

# Susceptibility of the endangered frog *Dendropsophus meridensis* to the pathogenic fungus *Batrachochytrium dendrobatidis*

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**ABSTRACT:** Chytridiomycosis is an emerging disease that has driven some amphibian species to extinction while leaving others apparently unharmed. Its causative agent, *Batrachochytrium dendrobatidis* (*Bd*), now persists endemically in many amphibian communities. Understanding host species response to *Bd* infection is critical for managing chytridiomycosis because the epidemiology of this disease is host-specific. *Dendropsophus meridensis* is an endangered hylid frog endemic to the Venezuelan Andes. This species is sympatric with the American bullfrog *Lithobates catesbeianus*, an introduced species known to act as a reservoir for *Bd*. High prevalence of infection and high zoospore burdens in wild populations of *D. meridensis* in the Venezuelan Andes suggested some tolerance for *Bd*. However, experimental exposure of post-metamorphic frogs resulted in 53% mortality, a value that represents a 14-fold increase in the odds of dying compared to control frogs. Repeated diagnostics using real-time polymerase chain reaction assays demonstrated that individuals that died accumulated a higher number of zoospores than those that survived, although this value was lower than the mean zoospore burdens observed in natural populations. Given the susceptibility of *D. meridensis* to a strain of *Bd* isolated from a nearby population of bullfrogs, we emphasize the need to limit the dispersion of this invasive species.

**KEY WORDS:** Amphibians · Chytrid fungus · Chytridiomycosis · American bullfrogs · Experimental infections · Venezuela

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## INTRODUCTION

Chytridiomycosis is an emerging infectious disease that has spread across amphibian communities worldwide (Skerratt et al. 2007, Fisher et al. 2009b, Crawford et al. 2010). Its causative agent, *Batrachochytrium dendrobatidis* (*Bd*; Berger et al. 1998, Longcore et al. 1999), is a fungal pathogen that can kill amphibians by disrupting the integrity of their skin and compromising their homeostasis (Berger et al. 1998, Voyles et al. 2009, Brutyn et al. 2012). While rapid population declines and local extinctions have been associated with the spread of *Bd*, this pathogen

now persists endemically in many amphibian populations (Retallick et al. 2004, Kriger & Hero 2006, Puschendorf et al. 2006, Rachowicz et al. 2006, Sánchez et al. 2008, Briggs et al. 2010, Vredenburg et al. 2010, Lampo et al. 2012).

The drivers of chytridiomycosis epizootics are not fully understood. The presence of *Bd* in declining and non-declining sympatric species suggests that the epidemiology of this disease is dependent on host-specific responses to the pathogen (McDonald et al. 2005, Woodhams & Alford 2005). Frog responses to *Bd* infection vary considerably among species; some die shortly after exposure (Berger et al.

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1998, 2004, Nichols et al. 2001, Lamirande & Nichols 2002, Woodhams et al. 2003, Rachowicz & Vredenburg 2004, McDonald et al. 2005, García et al. 2006, Retallick & Miera 2007) while others are able to rid themselves of the pathogen (Márquez et al. 2010) or live with aclinical infections for long periods (Reeder et al. 2012). Theoretical work suggests that the within-host dynamics can explain the different population-level outcomes of *Bd* infection (Briggs et al. 2010). Thus, understanding the infection progression in particular species is the first step for predicting epizootic scenarios.

Assessing frog susceptibility to chytridiomycosis in wild populations is difficult. Morbid individuals are rarely observed in the field because clinical signs often develop shortly before death and frog die-offs can occur unnoticed (Longo & Burrowes 2010, Woodhams et al. 2011, Greenspan et al. 2012). Thus, experimental infections are an important tool for determining the susceptibility of frogs to *Bd* infection and observing disease progression. Mortality of experimentally infected amphibian species can vary from 0 to 100% (see review by Kilpatrick et al. 2010), although this variation can be partially attributed to differences in experimental conditions among studies (i.e. pathogen strain, initial dose, host-stage, or temperature). However, species exposed to *Bd* under identical experimental conditions have shown substantial intraspecific variation in their susceptibility to chytridiomycosis (Searle et al. 2011). Given the complexity of the interaction between the pathogen, the host, and ambient conditions, studies on species susceptibility to *Bd* under controlled conditions are a first step for predicting the impact of chytridiomycosis in wild populations.

*Dendropsophus meridensis* is a frog species endemic to the Venezuelan Andes. It breeds in lentic waters, but adults are frequently found on the surrounding vegetation (Duellman & Trueb 1986). This species is considered Endangered by the IUCN. Its small geographic range, the increasing fragmentation of its distribution due to the expansion of agricultural activities, and the invasion of its habitat by the introduced American bullfrog *Lithobates catesbeianus* are among the major threats this species faces (La Marca 2004). The invasion of *D. meridensis* habitats by bullfrogs is concerning, because, in addition to being a voracious predator (Hecnar & M'Closkey 1997), bullfrogs may act as reservoir hosts to *Bd* that enable this pathogen to drive less tolerant hosts to local extinction (Daszak et al. 2004).

It is not known how vulnerable *Dendropsophus meridensis* is to developing lethal chytridiomycosis, but the prevalence of *Bd* infection in wild popula-

tions ( $\approx 27\%$ ; Sánchez et al. 2008) suggests some level of tolerance for *Bd*. Based on repeated diagnostics using quantitative real-time PCR (qPCR), we documented the course of infection and determined the susceptibility of *D. meridensis* to chytridiomycosis, after experimental exposure of post-metamorphic individuals to *Bd*.

## MATERIALS AND METHODS

### Specimen collection and acclimation

Fifty-one tadpoles of *Dendropsophus meridensis* were collected in November 2010 from an artificial pond at a locality in San Eusebio, Mérida State, Venezuela ( $8^{\circ}38'N$ ,  $71^{\circ}23'W$ , 2240 m a.s.l.). Tadpoles were preferred over post-metamorphic stages, because in some species, infection is eliminated by the loss of keratinized mouthparts during metamorphosis (Woodhams & Alford 2005), and reinfection in the laboratory can be minimized if the water is regularly changed. Live tadpoles were transported to the laboratory in individual containers following biosecurity guidelines to avoid cross-contamination (Aguirre & Lampo 2006). In the lab, tadpoles were maintained individually in  $13.5 \times 11$  cm diameter plastic containers filled with tap water in a Precision 818 Low Temperature Illuminated Incubator at  $19^{\circ}C$  and 12 h photoperiod. Tadpoles were fed every other day with Nutrafin Mix<sup>®</sup> fish flakes, and the water was totally or partially changed, depending on the amount of debris accumulated. After metamorphosis, all specimens were moved to individual  $19 \times 30 \times 9$  cm plastic terrariums with absorbent paper as a substratum. These were also kept in the incubator at  $19^{\circ}C$ , a temperature within the range reported in the area inhabited by *D. meridensis* ( $15\text{--}23^{\circ}C$ ), and 12 h photoperiod. Every 2 to 3 d, terrariums were sprayed with water to maintain humidity, and post-metamorphic frogs were fed with fruit flies *Drosophila melanogaster* and house crickets *Acheta domestica* sprayed with Rep-Cal<sup>®</sup> multi-vitamins and calcium.

### *Bd* isolation and culture

Skin samples were obtained from bullfrog tadpoles collected in October 2010 from Jají (Municipio Campo Elías, Mérida State), a locality near where *Dendropsophus meridensis* were collected (Hanselmann et al. 2004, Sánchez et al. 2008). *Bd* was isolated from infected oral discs using petri dishes with standard

TGhL nutrient agar medium (1% tryptone, 1% agar) with 0.2% penicillin (Pessier et al. 1999). Isolates were transferred to 1% tryptone broth and stored at 4°C. The isolate was labeled La Esmeralda-Catesbeianus-10-CG-1 (*Bd*LEcat10CG-1) according to the nomenclature suggested by Berger et al. (2005).

### Experimental infection

Thirty-seven petri dishes with standard TGhL medium were inoculated with 200 µl of the liquid culture of isolate *Bd*LEcat10CG-1 and kept at 18°C. After 2 wk, each petri dish was washed with 1 ml of water, and after 1 min, zoospores were harvested by collecting the water with the suspended zoospores and pooling them into a single solution. Zoospores were counted with a hemocytometer and diluted to obtain a *Bd* inoculum of  $3 \times 10^6$  zoospores ml<sup>-1</sup> for experimental infections. For controls, 17 petri dishes with standard TGhL medium (without *Bd*) were washed with 1 ml of water each, and after 1 min, the water was collected and pooled into a control inoculum.

Post-metamorphic frogs varied significantly in age at the time of exposure (4–14 wk old). All individuals were swabbed and tested for *Bd* using qPCR 2 wk before the beginning of the experiment. Although this test provides no information on previous exposures, it suggested that, except for 5 frogs that tested positive with low numbers of zoospores, all others were uninfected at the beginning of the experiment. Non-infected frogs were assigned randomly to treatment (17) or control groups (15). The 5 infected frogs were experimentally exposed and assigned to a separate treatment group (reinfected). All frogs were inoculated in plastic bags. Three milliliters of the *Bd* inoculum ( $9 \times 10^6$  zoospores) were added to each 25 × 30 cm plastic bag containing treatment frogs, while a similar volume of the control inoculum (no *Bd*) was used for control frogs. Frogs were kept in these bags for 24 h at 19°C. Because frogs were occasionally found in the sides of the bags away from the inoculum, we induced them to move and contact the inoculum every 2 h to maximize exposure. All frogs were transferred to their terrarium after the 24 h exposure period.

### *Bd* sampling and diagnosis

After exposure, samples were obtained from every individual in the control and treatment groups every 6 d during 66 d, by swabbing the groin, the ventral

surface of the limbs, and the interdigital membranes. Before swabbing, each frog was rinsed with tap water to minimize the probability of contamination with zoospore remains in the substrate of containers, or to eliminate detritus that could interfere with DNA amplification. Samples were stored at -4°C for 1 to 6 d before DNA was extracted. Animals that died were preserved in alcohol and kept as vouchers in the laboratory, and those that survived were used in subsequent experiments.

To diagnose *Bd* infection and estimate zoospore loads, qPCR Taqman assays were conducted using an Opticon Thermocycler (BIORAD) (Boyle et al. 2004). DNA was extracted from swabs or tissue samples using Prep-Man Ultra (Applied Biosystems by Life Technology) (Boyle et al. 2004). Standard curves were constructed using 100, 10, 1, and 0.1 *Bd* zoospore quantification standards (provided by A. Hyatt, Australian Animal Health Laboratory, Division of Lifestock Industries, CSIRO, Victoria, Australia) in each assay (Boyle et al. 2004). Negative and positive controls were included in each assay to detect DNA contamination or amplification inhibition. Each sample was run in duplicate. A sample was considered positive when both replicates had more than 0.1 zoospore DNA-equivalents. If both replicates had less than 0.1 zoospore DNA-equivalents, the sample was recorded as negative. The infection intensity in each sample was estimated as the mean number of zoospore DNA-equivalents in both replicates. If replicates of a single sample resulted in different diagnostics (i.e. 1 positive and 1 negative) or their estimated number of zoospores differed by 1 order of magnitude, the sample was diagnosed again from stored DNA. Diagnostic and zoospore estimates for the second run were unequivocal in all cases, and thus replaced the previous estimates. We considered a frog as having recovered from *Bd* infection if it tested negative in at least 2 consecutive samples.

### Statistical analyses

To determine whether *Bd* infection had a significant effect on the survival of *Dendropsophus meridensis*, we compared survival curves of treatment and control frogs using Cox's proportional hazards model on censored survival data using the Survival package (v. 2.36-5) implemented in the R statistical computing environment (R Development Core Team 2011). After a log-transformation to homogenize the sample variance, the number of zoospore DNA-equivalents in samples from deceased and surviving

animals was compared using a Wilcoxon signed paired test, to determine whether infection intensity was related to morbidity or mortality. The recovery time and time-to-death were estimated as the time between exposure and the time to the first of 2 consecutive negative tests or death, respectively.

## RESULTS

Thirty-seven tadpoles reached the juvenile stage in the lab between December and March 2011. Infection rates after experimental exposure were high; except for 1 reinfected frog that tested negative throughout the experiment, all other exposed frogs tested positive after 24 d. Negative PCR controls gave no evidence of contamination. In contrast, all but 2 control frogs tested negative during the course of the experiment. These were probably accidentally infected during manipulation or had initially subdetectable infections. As their initial dose of exposure was not known, they were omitted from the analysis.

The survival curve suggested an initial incubation phase (exposure to 24 d) during which no mortality or morbidity was observed, and a clinical phase (24 to 66 d) during which some frogs became ill and survival decreased (Fig. 1). Survival curves between experimentally infected and control frogs differed

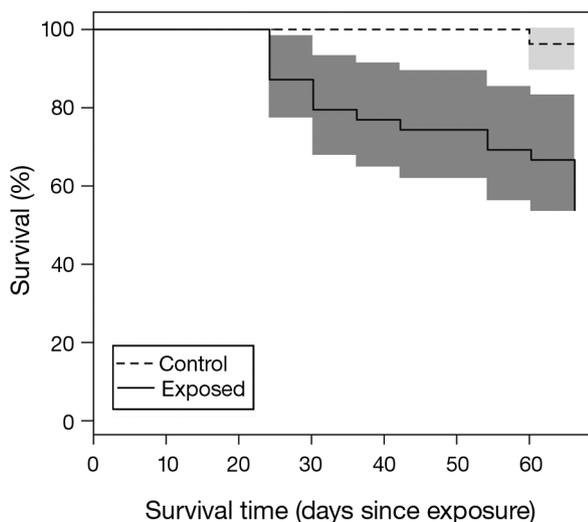


Fig. 1. *Dendropsophus meridensis*. Survival of adults after experimental exposure to  $9 \times 10^6$  zoospores of *Batrachochytrium dendrobatidis* (*Bd*). The 15 control frogs were non-exposed; the 22 exposed frogs included 5 individuals which tested positive for low numbers of *Bd* zoospores prior to this experiment, and were re-exposed. Gray areas: 95% confidence bands

significantly (logrank test = 9.26, df = 1,  $p = 0.0023$ ). However, because these curves did not differ between experimentally infected frogs and those naturally infected and re-infected in the laboratory (Logrank test = 0.66, df = 1,  $p = 0.4153$ ), these 2 groups were combined into a single treatment group of exposed frogs. The survival curve of this exposed group was also significantly different than that for the control group (Logrank test = 11.15, df = 1,  $p = 0.00084$ ; Fig. 1). The odds of infected frogs dying were 14 times greater than for non-infected control frogs. Ninety-three percent of frogs (14/15) in the control group survived while only 41% (9/22) of frogs in the exposed group (7 treatment + 2 reinfected) were alive at the end of the experiment. Despite the high incidence of infection of exposed frogs on Day 24, the fraction of surviving frogs that tested positive decreased thereafter. On Day 66, when the experiment terminated, only 1 out of the 9 exposed frogs that survived showed evidence of infection, and the number of zoospores in this animal decreased by 3 orders of magnitude between Days 60 and 66. This suggests that while *Bd* infection was lethal in some animals, others were capable of eliminating the pathogen before Day 60. The mean recovery time (41.3 d) of surviving frogs and the mean time to death (44.57 d) of infected frogs were similar, although these varied among individuals.

Infection progression varied greatly among frogs (Fig. 2). In some individuals, the number of zoospores varied by a factor of 100 during the course of the

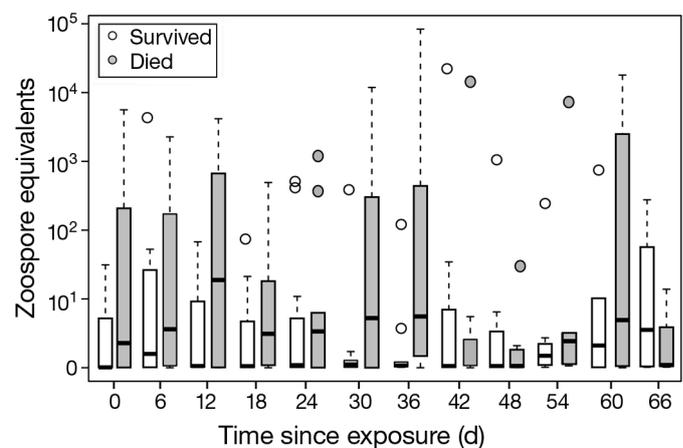


Fig. 2. *Dendropsophus meridensis*. *Batrachochytrium dendrobatidis* loads in 22 infected frogs after experimental exposure to  $9 \times 10^6$  zoospores. Bars show first and third quartiles with medians (black lines)  $\pm$  SD (whiskers) and outliers (circles) of zoospore equivalents determined from PCR analysis on skin swabs collected over the course of infection

experiment, while in others this variation did not exceed 1 order of magnitude. Possible explanations for these variations are differences in the age or body condition of post-metamorphic individuals, or uneven exposure of individuals to the inoculum while kept in plastic bags. Nonetheless, frogs that developed chytridiomycosis and died tended to show higher zoospore loads than those that survived with aclinical infections throughout the study (Wilcoxon signed paired rank = 67,  $p = 0.014$ ).

## DISCUSSION

Understanding frog species responses to *Bd* infection is a first step for predicting the impact this pathogen may have on frog communities. Experimental exposure of post-metamorphic frogs to *Bd* demonstrated that *Dendropsophus meridensis* is vulnerable to developing chytridiomycosis, but the outcome of infection varies among individuals. Some frogs develop lethal infections while others are capable of eliminating the pathogen. Although a 14-fold increase in the mortality odds of infected frogs compared to non-infected ones suggests a low tolerance under experimental conditions, these results do not necessarily describe disease progression in wild populations.

Frogs infected in this experiment appeared to have a lower tolerance to chytridiomycosis than some of their conspecifics from wild populations. Post-metamorphic individuals of *Dendropsophus meridensis* from natural populations tend to support higher zoospore burdens (2749 zoospores; Sánchez et al. 2008) than those observed during our experiments, including in animals that died (152 zoospores). One possible explanation is a higher vulnerability of frogs to *Bd* under captive conditions. Some species capable of coexisting with *Bd* in natural populations have shown 100% mortality upon experimental exposure in the laboratory (Briggs et al. 2005). Although the mechanisms associated with the recovery of *Bd*-infected frogs are not fully understood, the ability of frogs to change their body temperature by moving along temperature gradients appears to be important in reducing frog mortality due to chytridiomycosis (Richards-Zawacki 2010, Daskin et al. 2011). Such a response, however, was not possible in our experiments due to the absence of temperature gradients within the terrariums. Alternatively, the *Bd* strain used in this experiment could have a higher pathogenicity than some of the strains circulating in wild populations of *D. meridensis*. *Bd* strains vary in their

pathogenicity (Berger et al. 2005, Fisher et al. 2009a, Searle et al. 2011, Gahl et al. 2012, Doddington et al. 2013), and *Bd* maintained in culture can exhibit rapid shifts in pathogenicity, although successive passages in culture media tend to attenuate it (Fisher et al. 2009a, Brem et al. 2013). Thus, we need to investigate the possible sources of variation in *Bd* pathogenicity both *in vivo* and *in vitro*.

Despite potential variations in frog responses to infection between captive and wild frogs, our experiment demonstrates that *Dendropsophus meridensis* can be vulnerable to *Bd* strains isolated from nearby bullfrog populations. Theoretical work has shown that vulnerable hosts can be driven to extinction by pathogens in the presence of reservoir hosts (Holt & Lawton 1994, Hudson & Greenman 1998, McCallum & Dobson 2002, de Castro & Bolker 2005). Declines of several native frog species in Sierra Nevada National Park (California, USA) occurred in the presence of the Pacific chorus frog *Pseudacris regilla*, a *Bd* reservoir (Reeder et al. 2012). Although it is unclear whether such declines were driven by *P. regilla*, those authors suggested that this reservoir could have contributed by releasing high numbers of zoospores to which other species were exposed. In light of the susceptibility of *D. meridensis* to *Bd* strains isolated from bullfrogs, it is possible that bullfrogs could drive this endangered species to local extinction. Thus, we emphasize the importance of controlling bullfrog dispersion into *D. meridensis* habitats.

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