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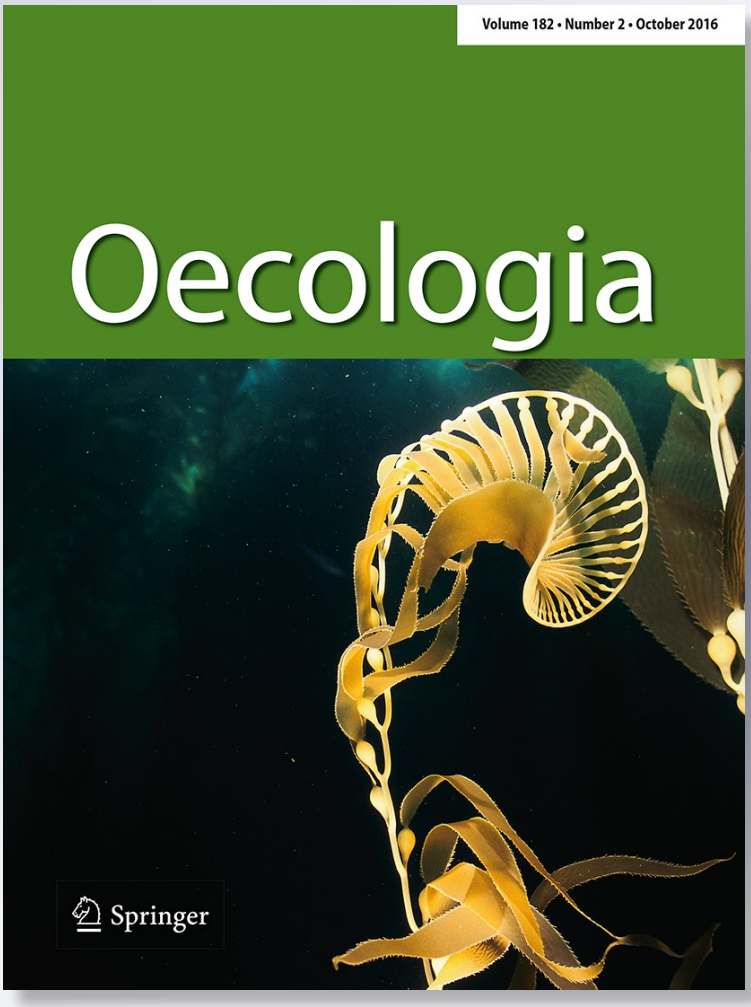

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CONSERVATION ECOLOGY – ORIGINAL RESEARCH

Ranavirus could facilitate local extinction of rare amphibian species

Julia E. Earl[1] · Jordan C. Chaney[2] · William B. Sutton[3] · Carson E. Lillard[2] · Andrew J. Kouba[4,8] · Cecilia Langhorne[5] · Jessi Krebs[6] · Rebecca P. Wilkes[7] · Rachel D. Hill[2] · Debra L. Miller[2,7] · Matthew J. Gray[2]

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Abstract There is growing evidence that pathogens play a role in population declines and species extinctions. For small populations, disease-induced extinction may be especially probable. We estimated the susceptibility of two amphibian species of conservation concern (the dusky gopher frog [*Lithobates sevosus*] and boreal toad [*Anaxyrus boreas boreas*]) to an emerging pathogen (ranavirus) using laboratory challenge experiments, and combined these data with published demographic parameter estimates to simulate the potential effects of ranavirus exposure on extinction risk. We included effects of life stage during pathogen exposure, pathogen exposure interval, hydroperiod of breeding habitat, population carrying capacity, and immigration in simulations. We found that both species were highly susceptible to ranavirus when exposed to the pathogen in water at environmentally relevant concentrations. Dusky gopher frogs experienced 100 % mortality in four of six life stages tested. Boreal toads experienced 100 % mortality when exposed as tadpoles or metamorphs, which were the only life stages tested. Simulations showed population declines, greater extinction probability, and faster times to extinction with ranavirus exposure. These effects were more evident with more frequent pathogen exposure intervals and lower carrying capacity. Immigration at natural rates did little to mitigate effects of ranavirus exposure unless immigration occurred every 2 years. Our results demonstrate that disease-induced extinction by emerging pathogens, such as ranavirus, is possible, and that threat may be especially high for species with small population sizes. For the species in this study, conservation organizations should incorporate ranavirus surveillance into monitoring programs and devise intervention strategies in the event that disease outbreaks occur.

Keywords Amphibian declines · *Anaxyrus boreas boreas* · Endangered species · Iridoviridae · *Lithobates sevosus* · Matrix model

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Introduction

Ecological stressors are major concerns for species threatened by extinction. Disease exposure represents one source of added stress and mortality, which can increase demographic stochasticity and facilitate extinction. Disease can theoretically cause extinction when host populations are small if transmission is density-independent even for brief periods (de Castro and Bolker 2005). In the past, many species that have suffered disease-related extinctions already had small population sizes (de Castro and Bolker 2005). To prevent or manage disease in species of conservation concern, it is important to know their susceptibility and the potential population consequences of exposure to emerging pathogens.

For amphibians, diseased-induced extinctions have been reported for the pathogen *Batrachochytrium dendrobatidis* (*Bd*, etiological agent of chytridiomycosis; Smith et al. 2006). While chytridiomycosis has clear risks, other amphibian pathogens, such as ranavirus, also have the potential to add stress and mortality to species already at risk of extinction. Ranaviruses are emerging pathogens linked to amphibian die-offs across the globe, with known cases of infection in >100 species in 18 taxonomic families (Duffus et al. 2015). Ranaviruses cause systemic hemorrhaging (Miller et al. 2015), with mortality occurring as fast as 3 days following exposure to the pathogen in water (Hoverman et al. 2011). There are multiple reasons why ranaviruses could cause species declines: ranaviruses infect multiple host species with different susceptibilities (Hoverman et al. 2011; Brenes et al. 2014), the virus particles can persist outside the host (Nazir et al. 2012; Johnson and Brunner 2014), and several of the host life stages naturally cluster (Wells 2007), all of which can result in density-independent transmission (Miller et al. 2011).

Ranaviruses have been shown to affect amphibian population dynamics. Two studies have been published that collected longitudinal data on populations with reoccurring ranavirus die-offs (Brunner et al. 2015). In England, ranavirus-related die-offs led to declines of the common frog (*Rana temporaria*) to only 19 % remaining of adult populations after 10 years (Teacher et al. 2010). In Spain, entire amphibian communities have declined over a 5-year period after ranavirus outbreaks. These declines included three species (*Alytes obstetricans*, *Bufo bufo*, and *Mesotriton alpestris*) from three families at multiple breeding sites (Price et al. 2014). In North America, no studies collecting longitudinal data in ranavirus-impacted populations have been published (Brunner et al. 2015); however, simulation models show that extinction of closed populations of wood frogs (*Lithobates sylvaticus*) from ranavirus exposure is possible (Earl and Gray 2014), although the conservation concern for this species currently is low according to the International Union for the Conservation of Nature (IUCN SSC Amphibian Specialist Group 2015). Disease-associated die-offs in amphibian communities are most frequently caused by ranaviruses in the US (43–58 % of die-offs; Green et al. 2002; Muths et al. 2006a), and some cases have involved large numbers of individuals (Wheelwright et al. 2014). In populations with reoccurring die-offs, recruitment may be non-existent (Petranka et al. 2007). However, it is possible that the negative effects of ranavirus outbreaks could be reduced in populations with a metapopulation structure through the rescue of affected populations by immigration (Petranka et al. 2007).

Our goal was to estimate the susceptibility of two amphibian species of conservation concern to ranavirus and simulate potential population changes due to the introduction of ranavirus. We conducted ranavirus challenge trials in the laboratory and applied survival estimates to stage-structured population matrix models (Caswell 2001) to simulate potential population consequences of ranavirus exposure. We examined two North American species: the federally endangered dusky gopher frog [(*Lithobates sevosus*), one of the rarest amphibians in North America] and the boreal toad (*Anaxyrus boreas boreas*), which has a population segment that is under review for listing under the US Endangered Species Act (ESA). In addition, we examined the effects of life stage during pathogen exposure, pathogen exposure interval, hydroperiod of the breeding habitat (gopher frogs only), population carrying capacity (boreal toads only), and immigration (boreal toads only). The inclusion of an immigration parameter addressed whether dispersal could rescue populations from any negative impacts of ranavirus introduction, which was not addressed in previous work (Earl and Gray 2014). Furthermore, we examined whether the release of captive-reared individuals could mitigate increased extinction risk for populations with ranavirus outbreaks. To our knowledge, ranavirus has never been detected in populations of dusky gopher frogs. A ranavirus-related mortality event was reported in recently metamorphosed boreal toads in Montana (Converse and Green 2005), and an outbreak of ranavirus occurred in a captive population of boreal toads in Colorado resulting in 91 % mortality (Cheng et al. 2014). The ranavirus challenge trials that we performed for dusky gopher frogs, including a previous study on adults (Sutton et al. 2014), are the first to assess susceptibility of all amphibian life stages to ranavirus in one species.

Materials and methods

Dusky gopher frogs and boreal toads are pond-breeding amphibians that are considered vulnerable to extinction. The dusky gopher frog is listed as federally endangered

under the ESA. Dusky gopher frogs occur at only one pond (Glen's pond) located in southern Mississippi, USA (Richter et al. 2003), though reintroduction attempts at other locations are in progress. Boreal toads occur in northwestern North America from Colorado and New Mexico north to southeastern Alaska. The boreal toad has one population segment (i.e., the eastern population covering Colorado, northern New Mexico, Utah, southern Wyoming, and southeastern Idaho) under review by the US Fish and Wildlife Service for listing under the ESA. Most of the boreal toad declines have been attributed to the introduction of *Bd* (Carey et al. 2005), although surveillance for other pathogens has been limited.

Ranavirus laboratory studies

We conducted laboratory challenges with ranavirus following standardized published protocols (Haislip et al. 2011, Hoverman et al. 2011). For the dusky gopher frog, we tested the egg, hatchling, larva, and metamorph stages, which corresponded to pathogen exposure starting at Gosner stage 11, 21, 30, and 41, respectively (Gosner 1960). We also tested the juvenile stage, which we defined as 1-month post-metamorphosis, and used survival data from Sutton et al. (2014) for the adult stage. For the boreal toad, we only tested the larva and metamorph stages due to a limited number of available animals. All animals were obtained as eggs from captive rearing facilities and raised in the laboratory to the appropriate life stage. Dusky gopher frog eggs were obtained from a captive assurance colony at the Henry Doorly Zoo and Aquarium in Omaha, NE (USFWS permit #TE171493-0), and boreal toad eggs came from captive-reared individuals at the Native Aquatic Species Restoration Facility in Alamosa, CO. These captive populations are maintained using methods to prevent inbreeding and maximize continued levels of genetic diversity.

Laboratory challenges were via a 72-h water bath exposure to a *Frog virus 3* (FV3)-like isolate (Miller et al. 2007) at 10^3 plaque forming units (PFU)/mL, which is considered an environmentally relevant dose (Brunner et al. 2015). The ranavirus strain we used was originally isolated from American bullfrogs (*Lithobates catesbeianus*) in a ranaculture facility in Georgia (Miller et al. 2007), and thus represents a geographically novel ranavirus for dusky gopher frogs and boreal toads that could be potentially introduced through the American bullfrog trade. In previous work (Hoverman et al. 2011), this FV3-like isolate generally caused greater mortality than the type species, FV3, originally isolated from morbid northern leopard frogs (*L. pipiens*) by Granoff et al. (1965). Eggs, hatchlings, tadpoles, and metamorphs were exposed individually to the virus in 2-L containers with 1 L of dechlorinated water. We exposed juvenile gopher frogs individually in 200 mL of water, which allowed the frog to sit in the water with at least half of its body immersed. For all age classes (except juvenile gopher frogs), we randomly selected 30 individuals; 20 were exposed to ranavirus and 10 were controls. Sample size was 15 exposed and 5 controls for juvenile gopher frogs. Control animals were held under the same laboratory conditions except their water was inoculated with the same quantity of Eagle's minimum essential medium. After the 72-h ranavirus challenge, animals were moved to new containers with the same amount of dechlorinated water, except for juveniles, which received 50 mL of water to prevent desiccation. As metamorphs resorbed their tails, we lowered the water and added foam platforms to allow them to crawl out of the water. We performed water changes and fed tadpoles and juveniles every 3 days. Tadpoles were fed pelleted commercial fish food at 10 % of their body mass and juveniles received one cricket. We maintained up to five non-experimental tadpoles held under the same laboratory conditions for body mass estimates. Anurans do not eat as embryos, hatchlings, or metamorphs (Wells 2007), so individuals were not fed during these stages. Survival was monitored twice per day for 21 days, which is sufficient to observe mortality due to ranavirus (Hoverman et al. 2011). As individuals died or at the end of the experiment, we performed necropsies and tested for ranavirus infection using quantitative PCR (Hoverman et al. 2011).

Population models

Stage-structured population models are useful for investigating the population consequences of changes in vital rates due to ecological stressors. For both species, we created stage-structured female only, discrete time (1-year increments) population matrix models (Caswell 2001) to examine effects of ranavirus on population dynamics and extinction risk (Earl and Gray 2014). We used females, because they generally produce one clutch per year, whereas males can fertilize multiple clutches and are thus not considered to be population limiting (Wells 2007). The models are post-breeding models, where 1 year in the model spanned from the end of one breeding season to the next. We parameterized models using published estimates from Richter et al. (2003) and Richter and Seigel (2002) for dusky gopher frogs and from Muths et al. (2010) and Biek et al. (2002) for boreal toads. For parameters with no published estimates available, we calibrated parameter estimates with published data on population trends (Table 1). Calibration was necessary for two parameters [juvenile survival (p_2) and the transition probability from juvenile to adult (p_3), see below] in the dusky gopher frog model. For calibration, we examined a range of plausible parameter values (p_2: 0.025, 0.05, 0.1, 0.2, and 0.3; p_3: 0.025, 0.05, 0.1, 0.3, 0.5,

and 0.7) and ran the model with all combinations of those parameter values for the years with data published in Richter et al. (2003). We chose the combination of parameter values that resulted in the greatest correlation between the published and simulated numbers of egg masses, breeding females, and emerging metamorphs.

In both models, for each parameter, random values were drawn from a normal distribution to simulate stochasticity (Table 1). To examine the effects of ranavirus introduction on the population, we used survival estimates from experiments described above (Table 1), and applied them at specific exposure intervals (none and every 50, 25, 10, 5, 2, and 1 years). Exposure intervals of every 10–50 years could represent reintroductions of the pathogen, while exposures every 1–5 years could represent the presence of reservoir hosts, such as reptile or other amphibian species or another life stage of the same species (Brunner et al. 2004, 2015). To simulate mortality in years with ranavirus exposure, we multiplied the demographic transition probability by the survival probability when exposed to ranavirus for the appropriate life stage. For these runs, we assumed that individuals that were exposed and survived were not exposed again during a subsequent life stage (Earl and Gray 2014). For the pathogen interval exposure, we treated intervals in a probabilistic manner, such that years with pathogen exposure were randomly determined using a binomial distribution appropriate for the exposure interval. For example, ranavirus was not introduced exactly every 10 years, but on average every 10 years for the 10-year exposure interval (Earl and Gray 2014).

Table 1 Parameter estimates used in dusky gopher frog and boreal toad population model simulations

Species/parameter	Parameter estimate	SD
Dusky gopher frog[a]		
p_1 (year 1 survival)	0.0151	0.0222
p_2 (probability to remain a juvenile)	0.1000[b]	0.0200
p_3 (transition from juvenile to adult)	0.0500[b]	0.0200
p_4 (probability to remain an adult)	0.1905	0.0250
F (fecundity)[c]	704	176
Ranavirus survival: egg	0.90	0
Ranavirus survival: hatchling	0	0
Ranavirus survival: tadpoles	0	0
Ranavirus survival: metamorph	0	0
Ranavirus survival: adult[d]	0	0
Boreal toad[e]		
p_1 (year 1 survival)	0.0293	0.0332
p_j (juvenile survival)	0.260	0.0400
p_a (adult survival)	0.870	0.0500
Ψ_{nb} (transition to stay a non-breeder)	0.640	0.1
Ψ_b (transition from non-breeder to breeder)	0.360	0.1
F (fecundity)[c]	3532	856
Ranavirus survival: tadpoles	0	0
Ranavirus survival: metamorph	0	0

[a] Population parameter estimates were derived from published estimates (Richter and Seigel 2002; Richter et al. 2003) and by calibrating the model to published population data (Richter et al. 2003)

[b] These estimates were derived by testing plausible values based on other species and choosing the combination that resulted in the highest correlation between simulated populations and published population data

[c] Fecundity is the average number of female eggs produced per female, accounting for the proportion of females breeding in a given year

[d] Experiment and results described in Sutton et al. (2014)

[e] Population parameter estimates were derived from published estimates (Biek et al. 2002; Muths et al. 2010)

Dusky gopher frog model

We created a three-stage matrix model for dusky gopher frogs. In this model, the population size for stage i (where i = pm, 1, and 2+ for pre-metamorphosis and years 1 and ≥2, respectively) at time t [$N_i(t)$] was calculated by multiplying the population matrix at time $t-1$ by the transition matrix containing parameter estimates for survival (p) and fecundity (F; Eq. 1, Table 1).

$$\begin{pmatrix} N_{\mathrm{pm}}(t) \\ N_1(t) \\ N_{2+}(t) \end{pmatrix} = \begin{pmatrix} 0 & 0 & F \\ p_1 & p_2 & 0 \\ 0 & p_3 & p_4 \end{pmatrix} \times \begin{pmatrix} N_{\mathrm{pm}}(t-1) \\ N_1(t-1) \\ N_{2+}(t-1) \end{pmatrix}. \quad (1)$$

We included hydroperiod in the model for the dusky gopher frog, because this is a major factor limiting metamorph production (Richter et al. 2003). We set a hydroperiod threshold at 81 days. When the hydroperiod is below this threshold, no metamorphs are able to emerge, because the pond dries before tadpoles are able to complete metamorphosis. Eighty-one days is suggested as the minimum amount of time required for dusky gopher frogs to reach metamorphosis (Richter et al. 2003). We examined two hydroperiod scenarios: a non-limiting hydroperiod (i.e., the hydroperiod was always 81 days or greater) and the Glen's pond average [133 ± 87 (SD)]. We did not include immigration or carrying capacity in the dusky gopher frog model, because Glen's pond is the only remaining viable population and the population size is so small that it is unlikely to be limited by carrying capacity.

Boreal toad model

For boreal toads, we created a model with seven life stages, where each age class was represented in the model until individuals reached maturity (year 6), at which point individuals were represented by reproductive status (breeders

or non-breeders). We constructed our model with parameter estimates and life history of the ESA candidate population segment, centered in Colorado, for populations unaffected by *Bd*. Boreal toads are known to be long-lived (9 or more years), and females reach maturity at ages five to six in Colorado (Mallawaarachchi et al. 2011). Females skip breeding at least every other year (Muths et al. 2010), which we adjusted for by grouping adults as breeders and non-breeders. In our model, the population size for stage i (where $i = 1, 2, 3, 4, 5$, b, and nb for years 1, 2, 3, 4, and 5 and breeders and non-breeders, respectively) at time t $[N_i(t)]$ was calculated by multiplying the population matrix at time $t - 1$ by the transition matrix containing parameter estimates for survival (p), fecundity (F), and transition probabilities (Ψ; Eq. 2, Table 1).

$$\begin{pmatrix} N_1(t) \\ N_2(t) \\ N_3(t) \\ N_4(t) \\ N_5(t) \\ N_b(t) \\ N_{nb}(t) \end{pmatrix} = \begin{pmatrix} 0 & 0 & 0 & 0 & 0 & F * p_a & 0 \\ p_1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & p_j & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & p_j & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & p_j & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & p_j & 0 & p_a * \Psi_b \\ 0 & 0 & 0 & 0 & 0 & p_a & p_a * \Psi_{nb} \end{pmatrix} \times \begin{pmatrix} N_1(t-1) \\ N_2(t-1) \\ N_3(t-1) \\ N_4(t-1) \\ N_5(t-1) \\ N_b(t-1) \\ N_{nb}(t-1) \end{pmatrix}. \quad (2)$$

Different survival probabilities were used for year 1 (p_1), juveniles (p_j), and adults (p_a, Table 1). Transition probabilities included the transition of a non-breeder to stay a non-breeder (Ψ_{nb}), and the transition of a non-breeder to a breeder (Ψ_b, Muths et al. 2010). The transition probability for a breeder becoming a non-breeder is one (Muths et al. 2010) and not included as a separate parameter. Parameter estimates for clutch size, survival from egg to year 1, and juvenile survival were based on Biek et al. (2002), and estimates for female survival and the transition probabilities were based on Muths et al. (2010).

Adult female carrying capacity was set as a population limit, where the combined abundance of breeding and non-breeding females could not exceed this level. Carrying capacities were estimated as the numbers of females detected at ponds during breeding (maximum of 107; Jackson 2008) and adjusted to account for non-breeding females. We examined carrying capacities of 250, 150, 100, and 50 adult females. In years when carrying capacity was exceeded, we set the number of adult females at the carrying capacity equally divided between breeding and non-breeding adults. We also included immigration in the boreal toad model. Many populations of boreal toads are isolated, but some dispersal between breeding sites has been reported (Muths et al. 2006b). For immigration, we added one breeding female to the population for each year that immigration occurred, and immigration occurred at a range of intervals: none and every 50, 25, 10, 5, and 2 years. These levels of immigration are reasonable for boreal toads as Muths et al. (2006b) found only 17 males and 3 females at a site different from their original capture location out of >1,900 captures over 15 years. Similar to ranavirus exposure interval, immigration was treated probabilistically with the years of immigration determined using a binomial distribution. In cases where the population went extinct the previous year, we precluded immigration, as it would represent recolonization not immigration.

Model output

Simulations were run in a factorial design varying five factors: life stage of ranavirus exposure (egg, hatchling, tadpoles, metamorph, or adult for dusky gopher frog; tadpoles or metamorph for boreal toads), exposure interval (none and every 50, 25, 10, 5, 2, and 1 years), hydroperiod (for dusky gopher frog only), carrying capacity (for boreal toads only), and immigration (boreal toad only). Life stages were delineated as in the laboratory experiment, where egg, hatchling, tadpoles, and metamorph corresponded to pathogen exposure starting at Gosner stage 11, 21, 30, and 41, respectively (Gosner 1960). Although we tested dusky gopher frog juveniles for ranavirus susceptibility, we did not include juveniles in model simulations, because juveniles typically do not visit the breeding pond where ranavirus exposure in water would most likely occur.

For each simulation scenario, we performed 1,000 model runs, which were run to extinction for the dusky gopher frogs and 150 years for boreal toads. We defined extinction in a very conservative manner, where there had to be zero individuals of any life stage remaining for extinction to occur (Earl and Gray 2014). For each run, we determined whether the population went extinct, the extinction time, and the final population size for adults. For dusky gopher frogs, all model runs started with the same initial population size, based on the most robust recorded female population size (130) derived from the highest number of egg masses laid at Glen's pond (Richter et al. 2003). For boreal toads, initial population sizes were set at the female carrying capacity. Population sizes for other life stages were calculated assuming stable population trajectories. Because we could generate a large amount of simulation data, we chose to present the data graphically and not perform statistical analyses (Earl and Gray 2014).

We were also interested in whether the introduction of captive-reared adults could mitigate the effects of ranavirus-related die-offs. We examined two approaches: the introduction of individuals the year following ranavirus exposure and the introduction of individuals at probabilistic intervals (i.e., every 25, 10, 5, and 2 years). We ran simulations increasing the number of individuals in each introduction event until the probability of extinction was equivalent (i.e., overlapping 95 % confidence intervals) to that with no

ranavirus exposure. In simulations, individuals that were introduced were always breeding females, and they were introduced prior to the mating season. In our model, all introduced, captive-reared individuals had the same survival and fecundity as wild individuals, which may not be realistic (see "Discussion"; Muths et al. 2014). Thus, our simulations represent optimistic reintroduction scenarios. All models were run in Matlab 2010a (Mathworks, Natick, MA, USA).

Sensitivity analyses were conducted to determine which model parameters influenced population dynamics most. We conducted sensitivity analyses by running 1,000 simulations of 150-year runs, where parameter values were randomly generated but fixed for all years of the model run. The total population size and whether the population went extinct in each 150-year period were used as the response variables. We assessed the influence of parameters on response variables by running simple and logistic regressions for population size and extinction, respectively, with R version 2.7.2 to produce R^2 values (R Development Core Team 2008).

Results

Laboratory experiments

Ranavirus was most pathogenic to the metamorph, larval, and hatchling stages for the dusky gopher frog, resulting in 100 % mortality in 5, 7, and 8 days post-exposure, respectively (Fig. 1a). Mortality was significantly slower for the juvenile stage, with 80 % mortality occurring over 12 days. Only 10 % mortality occurred in the embryo stage for dusky gopher frogs (Fig. 1a). There was 100 % mortality of boreal toad metamorphs and tadpoles in 5 and 7 days, respectively (Fig. 1b). All animals (except 1 gopher frog hatchling) that died tested positive for ranavirus (Online Appendix A, Table A1). There was no control mortality, except one juvenile gopher frog that tested negative for ranavirus. Animals of both species displayed lesions consistent with ranaviral disease (Figure A1).

Dusky gopher frog model simulations

All simulated populations of dusky gopher frogs went extinct quickly. For simulations without ranavirus exposure, extinction occurred in an average of 25 and 90 years for the Glen's pond average hydroperiod and the non-limiting hydroperiod, respectively (Fig. 2). Populations went extinct faster with shorter ranavirus exposure intervals. Extinction occurred more quickly when exposure occurred during the hatchling, larval, or metamorph stages than as adults, and exposure during the egg stage resulted in only a slight decrease in time to extinction relative to no ranavirus exposure. In the worst-case scenario of exposure during the larval or metamorph stages under the average hydroperiod, simulated populations went extinct in seven and 12 years for exposure every 1 and 2 years, respectively. The addition of 1–15 captive-reared breeding adult females was able to mitigate effects of exposure to ranavirus in most cases (Appendix A2). However, when ranavirus exposure occurred frequently (every 2 years) in all stages except the egg, it would be necessary to add more than 50 adult females or add one to four every other year (Appendix A2).

The sensitivity analysis showed dusky gopher frog populations were most sensitive to the transition probability from egg to year 1 (p_1), the transition from juvenile to adult (p_3), and fecundity (F). The results were similar for both hydroperiod scenarios. These parameters had a significant effect on determining whether populations went extinct (p_1: $R^2 = 0.30$–0.31; p_2: $R^2 = 0.17$–0.18; F: $R^2 = 0.10$–0.11; all $P < 0.001$) and on the final population size if they did not go extinct (p_1: $R^2 = 0.39$–0.46; p_2: $R^2 = 0.43$–0.44; F: $R^2 = 0.42$–0.46; all $P < 0.001$). Some of the sensitivities for

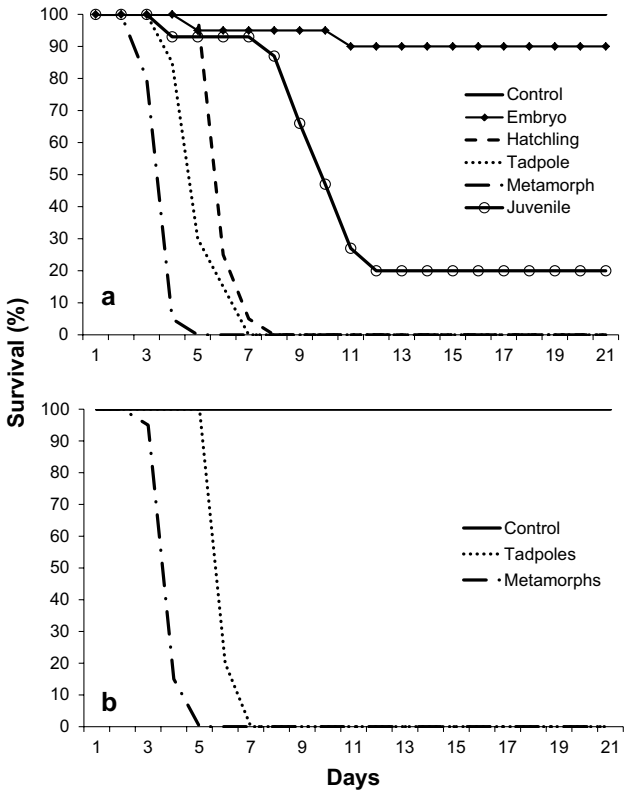

Fig. 1 Differences in survival among different life stages exposed to ranavirus in water (10^3 PFU/mL, $N = 20$ individuals exposed) for dusky gopher frogs (**a**) and boreal toads (**b**). Survival of control animals was 100 % for all experiments ($N = 10$ control individuals/life stage), except juvenile gopher frogs ($N = 15$ exposed individuals and 5 control); one control animal died, and it was negative for ranavirus

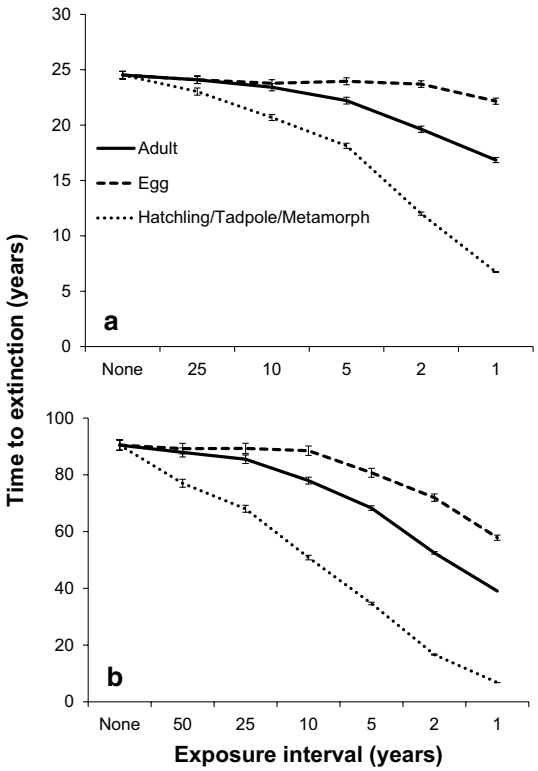

Fig. 2 Effects of the life stage of exposure to ranavirus and exposure interval on time to extinction in simulated dusky gopher frog populations ($N = 1{,}000$ simulations/scenario) under the average Glen's pond hydroperiod (133 days, **a**) and a non-limiting hydroperiod (**b**). Estimates for hatchlings, tadpoles, and metamorphs are the same, because they had the same mortality rate in laboratory experiments. *Error bars* represent standard error

the probability that a juvenile remains a juvenile and adult survival were statistically significant, but they were all very low (all $R^2 < 0.08$). The range of R^2 values indicates the range found between the two hydroperiod scenarios.

Boreal toad model simulations

Boreal toad populations had a small probability of extinction (<1 % of the simulations) over a 150-year period with no ranavirus exposure when carrying capacities were large ($K \geq 150$ adult females), but had moderate (14 %) to high (55 %) extinction probabilities with carrying capacities of 100 and 50 adult females, respectively (Fig. 3). The introduction of ranavirus during the larval or metamorph stages increased the probability of extinction, particularly with more frequent exposure intervals. Exposure to ranavirus every 1 or 2 years resulted in populations going extinct ≥94 % of the time, even with high levels of immigration. For populations with a small carrying capacity, exposure to ranavirus every 5 years also resulted in ≥94 % extinction in all cases except when immigration occurred every 2 years. Immigration decreased the probability of extinction somewhat, particularly for populations with smaller carrying capacities (Fig. 3).

Scenarios that resulted in greater extinction probabilities also reduced the time to extinction (Fig. 4) for populations that went extinct and final adult population size (Fig. A2) for populations that did not go extinct. Time to extinction was similar for no exposure to ranavirus up to exposure every 10 years, but occurred more quickly with ranavirus exposure intervals every 5 years or less. In the worst-case scenarios, extinction could occur in 39–70 years with ranavirus exposure every 2 years and in 19–30 years with exposure every year, depending on carrying capacity (Fig. 4). For populations that did not go extinct, adult population size (breeders and non-breeders combined) decreased with increasingly frequent ranavirus exposure intervals (Fig. A2). Declines were as great as 55–77 % over 150 years with exposure every 5 years, depending on carrying capacity. Immigration made a very little difference in the time to extinction or final adult population size unless it occurred every 2 years (Figs. 4, A2).

The addition of 1–15 captive-reared breeding adult females compensated for the effects of exposure to ranavirus when exposure was every 10 years or less frequent (Table A3). When ranavirus exposure occurred frequently (every 2–5 years), more than 50 adult females, or additions of 1–18 females every 2–5 years, would be necessary to prevent the high probabilities of extinction (Table A3). The sensitivity analysis showed that boreal toad population dynamics were most sensitive to the 1-year old survival (p_1) and juvenile survival (p_j) parameters. This was true for both the total population size (p_1: $R^2 = 0.27$, $p < 0.001$; p_j: $R^2 = 0.23$, $P < 0.001$) and the probability of extinction (p_1: $R^2 = 0.37$, $P < 0.001$; p_j: $R^2 = 0.19$, $P < 0.001$). Adult survival, fecundity, and transition probability from non-breeder to breeder all had very low sensitivities (all $R^2 < 0.05$).

Discussion

Our results suggest that ranaviruses can increase the probability of and decrease the time to extinction in rare amphibian species. The species we tested were highly susceptible to the FV3-like isolate we used. Of 35 amphibian species tested under similar laboratory conditions (Hoverman et al. 2011; Brenes 2013), the dusky gopher frog and boreal toad were the most susceptible. Dusky gopher frogs experienced 100 % mortality in four life stages (hatchling, tadpole, metamorph, and adult) and 80 % mortality in the juvenile stage when exposed to ranavirus in water at an environmentally relevant concentration for 3-day duration. Exposure to ranavirus for 3 days is reasonable for all life

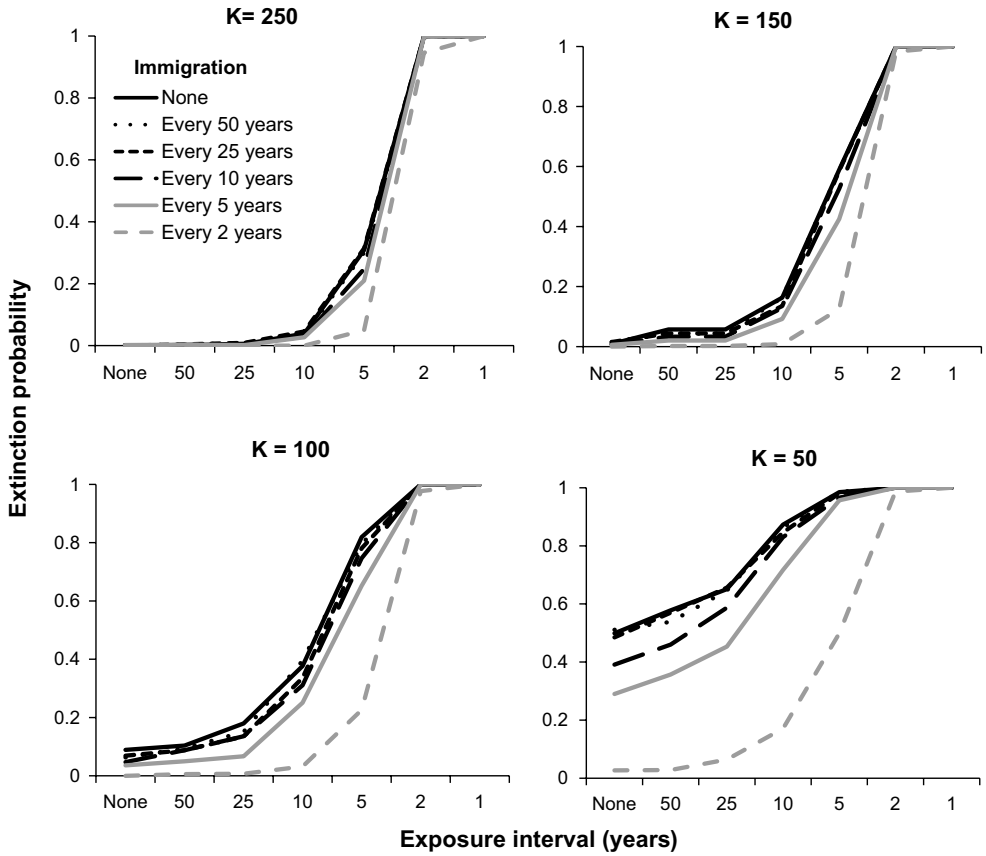

Fig. 3 Effects of the ranavirus exposure interval at the larval or metamorph stages, carrying capacity (*K*), and immigration frequency on probability of extinction over 150 years in simulated boreal toad populations ($N = 1{,}000$ simulations/scenario). Immigration always consisted of the introduction of one breeding female

stages, except the juvenile stage, which was not included in the simulations. Metamorph and adult gopher frogs usually stay at breeding sites for at least 3 days and 2 weeks, respectively (Richter and Seigel 2002), and hatchlings and tadpoles develop in water. Similar to other species, dusky gopher frog mortality was much lower in the egg stage than in other stages (Haislip et al. 2011). High mortality in most life stages puts the dusky gopher frog at high risk if ranavirus is introduced to the Glen's pond population.

Ranavirus susceptibility is generally higher in ranids, and species that breed in semi-permanent ponds, develop rapidly as tadpoles, and have limited geographic distributions (Hoverman et al. 2011). The geographic range for dusky gopher frogs is extremely restricted, and population isolation is typical for both dusky gopher frogs and boreal toads (Carey et al. 2005; Muths et al. 2006b; Richter et al. 2009). Population isolation tends to lead to reduced genetic diversity (Amos and Harwood 1998), which can affect susceptibility to ranavirus. For example, populations of the Italian agile frog (*Rana latastei*) were more susceptible to ranavirus if they were isolated and had lower genetic diversity (Pearman and Garner 2005). Low genetic diversity may be contributing to the high susceptibility of dusky gopher frogs and boreal toads to ranavirus. Dusky gopher frogs have experienced a strong population bottleneck resulting in low genetic diversity and high inbreeding (Richter et al. 2009). Boreal toads have lower allelic richness and observed heterozygosity in the Colorado and Utah populations, the candidate population segment, than other portions of the range (Switzer et al. 2009). Low genetic diversity may account for the high mortality of boreal toads in this study compared to western toad (*Anaxyrus boreas*) tadpoles from California, USA, which had only 5 % mortality when exposed to the same FV3-like isolate (Brenes 2013). However, it is unclear whether recent declines in genetic diversity have increased the susceptibility of these two species or if they would have been similarly susceptible prior to declines. The dusky gopher frog's sister species, the Carolina gopher frog (*Lithobates capito*), is also highly susceptible to ranavirus (85 % larval mortality with exposure to FV3; Hoverman et al. 2011), despite relatively higher levels of genetic diversity (Richter et al. 2009). The type of FV3-like ranavirus used in our study could also have contributed to the high mortality. This ranavirus was

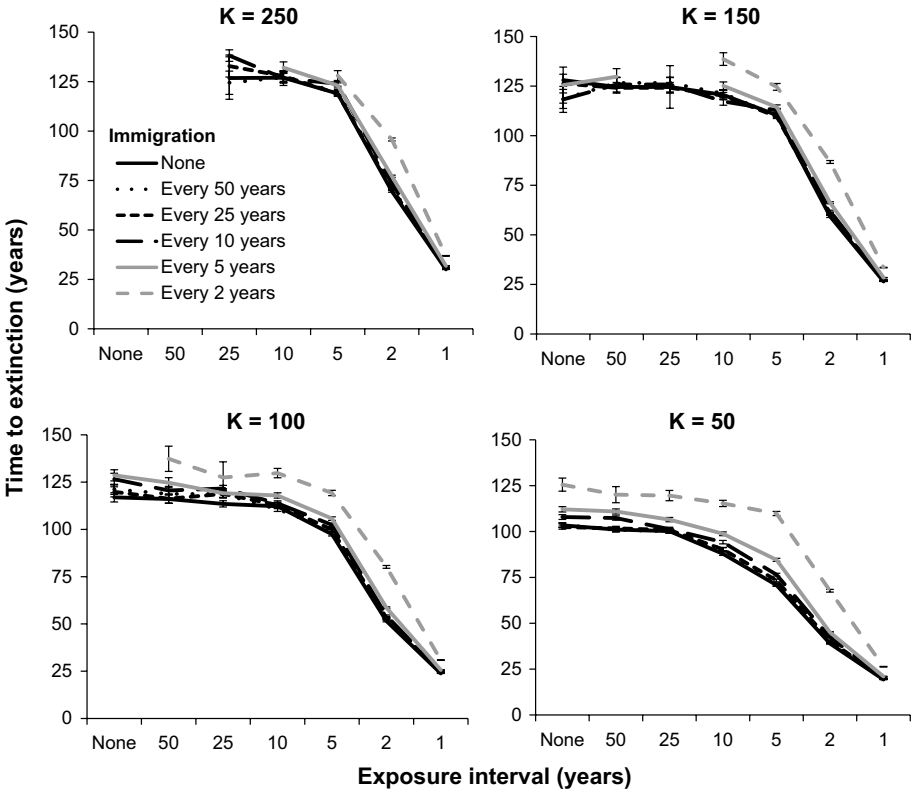

Fig. 4 Effects of the ranavirus exposure interval at the tadpole or metamorph stages, carrying capacity (*K*), and immigration frequency on time to extinction in simulated boreal toad populations ($N = 1{,}000$ simulations/scenario). Immigration always consisted of the introduction of one breeding female. *Error bars* represent standard error. Note that data points that appear missing on the graphs are for conditions where extinction never occurred in all of the simulations; there is, subsequently, no estimate of time to extinction

isolated in a ranaculture facility (i.e., American bullfrog farm) in Georgia, USA, and hence it may have represented a novel strain to the species we tested. Given that American bullfrogs are traded internationally and throughout the USA (Schloegel et al. 2009), our results also might represent introduction of a ranavirus facilitated by trade. Emergence of ranavirus, *Ambystoma tigrinum* virus, and *Batrachochytrium salamandrivorans* have been linked to the salamander trade (Storfer et al. 2007; Gray et al. 2015b).

The dusky gopher frog and boreal toad's high ranavirus susceptibility resulted in an increase in extinction probability, decrease in extinction time, and population declines with ranavirus exposure in simulated populations. For dusky gopher frogs, populations went extinct quickly without exposure to ranavirus and, under the worst-case scenarios, went extinct in seven and 12 years with ranavirus exposure in the hatchling, larval, or metamorph stages every 1 or 2 years, respectively. For boreal toads, probabilities of extinction within 150 years increased to 100 % with exposure to ranavirus every 1 or 2 years at all carrying capacities. The extinction probability was also 100 % with exposure every 5 years in smaller populations with a carrying capacity of 50 adult females and immigration every 5 years or less frequently. In the worst-case scenarios for boreal toads, populations went extinct in 21 and 45 years for exposure every 1 or 2 years, respectively, for populations with the smallest carrying capacity examined (50 adult females). These predictions are similar to closed populations of wood frogs (Earl and Gray 2014), which is another species that is highly susceptible to ranavirus.

Although the mortality rate from ranavirus exposure was the same for different life stages, the effects on the population sometimes varied. For dusky gopher frogs, extinction occurred faster with exposure in the hatchling, larval, or metamorph stages than in the adult stage. These results match our estimates of model parameter sensitivity, as would be expected with low sensitivity to adult survival and higher sensitivity to survival in year 1. This longer time to extinction when exposed to ranavirus in the adult stage likely occurred, because in the simulations, we assumed adult females were able to successfully breed prior to mortality by ranavirus. Sutton et al. (2014) reported that 100 % of adult dusky gopher frogs died between 11 and 17 days following exposure to ranavirus in water, which might be sufficient time to successfully breed. We also assumed that only one life stage was exposed to ranavirus in a particular year. However, given the multiple host species of ranavirus and environmental persistence of the virions (Brunner et al. 2015), exposure during more than one developmental stage is likely if individuals survive the first exposure, which was rare for the two species in this study. For the pre-juvenile life stages, robust adaptive immune response to subsequent ranavirus exposures is unlikely (Grayfer et al. 2015). Moreover, exposure to ranavirus during more than one life stage

can increase the likelihood of mortality compared to exposure only once (Echaubard et al. 2016). We were unable to test egg, hatchling, and adult stages for boreal toads; however, considering the strong correlation of high susceptibility among all stages except the egg for the dusky gopher frog, we would expect that hatchling and adult stages of boreal toads would be very susceptible as well. Thus, our simulations may represent optimistic scenarios.

Strong dispersal connectivity among populations might mitigate the effects of repeated disease-induced die-offs. A wood frog metapopulation in North Carolina, USA was found to persist despite frequent and high levels of ranavirus-induced mortality (Petranka et al. 2007). Immigration from nearby subpopulations not experiencing die-offs from ranavirus was hypothesized to constitute a rescue effect (Petranka et al. 2007). However, in our simulations, immigration at natural rates did not buffer boreal toad populations from the effects of ranavirus exposure unless it occurred every 2 years. In most cases, immigration frequencies of none to every 5 years did not substantially change extinction risk, time to extinction, or adult population size for populations not experiencing extinction. This result is consistent with theoretical models which suggest that higher connectivity within metapopulations will help buffer endangered species from the negative effects of disease (Gog et al. 2002; McCallum and Dobson 2002). In our simulations, each immigration event consisted of only one breeding adult female. Carey et al. (2005) suggested that most boreal toad populations are likely demographically and genetically isolated. Our results show that the low levels of dispersal connectivity typical for boreal toad populations, and likely other rare amphibian species, probably are insufficient to buffer against extinction if populations are subjected to high mortality events from stressors, such as ranavirus. Increased connectivity among subpopulations can increase metapopulation persistence in a variety of host–pathogen scenarios, including those with reservoir hosts (Gog et al. 2002; McCallum and Dobson 2002). Hence, the effects of connectivity on ranavirus disease outcomes might depend on dispersal rates, susceptibility of host species present, and pathogen exposure interval, which could be affected by the presence of reservoir hosts (i.e., subclinically infected carriers, McCallum and Dobson 2002). Inasmuch as ranavirus can infect multiple host species in three vertebrate classes (Duffus et al. 2015), estimating transmission dynamics of ranavirus in host metacommunities may be necessary to truly understand the effects of immigration. It is also possible that immigration of subclinically infected hosts could increase the probability of ranavirus outbreaks through multiple ranavirus reintroductions.

We further examined whether supplementing populations that have experienced ranavirus die-offs with captive-reared adult females could mitigate declines and prevent extinction. We found that this conservation strategy could potentially be useful when ranavirus exposure interval occurred rarely or with moderate frequency. When ranavirus exposure occurred very frequently (every 2–5 years), the introduction of captive-reared individuals would also have to occur very frequently (every 2 years) or involve large numbers of individuals (i.e., >50). For this analysis, we also assumed that wild and captive-reared females had the same survival and fecundity, which may not be the case. Previous work has shown that captive-reared boreal toad females lay egg masses with lower hatching success than wild boreal toads (Muths et al. 2014). If future work can estimate how equivalent wild and captive-reared individuals are in survival and reproduction, these estimates could be used to adjust our analyses. Our estimates were meant as a starting point to determine how to mitigate the severe population-level effects predicted from ranavirus exposure, and probably represent best-case scenarios. For real-conservation actions, simulations should be run to compare different available management options (e.g., Woodhams et al. 2011). For example, examining the effects of releasing other life stages, such as eggs or juveniles, could be considered. It may also be helpful to include a financial assessment to determine which actions would be most feasible given budget constraints. Gray et al. (2015a) provide some suggestions for performing a risk analysis for ranavirus introduction.

Our model results suggest that ranavirus represents a high risk for dusky gopher frog and boreal toad populations. Several assumptions were made during our simulations that likely resulted in conservative extinction outcomes. First, we set the extinction threshold very low, such that there had to be zero remaining individuals in all life stages for extinction to occur. Other studies frequently set the threshold just for adult females (e.g., Harper et al. 2008). Second, the initial population sizes represented the most robust years on record, which reduces the extinction probability and extends the time to extinction. In addition, as discussed, we assumed that ranavirus exposure occurred only during one developmental stage per annual cycle, which probably is unrealistic. All of these conditions result in more optimistic extinction scenarios.

We also made some assumptions that could decrease extinction probability. First, we assumed that the laboratory trials resulted in a similar mortality, as would be seen in the wild, and we treated survival with exposure to ranavirus as fixed. It is likely that there is some variability in survival with ranavirus exposure under the natural conditions. Natural conditions may allow for some behaviors or provide certain microhabitats that would increase individuals' ability to tolerate or clear infections. We also assumed that all individuals in the population were exposed to at least

10^3 PFU/mL of ranavirus. This environmental concentration of shed virus was estimated from a larval salamander in captivity with ranaviral disease (Rojas et al. 2005) and is known to result in infection and disease in multiple amphibian hosts (Hoverman et al. 2011). A recent study documented the concentration of ranavirus in the wild can exceed 10^3 PFU/mL during an outbreak (Hall et al. 2016). It is likely that ranavirus outbreaks initiate through direct contact of infected and uninfected individuals, with shed virions from infected and dead individuals playing a greater role as epidemics progress and ranavirus lethal dose (LD) concentrations are exceeded (Brunner et al. 2015). Our stage-structured model predictions could be improved by adding parameters for transmission and LD_{50} estimates for ranavirus (Gray et al. 2015a), which have not been estimated for the species we studied.

Our simulations also assume implementation of no disease intervention or conservation strategies. For the dusky gopher frogs, many different conservation actions (e.g., annual release of juveniles raised from cattle tanks) have been attempted that have increased the success of the Glen's pond population, and there is at least one additional population that may be viable due to repatriation efforts (e.g., Seigel et al. 2006). The data used to parameterize our model were from the years prior to these manipulations (Richter and Seigel 2002; Richter et al. 2003), and thus are most likely to represent the population dynamics without interventions by managers.

Our results suggest that ranavirus has the potential to cause local and global extinction if introduced into populations of highly susceptible host species. Risk of extinction from ranavirus exposure was not mitigated by low levels of immigration, suggesting that in some cases, the fate of open and closed populations may be similar. As such, preventing the introduction of ranavirus and other novel pathogens should be a priority and a component of species conservation plans. With species where risk is very high, as with the dusky gopher frog, it would be prudent to restrict public access to breeding ponds to minimize the likelihood of ranavirus introduction. Given our results, determining whether ranavirus is present may be a useful criterion when evaluating potential reintroduction sites for highly susceptible host species.

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Author contribution statement JEE and MJG conceived the study and led analyses and manuscript writing, JCC, WBS, and CEL performed the laboratory experiments, AJK, CL, and JK propagated the animals for the experiments, RPW replicated and titrated the virus, RDH and DLM lead the qPCR and histopathology, and all authors contributed to manuscript revision.

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