






REPORT

Experimental evidence that host species composition alters host–pathogen dynamics in a ranavirus–amphibian assemblage

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Abstract

Losses in biodiversity can alter disease risk through changes in host species composition. Host species vary in pathogen susceptibility and competence, yet how changes in diversity alter host–pathogen dynamics remains unclear in many systems, particularly with respect to generalist pathogens. Amphibians are experiencing worldwide population declines linked to generalist pathogens, such as ranavirus, and thus represent an ideal group to investigate how host species composition affects disease risk. We conducted experiments in which amphibian larvae of three native species (Pacific tree frogs, *Pseudacris regilla*; Cascades frogs, *Rana cascadae*; and Western toads, *Anaxyrus boreas*) were exposed to ranavirus individually (in the laboratory) or as assemblages (in outdoor mesocosms). In a laboratory experiment, we observed low survival and high viral loads in *P. regilla* compared to the other species, suggesting that this species was highly susceptible to the pathogen. In the mesocosm experiment, we observed 41% *A. boreas* mortality when alone and 98% mortality when maintained with *P. regilla* and *R. cascadae*. Our results suggest that the presence of highly susceptible species can alter disease dynamics across multiple species, potentially increasing infection risk and mortality in co-occurring species.

KEYWORDS

amphibians, amplification, biodiversity, host composition, ranavirus, susceptibility

INTRODUCTION

Ongoing losses in biodiversity have led to a sixth major extinction event (Ceballos et al., 2015; Wake & Vredenburg, 2008). Loss of biodiversity leads to changes in community structure, nutrient cycling, and ecosystem productivity, which affect the transmission of infectious diseases (Chapin et al., 2000; Hooper et al., 2012). Moreover, changes in host diversity may affect the dynamics of disease risk (Civitello et al., 2015; Johnson et al., 2013). Examinations of the relationship between species richness and disease risk have highlighted the importance of host competence, the ability of a host to maintain and transmit an infection, in multihost disease systems (Johnson et al., 2013). Since host species vary in competence, communities with a diverse assemblage of hosts should contain a range of host competencies, which may be absent in less diverse communities. Thus, a major research priority has focused on understanding how variation in host richness and species composition influence parasite transmission and the resulting patterns in disease risk (Johnson et al., 2015; Rohr et al., 2020).

Amphibians have experienced population declines and extinctions as a result of disease (Blaustein et al., 2012, 2018; Daszak et al., 2003; Rohr et al., 2008). Several globally distributed generalist pathogens have been implicated in large-scale amphibian population die-offs and population declines, including the chytrid fungi, *Batrachochytrium dendrobatidis* (Bd) (Voyles et al., 2009) and *B. salamandrivorans* (Bsal) (Grant et al., 2016), and the *iridovirus*, ranavirus (Gray et al., 2009; Price et al., 2014). Studies examining the relationship between amphibian diversity and chytridiomycosis have reported reductions in disease risk with increased host richness (Han et al., 2015; Searle et al., 2011; Venesky et al., 2014). Relationships between amphibian diversity and disease risk from other pathogens are less clear and have received less attention. This is the case regarding ranaviruses, which are globally distributed, generalist viruses that can infect amphibians, fishes, and reptiles (Gray et al., 2009). Ranavirus susceptibility and pathology vary with host species identity (Hoverman et al., 2010), viral strain (Schock et al., 2009), temperature (Brand et al., 2016; Sauer et al., 2019), and exposure dose (Brunner et al., 2005). Alterations of these parameters resulted in the mortality of ranavirus-infected species ranging from 0% to 100% (Blaustein et al., 2018; Cullen & Owens, 2002; Earl et al., 2016; Hoverman et al., 2010, 2011). How variability in host competencies to ranaviruses affects transmission rates within assemblages of multiple host species has not yet been examined.

Here we investigated a three-species amphibian guild to examine how community composition affects host–pathogen relationships. We focused our study on

Western toads (*Anaxyrus boreas*) because they are particularly susceptible to several pathogens (Blaustein et al., 2005; Earl et al., 2016; Searle et al., 2011) and have suffered mortality and population declines in parts of their range (Muths, 2003). In the Cascade Range of Oregon, *A. boreas* is sympatric with several amphibian species (Nussbaum et al., 1983) where pathogens are implicated in population declines. These include Cascades frogs (*Rana cascadae*) and Pacific tree frogs (*Pseudacris regilla*). *A. boreas* larvae aggregate at high densities either in single-species or mixed-species schools (O'Hara & Blaustein, 1982). Thus, this is an ideal system to study the interrelationships among biodiversity and disease. We experimentally examined the effects of ranavirus exposure on individual Western toads, Pacific tree frogs, and Cascades frogs in the laboratory to investigate how these species responded to ranavirus infection. Additionally, to better understand how host diversity affects disease outcomes (i.e., host survival), we conducted an outdoor mesocosm experiment that varied in ranavirus presence and host composition (one vs. three species).

METHODS

Animal collection and husbandry

Amphibian eggs were collected from naturally occurring breeding sites in Oregon in January 2015. For all species, eggs were collected from multiple egg masses over multiple collection events. Eggs of *P. regilla* (>20 egg masses) were collected in the Willamette Valley at two sites (44.572° N, –123.300° W and 44.691° N, –123.216° W). Eggs of *R. cascadae* (three egg masses) were collected at Parish Lake (44.522° N, –122.031° W), whereas those of *A. boreas* (collected from three breeding pairs) were collected in the Deschutes National Forest (44.032° N, –121.687°). We housed eggs in 40-L aquaria filled with dechlorinated water treated with AquaNova (Kordon LLC, Hayward, CA, Item 31161) and Amquel (Kordon LLC, Hayward, CA, Item 31261). Within 2 days of hatching, we moved larval amphibians into new aquaria (as described earlier) at densities of 1–2 animals/L and fed animals a mixture of rabbit chow, spirulina flakes, and shrimp flakes (3:1:1) ad libitum every other day. Complete water changes occurred weekly, and temperatures remained between 12°C and 15°C.

Ranavirus culture

We used a ranavirus isolate obtained from infected wood frog tadpoles (*R. sylvatica*) from Iron River, Michigan.

This isolate has 99% similarity to a ~500 bp fragment of the membrane cofactor protein gene of *Frog Virus 3* (GenBank Accession number: PRJNA504607; Hoverman et al., 2019). The virus was cultured using a protocol adapted from Hoverman et al. (2010) wherein the virus was passaged through fathead minnow cells incubated at 28°C without CO₂ and fed with Eagle's minimum essential medium with Hank's salts (HMEM) and 5% fetal bovine serum. The virus was stored at -80°C until the start of the experiments.

Laboratory experimental design

We experimentally exposed 28 larvae from all three species, *P. regilla*, *A. boreas*, and *R. cascadae*, to virus or a sham control. Forty-eight hours prior to exposure, amphibians between Gosner (1960) developmental Stages 26–30 were moved to individual 1000-ml containers with 800 ml water and acclimated to 23°C. For the virus treatment, animals were exposed to a total of 10⁵ plaque-forming units (PFU) in 500 µl HMEM, which equated to a final concentration of 10² PFU per ml. Previous studies used concentrations from 10² to 10⁶ PFU per ml and demonstrated sublethal effects on amphibians (Hoverman et al., 2010, 2011). Control animals received 500 µl HMEM without virus as a sham treatment. At Gosner Stage 42 (determined by the emergence of front limbs), animals were moved to individual plastic containers (33.5 × 20 × 9 cm) tilted at an ~30° angle to create a partially dry environment and secured with a mesh lid and elastic. Water was changed every 4 days. Postmetamorphic amphibians were fed three to five vitamin- and mineral-dusted crickets ad libitum. Animals were checked twice daily for mortality. Upon death we stored the animals at -20°C. Animals surviving 3 weeks after metamorphosis were euthanized via submersion in a neutrally buffered MS-222 solution, frozen, and stored at -20°C. Quantitative PCR was used to determine ranavirus loads from host livers, with a protocol modified from Picco et al. (2007). Infection severity was quantified in ranavirus-specific genome equivalents (GEs). Twenty-one unexposed *P. regilla*, nine exposed *A. boreas*, and nine unexposed *A. boreas* were untested due to logistical issues.

Mesocosm experimental design

To test how ranavirus transmission and mortality varied within single- and multiple-host species treatments, we used 120-L mesocosms at Oregon State University's Lewis-Brown Horticultural Farm (44.548° N, -123.215° W).

Mesocosms contained sterilized leaf litter covering the bottom of the tank, were filled with well water and then inoculated with an additional 1 L of water containing zooplankton, phytoplankton, and periphyton from a 900-L mesocosm free of fish and amphibians, then covered with mesh lids. Mesocosms were left for 4 weeks to establish algal growth prior to the experiment. Mesocosms included two levels of host composition: one host (*A. boreas* only, 36 tadpoles) or three hosts (*P. regilla*, *R. cascadae* and *A. boreas*, with 12 tadpoles of each species). Each of our four treatments was run with four replicates for 16 total mesocosms. The one-species treatment included *A. boreas* alone because they are the most commonly occurring amphibian species in the highest elevation sites in our Oregon study system and are sympatric with *P. regilla* and the *R. cascadae* (Nussbaum et al., 1983).

Our design was substitutive with a constant initial host density in every treatment. Hosts were exposed to either three ranavirus-exposed animals (ranavirus treatment) or three naïve animals (sham-inoculated control). In single-host treatments, these three additional animals were *A. boreas*, whereas in multihost treatments one animal of each species was added. Ranavirus exposures occurred in 10 L treated water to which either ranavirus or a sham solution was added. Ranavirus was thawed, and we added 5 ml of virus with HMEM to the 10-L aquaria for a final concentration of 10³ PFU/ml. To the control treatments were added 5 ml sterile HMEM. Animals were moved from the laboratory to the mesocosm location in 2-L collection jars.

Thirty-three unexposed larvae were added to each mesocosm on Day 0. On Day 1 of the experiment, we added an additional three animals to each mesocosm that had been exposed either to ranavirus or the sham inoculum as described earlier. At the experiment's onset, all animals were larvae: *A. boreas* (Gosner Stages 25–28), *P. regilla* (Gosner Stages 25–30), and *R. cascadae* (Gosner Stages 25–28). Well water was added to the mesocosms weekly to offset evaporation. Dead animals were not removed from mesocosms since dead conspecifics may be an important infection source (Gray et al., 2009; Miller et al., 2011). Foam floats were placed in the mesocosms to allow metamorphosing animals to leave the water. On Day 30, we measured survival by catching and counting each animal, briefly housing them in 10-L aquaria of water from the same mesocosm, then returning them immediately to the mesocosms. As the number of metamorphic animals utilizing floats increased, the decision was made to begin removing metamorphs. On Day 40, we removed and euthanized all metamorphic animals. Metamorphs were removed daily for the remainder of the experiment and removed animals were considered to have survived. On Day 60, we terminated the mesocosm

experiment by collecting and euthanizing all remaining animals, including larvae and metamorphs. A subset of surviving animals in each replicate ($n = 6$) was tested for ranavirus infection. Water temperatures in this experiment ranged from 18°C to 26°C.

Analysis

To examine survival of each species from the laboratory experiment, we analyzed Kaplan–Meier survival curves using the Cox–Mantel test in JMP 14.1 (Cox, 1972; Mantel & Haenszel, 1959). Body size and developmental stage were not measured, precluding any analyses of how these covaried with viral exposure.

For the mesocosm experiment, we used a two-way ANOVA to test for the main effects of virus and richness treatments and their interaction on both *A. boreas* and overall amphibian survival at both 30 and 60 days. Metamorph survival was compared across treatments via Tukey–Kramer honestly significant difference (HSD). Too few animals from the three-species treatments survived the 60-day trial to allow for any comparisons of additional response metrics, such as host body size, developmental stage, or viral prevalence and load.

RESULTS

In the laboratory experiment, *P. regilla* survival was reduced by ranavirus exposure ($\chi^2_1 = 34.31$, $p < 0.001$, $n = 28$, Figure 1a), with ranavirus-exposed *P. regilla* surviving an average of 7.32 days (SE = 0.56), compared with 31.11 days (SE = 1.12) for controls. Neither *A. boreas* nor *R. cascadae* survival was significantly different between ranavirus and control treatments (Figure 1b,c). Additionally, there was high mortality across species corresponding to the weeks after metamorphosis. Despite all species being exposed to and tested for ranavirus, only *P. regilla* tested positive for ranavirus infection. Of the exposed *P. regilla*, 27/28 tested positive with loads from 1.8 GE to 1.6×10^5 GE (mean 2.7×10^4 GE), and four of the seven tested unexposed *P. regilla* control animals tested positive with loads from 8.1 to 63.4 GE (mean 23.2 GE).

In the mesocosm experiment, amphibian mortality in the ranavirus-treated mesocosms exceeded the mortality of the control mesocosms across species compositions and time periods (Figure 2). There was a significant interaction between virus exposure and host composition on the survival of *A. boreas* at Day 30 (ANOVA $F_{1,12} = 43.89$, $p < 0.0001$) and

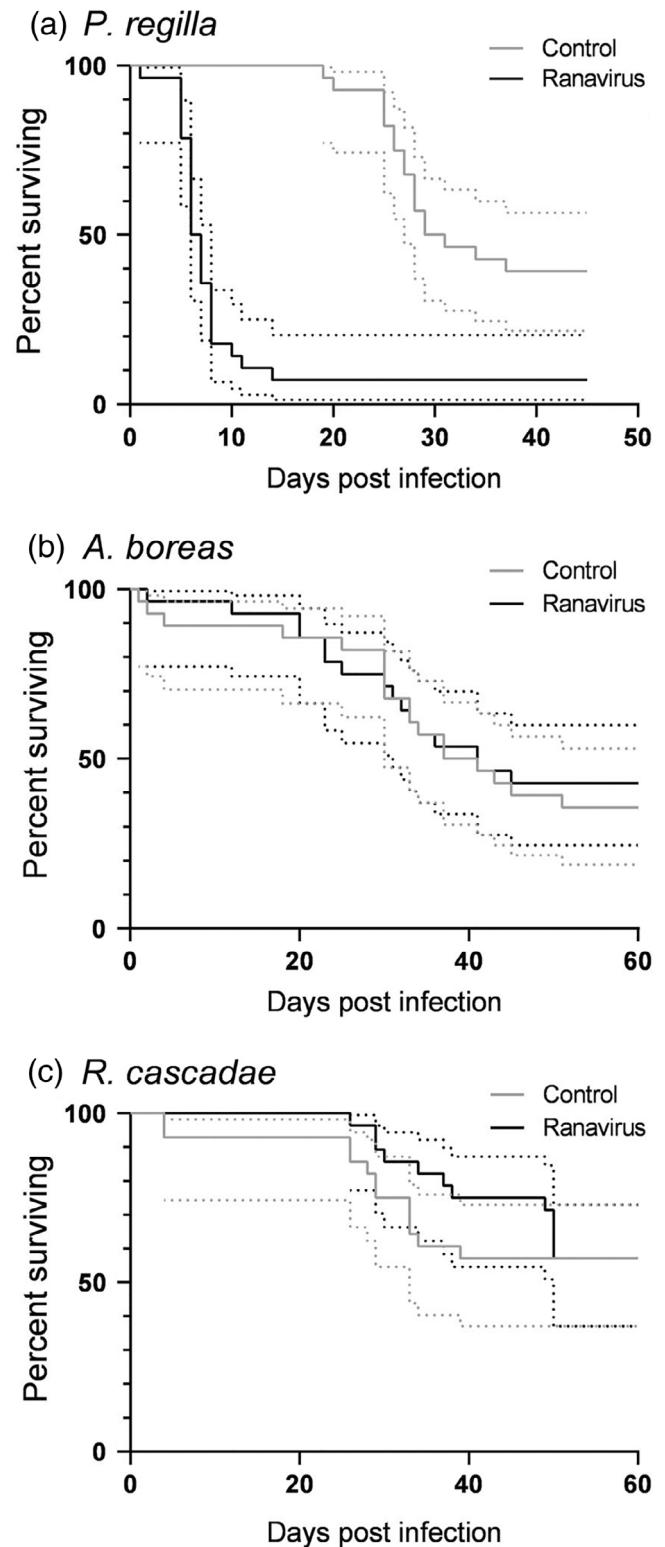


FIGURE 1 Laboratory experiment survival curves for (a) *Pseudacris regilla*, (b) *Anaxyrus boreas*, and (c) *Rana cascadae*, comparing ranavirus exposed (gray) and control (black) individuals. 95% confidence intervals represented by gray and black dotted lines, respectively.

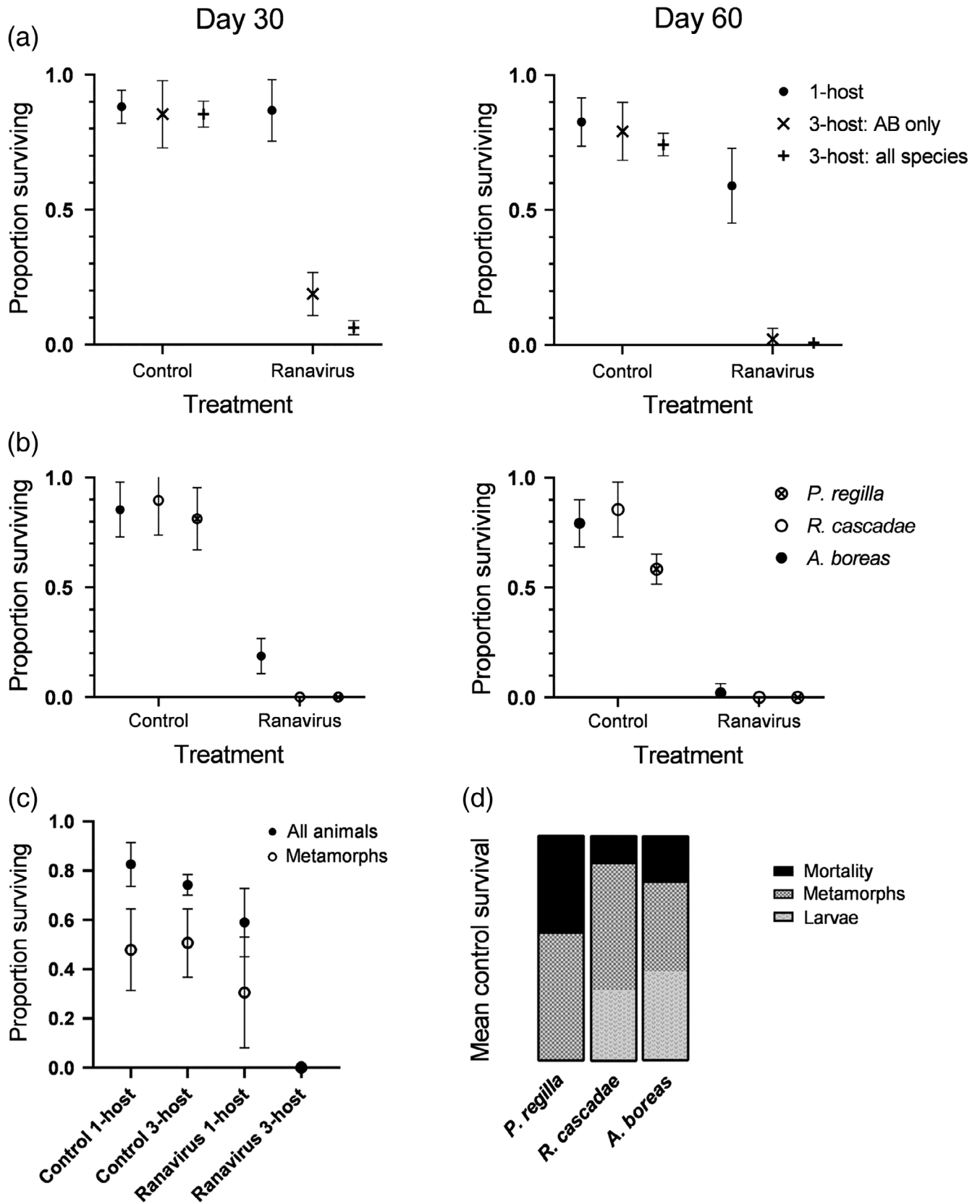


FIGURE 2 (a) Mean proportion *Anaxyrus boreas* and total mesocosm survival by treatment at 30 and 60 days and (b) mean proportion of three-host treatment survivors by species at 30 and 60 days. The lower panel shows the mean proportion of surviving postmetamorphic animals (c) by treatment and (d) by species for three-host control treatment. Error bars represent SD.

Day 60 (ANOVA $F_{1,12} = 30.74$, $p = 0.0001$). Although *A. boreas* survival was high (83%) in the control treatments (no ranavirus addition), in ranavirus-exposed treatments only 59% of toads in the single-species mesocosms, and <3% of those in the three-species mesocosms survived until Day 60 (Figure 2a). The other two amphibian species in the ranavirus treatments showed similarly low survival (Figure 2b); total survival of all species in the community was only 0.69% at Day 60 (Figure 2a). We observed a similar interaction between virus exposure and host richness when considering total host survival (i.e., the combined survival of all three hosts) at Days 30 (ANOVA $F_{1,12} = 122.18$, $p < 0.0001$) and 60 (ANOVA $F_{1,12} = 32.25$, $p = 0.0001$). There were no significant differences among the one-host control, three-host control, and one-host ranavirus treatments in the proportion of surviving animals that completed metamorphosis (57%, 68%, and 49%, respectively; Tukey–Kramer HSD [all p -values >0.05]). However, each of these three treatments were significantly different from the ranavirus-exposed, three-host mesocosms (three-host control $p = 0.0021$; one-host control $p = 0.0074$; one-host ranavirus $p = 0.0213$), which had no animals survive through metamorphosis (Figure 2c,d). No ranavirus infection was detected among the subset of exposed surviving animals tested at Day 60 ($n = 6$ per replicate), which included a total of 24 from *A. boreas* one-host treatments and the single surviving *A. boreas* from the three-host treatments.

DISCUSSION

Our laboratory experiment demonstrated variability in ranavirus susceptibility among amphibian species. This experiment suggests that of the three species tested in the laboratory, only *P. regilla* was successfully infected with ranavirus. Additionally, although some of our control *P. regilla* tested positive for ranavirus infection, suggesting cross contamination, *P. regilla* had significantly greater mortality in the ranavirus-exposed treatment relative to the sham-exposed controls, with most of the mortality occurring in the first 10 days after exposure (Figure 1a). Hence, of the three co-occurring species we examined, only *P. regilla* is prone to ranavirus-induced mortality when tested in isolation. In our mesocosm experiment, *P. regilla* also experienced high mortality when exposed to ranavirus in the presence of heterospecific larvae. Contrary to our laboratory experiment, however, *A. boreas* and *R. cascadae* experienced high mortality when exposed to ranavirus while in the presence of heterospecifics. Of the surviving animals that were tested in the mesocosm experiment, none tested positive for ranavirus infection, including the lone

A. boreas survivor of the three-host treatment. These animals either never became infected or cleared their infection during the experiment.

Despite not experiencing significant mortality in the laboratory, ranavirus-exposed *A. boreas* experienced greater mortality than controls in the mesocosm experiment. Increased diversity of host species in our mesocosms resulted in a higher mortality of *A. boreas*. This high mortality in *A. boreas* may be due to exposure to more viral particles than in the one-host treatment since there is evidence that a higher viral dose than used in our experiments can cause 100% mortality in *A. boreas* (Earl et al., 2016) and that dose affects ranavirus infection outcomes (Brunner et al., 2005). Additional viral particles could have been generated by the presence of the highly susceptible host *P. regilla*. Because our laboratory experiment only provided a proxy for host competence, it is possible that another host species was more competent than *P. regilla*, shedding a greater number of viral particles, but resistant to the experimental exposure dosage. The addition of *P. regilla*, a host species that is more susceptible than *A. boreas*, may have driven the increased mortality in our high-richness treatment by amplifying virus abundance in the mesocosms. It is also possible that other mechanisms led to this result. The deaths of *P. regilla* could have altered other factors in the mesocosms, resulting in increased mortality of *A. boreas* and *R. cascadae*, such as by altering water quality or competitive pressures. Previous work documented competition between these three species (Han et al., 2015), and infection with ranavirus could have affected interspecific competition that resulted in increased susceptibility to the pathogen. The reduced survival in the mesocosm experiment appears to be an interactive effect between viral presence and host species composition. Selecting a different single-host species (other than *A. boreas*) for comparison could change the relationship we found between species richness and ranavirus-induced mortality (Venesky et al., 2014).

Our results suggest an amplification effect (Faust et al., 2017). This result differs from those obtained in several studies that found that increased host richness diluted the disease risk of amphibian pathogens (e.g., Civitello et al., 2015; Han et al., 2015; Searle et al., 2011). The effect of diversity on ranavirus outcomes compared with other pathogens may be explained by differences in shared host/pathogen life history as hosts may be unable to detect and avoid conspecifics infected with a novel pathogen. The fungal pathogen Bd is also an aquatic generalist pathogen of amphibians, but experiments in the same amphibian system demonstrated a dilution of disease risk with increased host diversity (Han et al., 2015; Searle et al., 2011; Venesky et al., 2014).

Because losses in biodiversity can alter disease risk through changes in host species composition, and the dilution of disease risk with increased biodiversity is predominantly observed in systems experiencing biodiversity loss (Halliday et al., 2020), changes in biodiversity are often viewed through a lens of community disassembly. Of our three hosts, *P. regilla* inhabits the widest range of habitats and elevations (Nussbaum et al., 1983) and may be the least likely of our three hosts to be affected by drivers of biodiversity loss. However, factors such as climate change that induce large-scale changes in host habitat ranges (Lawler et al., 2009) could drive populations of *P. regilla* and *R. cascadae* into habitats in which *A. boreas* is the only amphibian species present. Thus, the amplification of disease risk observed in our mesocosm could occur as a result of community composition changes in *A. boreas*-dominated wetlands.

For species experiencing disease-driven population declines, it is paramount to understand the dynamics between decreasing biodiversity and disease risk. Here we demonstrated variation in host competence among three amphibian species and the potential effects of amphibian host species composition on ranavirus infection at the community scale. Our experimental results highlight how host identity and changes in community composition are important factors in understanding disease-associated population die-offs and suggest that increases in biodiversity do not guarantee a dilution of disease risk.

AUTHOR CONTRIBUTIONS

Andrew R. Blaustein, Cheryl J. Briggs, Jason Todd Hoverman, Jason R. Rohr, and Pieter T. J. Johnson planned the research. Paul W. Snyder designed the mesocosm experiment. Chloe T. Ramsay designed the laboratory experiment. Paul W. Snyder, Chloe T. Ramsay, and Carmen C. Harjoe collected the data. Paul W. Snyder conducted the analysis and drafted the manuscript. Emily S. Khazan revised the manuscript. All authors contributed to the editing and approval of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data (Snyder et al., 2022) are available in Dryad at <https://doi.org/10.5061/dryad.gtht76hqj>.

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