Physiological responses of Brazilian amphibians to an enzootic infection of the chytrid fungus *Batrachochytrium dendrobatidis*

Rafael P. Bovo[1],*, Denis V. Andrade[1], Luís Felipe Toledo[2], Ana V. Longo[3], David Rodriguez[4], Célio F. B. Haddad[1], Kelly R. Zamudio[3], C. Guilherme Becker[1]

[1]Departamento de Zoologia, c. p. 199, Universidade Estadual Paulista, 13506-900, Rio Claro, SP, Brazil
[2]Laboratório de História Natural de Anfíbios Brasileiros (LaHNAB), Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas, Rua Monteiro Lobato, 255, 13083-862, Campinas, SP, Brazil
[3]Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY 14853, USA
[4]Department of Biology, Texas State University, San Marcos, TX 78666, USA

ABSTRACT: Pathophysiological effects of clinical chytridiomycosis in amphibians include disorders of cutaneous osmoregulation and disruption of the ability to rehydrate, which can lead to decreased host fitness or mortality. Less attention has been given to physiological responses of hosts where enzootic infections of *Batrachochytrium dendrobatidis* (*Bd*) do not cause apparent population declines in the wild. Here, we experimentally tested whether an enzootic strain of *Bd* causes significant mortality and alters host water balance (evaporative water loss, EWL; skin resistance, R_s; and water uptake, WU) in individuals of 3 Brazilian amphibian species (*Dendropsophus minutus*, n = 19; *Ischnocnema parva*, n = 17; *Brachycephalus pitanga*, n = 15). Infections with enzootic *Bd* caused no significant mortality, but we found an increase in R_s in 1 host species concomitant with a reduction in EWL. These results suggest that enzootic *Bd* infections can indeed cause sub-lethal effects that could lead to reduction of host fitness in Brazilian frogs and that these effects vary among species. Thus, our findings underscore the need for further assessment of physiological responses to *Bd* infections in different host species, even in cases of sub-clinical chytridiomycosis and long-term enzootic infections in natural populations.

KEY WORDS: Chytridiomycosis · Amphibian declines · Fitness · Evaporative water loss · Water uptake · Amphibian skin · Mortality

INTRODUCTION

The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) causes chytridiomycosis and has led to amphibian population declines and extinctions in several parts of the globe (Berger et al. 1998, Lips et al. 2008, Vredenburg et al. 2010). Sub-lethal chytridiomycosis, however, is common where *Bd* is enzootic, such as in Brazil's Atlantic Forest, where multiple *Bd* strains have infected several hosts (Valencia-Aguilar et al. 2015) since at least the late 1800s (Rodriguez et al. 2014). Studies of *Bd* epidemiology in Brazil are on the rise (Becker & Zamudio 2011, Becker et al. 2014, 2015, Lambertini et al. 2016, Ruggeri et al. 2015, Valencia-Aguilar et al. 2015), but to date no records of amphibian declines in Atlantic Forest frogs have been attributed to *Bd* (Toledo et al. 2006, Haddad et al. 2013, James et al. 2015). Because the Atlantic Forest harbors ~10% of all described amphibian species (Haddad et al. 2013, Toledo et al. 2014, IUCN

2015), experimentally testing the effect of locally iso-
lated *Bd* on both mortality and physiological re-
sponses of local host species is critical for biodiversity
conservation.

Clinical infections of *Bd* often impair amphibians
from maintaining adequate homeostasis and directly
impact host physiological and functional responses
(Voyles et al. 2009, Salla et al. 2015). Specifically, *Bd*
infection causes hyperkeratosis (skin thickening)
and impairs host ability to absorb and regulate body
fluids, leading to electrolyte imbalance and cardiac
arrest in infected hosts (Voyles et al. 2011). Skin
permeability is critically important in regulating
water transport and exchange of electrolytes and
respiratory gases in amphibians (Wells 2007, Hill-
man et al. 2009, Voyles et al. 2011). In cases of
aclinical chytridiomycosis (i.e. when hosts do not
show gross pathological changes in the superficial
epidermis; Voyles et al. 2011), the potential impair-
ments in physiological function remain, for the most
part, unmeasured (but see also Salla et al. 2015).
However, previous studies indicate that even under
aclinical conditions, *Bd* may interfere with host
water balance (Carver et al. 2010, Wardziak et al.
2013). If that is the case, even minor shifts in host
osmoregulation — a physiological process intimately
associated with amphibian life history, habitat use,
and evolutionary ecology — could lead to long-term
decreases in host population fitness.

A number of studies have investigated the impact
of chytridiomycosis on host water balance (Voyles et
al. 2009, Carver et al. 2010, Voyles 2011, Campbell et
al. 2012, Wardziak et al. 2013). Water balance para-
meters are particularly relevant in amphibians due to
their highly permeable skin and the associated risk of
incurring excessive evaporative water loss (EWL;
Shoemaker et al. 1992, Spotila et al. 1992, Lillywhite
2006). While laboratory studies have shown impair-
ments of normal skin functions in *Bd*-infected
amphibians, the magnitude of these effects across
hosts depends on a complex array of factors, includ-
ing differences in *Bd* strains (Gahl et al. 2012), previ-
ous exposure of the host (Murphy et al. 2011), host
life history (Gervasi et al. 2013), and host susceptibil-
ity (Searle et al. 2011). It is uncertain whether low
infection intensities, such as those in localities where
Bd is an enzootic pathogen, can significantly inter-
fere with EWL rates and/or influence the rates of
water uptake (WU) through the ventral skin, and
whether such effects vary among host species.

In this study, we experimentally tested whether
infection by an enzootic strain of *Batrachochytrium
dendrobatidis* causes (1) significant mortality and (2)

alters water balance in 3 amphibian species native
to Brazil's Atlantic Forest. Specifically, we tested
whether experimental *Bd* infections affected 3 physi-
ological parameters key to host water balance: (1)
EWL, (2) skin resistance (R_s), and (3) WU. If endemic
Bd infection is detrimental for Brazilian amphibians,
we would expect to observe either some evidence
of disease-associated mortality (clinical chytridio-
mycosis) or some evidence of sub-clinical pathophy-
siology that may compromise host osmoregulatory
capabilities.

MATERIALS AND METHODS

Study species

We captured adult amphibians of 3 species in
October 2012 at Parque Estadual da Serra do
Mar–Núcleo Santa Virginia, municipality of São
Luiz do Paraitinga, state of São Paulo, Brazil
(23.35° S, 45.16° W). *Dendropsophus minutus* has
aquatic larvae, breeds in aquatic habitats, and occu-
pies the emergent vegetation at the edge of water
bodies. *Ischnocnema parva* and *Brachycephalus
pitanga* deposit eggs on land, undergo direct devel-
opment, and are active on the forest leaf-litter. By
using wild-caught amphibians in laboratory experi-
mental infections, we are not able to control for age,
previous exposure, and potential acquired resist-
ance (McMahon et al. 2014); however, randomly
assigning individual hosts for each treatment can
minimize these uncontrolled factors.

Experimental design

We treated field-collected animals with itracona-
zole 0.01% for 7 d to ensure study organisms were
Bd-negative prior to the beginning of the experiment
(Pessier & Mendelson 2010, Moreno et al. 2015);
treated hosts all tested negative prior to experimental
infections. We cultured an enzootic *Bd* strain isolated
from Minas Gerais, Brazil (CLFT 023; Schloegel et al.
2012, Longo et al. 2013) on tryptone agar Petri plates
at ~19°C for 7 d. We harvested *Bd* by flooding plates
with distilled water and waiting ~3 h for zoospore
release. The strain used in experiments belongs to
the global panzootic lineage (GPL), a highly virulent
strain causing amphibian declines and extinctions in
several regions of the globe (Rosenblum et al. 2013).
In Brazil, it has been present for at least a century
(Rodriguez et al. 2014). We then pooled inoculum

from plates and quantified zoospores with a hemocytometer.

We added 4 conspecific amphibians to rectangular experimental units measuring 40 × 29 × 13.5 cm and kept temperatures at 19.74°C (0.55 SD). These temperature records were measured by a Hobo® data logger at 30 min intervals and on a 12 h light:12 h dark cycle. Each experimental unit consisted of a plastic terrarium with terrestrial habitat covering one end of the container (i.e. autoclaved moist sphagnum) and aquatic habitat covering the other end of the container (i.e. 250 ml of *Bd* inoculum, 10^6 zoospores total, in the treatment group or 250 ml of distilled water in the control group).

We monitored amphibians daily and fed them pinhead crickets *ad libitum.* We swabbed all individuals after 18 d of exposure, a period encompassing several replication cycles of the pathogen (Longcore et al. 1999). Even though timing of *Bd* life-cycle depends on the isolate (Lambertini et al. 2016) and temperature (Murphy et al. 2011), 18 d is often enough time for animals exposed to very high zoospore counts to show signs of lethargy or mortality (Savage & Zamudio 2011, Gahl et al. 2012), thus also likely showing alterations in host physiology. We swabbed the single individual that died during the experiment and removed it from the experimental unit; water balance parameters were not measured for this individual. We tested samples for *Bd* in duplicate using Taqman quantitative PCR (qPCR) (Boyle et al. 2004, Lambertini et al. 2013) with CLFT 023 standards of 0.1, 1, 10, 100, and 1000 zoospore genomic equivalents (GE) to determine the infection intensity of *Bd* in each amphibian host. We used a total of 51 amphibians in downstream water balance analyses (Table 1).

Measurements of host physiological responses

All physiological measurements were taken within a window of 4 d following Day 18 post-inoculation. Prior to measuring evaporative water loss, we held animals in individual PVC containers (4 cm diameter) with 0.5 cm of water, in a climate-controlled chamber (122FC model, Eletrolab) at 20°C for 1 h to ensure that they were fully hydrated at the beginning of the experiment. Immediately after this period, each frog was carefully blotted with paper tissue, its urinary bladder was emptied by gently pressing the abdomen, and body mass was recorded (±0.0001 g). We placed each individual frog in a circular PVC chamber (8 cm diameter) connected to an automated open flow system that measures EWL. A pump combined to a mass flow meter (SS-3 Subsampler, Sable Systems) provided stable airflow current of 21.66 $cm^3 s^{-1}$, with relative humidity (RH) controlled at 30% by a RH/Dewpoint Controller (DG-4, Sable Systems). Airflow was directed into the experimental chamber, and relative humidity of excurrent air was continuously monitored by a water vapor analyzer (RH-300 RH/Dewpoint Analyzer, Sable Systems). All equipment was maintained inside the climate-controlled chamber at the experimental temperature of 20°C.

Changes in relative humidity were interfaced to a computer by an analog/digital unit (UI2, Sable Systems) and recorded/analyzed using the Expedata software (Sable Systems). The amount of water lost, per animal, per unit of time, was calculated from the increment in water content between the airflow upstream (controlled at 30% RH) and downstream of the animal chamber (measured using the water vapor analyzer). Measurements lasted for up to 30 min per individual, and for quantitative estimates we selected a period of steady-state readings lasting for at least 10 min, typically during the second half of the measurement period. For each animal, the total EWL was then corrected for unit area of exposed skin surface and expressed as µg H_2O cm^{-2} s^{-1}. We estimated the exposed surface area of individual amphibians on the basis of their body mass (McClanahan & Baldwin 1969, Young et al. 2005) and the posture assumed by the animals, which were monitored visually during the experiments. EWL through the respiratory system was assumed to be negligible (Spotila & Berman 1976, Bentley & Yorio 1979, Wygoda 1984) and not considered in our estimates.

Table 1. Summary of number of individuals, body mass (for all frogs), and infection loads (for *Bd*-infected individuals only) for the 3 host species. Data are means ± SD. Uninf: uninfected; GE: genomic equivalents

Species	Total (no.)	*Bd*-infected (no.)	Uninf. control control (no.)	Body mass (g)	*Bd*-infection loads (GE)
Dendropsophus minutus	19	12	7	0.51 ± 0.07	22.37 ± 49.44
Ischnocnema parva	17	10	7	0.32 ± 0.13	46.50 ± 60.74
Brachycephalus pitanga	15	8	7	0.2 ± 0.04	19.75 ± 45.75

Table 2. Effects of *Bd* infection on water balance in 3 Brazilian anuran species from the Atlantic Forest. Significant effects are highlighted in **bold**

Variable	F	p
Evaporative water loss ($\mu g\ cm^{-2}\ s^{-1}$)		
Treatment	14.73	**<0.001**
Species	9.34	**<0.001**
Species × Treatment	2.68	0.079
Body mass	2.08	0.155
Skin resistance ($s\ cm^{-1}$)		
Treatment	7.76	**<0.01**
Species	17.22	**<0.0001**
Species × Treatment	4.05	**<0.05**
Body mass	0.03	0.853
Water uptake ($\mu g\ cm^{-2}\ s^{-1}$)		
Treatment	−2.24	**<0.05**
Species	−0.05	0.956
Species × Treatment	−0.43	0.668
Body mass	1.18	0.246

For each individual frog that had its EWL rate measured, we also measured a similar-sized 3% agar replica to determine boundary layer resistance, which allowed us to calculate skin resistance to water efflux (R_s, expressed as $s\ cm^{-1}$) (see Shoemaker et al. 1992, Young et al. 2005). Total resistance to water efflux (R_t) was calculated as the sum of skin (R_s) and boundary layer (R_b) resistances and is expressed in terms of a mass transfer coefficient, i.e. as centimeters of water movement per second. The reciprocal of this coefficient is termed the resistance of water movement ($s\ cm^{-1}$; Shoemaker et al. 1992, Young et al. 2005).

Immediately after the EWL tests, each individual was placed in a Petri dish with water to a depth of 0.5 cm, sufficient to cover the ventral abdominal region (Cree 1988). Frogs were carefully blotted with paper tissue and weighed (±0.0001 g) 6 consecutive times at 2 min intervals. We calculated WU from the linear regression between body mass increments against time. Then, using the estimated surface area in contact with the water (same equations as above), we calculated the rate of WU per unit of area and expressed it as $\mu g\ H_2O\ cm^{-2}\ s^{-1}$. WU was not determined for *B. pitanga* individuals due to their diminutive body size, which precluded accurate measurements.

Statistical analyses

We compared mortality rates between treatment and control groups for each species using Wilcoxon tests. We compared host physiological responses (EWL, R_s, and WU) between *Bd* infection treatments (*Bd*-infected and *Bd*-uninfected) using general linear models (GLM) with standard least squares. We included in the model treatment crossed with species and the main effect of body mass. We performed GLMs to test the effect of *Bd* infection loads on each water balance parameter (EWL, R_s, and WU), independently for each species. We log$_{10}$-transformed *Bd* infection load data for this analysis. We did not include prevalence data in the analyses because all amphibians in the experimental infection tested positive for *Bd*.

RESULTS

Bd-infected hosts showed no significant increase in mortality when compared to uninfected controls; only 1 individual frog died during the course of the experiment (*B. pitanga*; *Bd*-infected; log-rank χ^2 = 0.875, df = 1, p = 0.349). However, the treefrog *Dendropsophus minutus* showed a significant increase in R_s associated with a decreased EWL (Table 2, Fig. 1). No such changes were observed for the other 2 species (Table 2, Fig. 1). WU did not differ significantly between *Bd*-infected and uninfected hosts in any of the species tested (Table 2, Fig. 1).

All individuals exposed to *Bd* tested positive at 18 d post initial exposure but showed consistently low infection loads (Fig. A1 in the Appendix). *Bd* infection loads did not differ among species (Kruskal-Wallis H = 2.68, df = 2, p = 0.26, Fig. A1, Table 1), and we also found no significant relationship between water balance measures and *Bd* infection loads after Bonferroni correction for multiple comparisons.

DISCUSSION

The global pandemic *Bd* strain (GPL-2) CLFT 023, enzootic in the Atlantic Forest for at least 100 yr (Rodriguez et al. 2014), did not cause significant mortality in any of our focal host species under experimental conditions but led to shifts in the water balance of 1 out of 3 of the tested species. Therefore, our results underscore that enzootic *Bd* GPL may cause negative effects in wild anuran populations, even in cases when hosts may have potentially evolved resistance or tolerance to the focal *Bd* isolate (Briggs et al. 2005, Carey et al. 2006, Woodhams et al. 2011, Rodriguez et al. 2014). Indeed, the observed host infection loads in our experimental study were 2 orders of magnitude lower than loads observed in
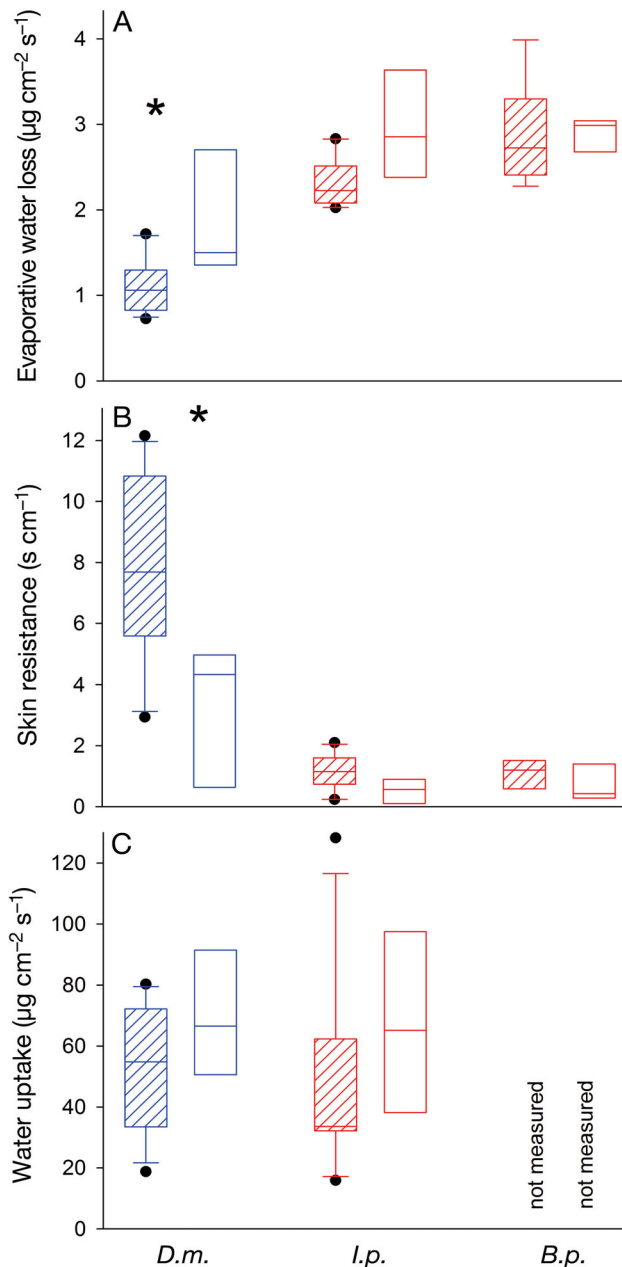
Fig. 1. Water balance parameters measured in our 3 focal host species; *Bd*-infected animals (striped boxes) and uninfected controls (open boxes). Horizontal lines: medians; boxes: interquartile ranges; whiskers: minimum and maximum values, excluding outliers (dots). Asterisks denote significant differences between *Bd*-infected and control treatments. Blue: host species with aquatic larval development; red: terrestrial species with direct development. *D.m.*: *Dendropsophus minutus*; *I.p.*: *Ischnocnema parva*; *B.p.*: *Brachycephalus pitanga*

clinical chytridiomycosis (Vredenburg et al. 2010). Moreover, our results show that these effects vary with species, although the mechanisms for these differences are not yet known.

For the species whose water balance was disrupted by *Bd* infection (*Dendropsophus minutus*), the main physiological response was an increase in R_s associated with a reduction in EWL. Whether such response was linked to *Bd*-induced disruptions in skin morphology (see Voyles et al. 2011) remains to be investigated, but this is a likely mechanism. We expected that *Bd* infection would lead to changes in WU because, in most anurans, uptake and regulation occur through specialized cells and tissues in the ventral region (Larsen et al. 1987, Wells 2007), and this is precisely the body region most severely infected by *Bd* (Berger et al. 1998, 2005, Pessier et al. 1999). Although disruptions in amphibian osmoregulation have not been clearly linked to hyperplasia or hyperkeratosis, our data suggest that some physiological aspects involving skin functions are compromised upon infection. None of our study species showed significant changes in WU as a result of infection (including *D. minutus*); however, we did find a trend of reduced WU in all infected species we measured, a pattern previously reported for other *Bd*-infected amphibians (Carver et al. 2010, Wardziak et al. 2013). Therefore, the 3 physiological parameters we measured changed in the direction expected in cases of clinical chytridiomycosis and thus may contribute to sub-lethal effects in an enzootic host–pathogen system. Our findings also support the epidermal dysfunction hypothesis (Rollins-Smith et al. 2009, Voyles et al. 2009, Carver et al. 2010), which suggests that *Bd* disrupts hosts rehydration and osmoregulatory processes.

Disruption in host rehydration and osmotic balance can also result in behavioral changes such as lethargic posture (Carver et al. 2010). Although we did not measure behavioral responses to *Bd* infections, we did not observe any obvious changes in posture during the course of the experiment and while measuring host physiological responses. An interesting question is whether aquatic and terrestrial hosts show different physiological responses as a result of infection. In general, aquatic amphibians are more likely to be naturally infected with *Bd* than terrestrial species due to greater exposure to the pathogen in aquatic reservoirs (Longcore et al. 2007, Rowley 2007, Bancroft et al. 2011). What is less clear is whether aquatic species exhibit faster acquired resistance and long-term adaptations due to prolonged contacts with the pathogen. Previous studies show that terrestrial species (e.g. *Anaxyrus boreas*; Carey et al. 2006) can sometimes be more susceptible to *Bd* than fully aquatic species (e.g. *Xenopus leavis*; Ramsey et al. 2010). *D. minutus*—the only study species

with aquatic larval development — was the only species showing significant changes in water balance parameters as a result of *Bd* infection. This could be explained, at least in part, by the fact that our enclosures mimicked both terrestrial and aquatic habitats, and thus more terrestrial species could be able to behaviorally escape infection in the terrestrial environment. Nevertheless, our 2 focal terrestrial host species (*Ischnocnema parva* and *Brachycephalus pitanga*) showed comparable low infection loads (see Fig. A1) but no significant changes in physiological responses. Further studies focusing on how physiological adaptations vary among host's life histories will provide valuable information about the effects of chytridiomycosis in the wild.

The fact that a *Bd* GPL strain isolated from Brazil did not cause significant mortality in 3 local host species definitely bodes well for the local anurofauna. Nonetheless, sub-lethal physiological changes in one of our focal species might reflect decreased fitness and lead to silent shifts in population abundances in the wild (Longo & Burrowes 2010). For example, individuals with impaired water balance might adopt behavioral changes to restore equilibrium between water loss and uptake, consequently leading to modifications in ecological interactions (new situations of predation or competition by use of new microhabitats), thus possibly compromising fitness.

It is also possible that such changes in host osmoregulatory functions could trigger population declines in the wild under special environmental circumstances (e.g. climate variability, global warming, and drought; Burrowes et al. 2004, Pounds et al. 2006). Even though amphibian declines in Brazil have not been linked to *Bd* (see Toledo et al. 2006, Ruggeri et al. 2015), a few enigmatic and isolated population crashes of stream-breeding frogs occurred in the Atlantic Forest during the late 1970s and early 1980s (Heyer et al. 1988, Weygoldt 1989), and *Bd* infection dynamics do vary seasonally (Ruggeri et al. 2015) and across habitat types (Becker & Zamudio 2011), suggesting that *Bd* outbreaks could indeed be a potential threat in local wild populations under specific conditions. Further studies testing how Brazilian amphibian species respond to multiple *Bd* strains under varying micro-environmental conditions will be especially fruitful for our understanding of *Bd* dynamics in nature (James et al. 2015).

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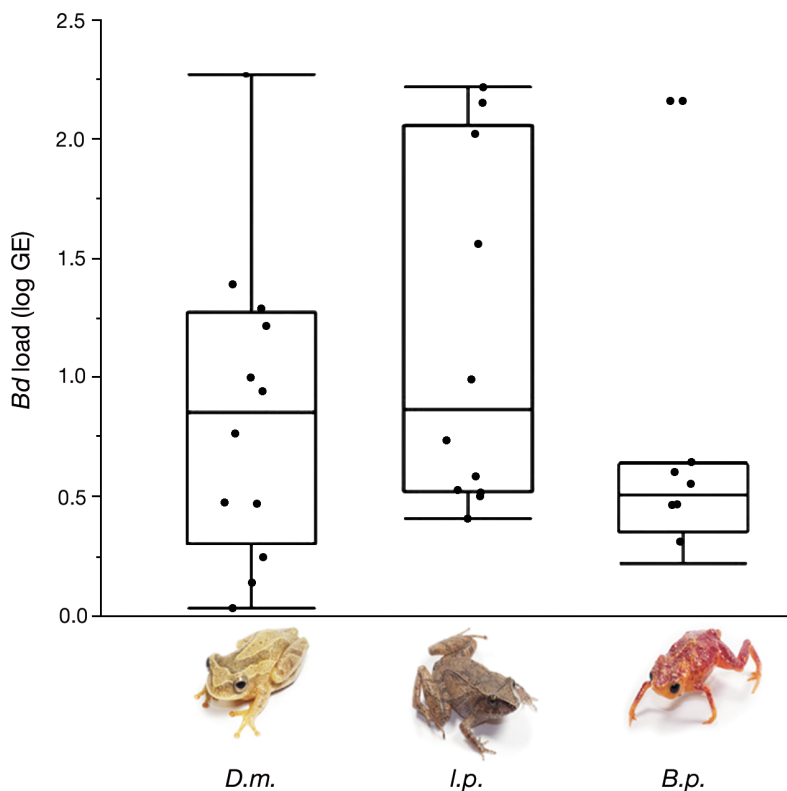
Appendix

Fig. A1. *Bd* infection loads (log genomic equivalents [GE]) of amphibians 18 d post exposure. Horizontal lines: medians; boxes: interquartile ranges; whiskers: minimum and maximum values, excluding outliers. Dots represent individual *Bd* load measurements. *D.m.*: *Dendropsophus minutus*; *I.p.*: *Ischnocnema parva*; *B.p.*: *Brachycephalus pitanga*. Points denote each observation