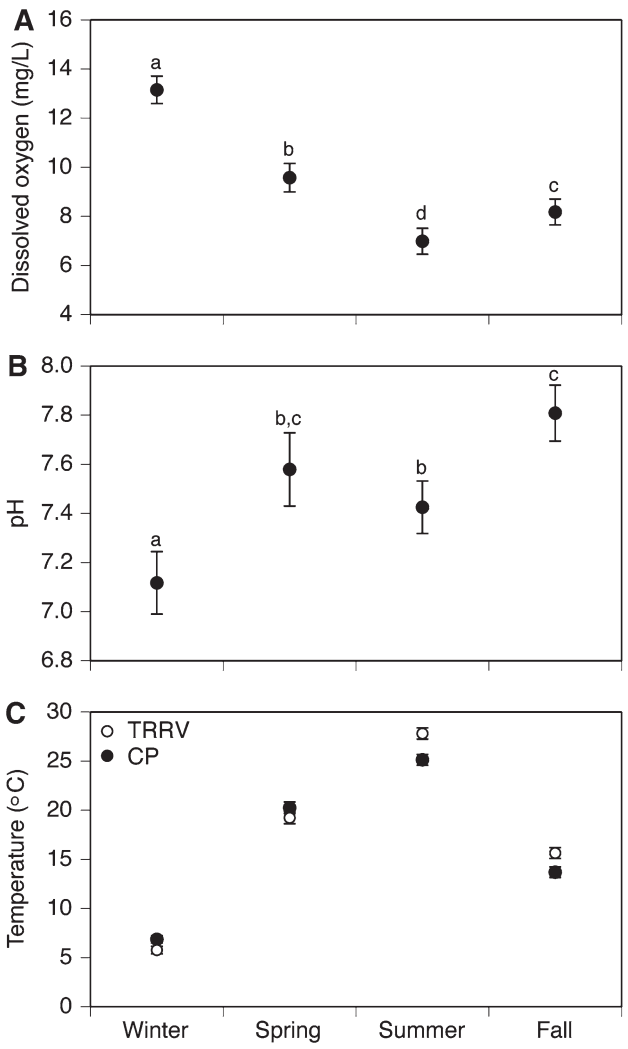

Fig. 4. Dissolved oxygen (**a**), pH (**b**), and temperature (**c**) across seasons in ponds sampled in eastern Tennessee. Data for dissolved oxygen and pH are averaged across cattle-access categories and regions. Seasons sharing lowercase letters are not significantly different based on Fisher's LSD ($P \geq 0.05$). Data for temperature are averaged across cattle-access categories but divided by physiographic regions (*CP* Cumberland Plateau and *TRRV* Tennessee River Ridge and Valley). Data are least-squares means + 1 SE.

Previous studies have documented increased prevalence of ranaviruses in some species inhabiting cattle-access ponds (Gray et al. 2007; Greer and Collins 2008). We found a significant positive association between cattle abundance and ranavirus prevalence in the summer sampling events; however, there was not an association in the remaining seasons. This suggests that the negative impact of cattle may be greatest during the summer when temperature is highest and dissolved oxygen is lowest across ponds on the landscape. As such, cattle access into wetlands may initiate changes in the abiotic characteristics of ponds that intensify and peak during the summer. The synergistic effect of multiple stressors on the landscape may play a significant role in ranavirus emergence.

There was limited support for other abiotic or biotic factors contributing to ranavirus prevalence. We only found a significant association between water temperature and ranavirus prevalence in the fall sampling events. However, the relationship was positive such that higher temperatures were associated with greater ranavirus prevalence. This result was counter to our expectation that lower temperatures would have immunosuppressive effects on amphibians and increase ranavirus prevalence as found in previous studies (Gray et al. 2007). However, ranavirus replication rates are greater at higher temperatures (Rojas et al. 2005), which may increase shedding rates into the aquatic environment and facilitate transmission rates. Given that this trend was not observed across all seasons, additional research is needed to assess water temperature as a possible mechanism of ranavirus emergence. Although there was no evidence that ranavirus prevalence differed across seasons, there was a general trend for low prevalence in the summer. Thus, summer may not be the best season for detecting ranavirus. We encourage future surveillance studies to sample amphibian populations during the entire course of the larval period to more accurately estimate ranavirus prevalence.

The recent emergence of infectious diseases across the globe has sparked considerable interest in understanding the spatial and temporal dynamics of diseases (Daszak et al. 2000; Cleaveland et al. 2001; Dobson and Foufopoulos 2001). While our study demonstrates that ranaviruses are common in amphibian communities, we have a limited understanding of the factors that trigger sudden population-level die-offs and the consequences of such die-offs on amphibian population dynamics (Petranka et al. 2007; Teacher et al. 2010). There is a great need for long-term monitoring studies that track local amphibian populations over time to identify potential factors resulting in disease emergence and quantify the impacts on amphibian recruitment and population size.

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Appendix

See Tables 4 and 5.

Table 4. Site information for the 40 ponds sampled in 2008 and 2009 in the CP and TRRV regions, Tennessee, USA

Pond	Year	Coordinates	Region	County	Cattle access	Mean cattle
R23	2008	36°04′02.4″ 83°52′29.2″	TRRV	Knox	A	2
R19	2008	36°01′49.6″ 83°45′55.1″	TRRV	Knox	A	67.5
R68	2008	36°03′05.2″ 83°41′35.8″	TRRV	Knox	NA	
R16	2008	36°02′59.2″ 83°45′17.4″	TRRV	Knox	NA	
R9	2008	36°07′23.0″ 83°43′07.9″	TRRV	Knox	NA	
R56	2008	35°41′19.7″ 84°29′33.7″	TRRV	Loudon	A	23
R51	2008	35°42′09.5″ 83°27′58.3″	TRRV	Loudon	A	85
R37	2008	35°39′09.2″ 84°11′18.5″	TRRV	Loudon	NA	
R44	2008	35°37′42.8″ 84°11′28.6″	TRRV	Loudon	NA	
R31	2008	35°44′55.3″ 84°14′06.4″	TRRV	Loudon	NA	
P16	2008	35°57′45.9″ 85°05′32.8″	CP	Cumberland	A	44
P44	2008	35°54′26.3″ 85°02′9.3″	CP	Cumberland	A	5
P14	2008	35°58′24.6″ 85°09′08.7″	CP	Cumberland	NA	
P26	2008	35°57′04.6″ 85°06′40.5″	CP	Cumberland	NA	
P37	2008	35°56′53.5″ 85°05′54.9″	CP	Cumberland	NA	
P82	2008	35°40′24.0″ 85°17′32.3″	CP	Bledsoe	A	65
P58	2008	35°42′46.0″ 85°10′58.7″	CP	Bledsoe	A	6
P75	2008	35°40′08.8″ 85°15′58.1″	CP	Bledsoe	NA	
P89	2008	35°40′38.4″ 85°12′07.2″	CP	Bledsoe	NA	
P50	2008	35°44′13.7″ 85°10′48.8″	CP	Bledsoe	NA	
K21	2009	36°01′46.6″ 83°48′42.9″	TRRV	Knox	A	6
K10	2009	36°04′19.4″ 83°47′32.4″	TRRV	Knox	A	30
K9	2009	36°03′59.7″ 83°48′0.8″	TRRV	Knox	A	10
K2	2009	36°01′30.3″ 83°47′30.6″	TRRV	Knox	NA	
K6	2009	36°05′3.9″ 83°46′24.8″	TRRV	Knox	NA	
N10	2009	35°38′38.6″ 84°10′25.4″	TRRV	Loudon	A	10
N9	2009	35°42′34.9″ 84°11′30.3″	TRRV	Loudon	A	35
N3	2009	35°38′48.7″ 84°10′25.7″	TRRV	Loudon	NA	
N8	2009	35°41′43.9″ 84°10′53.5″	TRRV	Loudon	NA	
N20	2009	35°40′01.9″ 84°26′21.4″	TRRV	Loudon	A	10
C26	2009	35°56′03.6″ 85°06′36.4″	CP	Cumberland	A	40
C23	2009	35°58′45.0″ 85°03′16.4″	CP	Cumberland	A	375
C25	2009	35°58′16.6″ 85°03′46.4″	CP	Cumberland	A	45
C12	2009	35°54′39.6″ 85°03′12.6″	CP	Cumberland	NA	
C10	2009	35°54′34.8″ 85°04′48.3″	CP	Cumberland	NA	
B19	2009	35°40′49.3″ 85°07′12.6″	CP	Bledsoe	A	8
B4	2009	35°37′42.7″ 85°16′24.9″	CP	Bledsoe	A	22.5
B10	2009	35°38′03.3″ 85°13′15.5″	CP	Bledsoe	A	37.5
B21	2009	35°37′35.8″ 85°16′57.2″	CP	Bledsoe	NA	
B12	2009	35°39′14.5″ 85°12′46.9″	CP	Bledsoe	NA	

A cattle access, *NA* non-access ponds.

Table 5. Ranavirus infection results for ponds that were sampled in eastern Tennessee, USA

	Species												
Pond ID	*L. palustris*	*L. sphenocephalus*	*L. catesbeianus*	*L. clamitans*	*Lithobates* hatchling	*H. chrysoscelis*	*P. feriarum*	*A. crepitans*	*A. americanus*	*G. carolinensis*	*Desmognathus*	*N. viridescens*	*A. talpoidum*	*A. tigrinum*
B10			2 (6)									1 (13)		
B12			0 (6)	5 (11)										
B19			0 (20)	0 (5)	0 (5)							0 (4)		
B21	1 (3)		0 (4)	0 (5)				1 (5)						
C10	0 (5)		0 (15)	1 (14)										
C12	2 (5)		9 (19)	6 (18)										
C23			2 (13)											
C26								0 (3)				0 (7)		
K10	2 (5)			3 (5)			0 (5)		0 (5)					
K2	0 (10)			4 (10)		0 (5)	0 (5)		0 (5)			0 (14)		
K21	5 (10)		0 (1)	4 (15)								0 (3)		
K6			0 (1)	1 (4)										
K9	1 (5)	0 (6)	1 (4)	6 (11)		0 (5)	0 (5)		1 (5)	0 (5)		0 (8)		
N10			1 (1)	0 (1)								1 (7)		
N20			0 (5)	3 (6)								0 (1)		
N3	0 (1)	2 (6)	0 (5)	0 (5)			1 (5)			0 (2)		0 (6)		
N8	0 (5)		1 (11)	3 (11)			0 (5)					4 (21)		
N9	0 (5)	0 (5)				0 (4)	1 (5)		0 (4)	0 (5)		0 (2)		
P14	0 (5)		3 (26)	9 (20)					0 (8)			0 (2)		
P16	0 (6)											0 (3)	0 (1)	
P26	1 (6)	1 (1)	0 (7)	4 (15)				0 (5)				1 (1)	0 (2)	
P37	0 (6)		1 (21)	2 (19)				0 (4)			0 (2)			
P44			2 (9)	2 (9)										
P50			0 (20)	1 (20)								0 (15)		
P58	3 (10)		0 (18)	2 (18)			0 (4)	3 (5)					0 (2)	
P75			0 (13)	5 (17)			0 (5)					0 (2)		
P82			0 (23)	3 (14)										
P89	0 (8)			2 (10)	0 (1)	0 (1)						0 (6)	0 (8)	
R16	0 (5)		5 (5)	4 (11)			0 (5)							
R23			2 (7)	1 (24)										
R31	1 (5)					0 (5)	0 (4)			0 (5)		0 (10)		1 (2)

Table 5. continued

Pond ID	Species												
	L. palustris	*L. sphenocephalus*	*L. catesbeianus*	*L. clamitans*	*Lithobates* hatchling	*H. chrysoscelis*	*P. feriarum*	*A. crepitans*	*A. americanus*	*G. carolinensis*	*Desmognathus*	*N. viridescens*	*A. talpoidum*	*A. tigrinum*
R37	1 (10)		0 (17)	2 (11)						0 (2)				
R44				0 (4)			0 (5)			0 (2)		0 (6)		
R51	0 (5)			1 (5)		0 (1)	0 (6)			0 (5)				5 (6)
R56	0 (5)		3 (11)	1 (21)	0 (5)									
R68	0 (7)		6 (24)	4 (19)								2 (26)		
R9	2 (13)		4 (15)	7 (19)			0 (3)		0 (5)					

Only ponds containing infection are shown. For each species, the total number of infected individuals (and total number of sampled individuals) across seasons is shown. See Table 4 for detailed pond information.

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