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Original Contribution

High Prevalence of *Batrachochytrium dendrobatidis* in Wild Populations of Lowland Leopard Frogs *Rana yavapaiensis* in Arizona

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Abstract: *Batrachochytrium dendrobatidis* (Bd) is a fungus that can potentially lead to chytridiomycosis, an amphibian disease implicated in die-offs and population declines in many regions of the world. Winter field surveys in the last decade have documented die-offs in populations of the lowland leopard frog *Rana yavapaiensis* with chytridiomycosis. To test whether the fungus persists in host populations between episodes of observed host mortality, we quantified field-based Bd infection rates during nonwinter months. We used PCR to sample for the presence of Bd in live individuals from nine seemingly healthy populations of the lowland leopard frog as well as four of the American bullfrog *R. catesbeiana* (a putative vector for Bd) from Arizona. We found Bd in 10 of 13 sampled populations. The overall prevalence of Bd was 43% in lowland leopard frogs and 18% in American bullfrogs. Our results suggest that Bd is widespread in Arizona during nonwinter months and may become virulent only in winter in conjunction with other cofactors, or is now benign in these species. The absence of Bd from two populations associated with thermal springs (water >30°C), despite its presence in nearby ambient waters, suggests that these microhabitats represent refugia from Bd and chytridiomycosis.

Keywords: disease, chytridiomycosis, thermal spring, frog, *Rana*, conservation

Introduction

Chytridiomycosis is an infectious amphibian disease caused by the fungal agent *Batrachochytrium dendrobatidis* (Bd) and has been implicated in catastrophic amphibian die-offs in North America, Central America, South America, Australia, Europe, and Oceania from the 1980s to present (Berger et al., 1998; Daszak et al., 1999; Weldon et al., 2004; Lips et al., 2006). Until recently, the prevailing perception based on these studies was that Bd represented a highly virulent fungal pathogen that typically induced massive die-offs in infected populations (e.g., Lips et al., 2006; Rachowicz et al., 2006). In the last few years, however, a more nuanced picture of the effects of Bd has emerged. Indeed, both retrospective studies based on preserved specimens (Ouellet et al., 2005; Puschendorf et al., 2006) and longitudinal population studies in the field (Briggs et al., 2005; Kriger and Hero, 2006; Longcore et al., 2007) have documented Bd-positive individuals without concomitant chytridiomycosis-induced mortality and without

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catastrophic population declines. These findings suggest that some host species are not susceptible to chytridiomycosis (e.g., Berger et al., 1998; Garner et al., 2006), have evolved resistance to the fungus, or occur in microhabitats that prevent the fungus from inducing chytridiomycosis (McCallum, 2005; McDonald et al., 2005; Woodhams and Alford, 2005). These results highlight the need for additional field studies to determine the relationship between Bd and its potential hosts in the wild.

The impetus for this study was to evaluate the threat that Bd represents for the lowland leopard frog (*Rana yavapaiensis*) in Arizona. Lowland leopard frogs have been absent since 1960 from large parts of the species' historical range (Clarkson and Rorabaugh, 1989), and populations continue to decline or disappear from additional sites throughout the species' range (Sredl, 2005). Several causes have been identified for these declines, including changes in hydrological regimes, introduced predators (Rosen and Schwalbe, 2002b), and chytridiomycosis, which has been detected primarily during the winter months (Bradley et al., 2002; Sredl et al., 2003). Several studies have established a correlation between chytridiomycosis and below-freezing temperatures in temperate anurans (Bosch et al., 2001; Bradley et al., 2002; Muths et al., 2003). It is not known, however, whether winter die-offs were caused by a seasonal return of a highly virulent form of Bd, whether Bd was endemic and acted in conjunction with other environmental factors, or whether Bd was persisting in other species such as the American bullfrog *Rana catesbeiana*, which is non-native to Arizona and a known Bd vector (Garner et al., 2006). The goals of this study were therefore to address two questions: (1) Does Bd infect lowland leopard frogs during nonwinter months? (2) Does Bd infect non-native American bullfrogs *R. catesbeiana* in Arizona? We also sampled multiple individuals from each population to explore possible correlations between estimated Bd prevalence and environmental conditions.

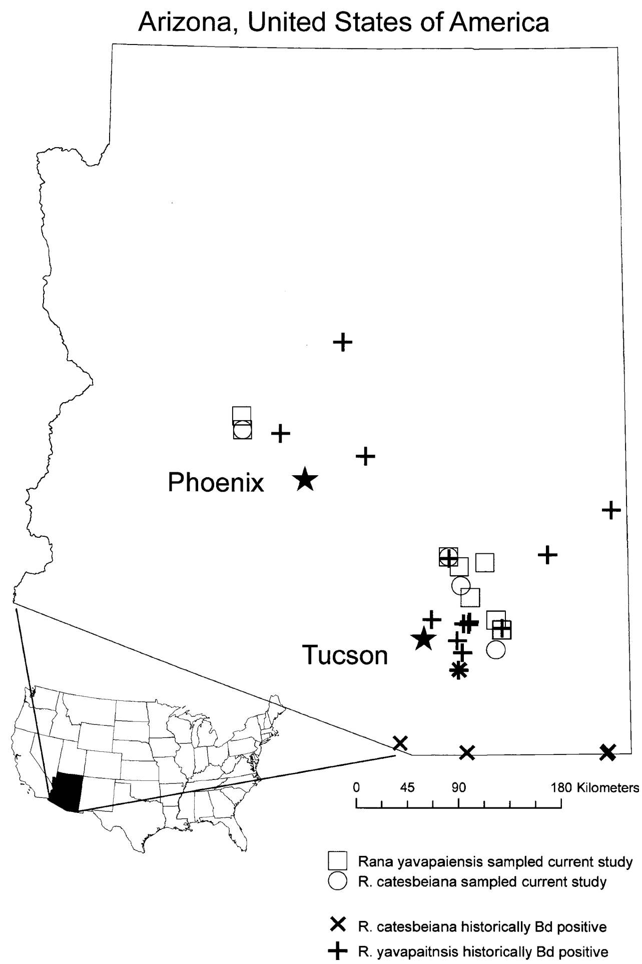

Figure 1. Geographic distribution of *Rana yavapaiensis* (open squares) and *R. catesbeiana* (open circles) populations in which at least one individual tested positive for *Batrachochytrium dendrobatidis* (Bd) in Arizona, United States, in this study. Bd-positive samples from prior studies from 1992 to 2003 (M. Sredl, P. Rosen, D. Caldwell, unpublished data; Bradley et al., 2002) from *R. yavapaiensis* and *R. catesbeiana* are marked with a "+" and "x," respectively, but were not included in the statistical analyses of this study. This figure illustrates that Bd has been confirmed throughout the state and is currently associated with persistent populations of both species of anurans.

Methods

In 2004 we sampled adult and juvenile lowland leopard frogs and American bullfrogs from 11 locations across Arizona (Fig. 1). These sites were chosen to represent a range of elevations and variable years of exposure to invasive species. A new pair of disposable latex gloves was used to capture each frog. A 35-mm wooden medical applicator (Puritan® Ref 807) was used to sample skin cells from the frog's venter, flanks, and groin. These areas were scraped a total of 25 times using the applicator, which was then placed in a 2-ml sterile tube (Laboratory Product Sales, Rochester, NY, #L233071) filled with 70% ethanol (made with HPCL-grade water). Skin scrapes were assayed within 2–4 months of being collected for the presence of *Batrachochytrium dendrobatidis* using PCR amplification by a commercial lab (Pisces Molecular, Boulder, CO). We also measured (snout-vent length, SVL) and sexed (male, female, or unknown) each frog prior to release. Control

Table 1. Populations of the Lowland Leopard Frog *Rana yavapaiensis* Sampled in 2004 for *Batrachochytrium dendrobatidis*

Location name	Prevalence, by site	Prevalence, by site and date	GPS (UTM Zone 12; NAD27)	Elevation (m)
San Pedro River, Dudleyville	0.33 (1/3)	14–16 March (1/3)	524960 mE 3642972 mN	611
San Pedro River, San Manuel	0.42 (5/12)	26 March (2/2) and 30 September (3/10)	543860 mE 3606677 mN	730
Muleshoe, Hotsprings	0.00 (0/15)	17 March (0/5) and 25 August (0/10)	571661 mE 3577927 mN	1241
Muleshoe, Secret Spring	0.33 (3/9)	17 March (1/4) and 25 August (2/5)	571193 mE 3578199 mN	1232
Cherry Springs	1.00 (4/4)	18 March (4/4)	566413 mE 3586743 mN	1302
Aravaipa Canyon, Upper	0.81 (13/16)	20–21 March (7/8) and 6 October (6/8)	556564 mE 3637826 mN	991
Aravaipa Canyon, Lower	0.33 (1/3)	25 March (1/3)	534080 mE 3634072 mN	702
Hassayampa River, Wickenburg	0.65 (11/17)	23–24 March (6/9) and 2 October (5/8)	343485 mE 3755638 mN	591
Hassayampa River, Wilderness Preserve	0.00 (0/6)	4 October (0/6)	342715 mE 3768033 mN	686

samples (involving all the steps of a normal sample but without first catching a frog) were collected at the end of each day to ensure the absence of cross-contamination between samples.

A binomial logistical regression was used to test for associations between the presence of Bd and sex, SVL, elevation, and date (Tabachnick and Fidell, 2001). For sites where Bd was not detected, we calculated the lowest prevalence we could expect to detect with 90% certainty given our sample size as follows: prevalence = $1-(1-0.9)^{1/n}$, where n is the sample size (DiGiacomo and Koepsell, 1986). In populations where Bd was not detected, we tested whether its prevalence differed statistically from that of the nearest-neighboring population and the species-wide (unweighted) mean (excluding the focal population). All tests were two-tailed and used $\alpha = 0.05$ and $\beta = 0.1$ for significance testing.

Results

We collected and processed 112 samples from 85 lowland leopard frogs and 27 American bullfrogs. Bd was detected in seven of nine lowland leopard frog locations sampled (Table 1) and the overall prevalence of Bd in the lowland leopard frog was 0.43 (unweighted mean of prevalence among all populations). The mean (unweighted) Bd prevalence of lowland leopard frog populations with at least one Bd-positive individual was 0.55 (Table 1). In other words, in populations where Bd was detected, more than half of all individuals were infected, on average. The only evidence of disease-related mortality during these sampling efforts was a single individual from Cherry Springs (later found to be Bd-positive) with typical symptoms associated with chytridiomycosis (lethargy, difficulty righting itself, and death within hours of being captured). There was no clear seasonal trend in Bd prevalence: infected individuals were prevalent in both late winter-early spring (67%) and later summer-early autumn (52%). We found no significant association between Bd infection and sex, elevation, SVL, or date (binomial logistical regression: all P values > 0.2; univariate tests: all P values > 0.2).

In two locations, Muleshoe Hotsprings and Hassayampa Wilderness Preserve, the estimated prevalence of Bd in lowland leopard frogs was 0.0 (Table 1). Given our sample sizes at these sites (n = 15 and 6), we had a 90% probability of detecting a Bd prevalence as low as 0.14 and 0.31, respectively (Table 2). Thus, the prevalence of Bd in these two sites was significantly lower than in the nearest-neighboring populations and the species-wide average (Table 2).

Bd was also found in three of four American bullfrog populations (Table 3). The overall prevalence of Bd in American bullfrogs was 0.18 (unweighted mean of prevalence among populations), which was lower than in lowland leopard frogs (0.43), but not significantly so (Kruskal-Wallis test, Mann-Whitney U = 8.5, P = 0.14). The estimated prevalence of Bd in American bullfrogs from the thermal

Table 2. Power Analyses for Sites at Which No *Batrachochytrium dendrobatidis* (Bd) Was Detected

Focal population	Sample size	Bd prevalence detectable with 90% confidence[a]	Bd prevalence (in neighbor population); probability (of detecting not one positive sample assuming Bd prevalence of neighbor population)[a]	Species-wide prevalence of Bd[b]; probability (of detecting not one positive sample assuming species-wide prevalence of Bd)[a]
Rana yavapaiensis				
Muleshoe Hotsprings	15	0.14	0.33 (Muleshoe, Secret Spring); $P = 0.002$	0.48; $P < 0.001$
Hassayampa River Wilderness Preserve	6	0.31	0.65 (Hassayampa River, Wickenburg); $P = 0.002$	0.48; $P = 0.020$
Rana catesbeiana				
Mammoth Hotsprings	10	0.21	0.18 (San Pedro, Dudleyville); $P = 0.137$	0.23; $P = 0.073$

[a] $C = (1 - P)^n$, where C = probability of detection, P = prevalence, and n = sample size (DiGiacomo and Koepsell, 1986)
[b] Species-wide average is unweighted and excludes focal population (see Methods section)

springs near Mammoth was 0.0 (Table 3). Given the sample size at this location ($n = 10$), there was a 90% probability of detecting a Bd prevalence as low as 0.21 (Table 2), which corresponds approximately to the mean prevalence in populations of American bullfrogs where it was detected (0.23; Table 3). Thus, if the true prevalence of Bd in Mammoth Hotsprings was typical for that species (0.21), then there was a 10% chance of sampling ten negative individuals.

There was a significant association in this study between the number of sampled thermal springs and the number of sites where Bd was not detected, pooling across seasons and species (Bd not detected and thermal water: 2, Bd not detected and ambient water: 1, Bd detected and thermal water: 0, Bd detected and ambient water: 10; Fisher's exact test, $P = 0.038$).

Discussion

Bd had a broad distribution and an overall prevalence of 43% in sampled populations of lowland leopard frogs. Three populations of lowland leopard frogs had observed infection rates between 0.65 and 1.0 (Table 1), which represent the highest ever recorded in wild populations of live, seemingly healthy anurans. Thus, it is apparent that Bd persists within lowland leopard frog populations during nonwinter months, although we did not detect any seasonal variation in infection rates.

Do populations with high Bd prevalence show evidence of ongoing catastrophic declines? We did not conduct quantitative surveys of every population, but available evidence is not consistent with catastrophic declines. For example, the population of lowland leopard frogs from Upper Aravaipa has been monitored every year (in October) using Visual Encounter Surveys (Crump and Scott, 1994) along a permanent 7-km stretch since 1977 (Fig. 2). These survey data suggest that despite the high prevalence of Bd (0.81) in 2004, the Upper Aravaipa population remained at relatively high densities and within typical variation of the last three decades in the two years following our survey for Bd. Furthermore, relatively high densities of recently laid egg clutches (up to 19 clutches in one 300-m segment of the San Pedro River at Dudleyville) and adults (up to 30 in a 50-m segment of Secret Spring) were observed in March 2007 (MAS, unpublished field notes). These field observations are potentially important because the long-term persistence of infected frog populations depends upon the ability of at least some adult frogs to survive and reproduce despite being infected with Bd (Briggs et al., 2005).

The oldest lowland leopard frog specimen with histological evidence of Bd in Arizona dates back to 1985 from nearby Secret Springs, Muleshoe (Rosen and Schwalbe, 2002a). Since then, Bd has been detected in histological samples of lowland leopard frogs museum specimens from many of the sites sampled for this study, including Dudleyville (1999) and Aravaipa Canyon (2000) (PCR, unpublished data). Collectively our evidence suggests that lowland leopard frog populations are capable of long-term persistence despite endemic Bd.

We cannot, however, assert that Bd is benign in this species. Indeed, effects of Bd could be sublethal or could

Table 3. Populations of the American Bullfrog *Rana catesbeiana* Sampled in 2004 for *Batrachochytrium dendrobatidis*

Location name	Prevalence, by site	Prevalence, by site and date	GPS (UTM Zone 12, NAD27)	Elevation (m)
San Pedro River, Dudleyville	0.17 (1/6)	14–16 March (1/5) and 26 March (0/1)	524960 mE 3642972 mN	611
Hassayampa River, Wickenburg	0.33 (2/6)	20–24 March (2/4) and 2 October (0/2)	343485 mE 3755638 mN	591
Mammoth Hotsprings	0.00 (0/10)	5 October (0/10)	535452 mE 3617252 mN	730
Cienega Creek	0.20 (1/5)	5 September (1/5)	539513 mE 3516675 mN	1343

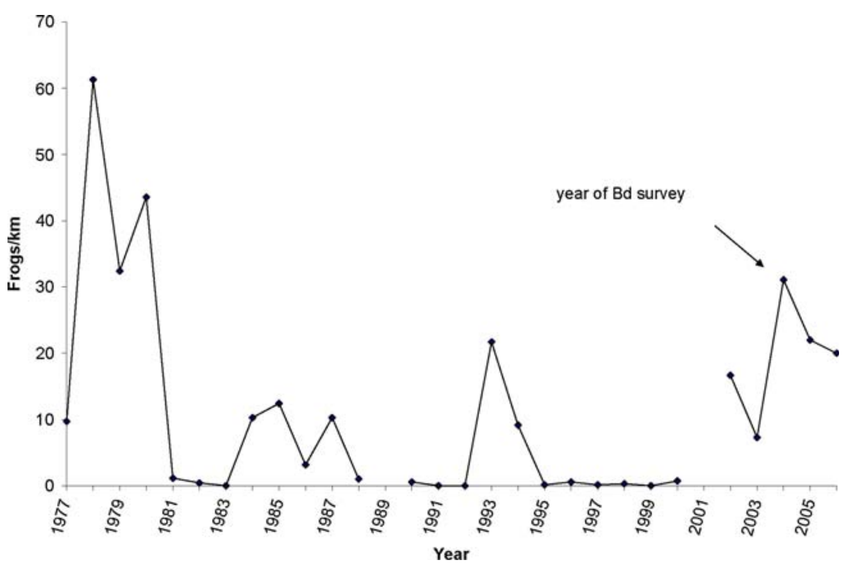

Figure 2. Number of lowland leopard frogs (*Rana yavapaiensisi*) per km detected along a 7-km river stretch at the head of Aravaipa River using Visual Encounter Surveys every October. No surveys were conducted in 1989 and 2001. This population suffered no catastrophic decline after the spring and fall of 2004, when 13 of 16 (81%) of sampled individuals were Bd-positive from this site. Data courtesy of Jay Schnell, Mark Haberstich, and the Nature Conservancy.

appear during other times of year. The discovery of one dying Bd-positive individual from Cherry Springs suggests that chytridiomycosis remains a threat to some individuals, even during nonwinter months. Furthermore, we cannot rule out the possibility that Bd-positive individuals suffered higher mortality during the winter, and that populations subsequently rebounded through a density-dependent process.

We also found Bd in three of four sampled American bullfrog populations. This result is consistent with previous reports that American bullfrogs may serve as vector for Bd (Daszak et al., 2004; Hanselmann et al., 2004; Garner et al., 2006). The single population where Bd was not detected (Mammoth Hotsprings) was associated with a thermal spring. The species-wide infection rate in American bullfrogs (0.18) was lower than that of lowland leopard frogs (0.43; Table 1), although not significantly different possibly because of low statistical power.

Over the course of this study, two of three ranid populations where Bd was not detected were associated with thermal springs. In vitro Bd is viable between 4°C and 29°C and has a preferred growing temperature—and greatest virulence—between 17°C and 25°C (Longcore et al., 1999; Piotrowski et al., 2004; Carey et al., 2006). Water temperature at the sources of Muleshoe and Mammoth thermal springs measured 51.2°C and 40.2°C, respectively. We estimated that approximately 10–50 m^2 of each pool surface was greater than 30°C, depending on the season. All of the lowland leopard frogs we sampled at Muleshoe were found sitting in or on land within 1 m of waters at 22.8–35.8°C. At Mammoth Hostprings, three American bullfrogs were sitting on land within 5 cm of waters at 35.8–37.2°C and the remaining seven individuals were in waters at 22.4–32.9°C.

Secret Spring presents a marked contrast with the nearby Muleshoe Hotsprings because its water temperatures were much cooler (ca. 20°C in March 2005, 24°C in August 2004). We assume that lowland leopard frogs can easily disperse between these two sites because they are separated from one another by only 550 m as the crow flies, and both sites are less than 50 m from a major stream channel with sustained flow after periods of rain. The presence of Bd during both March and August sampling periods in Secret Spring and its simultaneous absence from Muleshoe Hotsprings (Table 1) suggests that Bd is excluded by the thermal waters of Muleshoe Hotsprings. Likewise, we suspect that Bd is excluded from Mammoth Springs because Bd-positive individuals of both ranids have been found in the San Pedro River (Tables 1 and 3), which is only about 30 m away.

In sum, four lines of evidence suggest that Bd may be excluded from thermal springs: (1) the observed prevalence of Bd at both thermal springs was 0.0; (2) sample sizes at both thermal springs (n = 10 and 15) were larger than for most other sites, where Bd was readily detected; (3) Bd was repeatedly detected in ambient water nearby Muleshoe Hotsprings; and (4) there was a significant association between Bd-free populations and thermal springs. Only one population not associated with thermal waters was Bd-negative during our study, possibly due to hydrologic isolation or small sample size (see below). The absence of Bd from the thermal springs provides important field corroboration of in vitro studies, demonstrating that the fungus is not viable above 30°C (Longcore et al., 1999; Johnson et al., 2003; Woodhams et al., 2003; Piotrowski et al., 2004). Whether frogs can clear themselves of the chytrid fungus behaviorally by choosing to occupy warmer waters or whether this outcome occurs by chance remains to be investigated. In addition to adult ranids, we observed hundreds of lowland leopard frog tadpoles in 30–34°C waters in Muleshoe Hotsprings. (Lowland leopard frog egg clutches, in contrast, were generally found in cooler waters, below 29°C, although one clutch was found in 32°C.) If other species of anurans are found to be tolerant of warm water temperatures, wildlife managers may consider exploring the use of artificially warmed waters as a refuge from Bd for critical populations.

The only nonthermal site in which Bd was not detected was in the Hassayampa Wilderness Preserve. The small number of samples collected from this site (n = 6) implies a 10% chance that the true prevalence of Bd may have been as high as 0.31. Nevertheless, the prevalence at this site was significantly lower than that of the next nearest population (Hassaympa River at Wickenburg, 0.65) and significantly lower than the overall prevalence for the species (Table 2). We speculate that Bd may not have had the opportunity to reach (or reinvade) the site, which was hydrologically isolated at the time by several kilometers of dry river bed from the next nearest known population of ranid frogs.

We acknowledge that estimates of Bd prevalence in each population would have been more precise with larger sample sizes. In spring 2007 we attempted to resample American bullfrogs from Mammoth Hotsprings but the source of the spring had been capped and the pool of standing water had all but disappeared. We were also unable to locate additional thermal springs with waters warmer than 30°C and with resident populations of ranids in Arizona that could be sampled to corroborate the absence of Bd from thermal pools.

In summary, our results reveal that Bd is established in large areas of Arizona and infects a majority of lowland leopard frogs wherever it is found. Bd is confirmed to be present in ranid frogs at high prevalence, even during nonwinter seasons when it appears relatively benign. Future experimental field work should investigate whether warm water temperatures (30–35°C) can serve as an effective refuge from Bd and, by extension, chytridiomycosis.

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