Short communication

Widespread occurrence of an emerging pathogen in amphibian communities of the Venezuelan Andes

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A B S T R A C T

Many recent amphibian declines have been associated with chytridiomycosis, a cutaneous disease caused by the chytrid fungus *Batrachochytrium dendrobatidis*, but increasing evidence suggests that this pathogen may coexist with some species without causing declines. In the Venezuelan Andes, the disappearance of three anuran species during the late eighties was attributed to *B. dendrobatidis*. Recently, this pathogen was found to be prevalent in this region on the introduced American bullfrog, *Lithobates catesbeianus*. As a first step toward assessing the risk of amphibian communities to *B. dendrobatidis* in this region, we conducted a broad survey across multiple habitat types and an altitudinal gradient spanning over 2000 m. We diagnosed 649 frogs from 17 species using real time and conventional PCR assays, and recorded relevant abiotic characteristics of host habitats. Infection was detected in 10 native species of pond, stream and terrestrial habitats from 80–2600 m, representing nine new host records. *L. catesbeianus* was the most important reservoir with 79.9% of individuals infected and an average of 2299 zoospores. Among native frogs, *Dendropsophus meridensis,* an endangered species sympatric with *L. catesbeianus,* showed the highest infection prevalence and mean zoospore load (26.7%; 2749 zoospores). We did not detect clinical signs of disease in infected hosts; however, species such as *D. meridensis* may be at risk if environmental stress exacerbates vulnerability or pathogen loads. While surveillance is an effective strategy to identify highly exposed species and habitats, we need to understand species-specific responses to *B. dendrobatidis* to stratify risk in amphibian communities.

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1. Introduction

Increasing evidence suggests that several factors may be involved in the recent emergence of chytridiomycosis (Lips et al., 2006; Pounds et al., 2006, 2007; Alford et al., 2007; Di Rosa et al., 2007), a fungal disease linked to worldwide population declines and extinctions of several amphibians (Berger et al., 1998, 1999; Daszak et al., 1999; Longcore et al., 1999; Lips

et al., 2006). While some data suggests that the recent spread of the causative agent of this disease, *Batrachochytrium dendrobatidis* (*Bd*), has caused the mortality of susceptible species in naïve populations (Berger et al., 1999; Daszak et al., 1999; Lips et al., 2006; Skerratt et al., 2007), results from other studies indicate that climate shifts in recent decades have increased the vulnerability of amphibians to this disease at many highland localities (Pounds et al., 2006). Also, stressors such as crowding during dry episodes and physiological stress due to adverse climatic conditions or coinfections have also been hypothesized to promote disease development (Pounds et al., 1999; Lampo et al., 2006b; Alford et al., 2007; Di Rosa et al., 2007). However, the contribution of each of these factors in triggering chytridiomycosis outbreaks in several regions is still a matter of current debate (Alford et al., 2007; Di Rosa et al., 2007; Pounds et al., 2007; Skerratt et al., 2007; Lips et al., 2008; Pounds and Coloma, 2008).

Despite its negative impact on some frog species, *Bd* has been found in several non-declining frog species around the world (Beard and ÓNeill, 2005; Ouellet et al., 2005; Woodhams and Alford, 2005; Kriger and Hero, 2006; Lips et al., 2006; Puschendorf et al., 2006). The ability of some non-declining species to develop subclinical infection and maintain high infection prevalence (e.g., *Lithobates catesbeianus*) suggests that some host species are less vulnerable to this pathogen (Daszak et al., 2004; Hanselmann et al., 2004). Pathogens may also infect vulnerable populations without noticeable effects, if transmission rates are sufficiently low to keep mortality under recruitment rates. On the other hand, *Bd* currently persists in frog species that presumably suffered chytridiomycosis-induced declines in the past but whose populations are now recovering (e.g., *Taudactylus eungellensis* in Australia and *Atelopus cruciger* in Venezuela) (Retallick et al., 2004; Rodríguez-Contreras et al., 2008). Several explanations are possible: (1) virulence of the pathogen has decreased since the initial epidemic outbreak, (2) environmental stressors that compromised the vulnerability of frogs in the past are no longer present and (3) *Bd* transmission in recovering populations is still below the epidemic threshold. Understanding the factors affecting the prevalence and intensity of *Bd* infection among various host species, and what makes some hosts tolerant and others vulnerable, is crucial for predicting the impact this pathogen may have on amphibian communities.

In Central and South America, declines of at least 56 amphibian species have been associated with the presence of *Bd*; four of these are critically endangered species endemic to Venezuela (Berger et al., 1998; Lips, 1999; Ron and Merino-Viteri, 2000; Bonaccorso et al., 2003; Lips et al., 2003, 2004, 2006; Puschendorf, 2003; Burrowes et al., 2004; La Marca et al., 2005; Seimon et al., 2005; Carnaval et al., 2006; Lampo et al., 2006b). Although sampling of museum collections have not been extensive (431 museum specimens from 1920 to 2002 (Bonaccorso et al., 2003; Lampo et al., 2006b; Lampo and Señaris, 2006)), the earliest evidence of *Bd* in this country dates to 1986 from one *A. cruciger* from the Cordillera de la Costa (Bonaccorso et al., 2003). Also, seven specimens from three other declining *Atelopus* species collected during 1988 were found infected at the Cordillera de Mérida (Lampo et al., 2006b), a region that has 11 critically endangered species (IUCN, Conservation International and NatureServe, 2006. Global Amphibian Assess-

ment. www.globalamphibians.org (accessed on 8th August, 2007)). It was recently proposed that an epidemic wave swept across the Cordillera de La Costa and the Cordillera de Mérida in Venezuela between 1977 and 1988 (Lips et al., 2008). However, the spatiotemporal distribution of infection in *Atelopus* frogs at the Cordillera de Mérida and at the Cordillera de la Costa, with four positive localities temporally concentrated (1986–1988) but spatially separated (70–445 km) suggests that *Bd* could have been present endemically below detectable levels, and particular climatic conditions triggered synchronized epidemics in Venezuelan during the late 1980's (Lampo et al., 2006b). While there have been no reports of massive mortalities in Venezuela, all infected *Atelopus* species disappeared for, at least, one decade (La Marca and Lötters, 1997; Manzanilla and La Marca, 2004; Lampo et al., 2006b; Rodríguez-Contreras et al., 2008). In the Venezuelan Andes, *Bd* was also detected in one *Leptodactylus* sp. and one *Mannophryne cordilleriana* collected in 1996 and 2002, respectively (Lampo et al., 2006b) and more recently, in *L. catesbeianus* populations (the American bullfrog, previously *Rana catesbeiana*), a recently introduced exotic species that carries high pathogen loads without clinical symptoms of disease (Hanselmann et al., 2004). Currently, there are no other *Bd* reports in the country.

Although most of the critically endangered species of the Cordillera de Mérida have not been observed in the last two decades, one recent rediscovery presents the possibility that some populations may be recovering to detectable levels (Barrio-Amorós, 2004). However, *Bd* will continue to threaten amphibian populations in the Andes if the climatic scenarios that promoted epidemics in the past repeat (Pounds et al., 2006). The historical and potential threat of chytridiomycosis to amphibian hosts, especially in the Andean region, and the incipient state of knowledge about *Bd* epidemiology together reinforce the need for infection surveillance in wild host populations to monitor the impact of this disease in potentially vulnerable areas (Daszak et al., 2007). As a first step towards identifying conservation priorities and assessing epidemiological risks for Andean amphibians to chytridiomycosis, we screened 17 frog species for *Bd* to (1) identify host species and key reservoirs species, (2) explore the species-specific variations in prevalence and *Bd* zoospore loads, and (3) characterize the altitudinal ranges and breeding habitats of host species.

2. Materials and methods

The study was conducted at the Cordillera de Mérida (Mérida State, Venezuela) between 8°30′N–71°37′W and 8°45′N–71°15′W, in an area that covers an altitudinal gradient between 80 and 2600 m and includes several vegetation types (cloud forest, semi-deciduous montane forest, and submontane moist forest (Ataroff and Sarmiento, 2004)), although many have been degraded due to farming activities (Fig. 1). A predominant characteristic of this area between 1800 and 2600 m of altitude is the presence of many artificial water reservoirs for cattle that are mainly used by the introduced bullfrog, *L. catesbeianus*.

We sampled 444 adult frogs from 16 species found in 22 ponds and 19 rivers or streams. Each water body was visited for a minimum of one hour three times during the study for
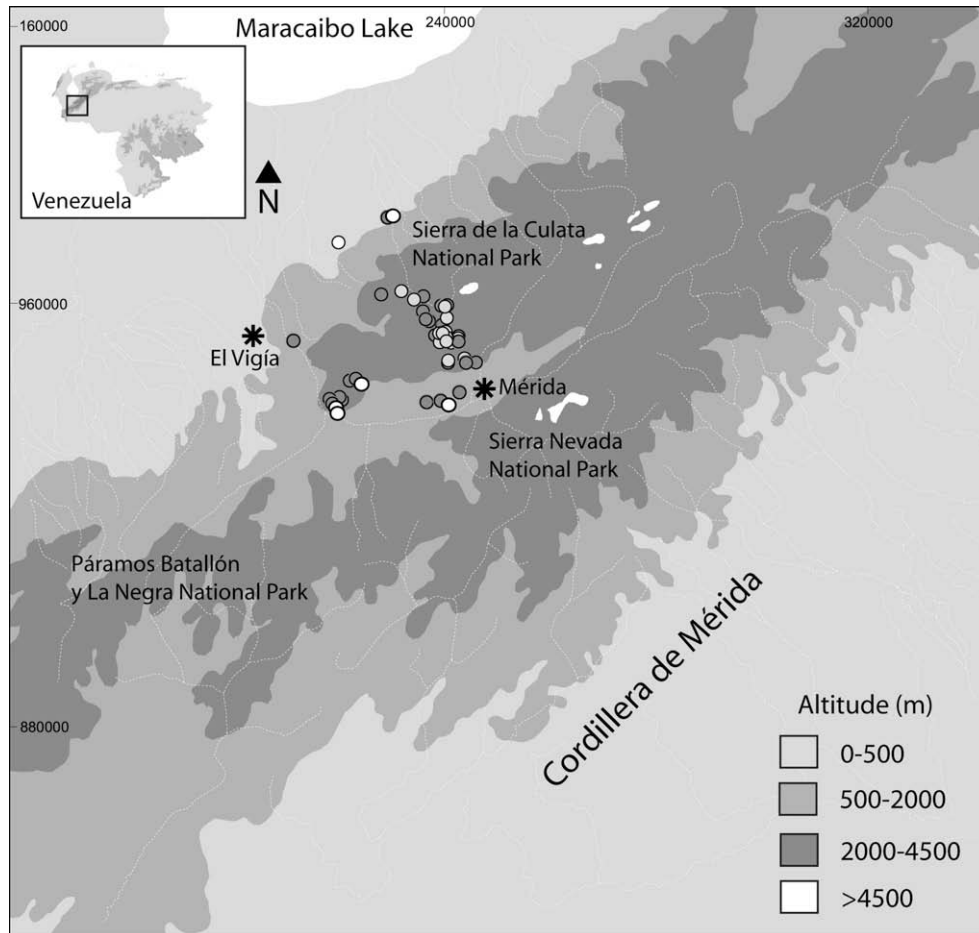
Fig. 1 – Chytrid screening in the Venezuelan Andes. Circles represent sites sampled by this study; white circles denote sites with infected frogs.

an approximate total of 390 man-hour of effort. Sampling covered 22 months between October 2003 and March 2006, although 88% of the samples were collected during the rainy season (April–November). Tissue samples from all adults were obtained by cutting a small piece of interdigital membrane and one V-toe tip. All adult frogs were immediately released except for bullfrogs, which are currently under an eradication program by government agencies (Ministerio de Ambiente-Minamb). To compare the prevalence of infection and zoospore concentrations between larval and post-metamorphic stages, we collected 85 larvae of *Dendropsophus meridensis* and 119 of *L. catesbeianus* over 2.5 years. Theoretical evidence suggests that high fecundity and strong density-dependent effects on larval survival tend to compensate for the removal of pre-metamorphic stages of many amphibian species (Lampo and De Leo, 1998). In addition, one larva of *Pseudis paradoxa* was also collected, as we had no other representative of this species. All larvae were sacrificed and their complete oral disk removed for diagnosis. Biosecurity protocols were followed to avoid cross infection between individuals or locations (Lips et al., 1999; Aguirre and Lampo, 2006). Except for one *Leptodactylus* currently under description (La Marca, personal communication), all specimens were identified to species.

For DNA detection, nucleic acids were extracted from 1–3 mg tissue samples with PrepMan Ultra according to estab-

lished protocol (Boyle et al., 2004). Real Time Taqman PCR assays were conducted for 390 samples (205 larvae and 185 adults) using an Opticon thermocycler and standard curves were constructed by using 100, 10, 1 and 0.1 *Bd* zoospore quantification standards provided by A. Hyatt (Australian Animal Health Laboratory-AAHL, Division of Livestock Industries, CSIRO, Victoria, Australia). Each sample was run in triplicate and negative controls consisting of the reaction mixture without tissue sample were used during the extraction and PCR assay to detect DNA contamination. Mean estimates for the cycle threshold of reaction (C_t value) for all internal standards $(0.1, 1, 10, 100)$ were similar to those reported by the AAHL (Table 1) (Hyatt et al., 2007). We considered a sample positive if all three readings of the triplicate showed greater than 0.01 zoospore DNA equivalents. Loads in each sample were estimated by averaging the number of zoospore DNA equivalents detected in the three replicates and multiplying these numbers by a factor equal to the dilutions between the extraction and the final sample (×80). The rest of tissue samples (259 bullfrogs) were diagnosed for *Bd* by PCR conventional assays (Annis et al., 2004).

Sample sizes were limited by the availability of each species. For those species with more than 20 sampled individuals, we estimated the prevalence of infection by dividing the number of infected individuals by the total number diagnosed

C_t	Internal standard (number of zoospores)			
	0.1	1	10	100
No. of cases	46	48	46	49
Mean	37.5	34.4	31.0	27.7
95% CI upper	38.0	34.9	31.4	28.1
95% CI lower	37.0	33.9	30.6	27.2
Standard error	0.3	0.3	0.2	0.2

(Table 2). This sample size guarantees an error <14% in the prevalence estimates (Digiacomo and Koepsell, 1986). *L. catesbeianus* samples diagnosed by real time-PCR (rt-PCR) and conventional PCR were analyzed separately as sensitivity of these assays is different (Kriger et al., 2006). Species- and stage-specific variations in prevalence of infection and zoospore concentrations were explored. Chi-square independence tests on two-way contingency tables were used to test whether infection was independent of species or stage (pre- vs. post-metamorphic). Because we found no significant differences between pre- and post-metamorphic prevalence of *L. catesbeianus* ($\chi^2 = 0.997$; $df = 1$; $p = 0.318$) or *D. meridensis* ($\chi^2 = 0.140$; $df = 1$; $p = 0.708$), we pooled these data to explore species variations. *Bd* zoospore distribution among hosts was determined in *L. catesbeianus* and *D. meridensis*, the two species with highest infection prevalence. The density distribution of hosts with different zoospore concentrations was fitted to a negative binomial function by maximum likelihood methods

(Maximum-likelihood Negative Binomial Distribution Fitting (v1.0.0) in Free Statistics Software (v1.1.21-r4). http://www.wessa.net/rwasp_fitdistrnegbin.wasp/). Zoospore loads of positive samples were log-transformed and data for pre- and post-metamorphic individuals were pooled as no significant differences were detected between theses stages in *L. catesbeianus* ($t = −0.644$, $df = 117$, $p = 0.521$) or *D. meridensis* ($t = 0.628$, $df = 26$, $p = 0.536$). Differences in zoospore loads between *L. catesbeianus* and *D. meridensis* were analyzed by *t*-tests on log-transformed data (Sokal and Rohlf, 1998). For all other infected species, we estimated only the mean zoospore load. The relation between the mean of log-zoospores for species and the altitude were explored by a linear regression (Sokal and Rohlf, 1998).

3. Results

B. dendrobatidis was found in 323 frogs out of a total of 649 samples; 44 of the *Bd*-positive specimens came from 10 native species, and 279 of the samples were bullfrogs (Table 2). Nine of the native species – *D. meridensis, Gastrotheca nicefori, Hypsiboas crepitans, Hyloscirtus jahni, Hyloscirtus platydactylus, Mannophryne collaris, Engystomops pustulosus, P. paradoxa*, and *Scarthyla vigilans* – are new host records for *Bd* infection. Of these, three species are endemic to the Venezuelan Andes and three are currently threatened (Table 2) (IUCN, Conservation International and NatureServe, 2006. Global Amphibian Assessment. www.globalamphibians.org (accessed on 8th August, 2007)). In contrast to some other countries where *Bd* has been detected, mass mortalities were not observed in any of the populations sampled.

Table 2 – Prevalence and zoospore load in frog species diagnosed for B. dendrobatidis by rt-PCR assay from La Cordillera de Mérida, Mérida state, Venezuela

Species	n	No of Positives	Prevalence (%) (95% CI)	Mean zoospores	Altitudinal range of positives	Breeding habitat
Dendropsophus meridensis∗ (EN)	105	28	26.7 (18.21–35.3)	2749	2330–2430	Permanent ponds
Dendropsophus microcephalus	20	0	∗∗∗	∗∗∗	∗∗∗	Permanent and ephemeral ponds
Dendropsophus minutus	8	0	∗∗∗	∗∗∗	∗∗∗	Permanent and ephemeral ponds
Pristimantis vanadise∗	1	0	∗∗∗	∗∗∗	∗∗∗	Terrestrial
Engystomops pustulosus	3	2	∗∗∗	12	98	Ephemeral ponds
Gastrotheca nicefori	2	2	∗∗∗	1701	1656–2327	Terrestrial (marsupial frog)
Hyalinobatrachium duranti∗	3	0	∗∗∗	–	–	Permanent streams
Hyloscirtus jahni∗	2	2	∗∗∗	2657	2390	Permanent streams
Hyloscirtus platydactylus (VU)	1	1	∗∗∗	1150	2000	Permanent streams
Hypsiboas crepitans	20	1	5.0 (0–14.5)	54	971	Permanent and ephemeral ponds and streams
Leptodactylus sp.∗	13	0	∗∗∗	∗∗∗	∗∗∗	Permanent and ephemeral ponds
Lithobates catesbeianus	149	119	79.9 (73.4–86.3)	2299	1837–2601	Permanent ponds
Mannophryne collaris∗ (EN)	3	1	∗∗∗	5	978	Permanent streams
Pseudis paradoxa	1	1	∗∗∗	26	127	Permanent ponds
Rhinella marina	20	2	10.0 (0–23.15)	11	861–1837	Permanent and ephemeral ponds and streams
Scinax flavidus	4	0	∗∗∗	∗∗∗	∗∗∗	Permanent ponds
Scarthyla vigilans	35	4	11.4 (0–21.9)	46	810–1837	Permanent ponds

Asterisk (∗) indicate species endemic to Venezuelan Andes and capital letters in parenthesis indicate the IUCN category for threatened species: EN, Endangered; VU, Vulnerable.

Differences in prevalence suggest that host exposure to *Bd* infection varied significantly among species. Infection prevalence was not independent of host species ($\chi^2 = 125.0$, $df = 4$; $p < 0.001$). Bullfrogs had the highest prevalence followed by its sympatric species, *D. meridensis*. Other host species, *Rhinella marina*, *H. crepitans* and *S. vigilans*, showed infection prevalence below 20% (Table 2). Bullfrog prevalence estimated from conventional PCR assays was lower (61.78%) than that estimated by rt-PCR assays (79.87%). These differences can be attributed to a higher sensitivity of the latter assay (Kriger et al., 2006); 11% of the positive specimens detected by rt-PCR lie below the detection threshold of the conventional PCR assay (1 zoospore DNA equivalent).

Zoospore loads in *L. catesbeianus* and *D. meridensis* showed a negative binomial distribution typical of macroparasites (Hudson and Dobson, 1995; Shaw et al., 1998). Both species show zoospores were highly aggregated in few individuals, with most frogs showing below 10^3 zoospores per tissue sample and very few with concentrations above 10^5. The negative binomial parameter (k) showed that zoospore aggregation was greater in *D. meridensis* ($k = 0.177$; $SD = 0.036$) than *L. catesbeianus* ($k = 0.255$; $SD = 0.026$). Also, the mean number of zoospores in *D. meridensis* (2749) was significantly greater than for *L. catesbeianus* (2299) ($t = -2.370$; $df = 145$; $p = 0.02$) in 1–3 g or tissue.

Bd showed a broad geographic distribution within the study area. Infected species were found across an area larger than 1000 km^2 between 98 and 2600 m (Fig. 1). Most infected individuals were collected above 1000 m (Table 2), and we detected a positive relationship between host zoospore load and altitude across all host species ($R^2 = 0.737$; $F = 25.177$; $p < 0.001$) (Fig. 2).

4.   Discussion

While the presence of *Bd* has been associated with the disappearance of many Andean species, including three *Atelopus* frogs from the Cordillera de Mérida, currently this pathogen appears to be widespread and endemic in this region. It was detected in 65% of the 17 examined species spanning six families (Aromobatidae, Amphignathodontidae, Bufonidae, Hylidae, Leiuperidae and Ranidae) found in ponds (ephemeral and permanent), streams and terrestrial habitats across an altitudinal gradient ranging from 98 to 2600 m. Although infection was detected in multiple species in every sampling occasion during the last 2.5 years, no mortalities were observed. Also, declines comparable with those recorded during the late 1980's have not been observed since then, despite historical evidence suggesting that *Bd* has persisted in the Cordillera de Mérida for more than 19 years (Hanselmann et al., 2004; Lampo et al., 2006a,b).

The heterogeneous distribution of *Bd* among species at the Cordillera de Mérida suggests variations in host exposures to this pathogen, and/or species-specific responses to infection. The highest infection prevalence was observed in *L. catesbeianus*, a likely reservoir species for *Bd* given its capacity to maintain subclinical infection (Daszak et al., 2004). Although the geographic distribution of this introduced species is still limited to 34 km^2 (Sánchez et al., unpublished data), we found evidence of infection in every sampled pond where this
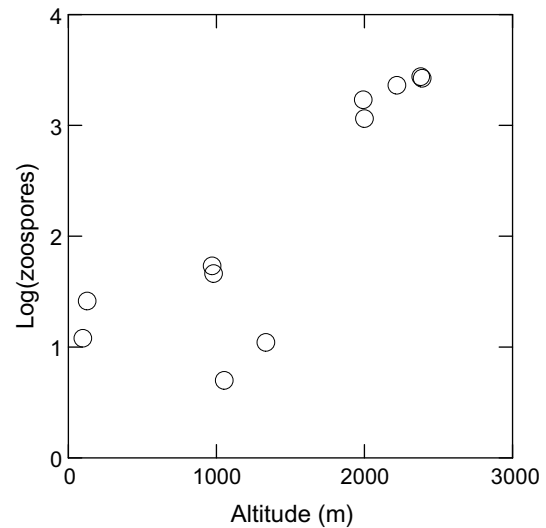

Fig. 2 – Relationship between mean zoospore load and the midpoint of the altitudinal distribution for eleven frog species infected by *B. dendrobatidis* in the Cordillera de Mérida, Venezuela.

species occurred. Among the native frogs, *D. meridensis* – the only species that shares habitat with *L. catesbeianus*, appeared to have the highest prevalence of infection. The high *Bd* exposure in *D. meridensis* could be driven by the presence of *L. catesbeianus*, a potentially important source of *Bd* infection through interspecific transmission. Interspecific transmission may play an important role in the infection dynamics of multi-host pathogens (Thorne and Williams, 1988; McCallum and Dobson, 1995). For example, the presence of prairie dogs increased the incidence of canine distemper virus on black-footed ferrets (McCallum and Dobson, 1995). Also, *Bd* exposure may be higher in permanent ponds where *L. catesbeianus* and *D. meridensis* are present. Aquatic amphibians associated with permanent water bodies may experience higher infection risk than those inhabiting ephemeral waterbodies (Lips, 1999; Stuart et al., 2004; Kriger and Hero, 2007).

Most permanent ponds in the study area occurred at higher altitudes, where temperatures are believed to be ideal for *Bd* growth (Daszak et al., 1999, 2003). While our results show that host species from higher altitudes had higher infection prevalence and zoospore loads, we cannot separate the altitudinal effects on prevalence from other confounding effects such as habitat-specific exposure risks or species-specific responses to *Bd* infection. For example, *L. catesbeianus*, a species capable of maintaining high levels of infection, inhabits permanent ponds at high elevations (between 1800 and 2800 m). It is therefore not surprising that this species has the highest infection prevalence and loads.

While high exposure to pathogens may result in high prevalence of infection, this can only be maintained in non-declining populations if the species is not highly vulnerable to the pathogen. We found no clinical signs of disease, such as abnormal posture, lethargy, or loss of righting reflex (Daszak et al., 1999) in any of the infected individuals of *D. meridensis*, despite its relatively high prevalence of infection (26.7%) and zoospore loads (2749 zoospores). This suggests that this

species could develop subclinical infection and may also act as a reservoir host. In Australia, *Litoria wilcoxi* with prevalence between 27% and 28% have been suggested as a reservoir (Retallick et al., 2004; Kriger and Hero, 2006).

Despite evidence suggesting that *D. meridensis* may currently act as a reservoir, its epidemiological status is a matter of conservation concern. As climatic or environmental deterioration may exacerbate infection and promote disease development, vulnerability of species may change over time. Our results show that *D. meridensis* harbors relatively high zoospore loads compared to other native species and that almost one third of the population could be at risk of developing chytridiomycosis. Due to the limited geographic distribution (<5000 km^2) and continuing fragmentation of its habitat, *D. meridensis* is considered "endangered" by the IUCN (*D. meridensis*. http://www.iucnredlist.org (accessed on 4th August, 2007)). The combination of these factors raises the possibility of increased threat in *D. meridensis* and warrants further monitoring of remaining populations.

While most other native species showed lower *Bd* loads and low infection prevalence compared to *D. meridensis* and *L. catesbeianus*, these patterns preclude inference about the vulnerability of these populations to developing chytridiomycosis. Some of these species may show innate resistance to infection or may lose *Bd* infection in the field. Infected frogs experimentally exposed to high temperatures in the laboratory cleared *Bd* infection (Woodhams et al., 2003), raising the interesting possibility that infected hosts could behaviorally mediate and eliminate infection. However, empirical evidence for behavioral responses to infection in amphibians exposed to *Bd* remains scarce (Han et al., in press). Mark-recapture studies of adult frogs have also documented infected frogs losing infection over time, presumably due to seasonal variations in temperature (Kriger and Hero, 2006). Alternatively, other species may be highly vulnerable but may experience such low transmission rates that mortality and other population-level impacts are difficult to detect. Highly vulnerable species are more likely to be at risk for chytridiomycosis if extrinsic factors lead to an increase in transmission rates.

Assessing the risk of anuran species to chytridiomycosis in areas where *Bd* occurs is a challenging task that requires a thorough understanding of the spatial and temporal variation in epidemiological parameters among various community assemblages. In the Cordillera de Mérida, the *Bd* distribution among frog species is heterogeneous. To accurately characterize disease risk within these frog communities, it is critical to understand the factors contributing to low infection prevalence and zoospore loads in most species. Furthermore, understanding the extrinsic factors that may mediate species vulnerability to this pathogen will be important for conservation management, particularly in those populations that may experience heightened pathogen exposure through co-existing reservoir species. While experimental infection trials are crucial to identify species-specific susceptibility to *Bd* infection and vulnerability to chytridiomycosis, identification of those variables that potentially mediate the exposure of frogs to this pathogen will contribute in important ways to the conservation of host species across heterogeneous landscapes.

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