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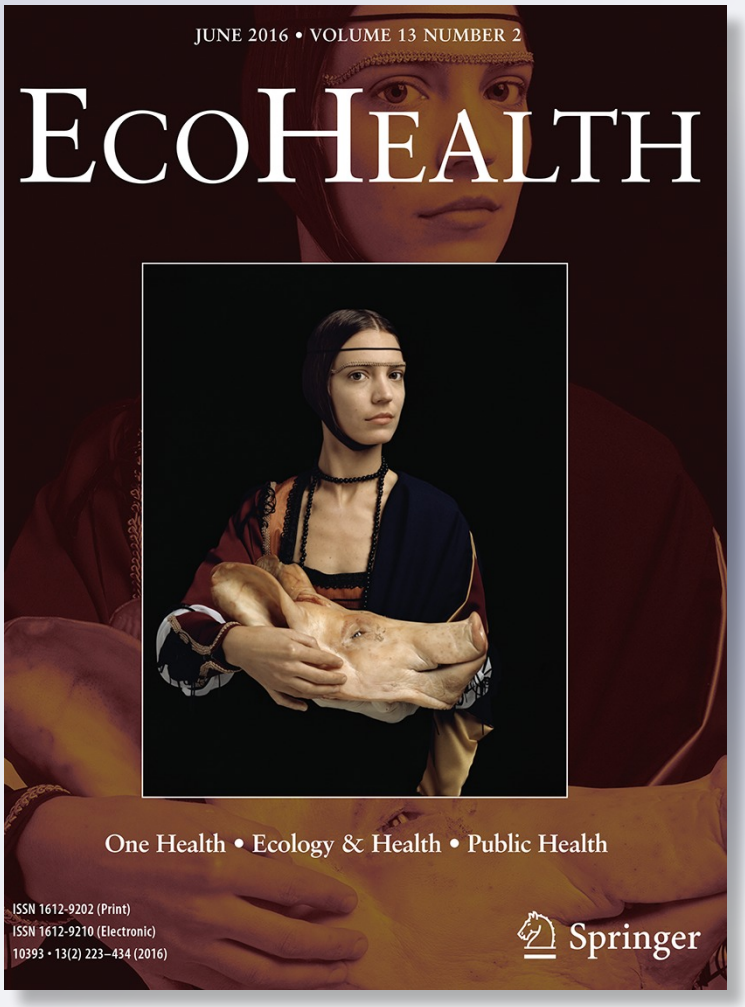

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Original Contribution

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Abstract: The occurrence of emerging infectious diseases in wildlife populations is increasing, and changes in environmental conditions have been hypothesized as a potential driver. For example, warmer ambient temperatures might favor pathogens by providing more ideal conditions for propagation or by stressing hosts. Our objective was to determine if water temperature played a role in the pathogenicity of an emerging pathogen (ranavirus) that infects ectothermic vertebrate species. We exposed larvae of four amphibian species to a *Frog Virus 3* (FV3)-like ranavirus at two temperatures (10 and 25°C). We found that FV3 copies in tissues and mortality due to ranaviral disease were greater at 25°C than at 10°C for all species. In a second experiment with wood frogs (*Lithobates sylvaticus*), we found that a 2°C change (10 vs. 12°C) affected ranaviral disease outcomes, with greater infection and mortality at 12°C. There was evidence that 10°C stressed Cope's gray tree frog (*Hyla chrysoscelis*) larvae, which is a species that breeds during summer—all individuals died at this temperature, but only 10% tested positive for FV3 infection. The greater pathogenicity of FV3 at 25°C might be related to faster viral replication, which in vitro studies have reported previously. Colder temperatures also may decrease systemic infection by reducing blood circulation and the proportion of phagocytes, which are known to disseminate FV3 through the body. Collectively, our results indicate that water temperature during larval development may play a role in the emergence of ranaviruses.

Keywords: amphibians, climate change, disease, pathogen, ranavirus, temperature

Introduction and Purpose

Atmospheric warming associated with global climate change has been hypothesized to affect wildlife populations via complex pathways (Gilman et al. 2010). Evidence is accumulating that changes in ambient temperature can affect breeding phenology (English et al. 2012; Li et al. 2013), reproductive success (Fisher et al. 2014), and survival of wildlife (Bromaghin et al. 2015). Temperature also may play a role in the emergence of infectious diseases (Rohr and Raffel 2010; Altizer et al. 2013). For example, increasing temperature is hypothesized to alter the distribution of the blacklegged tick (*Ixodes scapularis*), resulting in distribution shifts and emergence of tick-borne diseases

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in previously uninfected areas (Ogden et al. 2008). Presumably, increasing temperature also could affect the virulence of pathogens, by exposing hosts to conditions that are more optimum for the pathogen (Altizer et al. 2013). Pathogen propagation could be benefited if changes in temperature stress the host, thereby comprising immune function, or expose the host to thermal ranges optimal for pathogen replication and transmission (Altizer et al. 2013). If one of these relationships exists, one would expect that it would be most pronounced with pathogens that infect ectothermic vertebrate species, because their body temperature fluctuates with ambient conditions (Rohr and Raffel 2010).

Ranaviruses are emerging pathogens that infect amphibians, fish, and reptiles (Duffus et al. 2015). *Frog virus 3* (FV3) is the type species for the genus *Ranavirus* (Jancovich et al. 2015) and has been shown to replicate faster in host cells in vitro with increasing temperature (Ariel et al. 2009). Numerous cases of ranavirus die-offs have been reported during summer (Brunner et al. 2015), with favorable thermal conditions for ranavirus replication speculated as a driving mechanism. Bayley et al. (2013) reported that infection and mortality of common frog (*Rana temporaria*) larvae by FV3 was greater at 20°C compared to 15°C. However, several field and laboratory studies have shown infection by ranaviruses can be greater at cooler temperatures (Rojas et al. 2005; Gray et al. 2007; Allender et al. 2013), typically citing reduced immune function in the host. These conflicting reports highlight the uncertainty surrounding the potential effects of changes in ambient temperature on ranavirus-host interactions.

Our objective was to test for differences in FV3 pathogenicity among larvae of four amphibian host species exposed to ranavirus at two temperatures (10 and 25°C). We chose two species (wood frog, *Lithobates sylvaticus* and spotted salamander, *Ambystoma maculatum*) that breed traditionally during early spring in North America when water temperature is typically 5–10°C, and two species (Cope's gray tree frog, *Hyla chrysoscelis* and green frog, *L. clamitans*) that breed during summer when water temperature is typically 20–30°C. Our aim was to determine if viral replication or temperature-induced stress were driving mechanisms affecting pathogenicity to FV3. If the viral replication hypothesis is supported, one would expect greater viral copies in tissues and pathogenicity at 25°C for all species; however, if the latter is true, greater pathogenicity at 25°C should only be observed in the wood frog and spotted salamander. We also explored the consequence of small changes in water temperature (from 10 to 12°C) on ranavirus pathogenicity for the most susceptible host species that we tested with the largest geographic distribution (wood frog).

Methods

Experimental Challenges

We performed our research at an indoor controlled facility of the University of Tennessee Institute of Agriculture. We collected egg masses from nearby breeding populations in Tennessee and Kentucky, USA (TN Permit #1990 and KY Permit #SC1111075). Egg masses were hatched and raised in 324-L wading pools located outdoors and covered with 70% shade cloth lids that allowed larvae to experience natural temperature fluctuations and photoperiods. Because developmental stage can affect susceptibility to ranavirus (Haislip et al. 2011), we standardized the time of exposure at Gosner stage 30 for anuran species (Gosner 1960) and 1-month post-hatch for the caudate species following previous studies (Hoverman et al. 2011; Brenes 2013). To ensure that larvae were negative for ranavirus prior to experiment, we tested four random individuals per species for infection (Hoverman et al. 2010)—all of which were negative.

Larvae were moved into the controlled facility at the target developmental stage, allowed to acclimate indoors (23°C constant temperature with 12:12 artificial light photoperiod) for 24 h, and 80 larvae per species distributed equally ($n = 40$) between two environmental chambers (Conviron, Controlled Environments, Winnipeg, Manitoba, Canada) set at 25°C. Given the temporal variation for egg mass deposition, we were able to perform the experiments separately for each species. Each larva was housed individually in 2-L containers filled with 1 L of dechlorinated-aged tap water. Containers were arranged in a randomized complete block design with 10 containers placed on each of four shelves in the chamber. Temperature treatments were 25 and 10°C, because these correspond to average water temperature in amphibian breeding habitats in summer and spring, respectively, in Tennessee, USA (Schmutzer et al. 2008). After larvae were placed in chambers, the temperature in one of the chambers was decreased 2°C every day for the first 6 days and 3°C on the seventh day to reach the target temperature of 10°C. After 2 days, half of larvae in each chamber ($n = 20$) were

exposed to an FV3-like ranavirus (Miller et al. 2007) at 10^3 PFU/mL, while the other half were exposed to the same quantity of Eagle's minimum essential medium. Thus, total sample size per species per temperature was $n = 40$, with 20 exposed to virus and 20 controls. The concentration of virus we used is known to cause ranaviral disease in the species we tested at 22°C (Hoverman et al. 2011). We replicated the virus in fathead minnow cells and titrated it following standardized procedures reported in previous studies (Hoverman et al. 2010, 2011). The FV3-like virus we used was on its second cell passage following isolation by Miller et al. (2007).

During the experiments, tadpoles were fed ground fish flakes TetraMin® every 3 days at a ratio of 12% of body mass, which is sufficient for normal growth and development (Relyea 2002). We measured a separate sample of five non-experimental tadpoles that were placed in the bottom of the chambers and treated identical to controls to determine food ration amounts. The use of non-experimental tadpoles reduced the likelihood of cross contamination among experimental units and avoided introducing potential stress into the experiment associated with weighing individuals. Tadpole mass was measured at the beginning of each experiment and once per week thereafter to calculate food ration. Salamander larvae were fed 1 mL of brine shrimp daily.

Larvae were monitored twice daily for survival and morbidity. Larvae that exhibited morbidity consistent with ranaviral disease (i.e., petechial hemorrhages, edema, and loss of equilibrium; Miller et al. 2015) for greater than 24 h were humanely euthanized. Water was changed (100% of volume) every 3 days to maintain water quality (Hoverman et al. 2010). The duration for all trials was 4 weeks (28 days), which is sufficient duration for morbidity to be observed from ranavirus infection (Brunner et al. 2004; Hoverman et al. 2010). At the end of each experiment, all remaining larvae were humanely euthanized by immersion in benzocaine hydrochloride diluted in 90% ETOH, until cessation of breathing. All animal husbandry followed approved University of Tennessee IACUC protocol #2074.

After observing results from the first year of experiments, we performed a follow-up experiment with wood frog larvae, where target temperatures were 10 and 12°C. We followed the identical acclimation and husbandry procedures; however, this experiment lasted for 42 days. After 28 days, the 10°C chamber was increased to 12°C, while temperature in the 12°C chamber remained constant.

Ranavirus Infection and Viral Load

All individuals were necropsied and any gross signs of ranaviral disease recorded. Sections of liver and kidney were collected and stored at -80°C to test for the presence of ranavirus DNA (i.e., infection). Remaining tissues were collected and processed for routine histology as supportive evidence of ranaviral disease (Miller et al. 2015). Genomic DNA (gDNA) was extracted from a homogenate of the liver and kidney tissue using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA). We used a Qubit™ fluorometer and Quant-iT™ dsDNA BR Assay Kit to quantify concentration of gDNA in each sample (Invitrogen Corp., Carlsbad, CA, USA). Real-time quantitative PCR (qPCR) was performed targeting a 70-bp region of the virus' major capsid protein to detect infection and quantify viral copies as previously described by Picco et al. (2007) and Hoverman et al. (2010). In brief, 0.25 ug of DNA was added to a total reaction volume of 25 µL that included 2.5 µL of 5X buffer, 4 µL of 25 mM MgCl2, 0.625 µL of 10 mM of dNTPs, 1 µL of both 10uM Forward and Reverse primers, 0.25uL of 5uM probe, and 0.5 µL of 5u/µL GoTaq Flexi DNA polymerase. Samples were run in duplicate at 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min for 40 cycles. Four controls were used for the qPCR: two negative controls (i.e., DNA grade water and tissue from a known ranavirus-negative tadpole) and two positive controls (i.e., virus and tissue from a known ranavirus-positive tadpole).

We declared infection for samples when the average cycle threshold (CT) value between the two qPCR runs $\leq$31. This decision rule was based on a standard curve (i.e., linear model) that was generated by regressing CT values against extracted gDNA (0.25 µg) from known quantities of cultured virus titrated at 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 plaque forming units (PFU)/mL for our qPCR system (ABI 7900 Fast Real-Time PCR System; Life Technologies Corporation, Carlsbad, California). Three qPCR replicates were performed per titer, resulting in a standard curve with precise fit ($R^2 = 0.99$). The lower bound of the confidence interval of the standard curve for no virus was CT = 31.6, hence we conservatively chose 31 to declare infection. We used our standard curve to subsequently estimate viral copies in tissues and reported in units of PFU per 0.25 µg of gDNA, which has been recommend previously (Gray et al. 2015). These units are an index of viral load and represent viral copies per standardized mass of extracted gDNA from the liver and kidney tissue homogenate.

Statistical Analyses

We used a Fisher Exact Test to determine if differences existed in the proportion of individuals that became infected and died (i.e., clinical disease) between 10 and 25°C for each species (Gray et al. 2015). We performed the same analysis to test for differences in the proportion of individuals that became infected and survived (i.e., subclinical infection) between 10 and 25°C. For individuals that were infected, we tested for differences in mean viral copies between 10 and 25°C for each species using two-sample T-tests accounting for unequal variances. Shapiro–Wilk's test was used to verify normality of viral copy data, which was confirmed for all species ($P > 0.08$). We used Kaplan–Meier analysis (log-rank Chi square test statistic) to test for the differences in the survival curves between 10 and 25°C for each species (Allison 1995). All analyses were performed using SAS 9.3® JMP Pro v.11 (SAS Institute, Cary, NC) and conducted at $\alpha = 0.05$.

Results

Experiment 1: 25 vs. 10°C

Larvae exposed to ranavirus at 25°C were more likely to become infected and die than individuals at 10°C for all species (Fig. 1; Fisher $P < 0.04$). Wood frog and green frog larvae were more likely to become infected and survive (i.e., carry subclinical infections) at 10°C (Fig. 1; Fisher $P < 0.02$). Mean viral copies in tissues were greater at 25°C compared to 10°C (Table 1). Tissues from wood frog tadpoles had the greatest mean viral copies at 25°C, nearly 20–140 times greater than all other species, and showed significant splenic necrosis (Fig. 2). In general, less splenic necrosis was observed at 10°C (Fig. 2). Mortality was faster and greater at 25°C compared to 10°C for all species except Cope's gray tree frog (Fig. 3; $\chi^2_1 = 7.1–41$, $P < 0.008$). For this species, the opposite relationship existed. Substantial control mortality (65%) also occurred for Cope's gray tree frog tadpoles at 10°C but was 0% at 25°C. For all other species, control mortality was <5% in both chambers, and none of the control larvae tested positive for ranavirus.

Experiment 2: 12 vs. 10°C

After 28 days, mortality was greater for wood frog tadpoles at 12°C (90%) compared to 10°C (0%, Fig. 4; Fisher $P < 0.001$). On day 28, temperature in the 10°C chamber was increased to 12°C, and mortality began 4 days later. When the experiment ended at 42 days, there was no difference in mortality between the chamber at 12°C (100%) and the chamber that was changed from 10 to 12°C at 28 days (95%, Fig. 4; Fisher $P = 0.99$). Mean viral copies

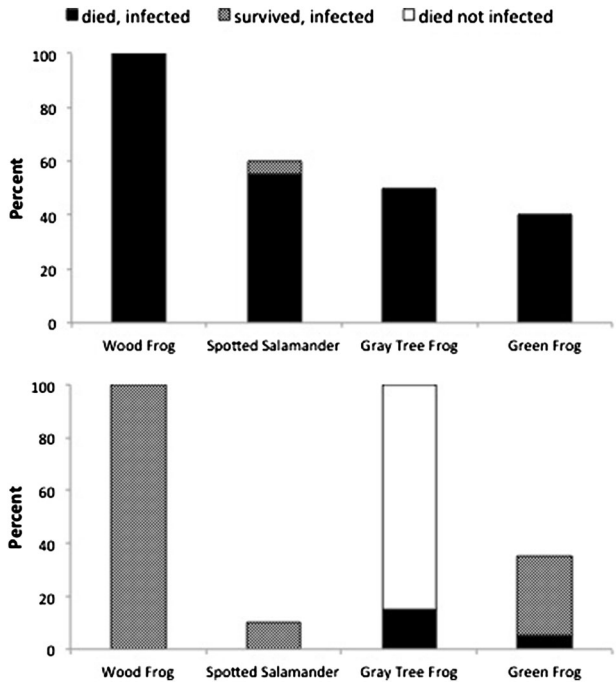

Figure 1. Survival and infection status after 28 days post-exposure (10^3 PFU/mL) to an FV3-like ranavirus at two temperatures (top = 25°C, bottom = 10°C) for four amphibian species

Table 1. Viral Copies (Plaque Forming Units [PFU] Per 0.25 µg of gDNA) in a Homogenate of Liver and Kidney Tissue from Infected Larvae of Four Amphibian Species Exposed to *Frog Virus 3* in Water at Two Temperatures.

Species[1]	25°C			10°C			$t_{0.05}$	P
	n^2	$\bar{X}$	SE	n^2	$\bar{X}$	SE		
AMMA	12	9476	3196	2	10	2	2.96	0.013
HYCR	10	1267	538	3	5	0.3	2.34	0.044
LICL	7	5781	1936	7	24	13	2.97	0.025
LISY	20	185,464	21,387	20	564	418	8.64	<0.001

[1]*AMMA = Ambystoma maculatum, HYCR = Hyla chrysoscelis, LICL = Lithobates clamitans, LISY = L. sylavaticus.*
[2]*Sample size is the number of infected larvae; total sample size for the experiment was n = 20 per species per temperature, with 20 additional controls for each species-temperature combination.*

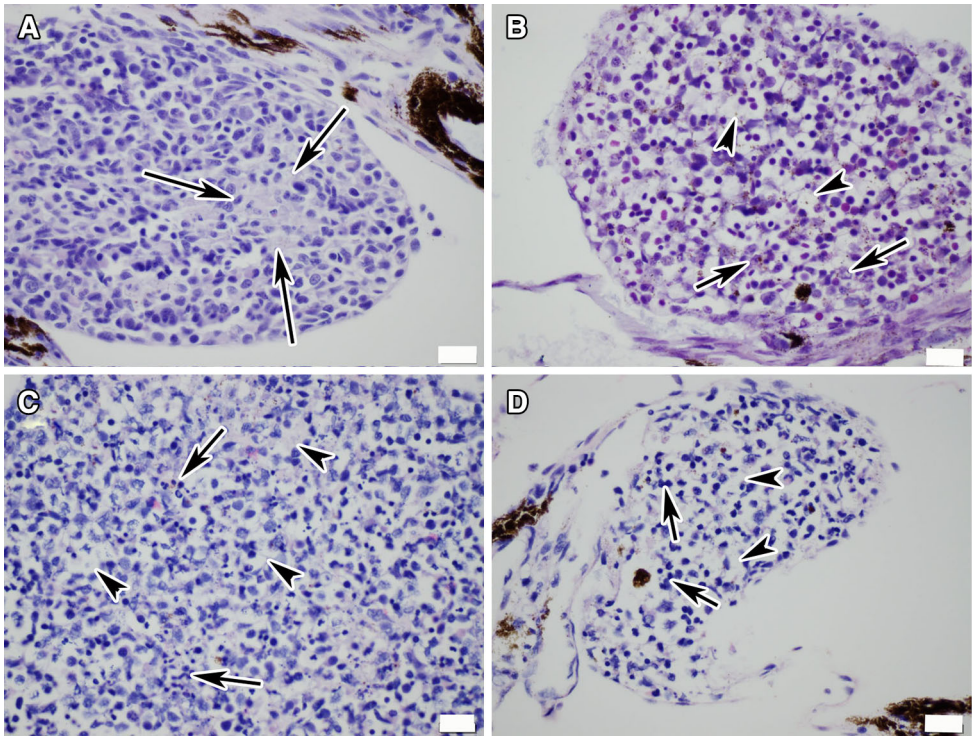

Figure 2. Hematoxylin and Eosin stain of spleens from wood frog tadpoles exposed to ranavirus. Bars equal 20 µm. (**a**) Focal necrosis (delineated by *arrows*) in the spleen of a wood frog tadpole exposed to ranavirus in a water bath at 10°C. This tadpole was infected with ranavirus but did not display clinical illness. (**b**) Diffuse splenic necrosis in a wood frog tadpole exposed to ranavirus in a water bath at 25°C. (**c**) Diffuse splenic necrosis in a wood frog tadpole exposed to ranavirus in a water bath held at 10°C for 28 days then changed to 12°C for an additional 14 days. (**d**) Diffuse splenic necrosis in a wood frog tadpole exposed to ranavirus in a water bath at 12°C. Animals in B-C succumbed to ranaviral disease. Fragmentations (*arrows*) and swelling (vacuolation; *arrowheads*) of cells are seen throughout the spleens

were about 20% higher in individuals held at 12°C for the entire experiment ($\bar{X}$ = 10,465; SE = 2777) compared to those where the temperature changed from 10 to 12°C ($\bar{X}$ = 8804; SE = 2600), but statistical differences were not detected ($t_{0.05}$ = 0.44, P = 0.67). Significant splenic necrosis was observed in both treatments 1(Fig. 2).

Discussion

We documented a positive relationship between temperature and viral copies for all species and both experiments. Additionally, mortality rate at 25°C was greater than at 10°C for 3 of 4 species and was greater at 12°C compared to

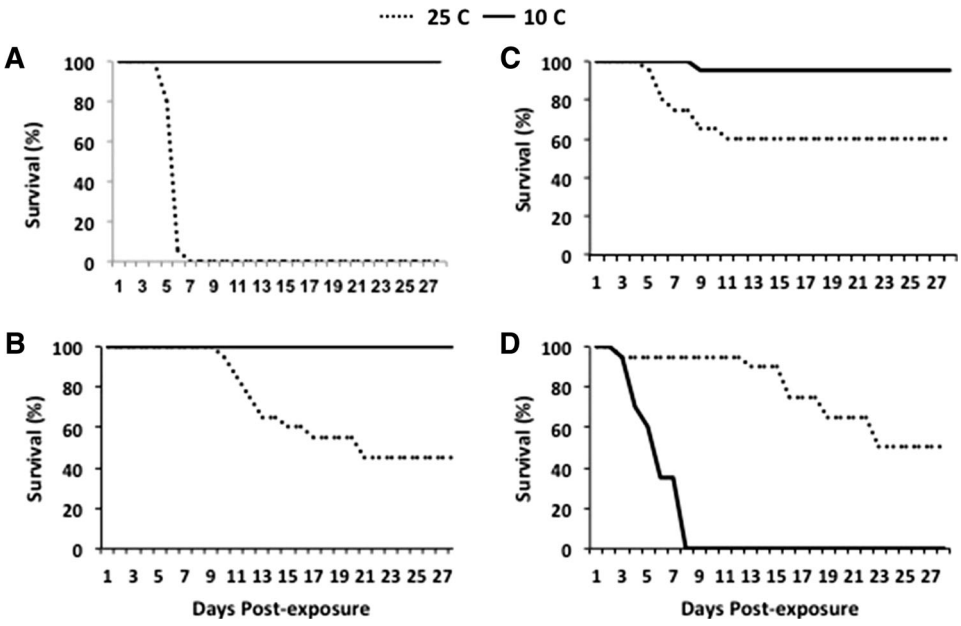

Figure 3. Survival of wood frog (**a**, *Lithobates sylvaticus*), spotted salamander (**b**, *Ambystoma maculatum*), green frog (**c**, *L. clamitans*), and Cope's gray tree frog (**d**, *Hyla chrysoscelis*) larvae following exposure (10^3 PFU/mL) to an FV3-like ranavirus at two temperatures

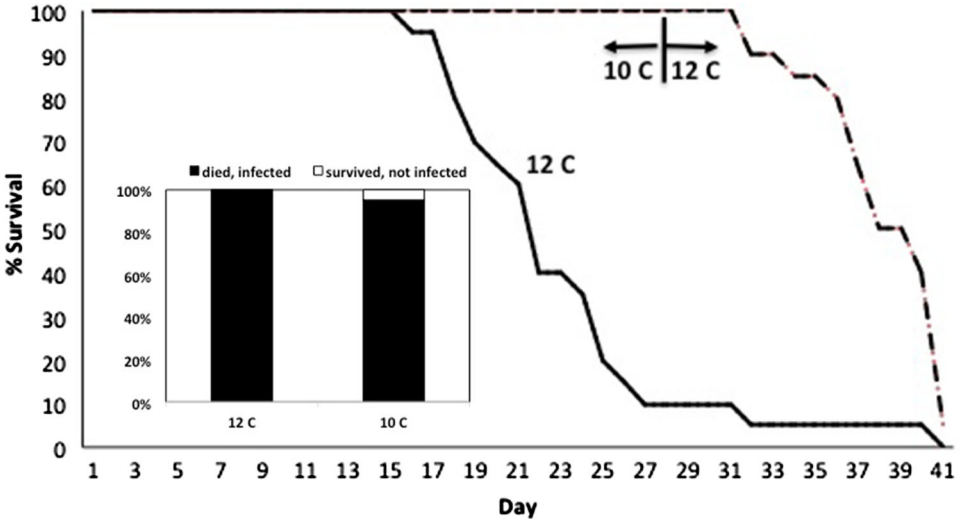

Figure 4. Survival of wood frog (*Lithobates sylvaticus*) tadpoles after 42 days at 12°C (*solid*) and 10°C (*dashed*). On day 28, the 10°C treatment was increased to 12°C. Inset = final survival and infection status

10°C for wood frogs. These results appear to support the viral replication hypothesis—that is, warmer water temperatures result in more favorable conditions for FV3 replication. Several in vitro studies have reported that FV3 replication increases with temperature up to 28–32°C (Granoff et al. 1966; Gravell and Granoff 1970; Chinchar 2002; Ariel et al. 2009). Similar in vitro replication trends were found for *Santee-Cooper Ranavirus*, which is a species of *Ranavirus* found in North America that commonly infects largemouth bass (*Micropterus salmoides*; Grant et al. 2003). Ranaviruses kill hosts by causing extensive necrosis in multiple organs, which reduces function (Miller et al. 2015). Given that ranaviruses can infect and cause cell death in <9 h (Chinchar 2002; Robert et al. 2011), faster replication should lead to more rapid disease progression and greater mortality (Brunner et al. 2015). In addition to our results, the positive trend between ranavirus pathogenicity and temperature has been reported in other controlled studies with FV3 (Bayley et al. 2013) and *Epizootic Haematopoietic Necrosis Virus* (EHNV)—a species of *Ranavirus* that infects fish in Australia (Whittington and Reddacliff 1995). Collectively, our results suggest that warmer water temperatures could facilitate the occurrence of FV3 outbreaks in amphibian populations.

Ranaviruses are a diverse group of pathogens, with six viral species recognized by the International Committee on Taxonomy of Viruses (Jancovich et al. 2015). It is unlikely

that the temperature patterns we report are representative of all *Ranavirus* species. For example, *Ambystoma tigrinum virus* (ATV) appears to be more pathogenic to salamander larvae at lower water temperatures (Rojas et al. 2005; D. Schock, Keyano College, unpublished data). There also may be host differences in response to FV3. Allender et al. (2013) reported that red-eared slider (*Trachemys scripta elegans*) mortality due to FV3 was greater at 22°C compared to 28°C. They speculated that turtle immune response probably was greater at the warmer temperature. Complex interactions with temperature also may exist among host and virus genotypes (Echaubard et al. 2014). Echaubard et al. (2014) reported that temperature-dependent virulence differed among three FV3-like strains and depended on host species and population. It also is possible that thermal optimums for ranavirus replication co-evolve with hosts and their habitats. For example, Ariel et al. (2009) reported that a ranavirus isolated from a short-finned eel (*Anguilla australis*) replicated better at 10–20°C compared to 28°C, perhaps because the host species lives in cold-water habitats. Given that the FV3-like ranavirus we used in our study was isolated from Georgia in the southern USA, its higher pathogenicity at 25°C could be due to the environment.

Although animal mortalities were lower in the colder treatment, individuals that died due to FV3 had lower viral copies. For example, viral copies in wood frog tadpoles that died at 12°C were 17 times lower than those that died at 25°C. Interestingly, similar observations have been reported in FV3-infected *Xenopus laevis* tadpoles, where animals pretreated with an antiviral type I interferon cytokine survived longer and had lower viral loads, but nonetheless incurred substantial tissue damage and died due to the infections (Grayfer et al. 2014). It is worth noting that FV3 infections in mice and rats result in extensive hepatic damage and animal mortalities (Gut et al. 1981; Kirn et al. 1972; Elharrar et al. 1973), despite the fact that FV3 does not replicate at these animals' body temperature of 37°C (Aubertin et al. 1973). FV3 also possesses potent prepackaged virulence determinants, which are sufficient to cause extensive host cell toxicity (Bingen-Brendel et al. 1972). From our study and as reported elsewhere (Grayfer et al. 2014), it appears that amphibian larvae are particularly sensitive to FV3-induced tissue damage, and even low FV3 loads may be sufficient to cause mortality.

Complex and multifactorial relationships between immune response and temperature have been well documented in ectothermic vertebrate species (Le Morvan et al. 1998; Carey et al. 1999; Zimmerman et al. 2010). Robust innate immune responses in *Xenopus laevis* larvae to FV3 typically occur 1–7 days post-infection at room temperature and include significant migration of macrophage-lineage cells to sites of viral infection (Morales et al. 2010; De Jesús Andino et al. 2012). Notably, FV3 is able to subvert the first waves of innate immune responders, with macrophage-lineage cells serving as dissemination vectors for the pathogen [reviewed in Grayfer et al. (2012)]. This phenomenon is supported by the observation that *X. laevis* tadpoles enriched for certain macrophage populations prior to an FV3 challenge succumb faster to the infections and bear greater viral burdens (Grayfer and Robert 2014). In our study, lower temperatures may have prevented FV3-infected phagocyte dissemination to distal organs, such as the kidney and liver, which normally serve as principal FV3 replication sites. Thus, FV3-infiltrated immune cell vectors would be relatively confined to animal peripheries, resulting in lower kidney and liver FV3 loads and greater animal survival, as we observed. Decreased blood circulation, which is known to occur in ectothermic vertebrates at lower temperatures (Engelsma et al. 2003; Maekawa et al. 2012), may also have contributed to reduced phagocyte, and hence FV3 dissemination.

Decreased temperatures also have been documented to result in significant shifts in the proportions and the activation states of immune cells such as macrophages (Maniero and Carey 1997; Kizaki et al. 1985; Sesti-Costa et al. 2012). Concurrently, it has been demonstrated that distinct amphibian macrophage populations confer increased tadpole host susceptibility to FV3 while other macrophage populations render these animals significantly more resistant to this pathogen (Grayfer and Robert 2014). It is possible that the proportions of FV3-susceptible and -resistant immune effector cells are skewed toward the latter at lower temperatures, resulting in increased resistance to this pathogen.

If warmer temperatures are more concordant to FV3 replication, then immune efficacies notwithstanding, lower temperatures would decrease viral loads and increase animal survival, as observed in our study. It also is possible that decreased temperatures result in decreased viral replication as well as decreased phagocyte dissemination or increased proportions of anti-FV3 immune effector cells.

An alternative, but not mutually exclusive explanation for greater pathogenicity of ranavirus at warmer temperatures is temperature-dependent activation of FV3 immune evasion genes. Ranaviruses persist and propagate through complex interactions between host cells and immune

responses (Grayfer et al. 2015). Cotter et al. (2008) reported >100 up- and down-regulated genes in *A. mexicanum* following exposure to ATV. Some proteins that are encoded by FV3 immune evasion genes include vIF-2α, vCARD, and dUPTase (Grayfer et al. 2015). Temperature-dependent synthesis of the immune evasion proteins has not been investigated for FV3; however, it is known to occur in other pathogens (Loh et al. 2013). Indeed, more research is needed on temperature-dependent immune responses to ranavirus infections.

Climate-driven disease emergence has been hypothesized for other pathogens (Rohr and Raffel 2010; Hoverman et al. 2013). Our wood frog results indicate that small changes in water temperature can lead to different disease outcomes. No mortality of wood frog tadpoles occurred at 10°C after 28 days post-exposure to FV3; however, survival was 10% at 12°C over the same duration. Moreover, changing the 10°C treatment to 12°C after 28 days resulted in 95% mortality in 13 days. This finding may be especially pertinent for wood frog populations at northern latitudes in North America (e.g., Canada, Alaska), where breeding sites might not currently exceed 10°C during tadpole development. Most climate change scenarios over the next 80 years predict a 2–6°C increase in atmospheric temperatures (National Research Council 2010). Thus, slight increases in temperatures might lead to the geographic spread of FV3, or increased occurrences of die-offs in wood frog populations.

Our results support previous in vitro experiments that FV3 replication is slow to nonexistent <12°C (Chinchar 2002). However, our results also suggest that ranaviruses can infect hosts at <12°C. Three of the four species we tested had individuals that survived at 10°C with ranavirus DNA detected in the liver and kidney. It is unknown whether the detected ranavirus DNA was viable. Given the results from the wood frog experiment where temperature was changed from 10 to 12°C, we hypothesize that FV3 can infect host cells at <12°C, but remains in a latent state until 12°C is exceeded. Latent infections by FV3 have been reported previously (Morales et al. 2010) and may be a strategy to avoid the host's immune system until conditions are more optimal for the pathogen. The occurrence of sub-lethal infections at colder water temperatures may contribute to ranavirus persistence in the environment (Gray et al. 2007). For example, Brunner et al. (2004) suggested that sub-lethally infected adult amphibians might shed ranavirus during breeding and infect larvae, which could manifest as disease later when water temperature increases.

Mortality of Cope's gray tree frog tadpoles was greater at 10°C than at 25°C. Of the 20 individuals that died, only two were infected with ranavirus and at very low levels. Virus in these individuals was on average 40 times lower than infected individuals of the other three species at 10°C. Moreover, 65% of Cope's gray tree frog control tadpoles died at 10°C. We hypothesize that cold-induced stress resulted in mortality of the Cope's gray tree frog tadpoles at 10°C. Cope's gray tree frogs breed during summer (May–August in Tennessee, USA) when water temperatures typically exceed 20°C (Burton 2007; Schmutzer 2007; Niemiller and Reynolds 2011). Cold-induced mortality has been reported for other amphibian species (Echaubard et al. 2014). Although these results were unexpected, they provide evidence that Cope's gray tree frog tadpoles likely were stressed at 10°C, but this condition did not result in greater infection as we hypothesized initially. Thus, these results further affirm that temperature impacts on viral replication, macrophage dissemination, or immune system evasion may be more important than temperature-mediated stress responses. Others have found that natural factors (e.g., food limitation, predation threat) that can act as stressors do not increase the probability of ranavirus infection (Haislip et al. 2011; Reeve et al. 2013).

Conclusion

Our study revealed a positive relationship between FV3 pathogenicity and water temperature in four amphibian species. The mechanisms driving this relationship are unknown, but may be linked to virus replication, phagocyte dissemination, and activation of host immune evasion genes. Future research should focus on these mechanisms and include a greater range of temperatures, host species, and ranavirus isolates. Modeling potential impacts of ranavirus under climate change scenarios are another important research direction.

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equipment, and logistical support. We thank two anonymous reviewers for improving our manuscript.

Compliance with Ethical Standards

Animal Ethics Statement All applicable institutional and/or national guidelines for the care and use of animals were followed. This work was approved under University of Tennessee Institutional Animal Care and Use Committee Protocol #2074.

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