

Amphibian chytrid infection is influenced by rainfall seasonality and water availability

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ABSTRACT: Amphibians suffer from a number of factors that make them the most threatened group of vertebrates. One threat is the fungal disease chytridiomycosis caused by the emerging pathogen *Batrachochytrium dendrobatidis* (*Bd*), which has rapidly spread and caused the loss of massive amphibian biodiversity worldwide. Recently, *Bd* was associated with a few amphibian population declines and extinctions in some areas of the Brazilian Atlantic Forest. However, the mechanisms underlying such declines are not fully understood. Therefore, it is essential to improve our knowledge of abiotic factors that can possibly influence *Bd* prevalence and chytridiomycosis disease severity. Herein we tested the hypothesis that water availability (such as in perennial streams, where *Bd* is frequently present in larvae) and rainfall would increase the prevalence of *Bd*. To test this, we sampled frogs from 6 transects with different numbers of perennial waterbodies, and we report that the more water available in the area, the higher the probability of *Bd* infection on anurans. Seasonality also influenced both the *Bd* prevalence in the area and the intensity of infection in infected frogs. However, *Bd* prevalence was higher during the rainy months whereas the infection burden was lower. We suggest that *Bd* is likely spread during the summer, when most anuran species gather near the water for spawning and when rainfall overfills ephemeral wetlands. On the other hand, during the drier months, a higher infection burden may be explained by increased disease susceptibility.

KEY WORDS: *Batrachochytrium dendrobatidis* · Chytridiomycosis · Pathogen dynamics · Waterborne disease · Highlands · Brazilian Atlantic forest

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INTRODUCTION

Emerging infectious diseases pose a major threat to wildlife (Fisher et al. 2012). Fungal diseases, in particular, are increasing in incidence (Fisher et al. 2012) and can have highly deleterious effects especially on naïve populations, such as the ones reported for bats (white-nose syndrome; Blehert et al. 2009, Gargas et al. 2009), snakes (snake fungal disease; Vissienon et al. 1999, Allender et al. 2015), and amphibians

(chytridiomycosis; Longcore et al. 1999, Martel et al. 2013). Chytridiomycosis has especially captured the attention of scientists and conservationists in the last 2 decades mainly because of its potential rapid spread and devastating consequences (Olson et al. 2013, James et al. 2015). Chytridiomycosis is caused by an aquatic fungus that usually prefers cooler temperatures (Longcore et al. 1999, Johnson et al. 2003, Piotrowski et al. 2004) and has a high prevalence in mountainous regions, where it has already caused

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declines of several populations worldwide (Berger et al. 1998, Lips 2016, Carvalho et al. 2017).

Batrachochytrium dendrobatidis (*Bd*) is comprised of a number of widely distributed genetic lineages (Olson et al. 2013, James et al. 2015). The wide-ranging lineage *Bd*-GPL is considered more virulent than endemic lineages (Farrer et al. 2011, Becker et al. 2017) and is the only lineage associated with declining populations thus far (Farrer et al. 2011, Bataille et al. 2013, Rosenblum et al. 2013). However, *Bd* is not always detrimental to amphibian assemblages (Ruggeri et al. 2015, Talley et al. 2015). Host susceptibility varies among species (Peterson & McKenzie 2014), and *Bd* dynamics are influenced by several factors such as seasonality (Kriger & Hero 2007a, Longo et al. 2010, Ruggeri et al. 2015), host genetics (Savage & Zamudio 2011, Ellison et al. 2014), and *Bd* genotype and phenotype (Berger et al. 2005a, Becker et al. 2017).

In Brazil, amphibians in the Atlantic Rainforest have experienced population declines in the past (Heyer et al. 1988, Weygoldt 1989) that were recently attributed to *Bd* (Carvalho et al. 2017). The Atlantic Forest is one of the 34 biodiversity hotspots in the world (Mittermeier et al. 2011) and harbors more than 500 anuran species, of which about 80% are endemic to this biome (Haddad et al. 2013). Currently, it contains a mixture of genetically distinct *Bd* lineages: 2 lineages of the *Bd*-GPL clade, an endemic strain (*Bd*-Brazil), and hybrids (Schloegel et al. 2012, Rosenblum et al. 2013, Jenkinson et al. 2016) that do not seem to be causing contemporary declines (e.g. Toledo et al. 2006, Preuss et al. 2016). However, the relative importance of chytridiomycosis as a conservation threat to this biome remains unclear. For example, although some studies have investigated the influence of habitat loss (Becker & Zamudio 2011), host diversity (Becker et al. 2014, 2015), and seasonality (Ruggeri et al. 2015) on *Bd* infections, we lack information about environmental attributes that can influence *Bd* dynamics, such as topographical parameters and rainfall seasonality.

As *Bd* and most anuran species rely on wet environments for completing their life cycle (Wells 1977, Longcore et al. 1999), we hypothesize that local hydrology plays an important role in regulating disease dynamics. A greater density of waterbodies in an area could increase the prevalence of *Bd* and its transmission, which might occur by frog-to-frog contact (Rowley & Alford 2007) and through waterborne zoospores (Rachowicz & Vredenburg 2004).

Therefore, in an attempt to improve our understanding of *Bd* dynamics in the Atlantic Forest, we

conducted a field study in a highland area (above 1000 m a.s.l.) where the genotype associated with die-offs in the tropics (Schloegel et al. 2012, James et al. 2015) is present (*Bd*-GPL-2; Jenkinson et al. 2016). We investigated whether the prevalence of *Bd* and infection loads found in this community of anurans could potentially be related to the density of water available in their habitats. We hypothesized that increased water availability in an area would be positively correlated to the probability of a frog being infected. We also expected to find higher infection rates (load and prevalence) during the rainy season, because the additional rainfall would also increase overall water availability.

MATERIALS AND METHODS

Study site

Serra dos Órgãos is located in the central portion of the Serra do Mar mountain range within the state of Rio de Janeiro, Brazil, and encompasses 2 important protected areas: Parque Estadual dos Três Picos (PETP) and Parque Nacional da Serra dos Órgãos (Parnaso). We surveyed 8 high elevation montane forest areas (above 1000 m a.s.l.): 5 transects inside the forest and 1 transect in an artificial pond at Parnaso, 1 transect inside the forest in PETP, and 1 transect in an artificial pond at the periphery of Parnaso (Table S1 in the Supplement at www.int-res.com/articles/suppl/d127p107_supp.pdf). Transects inside the forest varied from 300 to 700 m long and transects surrounding pond margins were 450 m. We sampled anurans found up to 30 m along the sides of each transect. All sites are located in the municipality of Teresópolis, which usually has a wet summer and dry winter (Santos 2000). Temperature and precipitation data for 2013 and 2014 (Fig. 1) were obtained from weather station A618, located in Parnaso (INMET 2015).

Sampling and *Bd* quantification

We grouped anurans according to their breeding habitat (aquatic when species breed in lotic or lentic habitats, and terrestrial when species breed on the forest floor, on bromeliads, and/or bamboos) and according to their development (indirect developers with tadpoles, or direct-developing species; Table S2 in the Supplement).

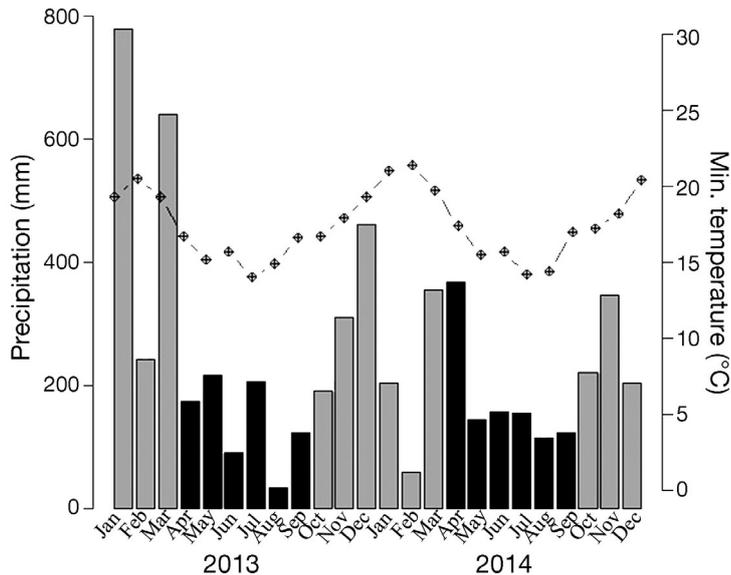


Fig. 1. Precipitation (bars) and minimum monthly temperature (line) at Serra dos Órgãos during the rainy (grey) and dry (black) seasons in 2013–2014

We conducted fieldtrips to the area during January, February, and November (rainy season) and during May and June (dry season) in 2013. However, we had difficulty finding frogs during the dry season and resampled the following year (May–June 2014) to achieve statistically meaningful sample sizes. We performed active and acoustic searches to locate and capture adult frogs. We captured frogs using individual non-powdered gloves to avoid contamination between sampled animals and followed standard field sampling protocols (Hyatt et al. 2007, Kriger et al. 2007a).

We swabbed a total of 182 frogs during the rainy season and 170 frogs during the dry season to quantify seasonal patterns on *Bd* infection prevalence (number of infected individuals/total number individuals sampled) and intensity (*Bd* load). Each swab was stored in sterile plastic tubes at -20°C . If frogs were identified to species, they were photographed and released at the site of capture. If the specimen could not be identified in the field, the frog was killed according to Brazilian regulations (Concea) and deposited in the zoological collection of the Universidade Federal do Rio de Janeiro (Table S2).

To quantify the presence and infection intensity of *Bd* in each sample, we extracted DNA from swabs using PrepMan

ULTRA[®] (Life Technologies), and then quantified infection intensities using a Taqman[®] qPCR Assay (Life Technologies) with standards of 0.1, 1, 10, 100, 1000, and 10000 zoospore genome equivalents (GE) (Boyle et al. 2004, Lambertini et al. 2013) using the strain CLFT023. We considered a sample as positive (*Bd*⁺) when the infection load was ≥ 1 GE (Kriger et al. 2007b).

Using ArcGIS we created a 300 m buffer around each transect sampled (excluding the 2 ponds due to different anuran composition) and included the hydrography layer of Serra dos Órgãos provided by managers from Parnaso and PETP (Fig. 2). We measured the length of streams in the hydrography layer that were inside the buffer we created for each transect and divided it by the buffer area to calculate waterbody density for each transect.

Statistical analyses

We calculated prevalence as the proportion of *Bd*⁺ samples for each sampled site, and after confirming the normality of our data via a Shapiro-Wilk test, we ran a linear regression between *Bd* prevalence (considering all seasons) and waterbody density. For this

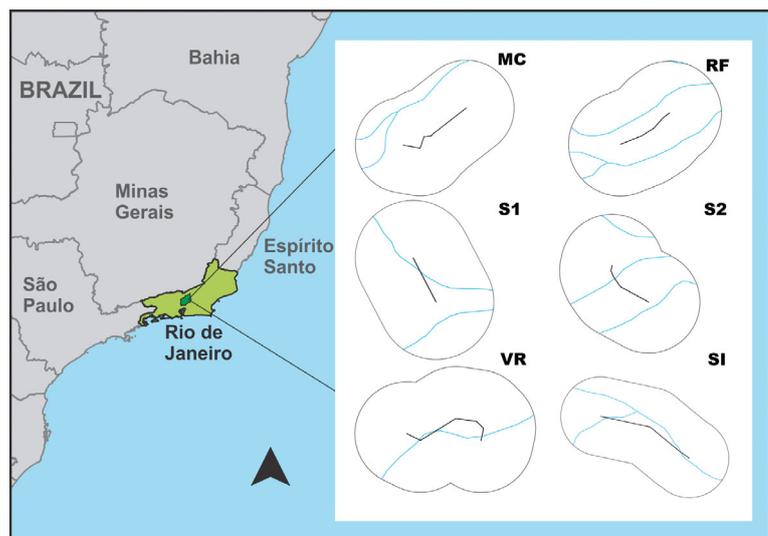


Fig. 2. Sampled transects (black lines) with buffers in the municipality of Teresópolis, Rio de Janeiro, Brazil. Blue lines inside buffers represent rivers from the local hydrography map (scale 1:10 000, UTM sad69). Transects MC (Mozart Catão), RF (Rancho Frio), S1 (Suspensa 1), S2 (Suspensa 2), and SI (Sino) are located in Parque Nacional da Serra dos Órgãos, and transect VR (Vale da Revolta) in Parque Estadual dos Três Picos

analysis, we excluded 2 transects that surround permanent ponds (LI and Pis; see Table S1), because they have a distinct anuran composition compared to the other transects (which are influenced by stream and terrestrial based anuran assemblages and present a similar frog composition). Thus, we considered as waterbodies only the streams/ponds from the hydrology map of the area (we did not consider ponds or ephemeral streams) in order to test whether the density of water available throughout the year would influence the prevalence of *Bd* in the area or not. We also conducted simple *t*-tests for calculating the statistical differences in prevalence and mean infection loads between the wet and dry seasons.

For testing which variables best explained *Bd* presence/absence and infection intensity, we performed a GLM using logistic and quasi-Poisson regressions, respectively. Using samples from all transects, we tested the effect of seasonality, anuran habitat, and mode of development. When analyzing infection intensity, we only used data from frogs that were *Bd*⁺. We also tested the effect of species composition on both *Bd* prevalence and infection intensity, but this variable was not considered in the fitted models. Statistical analyses were performed in R (R Core Team 2016). We used R packages AICcmodavg, lettuce, MASS, and pscl for statistics and ggplot2 for figures.

RESULTS

More than 30% of all anurans sampled were infected with *Bd*. Hylidae was the most infected family, and Brachycephalidae was the family with the highest infection load (Table 1). Overall *Bd* prevalence was 46% during the rainy season and 18% during the dry season ($p < 0.01$). Mean infection load of *Bd*⁺ specimens was higher in the dry season (3708 GE) than in the rainy season (442 GE; $p < 0.01$), and ranged from 1–51 313 to 1–9417 zoospores, respectively (Fig. 3). Both logistic and quasi-Poisson regressions revealed an effect of season on *Bd* prevalence and intensity of infection on frogs (Table S3 in the Supplement). The logistic model (Akaike's information criterion, AIC = 413.34) revealed a positive correlation between rainy season and *Bd* prevalence ($\beta = 1.36 \pm 0.25$ [SE], $Z = 5.45$, $p < 0.001$). On the other hand, our quasi-Poisson regression for *Bd*⁺ frogs showed a negative effect of rainy season on infection intensity ($\beta = -2.13 \pm 0.68$, $t = -3.11$, $p = 0.002$). Habitat and anuran develop-

Table 1. Anuran families tested for the presence/absence of *Batrachochytrium dendrobatidis*, showing the number of individuals collected, *Bd* prevalence within total samples ($n = 352$) and within families, and mean infection load for each family. GE: zoospore genome equivalents

Family	Sample size (n)	<i>Bd</i> prevalence (%)		Mean <i>Bd</i> load (GE)
		Total samples	Within family	
Brachycephalidae	165	11.1	23.6	2168.42
Bufo	7	0.9	42.9	909.86
Craugastoridae	7	1.1	57.1	643.12
Cycloramphidae	14	1.1	28.6	12.21
Hemiphractidae	9	1.7	66.7	29.52
Hylidae	139	14.2	36.0	1155.45
Leptodactylidae	1	0.3	100	7.28
Odontophrynidae	10	1.7	60	14.57

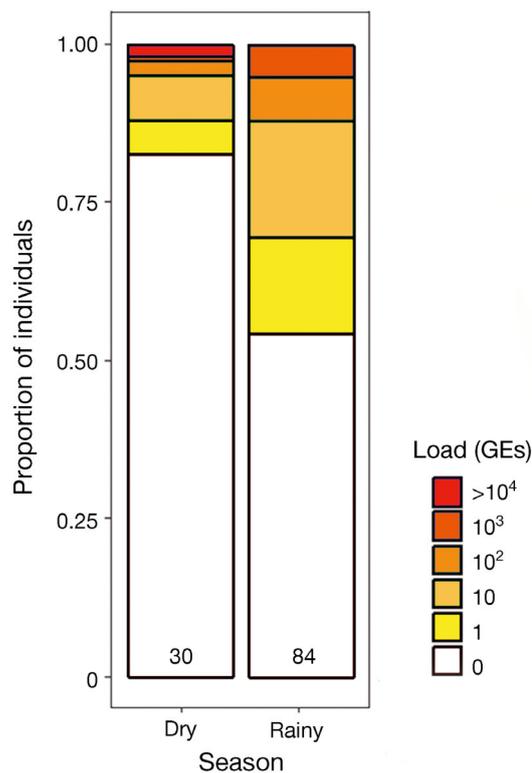


Fig. 3. Proportion of zoospore genomic equivalents (GE) on frogs from the rainy ($n = 182$) and dry ($n = 170$) seasons. Numbers inside bars represent *Batrachochytrium dendrobatidis*-positive (*Bd*⁺) samples. Note that during the dry season, infection load on specimens exceeded 10 000 zoospore genome equivalents (GE), whereas during the rainy season it did not reach 10 000 GE

mental mode did not explain the presence of *Bd* across frogs ($p > 0.05$; Table S3). The linear regression revealed that the prevalence of *Bd* increased with waterbody density in the area ($\beta = 8.6 \pm 3$, $T = 2.9$, $p = 0.05$, $r^2 = 0.672$; Fig. 4).

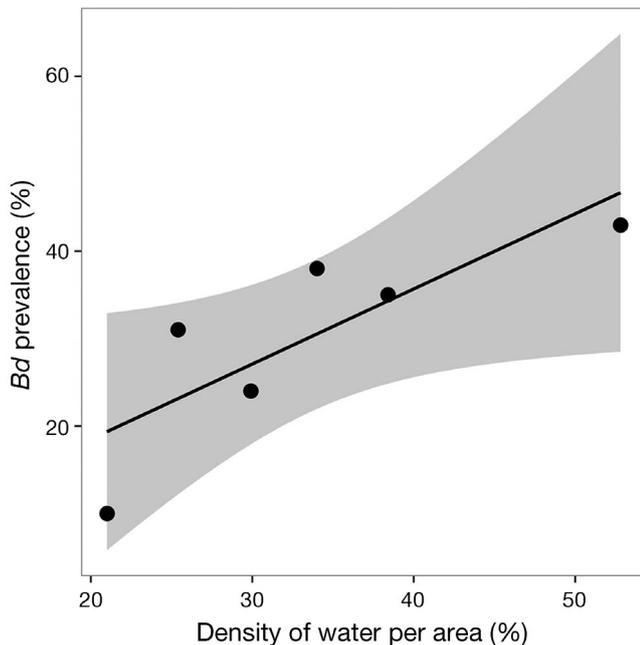


Fig. 4. Linear regression ($r^2 = 0.6715$) between the density of waterbodies available in the area and the prevalence of *Batrachochytrium dendrobatidis* (*Bd*) ($p = 0.046$), with 95% CI curve

DISCUSSION

As we hypothesized, waterbody density was positively correlated to *Bd* prevalence. This is the first time this variable has been tested as an explanatory factor for the presence of this pathogen. There are a few possible explanations for the influence of water availability on *Bd* distribution. First, *Bd* is a waterborne pathogen (Longcore et al. 1999) and depends mainly on water to disperse (Berger et al. 2005a). Second, the substrate-dependent phase of the *Bd* life cycle is on amphibian keratinized skin (Longcore et al. 1999, Kilpatrick et al. 2010), and *Bd* could potentially be transported between frogs via aquatic habitats. Finally, some chytrids have the ability to persist as saprophytes in aquatic habitats (Gleason et al. 2008), thus *Bd* could possibly grow in this habitat, which would allow the zoospores to also live in the absence of a host.

Aquatic dissemination of *Bd* zoospores in Serra dos Órgãos could be facilitated in 2 different ways: (1) by the connections of branching tributaries through the stream network (Campbell Grant et al. 2007), where the flowing water downstream would help zoospore dispersion through the area, as they are unable to swim more than 2 cm (Piotrowski et al. 2004); and (2) due to the region's topography (Silveira & Ramos

2007), rainfall overfills perennial streams, leaking water to ephemeral wetland habitats (authors' pers. obs.). In addition, *Bd* zoospores could potentially be found on different types of substrate such as moist sand, rocks, and muddy leaves (Johnson & Speare 2005, Rowley & Alford 2007, Kolby et al. 2015), and they could be carried downstream by the water to other sites (Hagman & Alford 2015). Therefore, an area with greater water availability would be likely to house more zoospores, consequently increasing the chances of encountering a potential host, and thus explaining the positive correlation between waterbody density and *Bd* prevalence.

Moreover, due to amphibian natural history (Duellman & Trueb 1994), greater water availability could be associated with an increased abundance of frogs in the area, and therefore more *Bd* zoospores in the environment. Especially during the breeding period, when most anuran species aggregate around waterbodies (Duellman & Trueb 1994), different types of contact happen, such as male–male combats and male–female amplexus (Wells 1977), facilitating dissemination of zoospores via contact among hosts (Rachowicz & Vredenburg 2004). Also, males from some species call from the riparian vegetation and could potentially deposit *Bd* zoospores on leaves, exposing other individuals to this pathogen (Kolby et al. 2015). Furthermore, as a consequence of reproduction, there will be more tadpoles in the waterbodies. During the larval stage, the species are susceptible to *Bd* but not prone to death in most cases and act as reservoirs for zoospores (Daszak et al. 2003, Blaustein et al. 2005, Briggs et al. 2010, Valencia-Aguilar et al. 2016), transmitting *Bd* within and between life stages (Rachowicz & Vredenburg 2004). Besides this, some of the species collected in Serra dos Órgãos have tadpoles that remain in the larval stage for long periods, such as *Aplastodiscus arildae*, *Bokermannohyla carvalhoi*, and *B. circumdata* (S. Potsch de Carvalho-e-Silva pers. obs.), and could potentially host *Bd* between the breeding seasons thus becoming a reservoir for zoospores (Bosch et al. 2001, Catenazzi et al. 2013).

Bd was found infecting more than 80% of the species sampled, confirming that it is a generalist pathogen in this biome (Valencia-Aguilar et al. 2015). However, our results revealed that season played different roles in its dynamics. On one hand, *Bd* was more prevalent during the rainy months, as expected. On the other hand, the mean infection load in positive frogs during the dry season (3708 GE) was higher than during the rainy season (442 GE), which is different from what we predicted. This pattern

between dry/cold vs. wet/warm seasons (Longo et al. 2010, this study) could be explained because zoospores are stimulated to produce more zoospores at lower temperatures (Woodhams et al. 2008), thus increasing the burden of infection on *Bd*⁺ individuals. However, because of lower precipitation and food availability during the winter, most anuran species remain inactive to reduce water loss and save energy (Duellman & Trueb 1994, Pough 2007), which would lower the probability of exposure to *Bd* zoospores.

Our results differ from what we have found for a nearby anuran assemblage in the Atlantic forest, where *Bd* prevalence was higher during the winter (Ruggeri et al. 2015). This difference might be due to the different anuran species in the 2 studies, as Ruggeri et al. (2015) only sampled stream-dwelling frogs and we sampled species from different habitats for this study. Since one of the main mechanisms of zoospore transmission is via water (Longcore et al. 1999, Berger et al. 2005b), stream-dwelling frogs would constantly get infected or re-infected (Woodhams & Alford 2005, Rowley & Alford 2007), but during the summer, the rainfall would overflow these streams, potentially spreading the zoospores and therefore decreasing the prevalence among frogs that inhabit streams and increasing the probability of arboreal and terrestrial anurans getting infected. Even though *Bd* seems more likely to infect stream-associated species rather than non-stream species (Hero et al. 2005, Kriger & Hero 2007b, Catenazzi et al. 2011, Gründler et al. 2012), in this study stream-associated species represent only 13% of *Bd*⁺ specimens. In contrast, direct-developers represent approximately 40% of our *Bd*⁺ samples, and the species *Ischnocnema parva* had the highest infection load. Because these direct-developer species are not directly associated with any type of aquatic habitat (Duellman & Trueb 1994, Haddad et al. 2013), they could potentially get infected when in contact with contaminated soil, leaves, and rocks, as *Bd* might persist on different types of substrate as a saprobe (Johnson & Speare 2005, Rowley & Alford 2007, Kolby et al. 2015), especially during the rainy season.

Bd has been present in amphibians of the Brazilian Atlantic forest at least since the 19th century (Rodríguez et al. 2014). Although it has been linked to anuran population declines in the past (Carvalho et al. 2017), currently specimens from this biome often show high loads of infection without presenting clinical signs of disease (Ruggeri et al. 2015, Preuss et al. 2016). During the period when we conducted this study, the infection prevalence was 32%, and no dying or dead frogs were observed even though

some individuals presented an infection burden of >10 000 zoospore GE, which is considered a lethal threshold for many host species (Vredenburg et al. 2010). Our data corroborate the ability of *Bd* to persist in a community without eradicating the hosts in the Atlantic forest (Ruggeri et al. 2015), although sub-lethal effects of the disease (e.g. Salla et al. 2015, Bovo et al. 2016) have not been measured. Given that this region of the Brazilian Atlantic Forest has seen some *Bd*-related local extinctions (e.g. Carvalho et al. 2017), it may be that the species that persist after the arrival of the panzootic are selected for tolerance or resistance to the pathogen.

However, even though the pathogen–host coexistence is currently stable in this biome, we show that prevalence is higher during the rainy season when most anuran species breed. If predictions of a future increase of 10 to 15% in rainfall in the austral hemisphere are correct (Stocker et al. 2013), it would extend the breeding period and the exposure to this pathogen. Therefore, future analyses should also consider the influence of hydrological factors (such as waterbodies and rainfall) in planning for conservation actions, since we have shown that the density of water from permanent waterbodies in the area would act on *Bd* dissemination, and precipitation in the area could increase *Bd* incidence among frogs. Finally, as *Bd* can be found in temporary habitats, we highlight the importance of learning about *Bd* movement and resilience outside its hosts.

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