

High susceptibility of the endangered dusky gopher frog to ranavirus

William B. Sutton^{1,2,*}, Matthew J. Gray¹, Rebecca H. Hardman¹, Rebecca P. Wilkes³,
Andrew J. Kouba⁴, Debra L. Miller^{1,3}

¹Center for Wildlife Health, Department of Forestry, Wildlife and Fisheries, University of Tennessee, Knoxville, TN 37996, USA

²Department of Agricultural and Environmental Sciences, Tennessee State University, Nashville, TN 37209, USA

³Department of Biomedical and Diagnostic Sciences, University of Tennessee Center of Veterinary Medicine, University of Tennessee, Knoxville, TN 37996, USA

⁴Memphis Zoo, Conservation and Research Department, Memphis, TN 38112, USA

ABSTRACT: Amphibians are one of the most imperiled vertebrate groups, with pathogens playing a role in the decline of some species. Rare species are particularly vulnerable to extinction because populations are often isolated and exist at low abundance. The potential impact of pathogens on rare amphibian species has seldom been investigated. The dusky gopher frog *Lithobates sevosus* is one of the most endangered amphibian species in North America, with 100–200 individuals remaining in the wild. Our goal was to determine whether adult *L. sevosus* were susceptible to ranavirus, a pathogen responsible for amphibian die-offs worldwide. We tested the relative susceptibility of adult *L. sevosus* to ranavirus (10^3 plaque-forming units) isolated from a morbid bullfrog via 3 routes of exposure: intra-coelomic (IC) injection, oral (OR) inoculation, and water bath (WB) exposure. We observed 100% mortality of adult *L. sevosus* in the IC and WB treatments after 10 and 19 d, respectively. Ninety-five percent mortality occurred in the OR treatment over the 28 d evaluation period. No mortality was observed in the control treatment after 28 d. Our results indicate that *L. sevosus* is susceptible to ranavirus, and if adults in the wild are exposed to this pathogen, significant mortality could occur. Additionally, our study demonstrates that some adult amphibian species can be very susceptible to ranavirus, which has been often overlooked in North American studies. We recommend that conservation planners consider testing the susceptibility of rare amphibian species to ranavirus and that the adult age class is included in future challenge experiments.

KEY WORDS: Anuran · Histopathology · Iridovirus · Ranidae

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INTRODUCTION

Amphibians are one of the most diverse vertebrate faunas worldwide, and based on recent estimates, possess the greatest proportion of species threatened with extinction (Stuart et al. 2004, IUCN 2013). It has been realized that pathogens can contribute to amphibian declines and may play a role in population extirpation and species extinction (Teacher et al. 2010, Vredenburg et al. 2010). Rana-

viruses are a group of double-stranded DNA viruses that are responsible for amphibian die-offs across the globe (Miller et al. 2011) and have contributed to declines of at least one species, the common frog *Rana temporaria* (Teacher et al. 2010). Amphibian species with limited geographic distributions tend to be more susceptible to ranavirus (Hoverman et al. 2011), which may be a consequence of the effects of genetic isolation on immune function (Pearman & Garner 2005). Thus,

rare amphibian species may be particularly susceptible to ranavirus (Gray et al. 2009).

The dusky gopher frog *Lithobates sevosus* is one of the most endangered amphibian species in North America. The historical distribution of *L. sevosus* included southwestern Alabama, Mississippi, and southeastern Louisiana, USA, but has been reduced to only a few known populations (ca. 100–200 breeding adults) in southern Mississippi (Richter et al. 2003, 2009). The IUCN Red List of Threatened Species classifies *L. sevosus* as Critically Endangered (Hammerson et al. 2004). *L. sevosus* and the closely related gopher frog *L. capito* are highly terrestrial species that commonly use gopher tortoise *Gopherus polyphemus* burrows and other subterranean habitats including stumpholes and abandoned small mammal burrows (Humphries & Sisson 2012, Tupy 2012), which likely reduce risks associated with predation or desiccation (Roznik & Johnson 2009). Periodic disturbance events (e.g. fire and wind disturbances) are necessary to maintain open canopy, early successional habitats for gopher frog breeding habitats. These conditions are essential for larvae to metamorphose from breeding ponds (Thurgate & Pechmann 2007).

Primary conservation concerns for *L. sevosus* communicated in the Gopher Frog Federal Recovery Plan include long-term impacts of limited genetic diversity, anthropogenic habitat alteration, and the effects of amphibian pathogens (USFWS 2002). Given the paucity of published data on the susceptibility of *L. sevosus* to pathogens and the known pathogenicity of ranaviruses to amphibians (Hoverman et al. 2011), we tested whether ranavirus could cause infection and mortality. Additionally, we performed histopathology on experimental individuals to obtain a better understanding of how ranavirus exposure causes morbidity in amphibians. Overall, our study provides an important case study for understanding the role that amphibian pathogens may play in extinction and guiding repatriation activities for rare amphibian species.

MATERIALS AND METHODS

We performed this experiment in a laboratory environment at the Johnson Animal Research and Teaching Unit at the University of Tennessee. We acquired 74 adult, captive-bred *Lithobates sevosus* from an assurance colony maintained by the Omaha Henry Doorly Zoo in Lincoln, Nebraska, USA. We should note that our study used extraneous individuals from

the *L. sevosus* breeding program that were destined for euthanasia. Zoos commonly cull portions of breeding programs without research application, thus our experiment made use of culled individuals and evaluated susceptibility to an emerging pathogen. Prior to the experiment, we randomly selected 2 individuals and used quantitative PCR (qPCR) to verify they were negative for ranavirus infection. We maintained frogs communally in 60 l plastic containers at a density of 7–8 frogs per container for 10 d to allow frogs to acclimate to laboratory conditions.

We randomly assigned 18 individuals to each of 3 ranavirus exposure routes (intracoelomic [IC], oral [OR], and water bath [WB]), and a matching control [C] group. The control group consisted of 18 total individuals allocated in groups of 6 individuals each to an IC, OR, and WB treatment. Control treatments were performed exactly the same as experimental treatments (described below), but only administering Eagle's minimum essential medium without virus. Prior to experimental inoculation, we obtained mass (g) and snout-vent length (mm) measurements of each study individual. Each ranavirus treatment consisted of a ranavirus dose (10^3 plaque forming units [PFUs] ml^{-1}) reported as the viral concentration in water shed by an infected salamander larvae in captivity (Rojas et al. 2005). We evaluated susceptibility of *L. sevosus* to a frog virus 3 (FV3)-like ranavirus isolated from a morbid American bullfrog (*L. catesbeianus*; Miller et al. 2007). We administered IC treatments by inserting a 0.5 ml syringe in the coelomic cavity near the distal end of the ventral surface of each experimental individual. Similarly, OR treatments were administered by inserting a 5 ml pipette tip into the oral cavity of each individual to ensure that the viral dose was swallowed. For the WB treatment, we exposed each individual singly in a 2 l tub with 400 ml of water to a final 10^3 PFUs ml^{-1} of virus for 3 d based on previous WB challenge experiments (Hoverman et al. 2010, 2011). This volume of water was enough to fully submerge the ventral and dorsal surface of most individuals. We treated all animals on the same day, but we administered control treatments first to prevent cross-contamination from the viral treatments. After treatments were administered, each frog was placed in a covered 12 l rectangular clear plastic container to complete the 28 d experimental exposure, similar to the methods used by Schock et al. (2008). We cleaned and sanitized containers every 3 d using 2% Nolvasan (Fort Dodge Animal Health; Bryan et al. 2009). After cleaning, we added 200 ml of aged water and provided each frog with 2–3 adult crickets. To provide frogs with access

to both dry and wet environments, we elevated one end of the container to allow water to pool at the lower end of the container.

We observed animals twice daily for gross signs of ranaviral disease (e.g. lesions, petechial hemorrhaging, loss of appetite, lethargy; Miller et al. 2011). If an animal presented gross signs for greater than 24 h, we declared it as a mortality event and the individual was euthanized humanely according to University of Tennessee IACUC protocol no. 2140 via WB exposure to benzocaine hydrochloride as described in Burton et al. (2008). From our previous experience during more than 50 challenge experiments with different amphibian species (Hoverman et al. 2010, 2011, Haislip et al. 2011, Brenes 2013), our point of euthanasia was indicative of imminent death and justified to avoid animal suffering. We are confident in our assessment of euthanasia serving as a proxy for true mortality (i.e. conditional mortality) in that we witnessed mortality of multiple (>20) study individuals prior to euthanasia. These mortality events occurred within 24 h of recording gross signs of ranavirus infection. All surviving individuals were likewise euthanized at the end of the 28 d experiment. Euthanized individuals were necropsied and gross lesions were noted. We collected sections of liver and kidney for qPCR to verify ranavirus infection. Sections of heart, liver, lung, gall bladder, stomach, large intestine, small intestine, spleen, kidney and gross lesions (e.g. ulcerations and hemorrhages of the skin and hemorrhages on tongue) were collected and stored in 10% formalin for later histopathological examination.

Diagnostic testing

To quantify ranavirus infection, we prepared a homogenate of the liver and kidney tissue from each individual. We chose liver and kidney as diagnostic tissues because these organs are targeted by ranavirus and provide reliable indication of infection (Robert et al. 2005, Green et al. 2009, Miller et al. 2009). We extracted DNA from each homogenate using a DNeasy Blood and Tissue Kit (Qiagen). Prior to qPCR analysis, we eluted 100 μ l of the extracted DNA and quantified the amount of DNA present in each sample. We used a model ABI 7900HT Fast Real-Time PCR System (Life Technologies) to test samples for ranavirus DNA using PCR primers amplifying an ~500 bp region of the ranavirus major capsid protein using primers 4 and 5 as described in Picco et

al. (2007). We considered a sample infected if the qPCR cycle threshold (CT) value was less than 30 based on standardized optimization with known quantities of ranavirus. For each qPCR analysis, we ran each extracted DNA sample in duplicate along with 2 positive controls (i.e. positive viral DNA and viral DNA from a ranavirus-positive amphibian) and 2 negative controls (i.e. DNA from a ranavirus negative amphibian and a sample containing only molecular grade water) and report mean (\pm SE) CT values for each treatment.

We processed a random sample of 6 individuals per treatment for histopathology. Formalin-fixed tissues were processed according to standard procedures, embedded in paraffin, sectioned at 5 μ m, placed onto glass slides, cover-slipped, and examined with light microscopy. For the purpose of this study, images that displayed representative diagnostic signs of morbidity were provided as illustrations and supportive evidence of ranaviral disease.

Statistical analysis

We used Kaplan-Meier analysis (PROC LIFETEST) in SAS version 9.3 to test for differences in conditional mortality rates of *L. sevosus* among ranavirus exposure treatments. In addition to ranavirus treatments, we tested the effect of body condition as a proxy of individual fitness on conditional mortality rates (Schulte-Hostedde et al. 2005). We estimated body condition as the standardized residuals produced by regressing mass by snout-vent length measurements obtained from each individual prior to study implementation (Schulte-Hostedde et al. 2005). We used a Tukey test for post-hoc comparisons and declared statistical significance at $\alpha = 0.05$.

RESULTS

Overall, we observed 98.1% conditional mortality of *Lithobates sevosus* that were administered a ranavirus treatment. Survival differed among ranavirus exposure routes ($\chi^2 = 99.8$, $p < 0.001$), with the IC route resulting in the fastest conditional mortality (Fig. 1). Conditional mortality rates were similar between OR and WB treatments ($\chi^2 = 0.19$, $p = 0.97$; Fig. 1). By 18 d into the experiment, we observed 100% conditional mortality in the WB treatment and 94% in the OR treatment (Fig. 1). Mean time (d) to mortality was lower in the IC treatment (8.61 ± 0.20 d) compared with both the OR (13.72 ± 0.90 d)

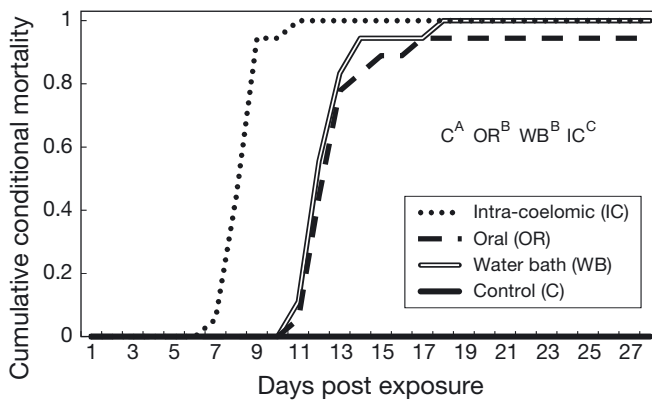


Fig. 1. *Lithobates sevosus*. Conditional mortality of adult dusky gopher frogs to 3 ranavirus exposure treatments (oral exposure [dashed line], intra-coelomic exposure [stippled line], and water bath exposure [hollow line]) and a matching control treatment (solid line) over a 28 d experimental period ($n = 18$ individuals per treatment). Mortality was 'conditional' because individuals with advanced signs of disease were euthanized prior to death to alleviate suffering. Different superscript letters above treatment designations indicate significant differences based on post-hoc comparisons

and WB (12.72 ± 0.37 d) treatments. Across all ranavirus exposure routes, only one individual in the OR treatment group survived the 28 d experimental period. We observed 100% survival of control individuals (Fig. 1) and did not detect an effect of body condition on conditional mortality ($\chi^2 = 0.54$, $p = 0.46$).

Gross and microscopic lesions of ranaviral disease were prominent (approximately 60% of morbid individuals). Grossly, blood vessels were diffusely congested throughout the body (Fig. 2A). Petechial haemorrhaging and ecchymosis were noted within the oral cavity (predominantly on the tongue; Fig. 2B) and occasionally on the serosa of the organs within the coelomic cavity (e.g. intestines, spleen; Fig. 2A). Erythema with occasional petechia were also prevalent (approximately 75% of morbid individuals) in the skin (Fig. 2C), primarily on the medial thighs and hind feet, and occasionally extended into the underlying skeletal muscle. Swelling (edema) and rare ulcerations were noted in the limbs, primarily the feet (Fig. 2D). Histopathological changes were generally severe. The most severe and consistent finding was massive splenic necrosis (Fig. 3A).

Vascular congestion was noted in all tissues and hemorrhages were often observed, primarily in the tongue (Fig. 3B). The liver was characterized by diffuse hepatocellular degeneration and occasionally foci of necrosis (Fig. 3C). Necrosis was randomly present within the kidneys and did not appear to be targeting a particular structure or cell type. Vascular necrosis was noted but was best viewed in areas that were least affected (Fig. 3D). Intracytoplasmic inclusion bodies were seen in multiple tissues but were most easily observed within the tongue (Fig. 3B inset).

We used qPCR as an additional indicator that ranavirus was the causative agent of morbidity and conditional mortality in our study. All morbid and deceased individuals were infected with ranavirus. We observed similar CT scores ($F_{2,51} = 0.27$, $p = 0.76$) among the 3 ranavirus treatments (IC, 13.58 ± 0.01 ; OR, 13.93 ± 0.06 ; and WB, 13.34 ± 0.06). For our qPCR system, these mean CT scores indicated high viral loads of approximately 10^5 PFUs per $0.25 \mu\text{g}$ of gDNA based on extrapolations from a ranavirus standard curve. Although the IC route caused conditional mortality 4 d sooner than the OR and WB routes, the viral load in deceased frogs was similar regardless of exposure route. The viral load for the one individual that survived in the OR treatment was 10^1 PFUs per $0.25 \mu\text{g}$ of gDNA.

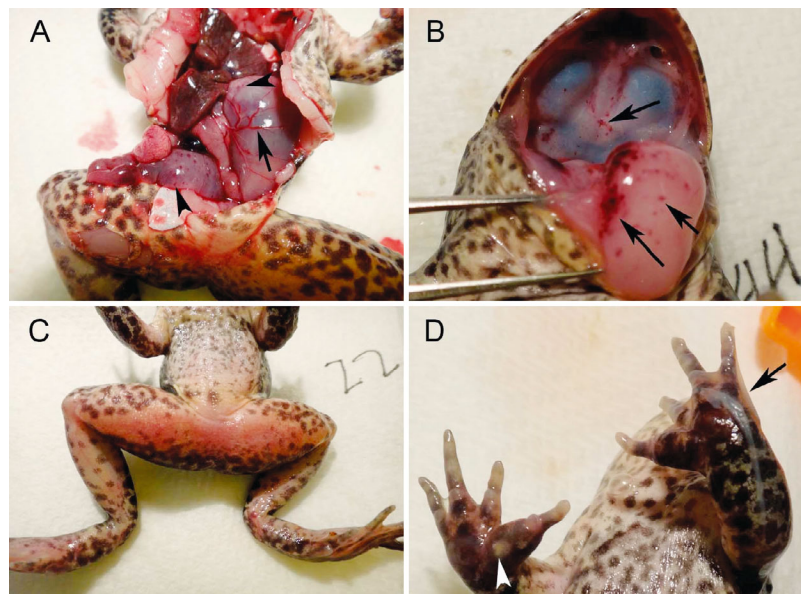


Fig. 2. *Lithobates sevosus*. Gross lesions observed in adult dusky gopher frogs experimentally infected with ranavirus. (A) Blood vessels were diffusely congested throughout the body (arrow) and petechia (arrowheads) were occasionally noted. (B) Hemorrhages (arrows) were observed in the oral cavity, especially on the tongue. (C) The thighs were diffusely erythemic on the medial aspect. (D) The limbs were often swollen and fluid (edema) could be discerned beneath the skin (arrow); ulcers (arrowhead) were rarely seen

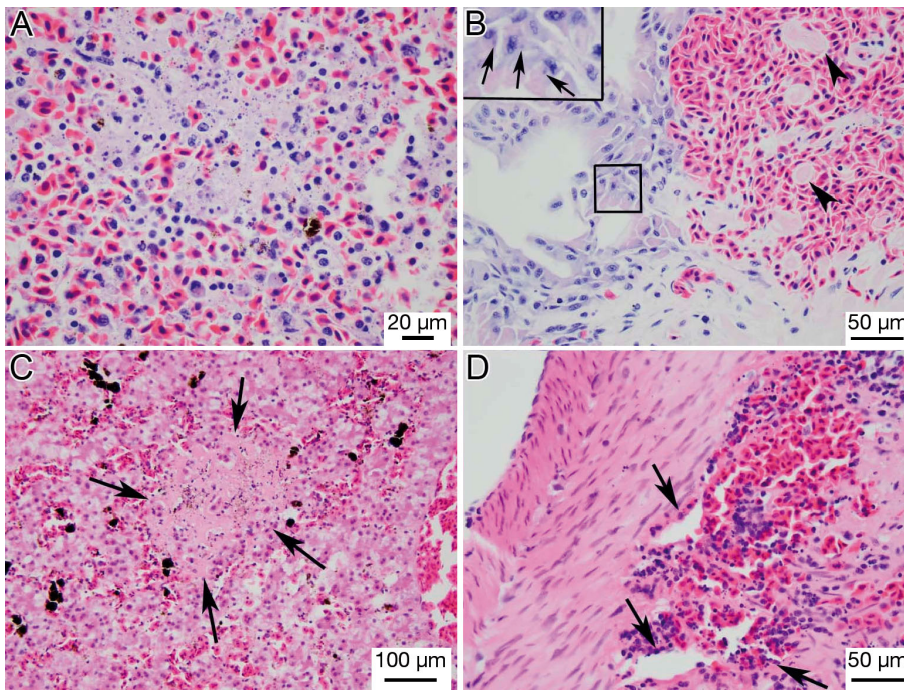


Fig. 3. *Lithobates sevosus*. Microscopic lesions observed in adult dusky gopher frogs experimentally infected with ranavirus. (A) The spleen was the most dramatic lesion with diffuse necrosis noted in all frogs. (B) Hemorrhage was observed within the muscle (arrowheads) of the tongue, and intracytoplasmic inclusion bodies (arrows) were seen within the epithelial cells of the mucosa. Inset in (B) shows higher magnification of outlined area. (C) Rare areas of necrosis (arrows) were observed within the liver. (D) Vascular (arrows) and perivascular necrosis were best discerned in tissues that were less affected, such as in the muscle wall of this section of intestine

DISCUSSION

Adult *Lithobates sevosus* were highly susceptible to ranavirus, with almost 100% conditional mortality regardless of ranavirus exposure route. Specifically, adult conditional mortality was 100% when exposed to ranavirus in a water bath, which is the highest level of mortality reported to date for any amphibian species during a WB challenge experiment. Unfortunately, few studies have evaluated adult anuran susceptibility to ranaviruses via WB exposure, which makes it difficult to compare relative susceptibility across species groups. Cullen & Owens (2002) observed mortality in 1 out of 3 adult ornate nursery frogs *Cophixalus ornatus* exposed to Bohle iridovirus via WB exposure. Of the 19 species of larval amphibians tested by Hoverman et al. (2011) with the same isolate and viral concentration as our study, no species experienced 100% mortality when exposed to ranavirus in water. Experimental inoculations of adult amphibians have often resulted in no response to ranavirus exposure or only mild signs of infection (Clark et al. 1968, Daszak et al. 1999, Gantress et al. 2003). However, Cunningham et al. (2007) and Picco et al. (2007) reported high susceptibility of *Rana temporaria* and tiger salamanders *Ambystoma tigrinum* to ranavirus via IC injection and acknowledged that exposure via environmentally relevant routes (e.g. WB) are necessary to gain a realistic understanding

of the effects that ranaviruses may have on adult amphibians in nature. Our results provide evidence that ranavirus can infect adult *L. sevosus* and potentially cause die-offs and accelerate declines in remaining populations.

The high susceptibility of *L. sevosus* to ranavirus may be a consequence of reduced genetic diversity. Pearman & Garner (2005) found that isolated populations of the Italian agile frog *R. latastei* were more susceptible to ranavirus than mixed populations that had greater heterozygosity. The historical distribution of *L. sevosus*, which previously included southwestern Alabama, southern Mississippi, and southeastern Louisiana, has been reduced to 2 isolated populations in close proximity to the De Soto National Forest in Mississippi. Richter et al. (2009) found considerably lower genetic variation and a strong signature of population bottleneck in *L. sevosus* when compared with the ecologically similar gopher frog *L. capito* and crawfish frog *L. areolatus*. Hoverman et al. (2011) reported that amphibian species with smaller geographic ranges generally were more susceptible to ranavirus than species with larger distributions. Experimental individuals examined in our study were bred from an assurance colony at the Omaha Zoo, with the founders of this colony sourced from the largest remaining historical *L. sevosus* population (i.e. Glen's Pond) in southern Mississippi. Therefore, individuals in our study likely possessed

genetic diversity similar to the remaining natural populations of *L. sevosus*.

Our study also demonstrated that the adult age class could experience high mortality when exposed to ranavirus, which has been reported infrequently. It is generally assumed that susceptibility to ranavirus increases throughout larval development, is highest during metamorphosis, and decreases post-metamorphosis (Chen & Robert 2011). These conclusions were developed based on experimental exposure studies of the African clawed frog *Xenopus laevis* (Robert et al. 2005), and correlate with the process of downregulation of immune function during metamorphosis (Rollins-Smith & Blair 1993, Warne et al. 2011). However, Haislip et al. (2011) found that amphibian mortality due to ranavirus varied by both life stage and species for 4 North American amphibian species. Die-offs of adult amphibians from ranavirus have been reported in Europe (Teacher et al. 2010, Kik et al. 2011), but infrequently reported in the USA (Green et al. 2002). Future experimental challenge studies should include adults, especially for uncommon species.

In our study, the OR and WB exposure treatments were environmentally relevant transmission routes for ranavirus (Gray et al. 2009), and represented the potential of *L. sevosus* to become infected after consuming infected prey or breeding at a site with virus shed by infected syntopic hosts. We did not detect a biologically relevant difference between these 2 treatments, which indicates that ranavirus has the potential to cause mortality of *L. sevosus* through multiple exposure routes. The exposure concentration (10^3 PFUs ml⁻¹) that we used during this study was reported as the viral concentration in water shed by an infected salamander larvae in captivity (Rojas et al. 2005) and is the best estimate to date of an environmentally relevant dose during a die-off (Gray et al. 2009).

To date, ranavirus has not been detected in wild *L. sevosus* populations; however, surveillance for the pathogen has not occurred. The presence of ranavirus in aquatic habitats may be linked with the permanence of water in aquatic breeding habitats. For example, ponds with relatively longer hydrology (i.e. semi-permanent and permanent ponds) tend to have a relatively high prevalence of ranavirus across multiple years compared with ponds that fill and dry completely (ephemeral ponds) in a given year (Hoverman et al. 2012). This trend may be related to the period of time that ranavirus can remain biologically active outside the host in aquatic environments. Nazir et al. (2012) found that ranaviruses can remain

active (i.e. time required for 90% reduction in virus titers) for 58–72 d in non-sterile pond water. Because *L. sevosus* breed in ephemeral habitats that dry each year, the risk of ranavirus becoming established may be lower than permanent and semi-permanent breeding habitats. However, an ephemeral pond (Gourley Pond) in the Great Smoky Mountains National Park has experienced re-occurring die-offs of wood frogs *L. sylvaticus* and spotted salamanders *Ambystoma maculatum* throughout the past decade (Green et al. 2002, Todd-Thompson 2010). Petranksa et al. (2007) also reported re-occurring die-offs of ephemeral pond-breeding amphibians at constructed wetland sites in North Carolina, USA. Sympatric species also may play an important role in introducing and maintaining ranavirus in amphibian communities (Gray & Miller 2013). For example, the southern toad *Anaxyrus terrestris* and ornate chorus frog *Pseudacris ornata* are sympatric with *L. sevosus*, and these species are susceptible to ranavirus (Brenes 2013). Collectively, these findings suggest that introduction of ranavirus into the remaining populations of *L. sevosus* is possible.

Efforts for long-term conservation of rare amphibians, such as *L. sevosus*, should include an appropriate disease management strategy with decontamination procedures. A disinfectant solution of $\geq 0.75\%$ chlorhexadine diacetate (i.e. Nolvasan) or $\geq 3\%$ bleach is effective at inactivating ranaviruses and should be used before and after sampling events to prevent translocation of pathogens (Bryan et al. 2009). Considering the high susceptibility of adult *L. sevosus* to ranavirus, it is important to understand the susceptibility of other life stages (e.g. egg, larvae, metamorph) to ranavirus (e.g. Haislip et al. 2011). These data will provide information to properly guide reintroduction, repatriation, and population augmentation efforts. Collectively, these efforts are essential to guide long-term conservation of threatened and declining amphibian species, including *L. sevosus*.

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*Editorial responsibility: Lee Skerratt,
Townsville, Queensland, Australia*

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