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Original Contribution

Batrachochytrium dendrobatidis and the Decline and Survival of the Relict Leopard Frog

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Abstract: Epizootic disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) is a major driver of amphibian declines, yet many amphibians declined before the pathogen was described. The Relict Leopard Frog, *Rana onca* (=*Lithobates onca*), was nearly extinct, with the exception of populations within a few geothermal springs. Growth of *Bd*, however, is limited by high water temperature, and geothermal springs may have provided refuge during outbreaks of chytridiomycosis. We conducted field surveys and laboratory experiments to assess the susceptibility of *R. onca* to *Bd*. In the field, we found *Bd* at one of the two areas where remnant populations of *R. onca* still occur, but not in the other. In the laboratory, we infected juvenile frogs from these two areas with two hypervirulent *Bd* isolates associated with declines in other ranid species. In our experiments, these *Bd* isolates did not affect survivorship of *R. onca* and most infections (64%) were cleared by the end of the experiments. We propose that *R. onca* either has inherent resistance to *Bd* or has recently evolved such resistance. These results may be important for conservation efforts aimed at establishing new populations of *R. onca* across a landscape where *Bd* exists. Resistance, however, varies among life stages, and we also did not assess *Bd* from the local environment. We caution that the resistance we observed for young frogs under laboratory conditions may not translate to the situation for *R. onca* in the wild.

Keywords: *Rana onca*, *Lithobates onca*, *Batrachochytrium dendrobatidis*, Chytridiomycosis, Environmental refuge

INTRODUCTION

Greater than 40% of amphibian species worldwide are estimated to be declining, with some already having gone extinct (Stuart et al. 2004). This amphibian crisis may foreshadow a potential sixth mass extinction, with emerging infectious disease as a significant factor in declines (Wake and Vredenburg 2008). The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) causes the amphibian disease chytridiomycosis and is definitively associated with epizootic dynamics and host population collapse (e.g., Lips et al. 2006; Vredenburg et al. 2010). Yet, many amphibian species declined or disappeared before this pathogen was discovered and described (Lips 1998; Berger et al. 1998). Thus, many studies have hypothesized that species that

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declined mysteriously in the decades before this discovery may have succumbed to chytridiomycosis outbreaks following *Bd* invasion (Cheng et al. 2011).

The Relict Leopard Frog, *Rana onca* (=*Lithobates onca*), underwent a dramatic historical decline that has little documentation (Jaeger et al. 2001; Bradford et al. 2004). While the species appears to be a narrow endemic known from southwestern Utah, southern Nevada, and northwestern Arizona (Oláh-Hemmings et al. 2010), by the latter part of the twentieth century its historical range and population size had been greatly reduced (Bradford et al. 2004). By 2001, only about 1100 adult frogs were estimated from a few sites in two general areas of southern Nevada, along the Northshore of Lake Mead within the former Virgin River drainage (Northshore) and within Black Canyon along the Colorado River below Lake Mead (Black Canyon; Figure 1). *Rana onca* is currently of conservation concern and managed under a voluntary conservation agreement and strategy (RLFCT 2016).

The causes for the decline of *R. onca* are not entirely clear, but evidence for *Bd* as a factor is lacking. Bradford et al. (2004) summarized presumed factors including: alteration and loss of habitat caused by agriculture and water development; loss of disturbance regimes that previously maintained more vegetatively open habitats; and introduction of exotic predators and competitors such as the American Bullfrog (*Rana catesbeiana* = *Lithobates catesbeianus*), Red Swamp Crayfish (*Procambarus clarkii*), and various predatory fishes. These authors noted that synergistic interactions among these factors were likely. They did not, however, speculate that disease might have been an additional important factor in the decline of the species.

More recently, Forrest and Schlaepfer (2011) noted that remaining historical populations of *R. onca* were all associated with geothermal springs with source temperatures exceeding 30°C, above the thermal limits for *Bd* growth (Piotrowski et al. 2004). The species, however, was historically known mostly from nonthermal springs, rivers, and wetlands (Bradford et al. 2004). These authors speculated that remnant populations of *R. onca* might persist because of protection from epizootic disease conferred by the elevated water temperatures. Perhaps, historical epizootics of chytridiomycosis contributed to the collapse of *R. onca* populations throughout its range and may still be a limiting factor for current populations.

The presence of *Bd* within the historical range of *R. onca* in the eastern Mojave Desert is not well documented nor is it established whether *R. onca* is susceptible to chytridiomycosis. In regions like the Mojave Desert where seasonal humidity is often low and ambient temperatures often well above those favored by *Bd*, the pathogen is expected to be substantially limited (Piotrowski et al. 2004; Puschendorf et al. 2009; although see Forrest et al. 2015). From a conservation perspective, understanding the susceptibility of *R. onca* to chytridiomycosis is important, because management strategy for the species has focused on establishing new populations across its former range (RLFCT 2016). If *R. onca* is highly susceptible to chytridiomycosis, then the regional presence of *Bd* may greatly limit translocation options to geothermal springs or to isolated sites where *Bd* is not present.

Herein, we provide data on the presence of *Bd* within the historical range and current populations of *R. onca* and describe laboratory experiments that assess the susceptibility of this species to *Bd* infection and chytridiomycosis in the absence of confounding environmental factors. In the susceptibility experiments, we exposed juvenile frogs to two highly virulent isolates of *Bd* associated with severe declines in other ranid species (Vredenburg et al. 2010; Piovia-Scott et al. 2014). The frogs we used originated from two geographically distinct areas (Figure 1) where we had detected *Bd* in *R. onca* (Northshore) and where we had not (Black Canyon). We maintained these two sample groups in the susceptibility experiments under the rationale that frogs from currently exposed populations may have evolved stronger resistance or tolerance to the pathogen than frogs from presumed unexposed populations.

Methods

Field Assessment

Pathogen Sampling

We conducted field surveys over four years (2010–2013) to detect *Bd* on anuran species within a portion of the historical range of *R. onca* in southern Nevada and northwestern Arizona (Figure 1; Table 1). We used a swabbing methodology (e.g., Brem et al. 2007) to sample frogs in the field, applying sterile techniques. We swabbed rear feet, ventral surface of thighs and abdominal surface (5 strokes for each of the 5 surfaces) using a Whatman Omni Swab (GE Healthcare Bio-Sciences, Pittsburg, PA, USA). We

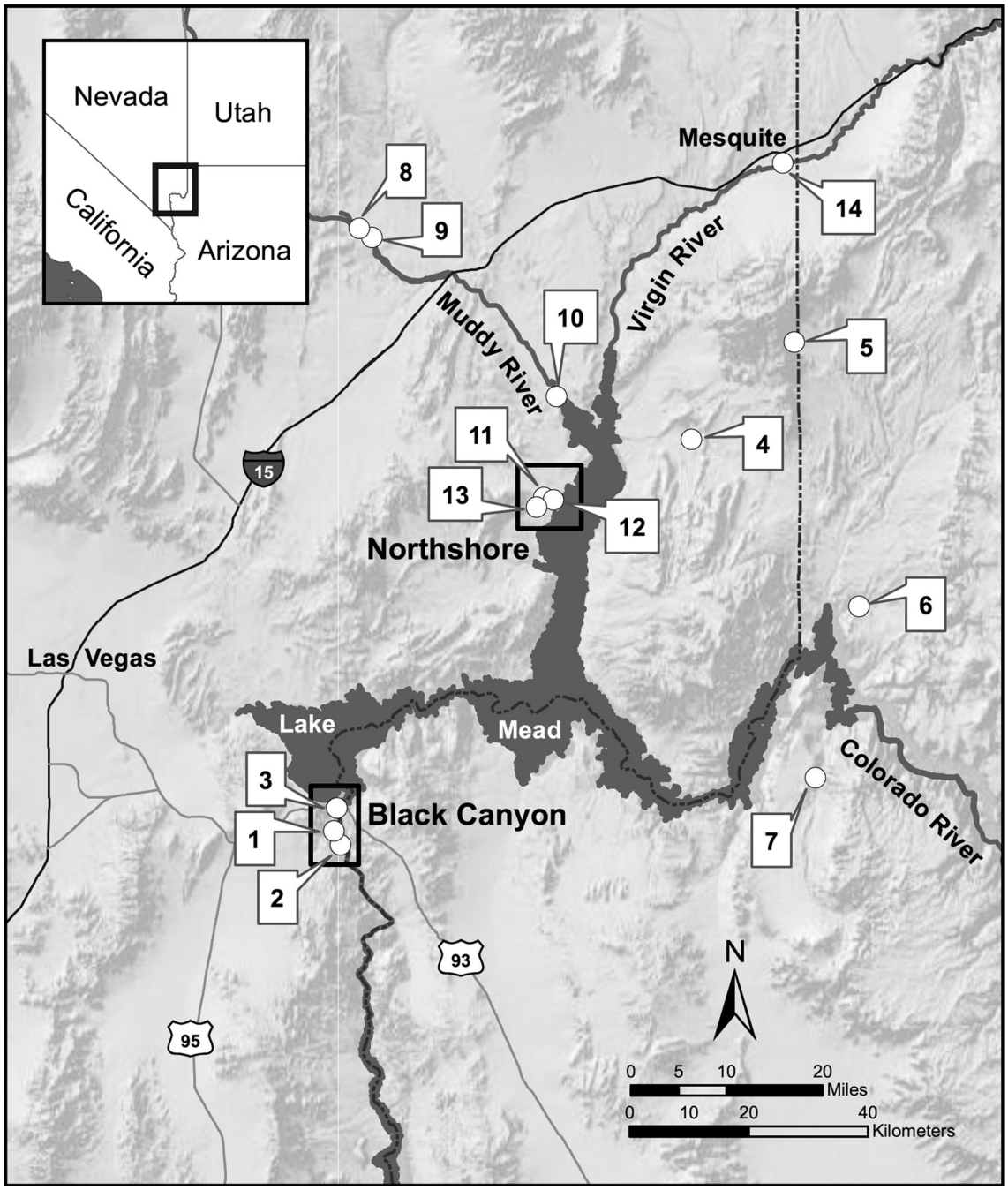

Figure 1. Locations of field sampling conducted for *Batrachochytrium dendrobatidis* within the historical range of *Rana onca*. Map numbers reference Table 1.

sampled at night predominately early in the year when ambient temperatures generally favored *Bd* growth (Piotrowski et al. 2004). Ambient air temperature was recorded at the beginning of capture events, and water temperature measured nearest to the point of each capture. At some sites, *R. onca* were individually marked with PIT tags associated with other monitoring efforts, and at these sites we scanned for tags which allowed observations of *Bd* infections in several individuals over time.

Pathogen Detection

We stored swab tips individually in 70% ethanol at 4°C until assayed at a commercial laboratory (Pisces Molecular,

Table 1. Locations and Species Sampled for *Batrachochytrium dendrobatidis* Within the Historical Range of *Rana onca* in Southern Nevada and Northwestern Arizona.

General area and site	Map no.	Date	T_A	T_W range	Species	Sample size	Proportion positive
Black Canyon, NV							
Boy Scout Canyon	1	05/06/2010	26.7	20.5–31.6	*RAON*	8	0
Black Canyon "Side" Spring	2	03/31/2011	25.0	18.1–22.0	*RAON*	20	0
Pupfish Refuge Spring	3	03/08/2012	18.9	15.6–30.2	*RAON*	30	0
Gold Butte, NV							
Red Rock Spring	4	02/29/2012	15.6	11.9	*RAON*	1	0
		03/22/2012	15.2	12.4–16.4	*ANPU*	9	0
					ANWO	4	0
					RAON	11	0
		03/26/2012	10.8	15.3–15.8	*RAON*	4	0
		03/30/2012	17.0	16.6–17.8	*RAON*	2	0
Virgin Mountains, NV							
Lime Spring	5	03/26/2013	9.6	7.9–12.5	*PSRE*	24	0
Grand Wash, AZ							
Tassi Spring	6	04/03/2010	21.1	18.1–24.5	*RAON*	8	0
		03/16/2011	25.9	18.4–24.6	*RAON*	28	0
Grapevine Mesa, AZ							
Grapevine Spring	7	03/14/2013	18.5	11.8–14.5	*RAON*	24	0
Muddy River, NV							
Muddy River—Warm Springs Natural Area	8	01/09/2011	7.9	11.7–31.3	*RACA*	13	0
		03/16/2011	24.0	17.6–31.0	*RACA*	7	0.29
Perkins Pond	9	02/27/2012	10.2	13.0–13.1	*PSRE*	3	0
		03/15/2012	10.6	N.T.	*PSRE*	2	0
		03/20/2012	11.3	11.1–15.0	*PSRE*	9	0
		03/25/2013	13.3	16.5–23.1	*PSRE*	12	0.25
Overton Wildlife Management Area	10	03/21/2012	12.1	15.7–20.2	*ANWO*	18	0
					PSRE	9	0.11
Northshore, Lake Mead, NV—Virgin River Drainage							
Blue Point Spring, upper	11	03/20/2010	14.7	19.7–29.7	*RAON*	10	0
		03/29/2011	18.2	23.4–29.9	*RAON*	10	0
		07/11/2011	32.3	25.5–29.7	*RAON*	8	0
		02/18/2012	14.0	19.2–27.9	*RAON*	12	0
		03/04/2012	13.4	19.7–28.9	*RAON*	6	0
Blue Point Spring, lower	12	03/21/2010	18.1	17.7–25.0	*RAON*	5	0.80
		03/29/2011	13.6	18.0–25.3	*RAON*	5	0.40
		07/16/2011	22.9	23.7–28.3	*RAON*	13	0
		02/21/2012	12.2	17.7–23.7	*RAON*	8	0.63
		03/07/2012	10.5	17.3–24.2	*RAON*	5	0.60
		03/22/2013	14.5	12.2–24.7	*RAON*	11	0.55

Table 1. continued

General area and site	Map no.	Date	T_A	T_W range	Species	Sample size	Proportion positive
Rogers Spring	13	03/16/2012	23.8	18.0–24.4	*RAON*	8	0
Virgin River, NV							
Mesquite—areas near Riverside Road Bridge	14	03/29/2012	16.0	13.6–15.2	*ANWO*	6	0
					PSRE	1	0
		03/30/2012	20.0	19.5–20.2	*ANWO*	2	0
		03/29/2012	16.0	15.3–20.0	*ANWO*	16	0
					PSRE	2	0
					RACA	2	0
Totals						376	0.07

Map numbers reference Figure 1. Also shown are sample dates, ambient air temperature at beginning of sampling (T_A), and range of water temperatures at points of capture (T_W range). Species designations are as follows: *Anaxyrus punctatus* = *ANPU*, *Anaxyrus Woodhousii* = *ANWO*, *Pseudacris regilla* = *PSRE*, *Rana catesbeiana* = *RACA*, *Rana onca* = *RAON*.

Boulder, CO, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was used to assay a 97 base pair fragment of *Bd* ribosomal RNA internal transcribed spacer 1 (ITS1; Kirshtein et al. 2007); laboratory protocols followed those outlined in Forrest et al. (2015). We pooled up to six samples for initial assays to determine the presence or absence of *Bd*, which allowed us to quickly process large numbers of animals. When *Bd* was detected, we then re-tested each sample individually to determine infection prevalence.

Susceptibility Experiments

Experimental Animals

We obtained juvenile frogs of *R. onca* from a conservation program (RLFCT 2016) where these animals were raised in tanks and raceways from eggs collected each year from wild populations. In the first experiment conducted in 2013, we used 60 frogs that were 15–23 weeks post-metamorphosis derived from three egg masses collected in the wild, one from Northshore and two from Black Canyon. For the second experiment conducted in 2014, we used 54 frogs that were 2–13 weeks post-metamorphosis derived from six egg masses, three collected from Northshore and three from Black Canyon. In both experiments, we divided the frogs into three treatment groups: (1) Northshore frogs exposed to *Bd*, (2) Black Canyon frogs exposed to *Bd*, and (3) an unexposed control group consisting of frogs from both areas (Table 2).

Housing

We conducted the experiments in an environmental chamber, with a 12-h photoperiod. For the first experiment, we maintained the temperature at 22°C, but we lowered the temperature to 19–20°C for the second experiment; these temperatures were well within the thermal preference for *Bd* (Piotrowski et al. 2004) and also acceptable for the frogs. We housed frogs individually in rigid, clear plastic containers with lids (20 cm W × 36 cm L × 12 cm H) containing ~950 ml of aged, dechlorinated tap water. Each container contained a small plastic platform that extended above the water which provided cover and a dry surface area. We transferred frogs weekly along with their platforms to clean (sterilized) containers with fresh water. Frogs were fed ~five juvenile crickets twice weekly; crickets were maintained on commercial cricket food.

Bd Isolates and Exposure

For the first experiment, we used a *Bd* isolate (CJB7) collected in 2009 from *Rana muscosa* during an epizootic at Sixty Lake Basin in the southern Sierra Nevada, California (see Vredenburg et al. 2010). For the second experiment, we used a *Bd* isolate (SLL) collected in 2011 from *Rana cascadae* during a period of high mortality in juvenile frogs at Section Line Lake in the Klamath Mountains, California (Piovia-Scott et al. 2014). Both isolates were identified as part of the highly virulent Bd Global Panzootic Lineage

Table 2. Design of *Batrachochytrium dendrobatidis* (*Bd*) Challenge Experiments on *Rana onca*.

Group	Source area	Experiment 1 No. frogs (no. egg masses)	Experiment 2 No. frogs (no. egg masses)
Group 1 (exposed)	Northshore	20 (1)	18 (3)
Group 2 (exposed)	Black Canyon	20 (2)	18 (3)
Control (not exposed)	Northshore	10 (1)	9 (3)
	Black Canyon	10 (2)	9 (3)

Each experiment consisted of three treatment groups representing frogs from two distinct areas where *Bd* was detected (Northshore) and where it was not (Black Canyon). Shown for each experiment are the number of frogs in each group and the number of egg masses from which the frogs were derived. In general, frogs in each group were drawn proportionately from the associated egg masses.

(*Bd*-GPL1; James et al. 2015; Piovia-Scott et al. 2014; Rosenblum et al. 2013), and under experimental conditions SLL resulted in the death of 95% of exposed *R. cascadae* frogs (Piovia-Scott et al. 2014). Prior to our experiments, both isolates had been cryopreserved and had low passage histories (CJB7 = 14 passages, SLL = 12 passages).

We prepared inoculums from *Bd* stock cultures by plating onto 5-cm plates of TGhL agar (16 g tryptone, 4 g gelatin hydrolysate, 2 g lactose and 10 g agar per liter of distilled water) and incubating at room temperature (23°C) for five days until large numbers of active zoospores were visible under magnification. On the days of animal exposure, we flooded the plates with 2 ml of sterile water, then decanted into a single volume and estimated zoospore density using a hemocytometer. We then exposed each frog to ~1 million zoospores per day over three consecutive days for a total dosage of ~3 million zoospores. The process consisted of holding each frog by the legs and pipetting the liquid containing zoospores (dosage volumes ~1.5–2 ml) onto the ventral groin and thighs, and into the water within its container.

Infection Detection and Intensity

We conducted each experiment for 15 weeks, sampling weekly for *Bd*; frogs were tested for *Bd* prior to experiments to confirm that they were not infected. We systematically swabbed each frog 10 times down each side of the ventral surface from armpits to bottom of thighs, and then five times on the webbing of each foot using a Dryswab (Medical Wire and Equipment, Corsham, Wiltshire, UK) and sterile techniques. We stored swab tips individually in tubes following air-drying, with samples refrigerated (first experiment) or frozen (second experiment) until assayed. In these experiments, we used a common assay based on qRT-PCR that followed the methods of Boyle et al. (2004), also used by Vredenburg et al. (2010) and many others. As described by those authors, we interpreted infection intensity (or *Bd* load) from the qRT-PCR data as the number of *Bd* zoospore equivalents (ZE) per swab by comparing the results of the reactions to known quantities of a *Bd* standard. We recognize, however, that the standard we used may not reflect actual zoospore counts in our isolates because of variable genomic content among *Bd* strains (Longo et al. 2013), but the patterns of infection intensity within experiments should be informative.

Statistical Analyses

We assessed each of the experiments separately (using Prism 6.0f, GraphPad Software Inc., La Jolla, CA, USA). We evaluated the effects of *Bd* treatment and collection site on survivorship using log-rank tests (Mantel–Cox tests). We evaluated infection intensities across the course of experiments using repeated measures two-way analysis of variance (ANOVA), followed by Tukey's post hoc analyses. We confirmed if there were overall differences in infection intensities between collection sites using Wilcoxon sign-rank tests. We used Fisher's exact tests to evaluate effect of collection site on the proportions of frogs that cleared *Bd*, defining clearance as non-detection of *Bd* on the last day of an experiment.

Results

Field Assessment

We sampled a total of 376 anurans representing five species within the historical range of *R. onca*, and detected *Bd* in Pacific treefrog (*Pseudacris regilla* = *Hyliola regilla*), *R. catesbeiana* and *R. onca* (Table 1). We found that *Bd* was

present at several sites within the historical range of *R. onca* (Figure 1). At sites still occupied by historical populations of *R. onca*, we detected *Bd* at the Northshore of Lake Mead, along the former Virgin River, but we did not detect *Bd* in Black Canyon along the Colorado River (earlier unpublished sampling in 2008 and 2009 also failed to detect *Bd* in the canyon).

The Northshore site where *Bd* was detected consists of a geothermally influenced spring and associated stream comprised of 'upper' and 'lower' segments separated by several hundreds of meters where the stream has tunneled underground. Water was hot (30°C at source), particularly along the upper stretch, and heavily laden with minerals, especially gypsum (Bradford et al. 2004). We did not detect *Bd* in any of the 46 *R. onca* sampled across three years from the upper segment of the stream, where frogs were captured at water temperatures as high as 29.9°C during our sampling in February and March (Table 1). In contrast, we consistently detected *Bd* across three years in a small population of *R. onca* occupying the lower segment where water was substantially cooler during the same period (≤25.3°C; Table 1). At this site, we did not observe any clinical signs of chytridiomycosis in frogs that tested positive, and we observed three marked adult frogs that survived at least 8–13 months after initially testing positive for *Bd*; upon recapture, one frog tested positive for *Bd*, one frog tested negative and the other was not retested but appeared healthy.

Susceptibility Experiments

Infectability

In the first experiment using *Bd* isolate CJB7, all frogs exposed to the isolate tested positive for *Bd*, with detection of *Bd* in each animal across at least several weeks. In the second experiment using *Bd* isolate SLL, exposed frogs similarly tested positive for *Bd*, except for two frogs from Black Canyon, one of which tested positive only on the first week following exposure and the other frog only once three weeks after exposure. None of the individuals in control groups in either experiment tested positive for *Bd*.

Infection Intensities

Mean weekly infection intensities in the first experiment reached 2889 ± 1001 ZE in Black Canyon frogs, with the highest individual infection intensity over 16,000 ZE. In Northshore frogs, however, mean weekly infection intensities reached only 365 ± 299 ZE, with the upper range of individual values not much higher (Figure 2a). In the second experiment, the pattern was reversed. The highest mean weekly infection intensity in Northshore frogs was 10,727 ± 2828 ZE, with the highest individual infection intensity over 40,000 ZE. Mean weekly infection intensities in Black Canyon frogs attained only 1887 ± 682 ZE, but with a maximum individual infection intensity of 9672 ZE (Figure 2b).

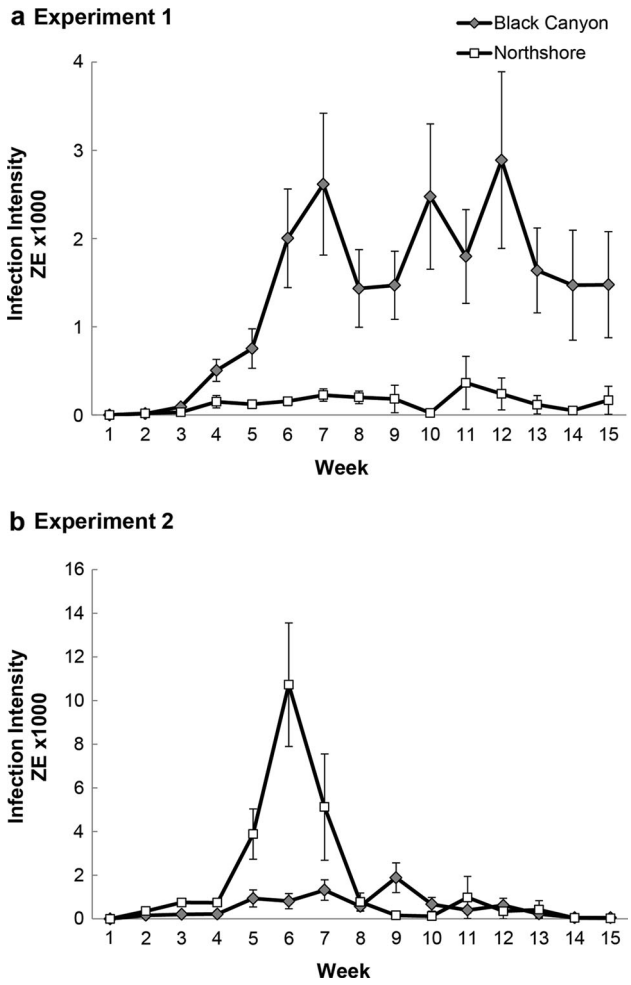

Figure 2. Mean and standard error of weekly infection intensities of *Batrachochytrium dendrobatidis* (*Bd*) in juvenile *Rana onca* measured as zoospore equivalents from skin swabs during two challenge experiments using different isolates of a hypervirulent global panzootic *Bd* lineage.

There was a significant interaction for infection intensity between groups (exposure and collection site) and time (week sampled) in each of the experiments (Experiment I, $F_{28,770} = 2.998$, $P < 0.001$; Experiment 2 $F_{28,700} = 8.080$, $P < 0.001$). There was also a significant

interaction for infection intensity in both experiments independently by group (Experiment I, $F_{2,55} = 27.66$, $P < 0.001$; Experiment 2 $F_{2,50} = 13.76$, $P < 0.001$) and by time (Experiment I, $F_{14,770} = 3.524$, $P < 0.001$; Experiment 2 $F_{14,700} = 9.774$, $P < 0.001$). In the first experiment, mean infection intensities in frogs from Black Canyon were significantly higher in weeks 6–15 (Figure 2a) than in frogs from Northshore or the control group (post hoc test, $P < 0.05$). Northshore frogs, however, never attained mean levels of infection high enough to significantly differentiate from non-infected frogs in the control group. In the second experiment, mean infection intensities for Northshore frogs in weeks 5–7 (Figure 2b) were significantly higher than in frogs from Black Canyon or the control group (post hoc test, $P < 0.05$), and the low mean infection intensities in Black Canyon frogs were never significantly different from the control group. Overall differences in infection intensity between groups exposed to *Bd* from Northshore and Black Canyon were confirmed in both experiments (Experiment I, $W = 105$, $P < 0.001$; Experiment 2, $W = 105$, $P < 0.001$).

Infection Clearance and Survivorship

Over the course of both experiments, overall survivorship was very high (>96% across experiments and groups). Survivorship was not significantly affected by *Bd* exposure or the collection site from which frogs were derived (Experiment 1, $\chi^2 = 4.104$, $df = 2$, $P = 0.1285$; Experiment 2, $\chi^2 = 4.116$, $df = 2$, $P = 0.1277$). In the first experiment, two frogs in the control group died from unknown causes. In the second experiment, two frogs died in the Northshore group exposed to *Bd*. One of these frogs died in week seven at the height of *Bd* infection intensity for the group (Figure 2b), but the infection intensity of this frog before its death was only 2335 ZE. The other death happened in week 15 after the frog had cleared *Bd* five weeks earlier.

Across both experiments, 48 of 75 surviving frogs exposed to *Bd* appeared to clear infections by the 15th week (we include in this analysis the frog that died during week 15 after having cleared *Bd*). Among frogs that tested positive for *Bd* more than once, individuals began clearing infections around weeks 6–7. In the first experiment, a higher proportion of exposed frogs from Northshore cleared infections (90% clearance) than from Black Canyon (20% clearance; $P = 0.0001$). In the second experiment, however, there was no significant difference ($P = 0.1212$) in clearance proportions between the treatment groups, with clearance being high for both Northshore (88% clearance) and Black Canyon (61% clearance) frogs.

Discussion

Our field surveys and laboratory experiments provide insight regarding the pathogen emergence hypothesis for a species of frog from the American Southwest that declined dramatically before the fungal pathogen *Bd* was discovered. In the field, we detected *Bd* in *R. onca* within one of the remaining two areas containing remnant historical populations. We also detected *Bd* within the historical range of *R. onca* in two other anuran species known to be vectors of the pathogen (Daszak et al. 2004; Reeder et al. 2012). In the laboratory, we determined whether two isolates of a highly virulent *Bd* lineage known to affect other western North American ranid species would infect *R. onca* and affect survivorship. We found that *R. onca* was susceptible to *Bd* infection under temperatures that favored the pathogen. All exposed frogs showed evidence of infection within the first few weeks following exposure, and all but two of these frogs developed clear patterns of infection over time. Infection by either of the two isolates, however, did not affect survivorship of *R. onca*. Only a single frog died while infected with *Bd* (isolate SLL), and the infection intensity prior to its death was relatively low when compared to other frogs that survived infections in that experiment. High infection intensities are associated with *Bd* epizootics (Vredenburg et al. 2010), while low infection intensities are associated with *Bd* enzootics (Briggs et al. 2010). Infection intensities of >10,000 ZE in both *Bd* isolates CJB7 and SLL were associated with mortality in other ranid species using the same standard as in our experiment (Vredenburg et al. 2010; Piovia-Scott et al. 2014). Average infection intensities in *R. onca* reached such levels with isolate SLL but not CJB7. For both isolates, however, we observed no clinical signs of chytridiomycosis and most (64%) frogs cleared infections by the end of experiments. In the field, we also observed evidence of resistance or tolerance to *Bd* in a few individual frogs that survived infections and in one case apparently cleared infection.

We asked whether population-level exposure to *Bd*, as determined by our field surveys, would affect the susceptibility of offspring from exposed populations to new *Bd* infection. We observed no consistent differences in infection intensities between frogs representing populations from the geographic areas where *Bd* had been detected

(Northshore) and where it had not (Black Canyon). As might be predicted, infection intensities during the first experiment reached significantly higher levels in frogs from Black Canyon than those from Northshore, and by the end of the experiment significantly more frogs from Northshore cleared infections than from Black Canyon. In the second experiment, however, the pattern of infection intensities among the groups was reversed, and while the pattern of clearance remained the same as in the first experiment there was no significant difference in clearance, with most frogs from both groups clearing infections. These idiosyncratic patterns could have resulted from the particularly high levels of genetic similarity among our samples in the first experiment, but our broader sampling of egg masses in the second experiment mitigated this issue. Given the pattern observed during the second experiment, our data provided no strong evidence that current population-level exposure has an impact on the course of *Bd* infection in offspring. We cannot, however, rule out the possibility that populations in Black Canyon may have been exposed to *Bd* sometime in the past.

Resistance to *Bd* infection varies among and within amphibian species (reviewed in James et al. 2015), and while environmental conditions appear to be a major component of the variation, some of the resistance has genomic underpinnings, allowing for the possibility of selection (Savage and Zamudio 2011; Ellison et al. 2015). Immunological resistance to *Bd* appears to involve integrated responses of both innate and adaptive immune systems (Richmond et al. 2009; McMahon et al. 2014). In *Rana yavapaiensis*, outcomes for *Bd* infected individuals and populations were positively associated with heterozygosity of genes and to particular alleles within the major histocompatibility complex (Savage and Zamudio 2011). The apparent resistance of *R. onca* to *Bd* may have several plausible explanations, the most parsimonious is that *R. onca* simply has inherent, immunological resistance to *Bd* and may never have been very susceptible to chytridiomycosis. Alternatively, the current populations of *R. onca* may be descended from individuals that survived a previous epizootic allowing selection for greater resistance to the pathogen. Since then, the pathogen–host relationship may have evolved and *Bd* may now be enzootic (see James et al. 2015).

When we initiated our experiments, we had expected *R. onca* to be highly susceptible to chytridiomycosis, which would explain the current remnant distribution within geothermal springs, as well as lend support to a scenario that *Bd* was associated with the historical decline of the species. The distribution of remaining historical populations within geothermal spring systems certainly is consistent with these sites being environmental refuges from disease because of high water temperatures or other characteristics of water quality (Forrest and Schlaepfer 2011), although the mechanism by which resistance to *Bd* could evolve within such systems has been questioned (Savage et al. 2015). While our results do not elucidate the historical question of *R. onca's* decline, the evidence suggests that *R. onca* currently shows a high level of resistance to two isolates of *Bd*-GPL1.

Limitations to accepting either of these resistance scenarios are that the *Bd* isolates we used may not be inherently virulent toward *R. onca* or had attenuated in the laboratory to anuran hosts. Although these isolates were linked to severe anuran population declines in the wild, pathogens maintained in vitro through serial passages may have diminished virulence in former hosts (Ebert 1998). Attenuation in cultured *Bd* has been reported (Brem et al. 2013; Langhammer et al. 2013; Refsnider et al. 2015), although such changes in *Bd* virulence may depend on culturing practices (Voyles et al. 2014). The isolates we used had recently been brought out of cryopreservation and had relatively low passage histories when compared to those showing attenuation (Brem et al. 2013; Langhammer et al. 2013). Although, it seems unlikely that both isolates would have attenuated, the lack of mortality and high levels of infection clearance in our first experiment led us to question the virulence of that specific isolate to *R. onca.* Our second experiment using a different isolate was intended to address our concerns, and we ran that experiment with slightly younger frogs and at a slightly cooler temperature, changes that we thought should favor *Bd*.

Conclusions

We found that *Bd* occurs in anurans within the historical range of *R. onca* and in one remnant historical population of this species. In a few individually marked *R. onca*, we observed evidence of *Bd* clearance and long-term survival of infected frogs in the wild. We observed in laboratory experiments that *R. onca* was resistant to *Bd* isolates known to be highly virulent in other ranid species, showing little evidence of mortality associated with infection and high incidence of infection clearance. We think it is unlikely that the apparent resistance resulted from attenuation or lack of

innate virulence to *R. onca* in the two *Bd* isolates we used. Instead, we suspect that *R. onca* has inherent resistance to *Bd* and chytridiomycosis, or has evolved such resistance. These results may bode well for conservation efforts aimed at establishing *R. onca* populations across a landscape where *Bd* exists. We did not, however, assess the susceptibility of *R. onca* to *Bd* from the local environment which may differ in virulence from those we used in our experiments. Furthermore, *Bd* susceptibility varies with life history stages (Rollins-Smith et al. 2011) and is likely influenced by health and body condition. Our frogs were post-metamorphic, captive raised and well fed, and as a group gained size and weight over the experiments. We caution that the resistance to *Bd* we observed under relatively benign laboratory conditions in such frogs may not translate to situations in the wild.

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