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Original Contribution

Phylogeny, Life History, and Ecology Contribute to Differences in Amphibian Susceptibility to Ranaviruses

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Abstract: Research that identifies the potential host range of generalist pathogens as well as variation in host susceptibility is critical for understanding and predicting the dynamics of infectious diseases within ecological communities. Ranaviruses have been linked to amphibian die-off events worldwide with the greatest number of reported mortality events occurring in the United States. While reports of ranavirus-associated mortality events continue to accumulate, few data exist comparing the relative susceptibility of different species. Using a series of laboratory exposure experiments and comparative phylogenetics, we compared the susceptibilities of 19 amphibian species from two salamander families and five anurans families for two ranavirus isolates: *frog virus* 3 (FV3) and an FV3-like isolate from an American bullfrog culture facility. We discovered that ranaviruses were capable of infecting 17 of the 19 larval amphibian species tested with mortality ranging from 0 to 100%. Phylogenetic comparative methods demonstrated that species within the anuran family Ranidae were generally more susceptible to ranavirus infection compared to species from the other five families. We also found that susceptibility to infection was associated with species that breed in semi-permanent ponds, develop rapidly as larvae, and have limited range sizes. Collectively, these results suggest that phylogeny, life history characteristics, and habitat associations of amphibians have the potential to impact susceptibility to ranaviruses.

Keywords: Anura, Caudata, Emerging infectious disease, Frog virus 3, Iridoviridae, Novel strain, Phylogeny, Reservoir

INTRODUCTION

While epidemiological research has historically focused on the interaction between a single host and a single pathogen, there is a growing appreciation that pathogens are embedded within complex ecological communities containing multiple host species (Pedersen and Fenton 2007; Begon 2008; Ostfeld et al. 2008; Telfer et al. 2008; Rigaud et al. 2010). Indeed, many emerging infectious diseases are linked to pathogens that are capable of infecting multiple hosts species within communities (Daszak et al. 2000; Dobson and Foufopoulos 2001; Woolhouse and Gowtage-Sequeria 2005; Craft et al. 2008). Given that natural communities contain a diversity of host species, research that identifies the potential host range of pathogens as well as host susceptibility is critical for

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understanding and predicting the dynamics of infectious diseases within ecological communities (Collinge and Ray 2006; Craft et al. 2008).

Empirical studies that document variation in host susceptibility (e.g., infection prevalence, mortality rates) to pathogens are accumulating in the literature (Craft et al. 2008; Cronin et al. 2010; Searle et al. 2011). An emerging question from this research is what drives variation in host susceptibility to generalist pathogens? Numerous factors are likely to influence host susceptibility including host life history characteristics (e.g., development rate, body size), habitat preferences, and host phylogenetic relatedness (Cronin et al. 2010). A novel and powerful approach that can expand our understanding of host susceptibility to multi-host pathogens is the integration of experimental exposure studies and comparative phylogenetics. Experimental exposure studies provide standardized conditions that remove environmental co-factors typical of field conditions (e.g., variation in temperature, host age, host development) and enable among-species comparisons in susceptibility to pathogens. These results can be combined with comparative phylogenetic methods to identify patterns of susceptibility due to shared evolutionary history (Felsenstein 1985; Harvey and Pagel 1991; Price et al. 2000; Stephens and Wiens 2004). Moreover, by incorporating host traits (e.g., life history characteristics, habitat preferences) into the analysis, potential correlates with host susceptibility can be identified. Collectively, these data can dramatically increase our ability to predict species that are at the greatest risk of infection and disease.

Amphibians have received considerable attention lately, because their populations are declining globally (Houlahan et al. 2001; Stuart et al. 2004). A number of factors have been hypothesized as contributors to these declines including the emergence of infectious diseases (Wake and Vredenburg 2008). Ranaviruses are one group of amphibian pathogens that have been identified as the etiological agent in die-off events around the globe (Green et al. 2002; Carey et al. 2003; Muths et al. 2006; Gray et al. 2009). In North America, over 20 species across five families have been reported in die-off events (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). Of these reported mortality events, the majority have occurred in the families Ranidae and Ambystomatidae. If field patterns are reflective of differences in species susceptibility, it is likely that species in the families Ranidae and Ambystomatidae are highly susceptible to ranavirus infections. While these field patterns suggest that phylogenetic relatedness may be a driver of susceptibility, an additional mechanism may be life history characteristics. For example, ranids generally develop for longer durations and are strongly associated with permanent water (Lannoo 2005), which may increase the likelihood of exposure to the pathogen given that water is an effective transmission route. Alternatively, species with larvae that develop over long durations in more permanent breeding sites may evolve resistance to ranavirus infection. It is also possible that species that breed in temporary wetlands and have short developmental times may be exposed to ranaviruses less frequently leading to greater susceptibility to the pathogen. Clearly, these hypotheses are contradictory, which emphasizes the need for controlled experiments to elucidate the patterns of ranavirus susceptibility and their relationship with host characteristics and phylogeny. To date, however, there have been no attempts to assess susceptibility under controlled conditions using a comparative phylogenetic approach. Thus, our objective was to compare the relative susceptibility (i.e., infection prevalence and mortality) of 19 larval amphibian species across seven families to a common set of ranavirus isolates and use comparative phylogenetic methods to quantify the importance of host characteristics and phylogeny in understanding patterns of susceptibility.

Methods

The 19 amphibian species used in the experiments were wood frogs (*Rana sylvatica*), gopher frogs (*R. capito*), southern leopard frogs (*R. sphenocephala*), northern leopard frogs (*R. pipiens*), pickerel frogs (*R. palustris*), green frogs (*R. clamitans*), American bullfrogs (*R. catesbeiana*), Cope's gray tree frogs (*Hyla chrysoscelis*), upland chorus frogs (*Pseudacris feriarum*), western chorus frogs (*P. triseriata*), mountain chorus frogs (*P. brachyphona*), American toads (*Bufo americanus*), eastern narrow-mouthed toads (*Gastrophryne carolinensis*), eastern spadefoots (*Scaphiopus holbrookii*), tiger salamanders (*Ambystoma tigrinum*), marbled salamanders (*A. opacum*), spotted salamanders (*A. maculatum*), mole salamanders (*A. talpoideum*), and red-spotted newts (*Notophthalmus viridescens*). These

species were chosen because they provided a broad representation of the pond breeding amphibian community in eastern North America. For each species, egg masses were collected from natural populations from 2008 to 2010 (Appendix Table 1). For Cope's gray tree frogs and eastern narrow-mouthed toads, we used the results from a previous study that used identical methods (Hoverman et al. 2010). All egg masses were transported to the controlled aquatic facility at The University of Tennessee Joe Johnson Animal Research and Teaching Unit (JARTU) and maintained in covered 300-l outdoor wading pools containing aged tap water until hatching. The exception was marbled salamanders, which oviposits on terrestrial moss. For this species, we transported the egg masses including the attached moss to the JARTU facility. Eggs were maintained indoors at 22°C for 2 months then inundated with water to hatch. After hatching, anuran larvae were fed ground TetraMin fish flakes (Tetra, Blacksburg, VA) ad libitum whereas salamander larvae were fed zooplankton (predominately *Daphnia* sp.) ad libitum until used in the experiments. Zooplankton were reared in 1,000-l outdoor cattle tanks inoculated with phytoplankton, eliminating the need to collect them from natural ponds where ranaviruses could have occurred. Each species was cultured individually and at densities ($\leq$1 individual l^{-1}) that are unlikely to induce stress (Relyea 2002). Prior to starting each experiment, we euthanized ten randomly selected individuals per species and tested tissue samples for ranavirus using PCR; all pre-experiment samples were negative for ranaviruses. In addition, all controls animals (n = 20 per species) from the experiments tested negative.

We used two ranavirus isolates in the experiments, *frog virus* 3 (FV3) and an FV3-like isolate (hereafter, the ranaculture isolate). The FV3 isolate was originally cultured by Allan Granoff from adult northern leopard frogs in Illinois, USA (Granoff et al. 1965); we obtained the isolate from Gregory Chinchar of The University of Mississippi. The ranaculture isolate was obtained in 2006 from a recently metamorphosed American bullfrog that died during a massive mortality event (>50%) inside a commercial ranaculture facility in southern Georgia, USA (GenBank accession no. EF101698; Miller et al. 2007a). In a previous study, we found that the ranaculture isolate was more infectious and caused greater mortality than the FV3 isolate (Hoverman et al. 2010). Our objectives were to determine if these results were consistent across a larger number of species and to determine whether correlates of species susceptibility were similar across isolates. Virus culturing was performed at the University of Georgia and details on these methods can be found in Hoverman et al. (2010). For both isolates, titered virus was sent from the University of Georgia Veterinary Diagnostic and Investigational Laboratory overnight to JARTU and stored at −80°C until used in the study.

For each anuran species, we conducted a single experiment when tadpoles reached Gosner (1960) stage 30. We standardized across developmental stage to facilitate species comparisons and because susceptibility to ranaviruses is known to vary among developmental stages (Haislip et al. 2011). Each experiment was conducted under identical laboratory conditions (23°C and a 12:12 day:night photoperiod). Our experimental units were 2-l plastic tubs filled with 1 l of aged dechlorinated tap water placed on 4 × 8-ft shelving units at uniform height. The water was passed through a dechlorination filter and aged for at least 24 h in indoor 700-l tanks prior to use. We randomly assigned a single individual to each experimental unit from a mixture of the collected egg masses (Appendix Table 1). Each experiment consisted of three treatments with 20 replicate tadpoles per treatment, totaling 60 experimental units. The treatments were virus-free control, bath exposure to the FV3 isolate, or bath exposure to the ranaculture isolate. We added 10^6 plaque-forming units (PFUs) of the appropriate virus isolate suspended in 60 µl of Eagle's minimum essential media (MEM) to each tub for the virus isolate treatments to reach a final concentration of 10^3 PFUs ml^{-1}. We added 60 µl of MEM to the control treatment to control for the addition of media to the experimental units. Based on previous study, doses between 10^2 and 10^6 PFUs of FV3-like ranavirus are sufficient to induce sublethal effects or morbidity in tadpoles (Tweedell and Granoff 1968; Pearman et al. 2004; Pearman and Garner 2005; Morales and Robert 2007; Hoverman et al. 2010).

During the experiments, water was changed every 3 days to maintain quality. We handled the control treatments first, followed by the virus treatments. In addition, we used new nets, changed gloves and rinsed all surfaces with 0.75% Nolvasan® (Fort Dodge Animal Health) between all treatments to prevent cross contamination (Bryan et al. 2009). Following the first water change, new experimental tubs were used and virus was not reapplied. Following each water change, tadpoles were fed a single 3-day ration of ground TetraMin at a rate of 12% of body mass per day (Relyea 2002). The food ration was calculated based on the mean mass of an independent set of ten tadpoles that were treated identically to the control treatment.

For the salamanders, the experimental design was slightly different from the anuran experiments. Experiments were started 30-day post hatching. A lack of laboratory space prevented us from starting the marbled salamanders until 60-day post hatching. The experimental units were 1-l tubs containing 0.5 l of aged dechlorinated tap water. Each experiment consisted of the same three treatments as described above (control and bath exposure to FV3 or the ranaculture isolate) with 20 replicates per treatment, totaling 60 experimental units. For tiger salamanders, we had enough larvae for only seven replicates of the control treatment. We added 5×10^5 PFUs of the appropriate virus isolate suspended in 30 µl of Eagle's MEM to each tub for the virus treatments (final concentration = 10^3 PFUs ml^{-1}). During the experiments, water was not changed because water quality remained high, thus, the virus challenge represented continuous exposure. Every 3 days during the experiments, we added 3 ml of concentrated zooplankton from the outdoor cattle tanks starting with the control treatment.

Although the experimental protocols slightly differed for the anuran and salamander larvae, we decided to analyze the groups together to provide a more comprehensive perspective of susceptibility. The greatest deviation in protocol between the two groups was the duration of exposure to the virus; the salamanders were continuously exposed because no water changes were conducted whereas the anurans were exposed for 3 days. Our previous study demonstrated that there is no difference in infection outcomes (infection prevalence or mortality) for individuals that are exposed continuously compared to those exposed for 3 days (Hoverman et al. 2010). Thus, the duration of exposure likely did not influence disease outcomes in our experiments. The experimental groups also differed when the experiments were started; the salamander experiments were started 30-day or 60-day post hatching while the anuran experiments were started at Gosner (1960) stage 30, which requires different amounts of time to reach depending on species. Although we have demonstrated that stage 30 anuran larvae are highly susceptible to ranavirus (Haislip et al. 2011), comparable studies in salamander larvae are lacking. However, laboratory experiments with 1- and 3-month-old salamander larvae have demonstrated these age classes are susceptible to ranavirus infections (Brunner et al. 2007; Kerby and Storfer 2009; Schock et al. 2009). While we acknowledge the differences in protocol between the anuran and salamander larvae, we feel that these differences were minor and likely did not impact the interpretation of our results.

The experiments were monitored daily for survival. For individuals that died during the experiment, we removed sections of the liver and kidneys and froze the tissues at −80°C for PCR analysis. We extracted DNA from the liver and kidney because these organs are known sites of ranavirus infections (Tweedell and Granoff 1968; Gantress et al. 2003; Converse and Green 2005; Miller et al. 2007a). For tadpoles of the chorus frogs and all salamander species, individuals were too small to be reliably necropsied. Consequently, individuals were rinsed with sterile water to remove virions and whole bodies frozen. All experiments were terminated after 21 days, which is sufficient time for ranavirus infection and morbidity to occur (Pearman et al. 2004; Pearman and Garner 2005; Brunner et al. 2007; Hoverman et al. 2010). Live individuals remaining at the end of the experiments were euthanized by immersion in benzocaine hydrochloride and the same post-mortem procedures followed. For ranavirus testing, we prepared a tissue homogenate from the liver and kidney or whole body and extracted DNA using a DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA). We used the real-time PCR methods of Picco et al. (2007) for virus detection.

The response variables for each species included mortality and infection prevalence. We did not detect infections in the individuals from the virus-free control treatments, thus, we excluded this treatment from the infection analyses. Given that some individuals died without detectable infections (Fig. 2), we used the number of infected individuals that died as our mortality response variable in the analyses. For each species, Pearson's Chi-square test was used to test for differences in mortality and infection prevalence among treatments. For each ranavirus isolate, we tested for species-level differences in mortality and infection prevalence using Pearson's Chi-square test. We also tested for differences in mortality rates among treatments using Wilcoxon–Gehan D test (Pyke and Thompson 1986; Hoverman et al. 2010). Given that the results from this analysis were similar to the analysis of final mortality, we included them in Appendix Tables 3, 4 and Figures 4, 5, and 6, but they are not discussed further. We used multiple regression analysis to examine whether case mortality differed between the virus isolates. For this analysis, we only included species with infected individuals (n = 11 for FV3 and n = 17 for the ranaculture isolate).

Analyses were conducted using SPSS 19.0 (SPSS Inc., Chicago, IL, USA) at $\alpha = 0.05$.

Phylogenetic Comparative Methods

We collected data from the literature on nine species-level characteristics to explore their potential association with ranavirus susceptibility using phylogenetic comparative methods (Conant and Collins 1998; Petranka 1998; Lannoo 2005; Elliot et al. 2009). The characteristics included hydroperiod of the breeding habitat, seasonal breeding time, duration of egg stage, duration of larval stage, size at metamorphosis, clutch size, adult body size, time to maturity, and species range size (Appendix Table 2). Breeding habitat hydroperiod was coded from 1 to 3 with 1 = temporary ponds that dry in the early summer, 2 = semi-permanent ponds that dry in certain years, and 3 = permanent water bodies. Breeding time was coded from 1 to 3 with 1 = winter to early spring breeding, 2 = late spring, and 3 = summer. Species range size was estimated from available distribution maps. For the remaining characteristics, there was substantial variation in the literature as would be expected for ectothermic vertebrates that are broadly distributed across latitudes (i.e., temperature gradients) that influence growth and development. Given that the data were often presented as a range of values as a consequence of this variation, we calculated the midpoint of the reported range as our response for each species in the analyses.

To conduct statistical analyses of the interspecific data, we obtained phylogenetic information on the relationships among the species (i.e., tree topology) from multiple studies (Shaffer et al. 1991; Hillis and Wilcox 2005; Wiens et al. 2005; Lemmon et al. 2007; Wells 2007; Fig. 1). Branch lengths representing the amount of change that has occurred along the tree (i.e., divergence times) were obtained using TimeTree (Hedges et al. 2006) or published studies (Lemmon et al. 2007). Given that divergence times were

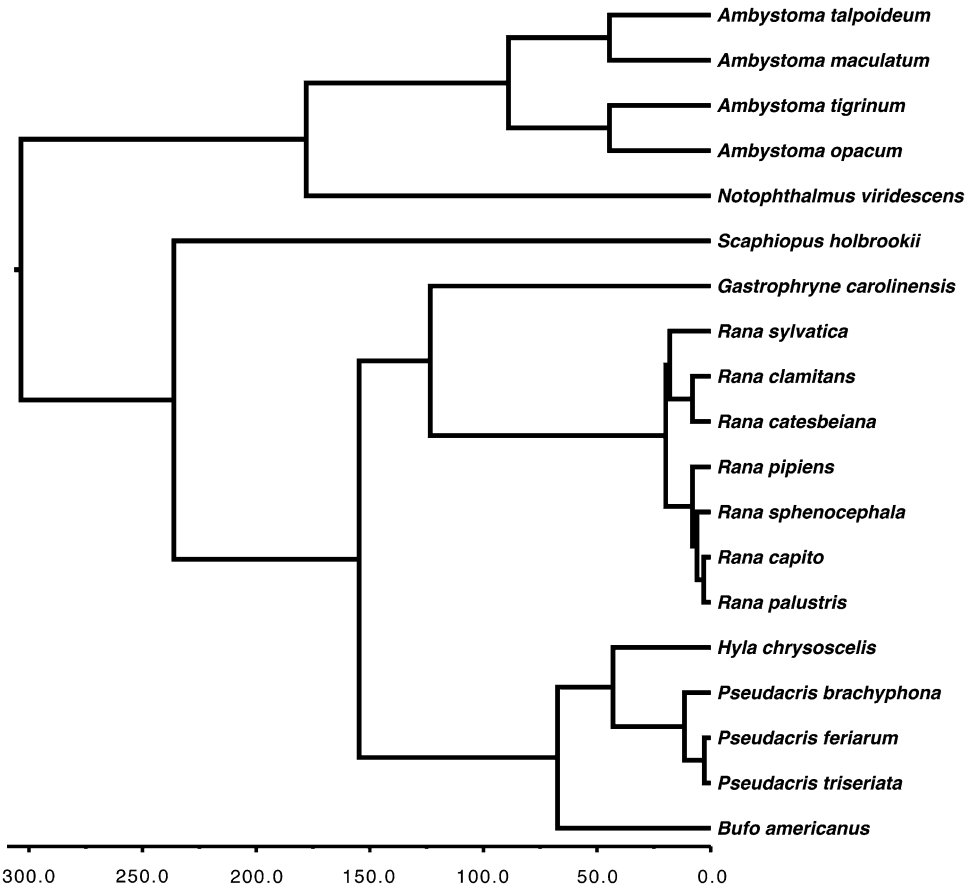

Figure 1. Phylogenetic hypothesis depicting relationships among the 19 amphibian species included in the study. Branch lengths are presented as divergence times (millions of years). We divided the branch lengths equally among the ambystomatids because divergence times were not available.

not available for the ambystomatids, we divided the branch lengths equally among the taxa.

We tested for phylogenetic signal in species susceptibility to the virus isolates using the "Kcalc" function in the "picante" package of R statistical software (R Development Core Team 2008). This analysis addresses the tendency for closely related species to resemble each other in trait values (Blomberg et al. 2003). As our measure of susceptibility, we used infection prevalence rather than mortality because there were cases where individuals died without detectable infections. To supplement this analysis, we tested for family-level variation in susceptibility for each virus isolate using a non-parametric Kruskal–Wallis test. We focused our analysis on the families Ambystomatidae, Ranidae, and Hylidae because they contained adequate representation (i.e., $\geq$4 species). We used a non-parametric test because the assumptions of normality and homogeneity of variance were violated.

We tested for correlations between species susceptibility (i.e., infection prevalence) to each ranavirus isolate and species characteristics using phylogenetic generalized least squares (PGLS) for categorical species traits and phylogenetic independent contrasts (PIC) for continuous traits. These analyses account for the shared evolutionary history between species. A fundamental assumption of comparative methods is that the contrasts are standardized (i.e., not correlated with branch lengths; Garland et al. 1992). Standardization was accomplished using Grafen's rho ($\rho = 0.5$) transformation for the phylogenetic tree combined with trait transformations. We $\log_{10}$-transformed species range size, duration of egg and larval stage, size at metamorphosis, adult size, and clutch size. The remaining continuous variables did not require transformation to achieve standardization. For our two categorical variables (breeding habitat and breeding time), we used two dichotomous dummy variables to represent the three categories (Ord and Martins 2006), and these were regressed against infection prevalence. Given that the coefficient of determination generated by the PGLS is roughly equivalent Pearson's product–moment correlation coefficient (Ord and Martins 2006; Stephens and Wiens 2008), we used a t test to assess the statistical significance of the coefficient at $\alpha = 0.05$. We used the program COMPARE for analyses of categorical variables (Martins 2004), and the "crunch" function in the "caper" package of R statistical software for analyses of continuous variables (R Development Core Team 2008).

Results

Infections were detected in 11 and 17 of the 19 species exposed to FV3 or the ranaculture isolate, respectively (Fig. 2). No infections were detected in the virus-free control treatments. Infection prevalence was on average 48% greater with the ranaculture isolate compared to FV3 for nine species (southern leopard frogs, northern leopard frogs, pickerel frogs, green frogs, Cope's gray tree frogs, mountain chorus frogs, eastern spadefoots, tiger salamanders, and spotted salamanders). Infection prevalence was 30% greater with FV3 compared to the ranaculture isolate for gopher frogs ($P = 0.028$). There was no difference in infection prevalence between the two virus treatments for the remaining nine species.

Mortality was significantly affected by the virus treatments for all species except American bullfrogs, upland chorus frogs, American toads, eastern narrow-mouthed toads, mole salamanders, and red-spotted newts (Appendix Tables 3, 4; Fig. 2). Of the 13 species affected by the virus treatments, wood frogs, gopher frogs, southern leopard frogs, and the three tree frog species experienced greater mortality with FV3 (mean mortality = 54%) compared to the control. In contrast, 13 species experienced greater mortality with the ranaculture isolate (mean mortality = 64%) compared to the control treatment. For eight of the 13 species (southern leopard frogs, northern leopard frogs, pickerel frogs, green frogs, mountain chorus frogs, gray tree frogs, eastern spadefoots, and tiger salamanders), final mortality was greater with the ranaculture isolate compared to FV3 (51% increase in final mortality).

We note that there were several species for which mortality was observed but infections were not detected in all individuals. For northern leopard frogs and eastern narrow-mouthed toads, there was mortality observed in the control treatment suggesting that background mortality may explain mortality without infection in the virus treatments. However, there was no mortality in the control treatment for the remaining species suggesting that background mortality was not an underlying cause for mortality without infection. Although it is unclear why all individuals

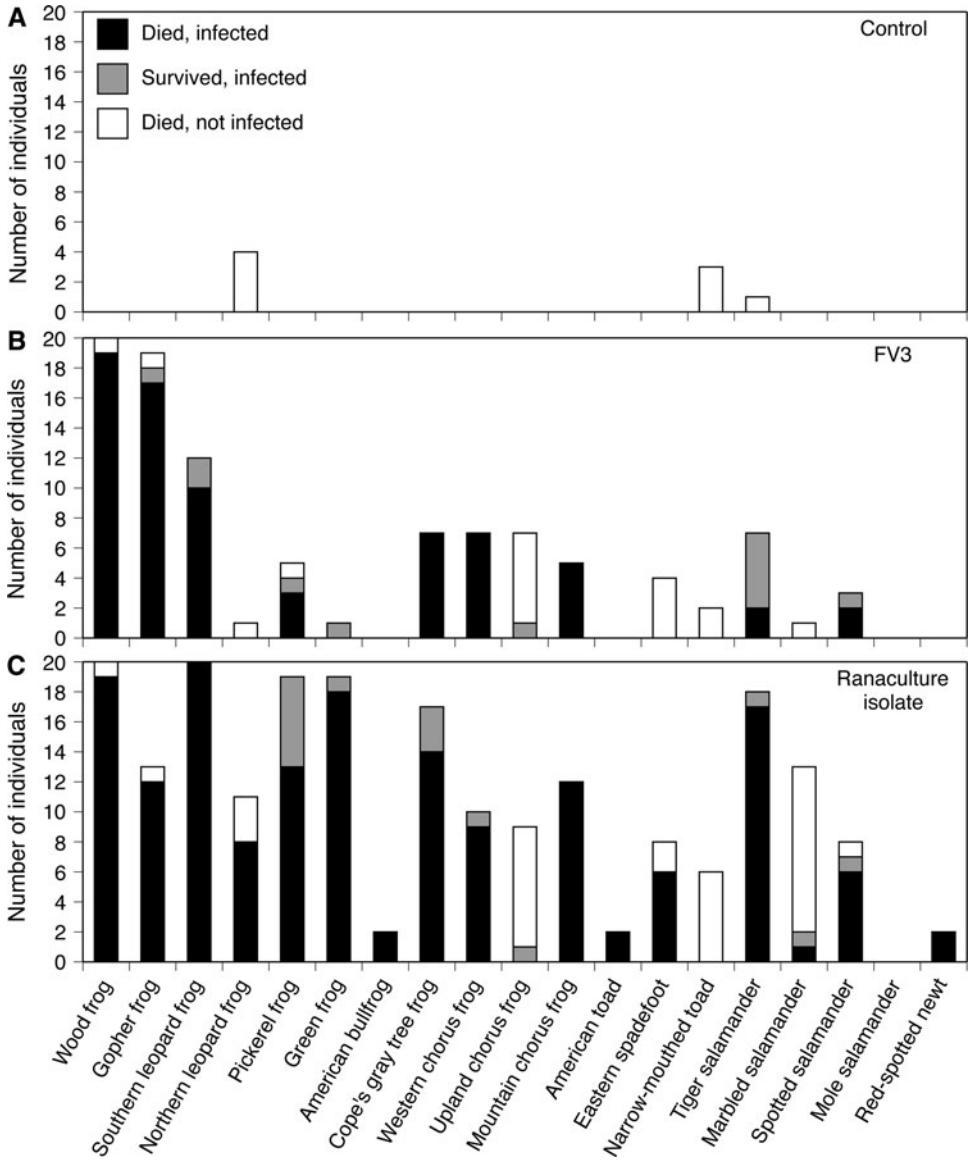

Figure 2. Infection and mortality results at the end of the experiment (21 days) for 19 North American larval amphibian species exposed to the virus-free control (**a**), *frog virus* 3 (**b**), or the ranaculture isolate (**c**). The status of the individuals within each treatment is divided into the number that died with infection (*black*), survived with infection (*gray*), and died without infection (*white*). The sample size ($n = 20$) was identical for all treatments except the control treatment for tiger salamanders ($n = 7$).

that died did not test positive for infection, it is possible that some individuals died from minor infections that were not detected by the PCR assay.

Among-Species Differences in Susceptibility

Within both virus treatments, there were significant differences in infection prevalence across the 19 species ($\chi^2_{18} \geq 190.7$, $P < 0.001$). With FV3, wood frogs and gopher frogs experienced the greatest infection prevalence ($\geq$90%); southern leopard frogs, Cope's gray tree frog, western chorus frogs, mountain chorus frogs, pickerel frogs, and tiger salamanders experienced intermediate infection prevalence (20–60%); and the remaining species experienced low infection prevalence or no infection ($\leq$10%). With the ranaculture isolate, wood frogs, southern leopard frogs, pickerel frogs, green frogs, Cope's gray tree frog, and tiger salamanders experienced the greatest infection prevalence ($\geq$85%); northern leopard frogs, gopher frog, mountain chorus frogs, western chorus frogs, eastern spadefoots, and spotted salamanders experienced intermediate infection rates (30–60%); and the remaining species experienced low infection prevalence ($\leq$10%).

Within both virus treatments, there were significant differences in mortality across the 19 species ($\chi^2_{18} \geq 138.2$, $P < 0.001$). With FV3, wood frogs and gopher frogs experienced the greatest mortality ($\geq$90%); southern leopard frogs, Cope's gray tree frogs, western chorus frogs, and mountain chorus frogs experienced intermediate

mortality (25–50%); and the remaining species experienced low or no mortality (≤15%). With the ranaculture isolate, wood frogs, southern leopard frogs, green frogs, and tiger salamanders experienced the greatest mortality (≥85%); northern leopard frogs, gopher frog, pickerel frogs, eastern spadefoots, eastern narrow-mouthed toad, Cope's gray tree frogs, western chorus frogs, mountain chorus frogs, and spotted salamanders, experienced intermediate mortality (30–70%); and the remaining species experienced low mortality (≤10%). The most significant predictor of mortality was the model composed solely of infection ($F_{1,26} = 531.2$, $P = 0.001$, adjusted $R^2 = 0.952$, Beta = 0.976). Thus, case mortality was high and did not differ among virus isolates. However, a few notable exceptions were pickerel frogs ($n = 6$) and Cope's gray tree frogs ($n = 3$) in the ranaculture treatment and tiger salamanders ($n = 5$) in the FV3 treatment in which infected individuals did not die.

There was no evidence that susceptibility to FV3 ($K = 0.322$, $Z = 0.626$, $P = 0.734$) or the ranaculture isolate ($K = 0.430$, $Z = -0.567$, $P = 0.298$) displayed phylogenetic signal. In addition, members of the families Ambystomatidae, Ranidae, and Hylidae did not differ in susceptibility to the virus isolates ($P \geq 0.352$). However, there was a general trend of greater susceptibility to infection for members of the Ranidae compared to the other families (Fig. 3a).

We used PIC and PGLS to test for evolutionary associations between susceptibility to ranaviruses (measured as infection prevalence) and species characteristics. For FV3, species breeding in semi-permanent ponds generally had greater susceptibility to infection compared to species breeding in temporary and permanent ponds ($r = 0.490$, $n = 17$, $P = 0.046$, Fig. 3b). In addition, evolutionary trends for greater susceptibility were associated with smaller species distributions (Pearson's $r = -0.510$, $n = 17$, $P = 0.026$). Similar to FV3, species breeding in semi-permanent ponds generally had greater susceptibility to the ranaculture isolate compared to species breeding in temporary and permanent ponds ($r = 0.600$, $n = 17$, $P = 0.011$, Fig. 3b). We also found that evolutionary trends for faster larval development were associated with greater susceptibility to the ranaculture isolate (Pearson's $r = -0.460$, $n = 17$, $P = 0.046$). There were no other significant associations between susceptibility to the virus isolates and species characteristics ($P \geq 0.166$).

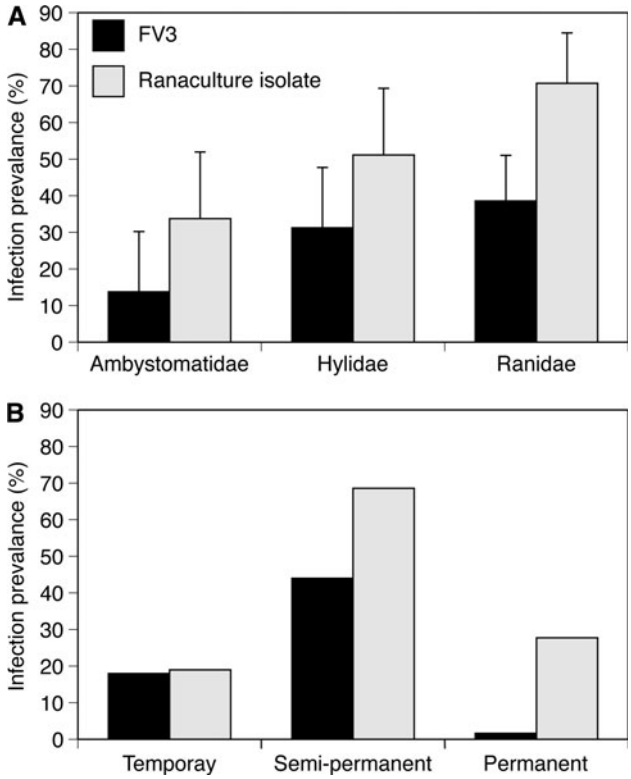

Figure 3. **a** Mean infection prevalence (+1SE) of the families Ambystomatidae ($n = 4$ species), Hylidae ($n = 4$ species), and Ranidae ($n = 7$ species) following exposure to *frog virus* 3 (FV3, *black bars*) or the ranaculture isolate (*gray bars*). **b** Infection prevalence of species associated with temporary, semi-permanent, or permanent ponds following exposure to *frog virus* 3 (FV3, *black bars*) or the ranaculture isolate (*gray bars*). Infection prevalence was estimated from the parameters generated using phylogenetic generalized least squares.

Discussion

We discovered that ranaviruses were capable of infecting 17 of the 19 larval amphibian species tested. These 17 species represented six amphibian families including Ranidae, Hylidae, Bufonidae, Scaphiopodidae, Ambystomatidae, and Salamandridae. This is the first study to document the remarkably wide host range of ranaviruses in Amphibia under standardized laboratory conditions. While our results demonstrate that ranaviruses infect multiple species of larval amphibians, it was clear that species vary in their susceptibility. The tested species represented a diversity of amphibian families in North America as well as differences in ecology that were used to infer factors associated with susceptibility.

It is logical to assume that species within the same family would show similar levels of susceptibility to ranavirus infection due to potential similarities in innate and adaptive immune responses to pathogens. However, there was no support for phylogenetic signal in species susceptibility suggesting a lack of association between species relatedness and susceptibility. Moreover, there was no difference in family-level susceptibility to infection among the Ranidae, Hylidae, and Ambystomatidae. Although the low sample size for each family may have limited our ability to adequately address family-level variation in susceptibility, there were some general trends worth discussing.

In general, species within the Ambystomatidae, Hylidae, and Ranidae tended to have low, intermediate, and high susceptibility to infection by FV3-like ranaviruses, respectively. The intermediate to high susceptibility of Hylidae and Ranidae was expected based on die-off reports from the field (Green et al. 2002; Docherty et al. 2003; Petranka et al. 2003; Duffus et al. 2008; Gahl and Calhoun 2008; Torrence et al. 2010; Brunner et al. 2011). However, the relatively low susceptibility of Ambystomatidae was counter to our expectation of high susceptibility based on field patterns of frequently observed die-off events in species from this family (Green et al. 2002). In wild populations of ambystomatids, two species of ranavirus have been detected; an FV3-like strain has been isolated from die-offs in the eastern United States (Docherty et al. 2003; Duffus et al. 2008; Brunner et al. 2011) and *A. tigrinum* virus (ATV) has been isolated from die-offs in the western United States (Jancovich et al. 1997; Bollinger et al. 1999). Under laboratory conditions, Schock et al. (2008) demonstrated that ATV caused 100% mortality in metamorphic tiger salamanders while FV3 only caused 20% mortality. Given that infections were detected in all exposed individuals, these results suggest that two ranavirus species differ in their virulence within tiger salamanders. To date, ATV has not been detected in ambystomatid populations in the eastern United States suggesting that FV3/FV3-like isolates may be the dominant ranavirus in the region where our species were collected. While this is the first study to examine ambystomatid salamanders from the eastern United States for their susceptibility to ranaviruses, FV3 does not appear to be as infectious as ATV to salamanders. However, it is important to note that the isolate of FV3 we used in our study has been in cell culture for >50 years and is attenuated. Despite the long history in cell culture, the FV3 that was isolated by Granoff et al. (1965) has been used in dozens of challenge experiments (e.g., Pearman et al. 2004; Schock et al. 2008; Hoverman et al. 2010) as a standard for comparison. Despite low susceptibility to FV3, ambystomatid salamanders tended to show higher susceptibility to the FV3-like ranaculture isolate. Although tiger and spotted salamanders largely drove this result, it appears that FV3-like isolates are infectious for certain species within this family.

For the remaining families (Bufonidae, Microhylidae, Scaphiopodidae, and Salamandridae), we did not have adequate representation to examine family-level variation in susceptibility. However, susceptibility was relatively low across the representatives of these families with the exception of the eastern spadefoots (Scaphiopodidae). Interestingly, red-spotted newts have been reported in die-off events (Green et al. 2002), but the species displayed very low susceptibility to infection. Although our results suggest that the susceptibility of the Bufonidae, Microhylidae, and Salamandridae to ranaviruses is relatively low, our limited sample size restricts our ability to generalize for these families.

We found that increased susceptibility to infection by FV3 and the ranaculture isolate was associated with evolutionary trends for breeding in semi-permanent ponds. Semi-permanent ponds are characterized by high amphibian diversity that displays substantial turnover (Werner et al. 2007a, b). This dynamic and diverse amphibian assemblage could lead to the introduction of multiple ranavirus types or strains as host species emigrate and immigrate from the system. If amphibians are intermittently exposed to selective pressure from different ranavirus types or strains, the evolution of host defenses to infection and pathology could be constrained. In contrast, the amphibian assemblage typical of permanent ponds is relatively consistent over time (i.e., dominated by green frogs, American bullfrogs, and red-spotted newts; Wellborn et al. 1996; Werner et al. 2007a, b). If the stability of this assemblage leads to frequent exposure to the same virus type or strain, the evolution of host defenses that limit infection could be favored. Within temporary ponds, ranavirus is not likely to persist across multiple years because pond drying eliminates the virus (Brunner et al. 2007). This could constrain evolutionary adaptations to the hosts that utilize these habitats (Daszak et al. 1999). The notable exception to this hypothesis was wood frogs, which breed in temporary ponds yet displayed high susceptibility. However, this species is a member of the most susceptible

family (Ranidae) in our experiments, which may partially account for the observed trend. Long-term monitoring of sites along the hydroperiod gradient coupled with ranavirus type or strain identification will be essential for determining if semi-permanent ponds are characterized by high ranavirus diversity. Using the identified ranavirus isolates from points along the hydroperiod gradient, challenge experiments can be used to test our hypotheses regarding host-pathogen co-evolution.

We also found an association between susceptibility to FV3 infection and evolutionary trends for smaller species range size. While these results suggest that uncommon species are relatively vulnerable to FV3, the mechanisms underlying this association warrant further study. For example, previous research has shown that reduced levels of genetic diversity in populations of the Italian agile frog (*Rana latastei*) were associated with greater mortality with FV3 (Pearman and Garner 2005). If amphibian species with small range sizes have reduced levels of genetic diversity, they may be particularly vulnerable to ranavirus infection. Alternatively, the likelihood of exposure to ranaviruses may be low for species with small range sizes. Consequently, these species may be more susceptible to FV3 due to the lack of a co-evolutionary history with the pathogen.

We also found that increased susceptibility to infection by the ranaculture isolate was associated with evolutionary trends for faster larval development. Due to nutritional and energetic limitations and physiological trade-offs, host life history characteristics such as fast development, short life span, and high fecundity can be associated with increased susceptibility to pathogens (Lochmiller and Deerenberg 2000; Zuk and Stoehr 2002; Miller et al. 2007b; Cronin et al. 2010; Warne et al. 2011). Ultimately, studies addressing the innate and adaptive immune responses of larval amphibians to ranaviruses will be necessary to determine if accelerated development is a general indicator of host susceptibility to ranavirus, especially given that other host life history characteristics such as duration of egg stage, clutch size, time to maturity, and adult size were not associated with susceptibility to ranavirus infection.

An intriguing result from these analyses is that the associations between species traits and susceptibility differed slightly between the virus isolates. We suspect that these differences could be the result of novel selective pressures that exist within the ranaculture facility. Our results demonstrate that the ranaculture isolate is more infectious compared to FV3. Given that the virus was isolated from an American bullfrog facility and American bullfrogs were highly resistant to infection in our experiments, the isolate could have evolved greater infectiousness to counter host defenses. As a consequence of these novel selective pressures on the virus, the associations between species traits and susceptibility typical of endemic isolates such as FV3 could have been broken. However, as discussed, the FV3 isolate used in our study has been in culture for over 50 years (Granoff et al. 1965), which can influence virus evolution and, thereby, associations with species characteristics.

In our study, we were only able to test disease outcomes with two ranavirus isolates and a single population from each species. Previous studies have demonstrated variation among ranavirus isolates and species in infectiousness and virulence (Cunningham et al. 2007; Schock et al. 2008, 2009) as well as population-level variation in amphibian host susceptibility to ranaviruses (Pearman et al. 2004; Brunner et al. 2005; Pearman and Garner 2005; Schock et al. 2008, 2009). Given that such variation has the potential to influence disease outcomes, we encourage future studies to test additional ranavirus species/isolates and host populations within a phylogenetic framework to assess the generality of our results.

The dynamic interaction between hosts and pathogens over ecological and evolutionary time scales presents a significant challenge for assessing the factors contributing to interspecific variation in susceptibility. By integrating controlled experiments with comparative phylogenetic methods, we demonstrate that a combination of factors including host phylogeny, life history characteristics, and habitat associations contribute to species differences in susceptibility. Based on these results, we predict high susceptibility to ranavirus infections for species within the family Ranidae that breed in semi-permanent ponds, with rapid development, and with limited range sizes. If future field surveillance studies and laboratory research confirm these predictions, conservation initiatives would have a powerful framework for understanding, predicting, and managing ranavirus disease dynamics in wild populations.

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Appendix

See Tables 1, 2, 3, and 4; Figures 4, 5, and 6.

Table 1. Egg mass collection site and number of egg masses collected for each of the 19 amphibian species used in the experiments

Scientific name	Common name	State	County	Location	Lat–Long	Egg masses
Rana sylvatica	Wood frog	TN	Sullivan	South Holston Weir Dam	36°31′25″N, 82°06′40″W	20
Rana capito	Gopher frog	GA	Taylor	Fall Line Sandhills	32°35′1″N, 84°13′32″W	6
Rana sphenocephala	Southern leopard frog	NC	Craven	Croatan National Forest	34°54′38″N, 76°59′15″W	10
Rana pipiens	Northern leopard frog	PA	Crawford	Pymatuning State Park	41°41′30″N, 80°30′20″W	10
Rana palustris	Pickerel frog	TN	Knox	Seven Islands Wildlife Refuge	35°57′14″N, 83°4′31″W	10
Rana clamitans	Green frog	TN	Union	Chuck Swan WMA	36°21′29″N, 83°54′49″W	4
Rana catesbeiana	American bullfrog	NC	Alamance	Private landowner	36° 6′12″N, 79°28′56″W	1
Hyla chrysoscelis	Cope's gray tree frog	TN	Knox	Seven Islands Wildlife Refuge	35°57′1″N, 83°41′41″W	3
Pseudacris triseriata	Western chorus frog	MI	Livingston	E.S. George Reserve	42°27′52″N, 84°0′12″W	15
Pseudacris feriarum	Upland chorus frog	TN	Knox	Seven Islands Wildlife Refuge	35°57′1″N, 83°41′41″W	50
Pseudacris brachyphona	Mountain chorus frog	TN	Franklin	Bear Hollow Mountain WMA	35°11′40″N, 86°3′10″W	5
Bufo americanus	American toad	PA	Crawford	Pymatuning State Park	41°34′10″N, 80°27′20″W	10
Gastrophryne carolinensis	Eastern narrow-mouthed toad	TN	Loudon	Private landowner	35°44′54″N, 84°14′5″W	10
Scaphiopus holbrookii	Eastern spadefoot	TN	Union	Chuck Swan WMA	36°21′29″N, 83°54′49″W	30
Ambystoma tigrinum	Tiger salamander	TN	Jefferson	Henderson Island WMA	35°59′55″N, 83°25′55″W	8
Ambystoma opacum	Marbled salamander	TN	Sullivan	South Holston Weir Dam	36°31′25″N, 82°06′40″W	8
Ambystoma maculatum	Spotted salamander	TN	Gibson	Private landowner	35°51′58″N, 88°43′52″W	11
Ambystoma talpoideum	Mole salamander	TN	Gibson	Private landowner	35°54′02″N, 88°43′31″W	6
Notophthalmus viridescens	Red-spotted newt	TN	Knox	Private landowner	36°02′10″N, 83°51′19″W	12

Table 2. Species-level characteristics for the 19 amphibian species used in the experiments

Common name	Breeding habitat	Breeding time	Duration of larval stage (days)	Size at metamorphosis (mm)	Clutch size	Duration of egg stage (days)	Adult body size (mm)	Time to maturity (year)	Species range (km^2)
Wood frog	1	1	65.0	17.0	1,200	12.0	52.5	2.0	8,884,571
Gopher frog	2	2	161.5	32.5	1,244	8.5	77.0	1.8	146,967
Southern leopard frog	2	2	62.5	26.5	1,500	14.5	70.5	2.0	1,649,839
Northern leopard frog	2	2	100.0	34.0	3,000	14.5	70.5	2.0	6,852,031
Pickerel frog	2	2	75.0	23.0	1,000	14.5	59.5	2.0	3,348,573
Green frog	3	3	242.5	29.5	4,000	8.0	73.5	1.0	3,541,109
American bullfrog	3	3	547.5	60.0	11,000	8.0	121.0	2.0	4,432,306
Cope's gray tree frog	2	3	55.0	16.5	1,500	5.0	41.5	2.0	2,484,281
Western chorus frog	1	1	55.0	10.0	1,000	8.5	27.0	1.0	868,550
Upland chorus frog	1	1	55.0	10.0	1,000	8.5	29.0	1.0	698,650
Mountain chorus frog	1	1	47.0	12.0	900	8.5	28.5	1.0	255,456
American toad	1	2	57.5	9.5	7,000	7.5	70.5	3.0	4,928,210
Eastern narrow-mouthed toad	1	3	37.0	10.3	950	3.0	50.5	1.5	1,003,376
Eastern spadefoot	1	3	45.0	10.3	1,500	1.3	27.0	1.5	1,516,976
Tiger salamander	2	1	100.0	62.0	550	34.5	195.0	2.0	1,857,253
Marbled salamander	1	1	150.0	57.5	119	100.0	98.5	2.0	1,637,972
Spotted salamander	1	1	203.5	51.0	625	34.0	154.5	2.5	3,557,209
Mole salamander	1	1	105.0	49.0	445	33.0	87.5	2.0	546,868
Red-spotted newt	3	3	90.0	20.0	350	27.5	89.5	1.4	2,373,532

Data were gathered from multiple literature sources (Conant and Collins 1998; Petranka 1998; Lannoo 2005; Elliot et al. 2009).

Table 3. Results of analyses testing the effects of virus exposure on the mortality of exposed individuals for each of the 19 larval amphibian species tested

Species	Mortality of infected individuals				
	χ^2_2	*P* value	Pairwise comparisons		
			Control–FV3	Control–RI	FV3–RI
Wood frog	51.8	0.001	0.001	0.001	1
Gopher frog	30.6	0.001	0.001	0.001	0.077
Southern leopard frog	40	0.001	0.001	0.001	0.001
Northern leopard frog	18.5	0.001	0.342	0.022	0.001
Pickerel frog	23.7	0.001	0.072	0.001	0.001
Green frog	51.4	0.001	1	0.001	0.001
Bullfrog	4.1	0.126	NT	NT	NT
Cope's gray tree frog	21.5	0.003	0.004	0.001	0.027
Western chorus frog	11.4	0.003	0.004	0.001	0.519
Upland chorus frog	NT	NT	NT	NT	NT
Mountain chorus frog	17.9	0.001	0.017	0.001	0.025
American toad	4.1	0.126	NT	NT	NT
Eastern spadefoot	13.3	0.001	1	0.008	0.008
Eastern narrow-mouthed toad	NT	NT	NT	NT	NT
Tiger salamander	28.9	0.001	0.756	0.001	0.001
Marbled salamander	2	0.362	NT	NT	NT
Spotted salamander	8.1	0.018	0.147	0.007	0.114
Mole salamander	NT	NT	NT	NT	NT
Red-spotted newt	4.1	0.126	NT	NT	NT

Pairwise comparisons (Fisher's exact test) among the treatments (*P* values) also are presented.
NT no test performed due to the lack of a significant χ^2 test. Given that no mole salamanders, eastern narrow-mouthed toads, or upland chorus frogs died with detectable infections, no tests were conducted for these species.

Table 4. Results of analyses testing the effects of virus exposure on the daily survival for each of the 19 larval amphibian species tested

Species	Wilcoxon–Gehan D_2	P value	Mean survival time			Pairwise comparisons		
			Control	FV3	RI	Control–FV3	Control–RI	FV3–RI
Wood frog	53.5	0.001	21.0	12.4	6.3	0.001	0.001	0.001
Gopher frog	26.8	0.001	21.0	11.4	12.3	0.001	0.001	0.630
Southern leopard frog	44.5	0.001	21.0	16.5	8.2	0.001	0.001	0.001
Northern leopard frog	12.9	0.002	18.4	20.5	14.0	0.144	0.040	0.001
Pickerel frog	22.4	0.001	21.0	19.0	13.1	0.038	0.001	0.002
Green frog	49.1	0.001	21.0	21.0	10.7	1.000	0.001	0.001
Bullfrog	4.1	0.131	21.0	21.0	20.5	NT	NT	NT
Cope's gray tree frog	26.6	0.001	21.0	19.3	14.7	0.004	0.001	0.001
Western chorus frog	12.0	0.002	21.0	18.1	15.9	0.004	0.001	0.178
Upland chorus frog	9.6	0.008	21.0	16.8	14.6	0.009	0.002	0.408
Mountain chorus frog	17.5	0.001	21.0	19.1	15.9	0.019	0.001	0.025
American toad	4.1	0.131	21.0	21.0	20.0	NT	NT	NT
Eastern spadefoot	9.3	0.009	21.0	18.1	15.8	0.038	0.002	0.236
Eastern narrow-mouthed toad	3.1	0.210	19.4	20.2	17.6	NT	NT	NT
Tiger salamander	33.4	0.001	18.4	21.0	19.2	0.685	0.02	0.001
Marbled salamander	30.8	0.001	21.0	20.1	17.9	0.317	0.001	0.001
Spotted salamander	11.4	0.010	21.0	20.9	19.7	0.152	0.004	0.052
Mole salamander	NT	NT	21.0	21.0	21.0	NT	NT	NT
Red-spotted newt	6.1	0.108	21.0	21.0	20.4	NT	NT	NT

Pairwise comparisons (Wilcoxon–Gehan D statistic) among the treatments (P values) also are presented.
NT no test performed due to the lack of a significant Wilcoxon–Gehan test. Given that no mole salamanders died in our experimental treatments, no tests were conducted for this species.

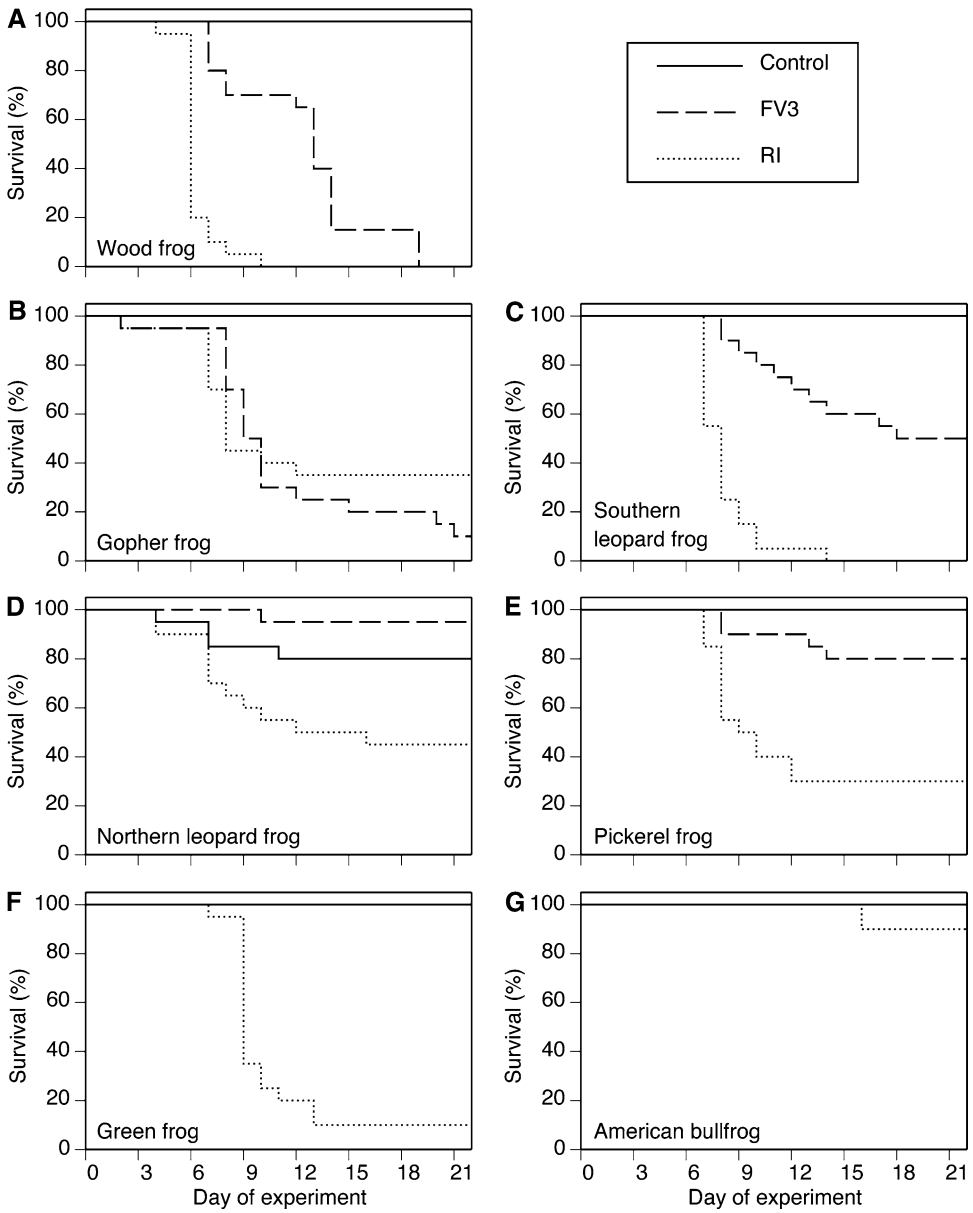

Figure 4. Survival curves for wood frog (**a**), gopher frog (**b**), southern leopard frog (**c**), northern leopard frog (**d**), pickerel frog (**e**), green frog (**f**), and American bullfrog (**g**) tadpoles exposed to three ranavirus treatments. Tadpoles were exposed to virus-free media (Control), frog virus 3 (FV3), or an FV3-like isolate from a Georgia ranaculture facility (RI). For each treatment, $n = 20$ tadpoles.

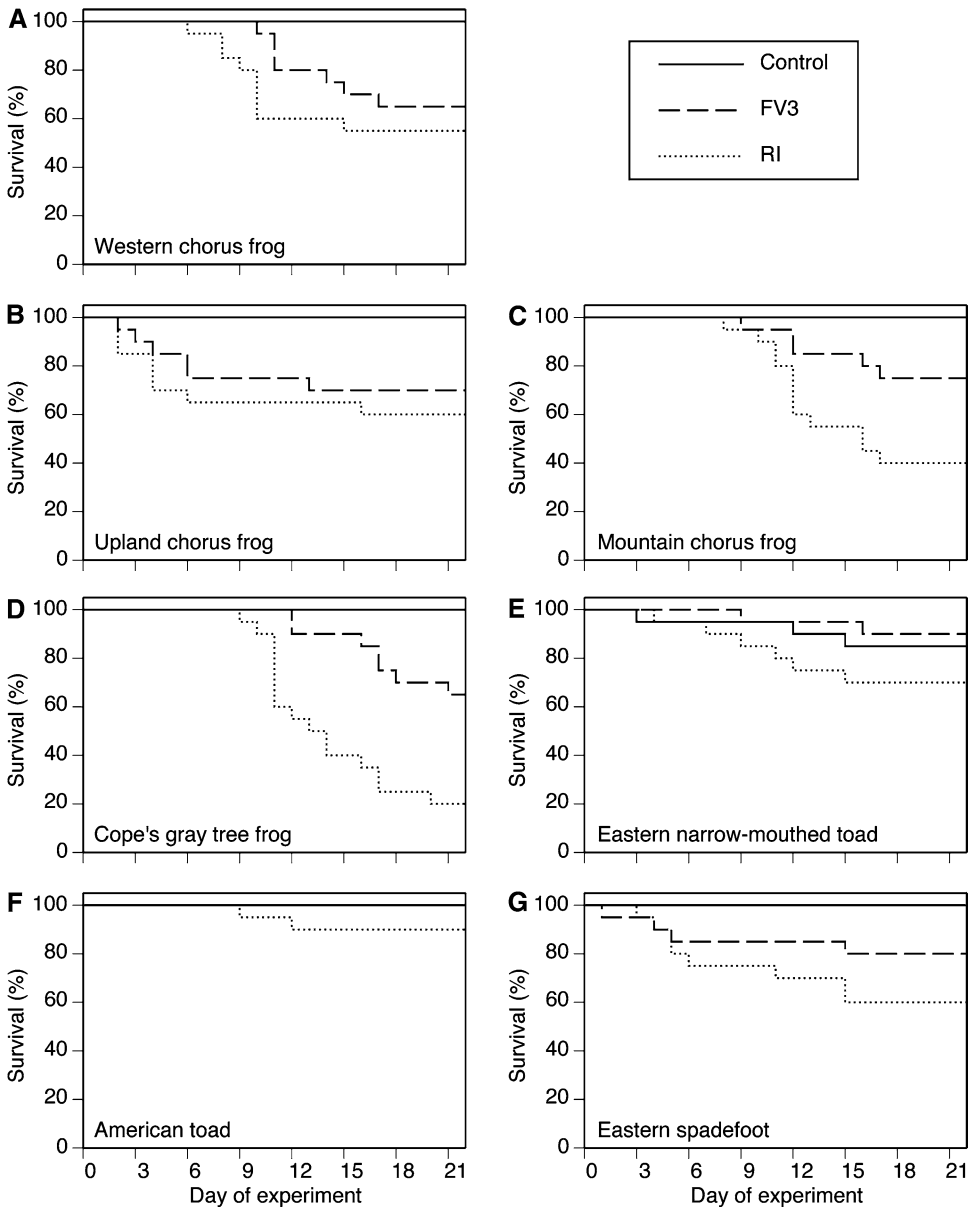

Figure 5. Survival curves for western chorus frog (**a**), upland chorus frog (**b**), mountain chorus frog (**c**), Cope's gray tree frog (**d**), eastern narrow-mouthed toad (**e**), American toad (**f**), and eastern spadefoot (**g**) tadpoles exposed to three ranavirus treatments. Tadpoles were exposed to virus-free media (Control), frog virus 3 (FV3), or an FV3-like isolate from a Georgia ranaculture facility (RI). For each treatment, $n = 20$ tadpoles.

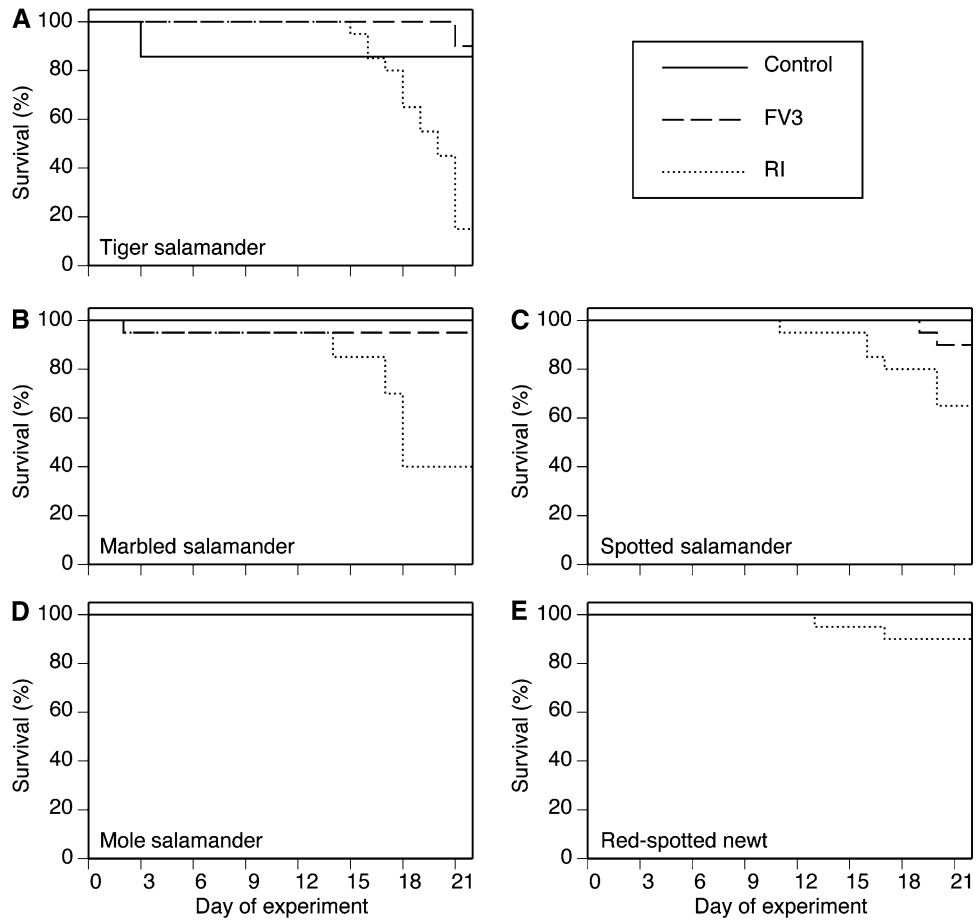

Figure 6. Survival curves for tiger salamander (**a**), marbled salamander (**b**), spotted salamander (**c**), mole salamander (**d**), and red-spotted newt (**e**) larvae exposed to three ranavirus treatments. Larvae were exposed to virus-free media (Control), frog virus 3 (FV3), or an FV3-like isolate from a Georgia ranaculture facility (RI). For each treatment, $n = 20$ larvae except for the tiger salamander control treatment, which contained $n = 7$.

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