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Alba Navarro Lozano

Ecology and Biogeography of *Batrachochytrium dendrobatidis* in the Brazilian Atlantic Forest

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Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Biologia Animal, junto ao Programa de Pós-Graduação em Biologia Animal, Área de Concentração – Ecologia e Comportamento, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquite Filho", Campus São José do Rio Preto.

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"It doesn't matter whether you're the lion or a gazelle, when the sun comes up, you'd better be running".

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RESUMO

O fungo quitridio *Batrachochytrium dendrobatidis* (*Bd*) é considerado uma das principais causas do declínio global dos anfíbios. *Bd* já foi encontrado em quase 700 espécies de anfíbios de todo o mundo. Embora muitos estudos tenham sido publicados desde a sua descrição em 1999, ainda há muito a ser conhecido, particularmente em relação à sua ecologia. Nossos objetivos neste estudo foram: (i) analisar os fatores bióticos e abióticos que regulam a prevalência e a intensidade de infecção de *Bd* em girinos que habitam poças e riachos no Núcleo Curucutu do Parque Estadual da Serra do Mar, sudeste da Mata Atlântica brasileira; (ii) compreender qual será o efeito das mudanças climáticas na distribuição potencial de *Bd* na Mata Atlântica brasileira; (iii) avaliar o uso de deformidades orais em girinos como indicadores confiáveis para a determinação de infecção por *Bd*; e (iv) criar uma ferramenta de divulgação para promover a transferência de conhecimento sobre *Bd* entre a universidade e a sociedade brasileira. Detectamos que a prevalência e a intensidade de *Bd* não diferiu entre poças e riachos, mas são regulados por diferentes fatores ambientais. Em riachos, a velocidade da água e a profundidade explicaram a variabilidade na prevalência de *Bd*, mas a intensidade permaneceu inexplicada. Por outro lado, a densidade do zooplâncton foi o fator chave para explicar a prevalência e a intensidade em poças. Estimamos que, hoje, cerca do 60% da área da Mata Atlântica brasileira apresenta condições climáticas adequadas para a ocorrência de *Bd*, porém diferentes cenários de aquecimento global para o ano de 2070 diminuiriam essas áreas entre um 27.5 e 42.6%. Também demonstramos que as deformidades orais em girinos não são um indicador confiável para o diagnóstico da quitridiomicose em girinos de anfíbios anuros, e que não devem ser utilizadas como ferramenta de diagnóstico isoladamente. Desenvolvimos uma página de internet (www.quitribrasil.com) para a divulgação de todos os casos de *Bd* relatados no Brasil. As informações geradas por estes trabalhos podem melhorar a compreensão da ecologia e distribuição de *Batrachochytrium dendrobatidis* em um dos biomas mais ricos e com as maiores taxas de endemismo de anfíbios no mundo.

Palavras-chave: *Batrachochytrium dendrobatidis,* Amphibia, Girino, Ecologia, Conservação, Distribuição geográfica, Mudança climática, Mata Atlântica, Morfologia

ABSTRACT

*The chytrid fungus Batrachochytrium dendrobatidis (*Bd*) is considered one of the main cause of global amphibian declines.* Bd *has been reported in nearly 700 species of amphibian worldwide. Although many studies have been published since its description in 1999, there is still much to be known, particularly regarding to its ecology. In this study, we aim to: i) analize biotic and abiotic factors that regulate prevalence and intensity of* Bd *infection in tadpoles inhabiting lentic and lotic habitats, in Nucleo Curucutu of the Parque Estadual da Serra do Mar, southeastern Brazilian Atlantic Forest; ii) understand the effect of climate change in potential future distribution of* Bd *in the Brazilian Atlantic Forest; iii) evaluate the use of oral deformities in tadpoles as reliable proxies for the determination of infection by* Bd*; and iv) create an outreach tool to communicate the knowledge of* Bd *from university to Brazilian society. We detected that the prevalence and intensity of* Bd *did not differ between ponds and streams but were driven by different environmental factors in the two habitats. In streams, water velocity and depth explained variability in* Bd *prevalence, but* Bd *intensities remained unexplained. On the other hand, zooplankton density was the key factor in explaining* Bd *prevalence and intensity in ponds. We estimated that currently, about 60% of the Atlantic Forest area shows climatic suitability for* Bd *occurrence; but in global warming scenarios for 2070 those suitable areas will decrease by between 27.5 and 42.6%. We also showed that oral deformities in tadpoles are not a trustworthy indicator for chytridiomycosis diagnosis in tadpoles of anuran amphibians, and should not be used in isolation as a diagnosis tool. We developed and launched one internet webpage (www.quitribrasil.com) to communicate all* Bd *cases reported in Brazil. Information generated by our study could improve the knowledge of ecology and distribution of Batrachochytrium dendrobatidis in one of the richest biomes with greatest values of endemism of anuran amphibians in the world.*

Keywords: *Batrachochytrium dendrobatidis, Amphibia, Tadpole, Ecology, Conservation, Geograohic distribution, Climatic change, Atlantic Forest, Morphology*

INTRODUCTION

Global loss of biodiversity is one of the most serious problem of our time (Baillie et al., 2010). Data from the International Union for Conservation of Nature (IUCN) warns that approximately 41% of the world's amphibians species are endangered, making them the most vulnerable group among vertebrates (IUCN, 2016). Whereas it is true that amphibian populations present natural abundance fluctuations (Pechmann et al., 1991; Alford & Richards, 1999), population declines occurred in the last decades have not being balanced with equivalent increases (Houlahan et al., 2000). Different studies have shown that amphibian population declines are the outcome of complex interactions of factors such as habitat loss and land-use changes (Bradford et al., 1993; Stuart et al., 2004; Cushmann, 2006), exotic species introduction (Fisher & Shaffer, 1996; Bradford 1989; Kiesecker et al., 2001), environmental contamination (Harte & Hoffman, 1989; Rohr et al., 2004; Blaustein et al., 2003), UV-radiation increase (Blaustein et al 1994, 1997; Anzalone et al 1998; Lizana & Pedraza 1998), climatic changes (Heyer et al., 1988; Laurance et al., 1996; Pounds et al, 1999, 2006; Pounds, 2001), and the emergence infectious diseases (Berger et al., 1998, 1999; Lips, 1999, 1998).

From the end of 1970's and during the 1990's, dramatic amphibian death events occurred in Central America and Australia (Laurence et al., 1996; Campbell et al., 1999; Pounds et al., 1997; Crump et al., 1992). What researchers studying these cases found most striking was that both events occurred in well conserved pristine forest areas. They suggested that climate change was responsible for them (Pounds et al., 1999). However, at the end of the 1990's it was detected a link between mass mortality events in Central America and Australia: the presence of fungal sporangium in upper layers of amphibians skin, a, infection called chytridiomycosis (Berger et al., 1998). Shortly after this discovery, Longcore *et al.* (1999) described *Batrachochytrium dendrobatidis* (*Bd*), a chytrid fungus responsible for those death events. Since *Bd* was first described, several dramatic declines of amphibian populations worldwide have been explained by *Bd* infections (*e.g.* Berger et al, 1998, 1999; Lips, 1999, 1998; Bosch et al., 2001; Ouellet et al., 2005; Weldon et al., 2004; Garner et al., 2005; Lips et al., 2006). Nowadays is known that *Bd* is spread worldwide, already found in 56 countries and affecting nearly 700 species of all three amphibian orders (*http://www.bd-maps.net*).

BOX 1

In 2013, Martel et al. discovered a second species of chytrid fungus, ***Batrachochytrium salamandrivorans*** (*Bsal*), which was causing lethal infections and population declines in the European *Salamandra salamandra*. Shortly after, the same research group pointed Oriental Asia as the origin of Bsal, since unlike with Bsal in Europe, the presence of the fungus do not seem to be affecting Asian urodels (Martel et al., 2014). To date, Bsal only occurs in urodeles (newts and salamanders) and not in anurans (frogs and toads) or caecilians.

Who is *Batrachochytrium dendrobatidis*? And, how does it affect amphibians?

Bd is a non-hyphal zoosporic chytrid aquatic fungus belonging to the class Chytridiomycetes and order Rhizophydiales (Longcore et al., 1999; Letcher et al., 2006). Although a saprobic phase has been suggested for *Bd*, to date only two life cycle phases has been described, a free-swimming zoosporic phase that allows *Bd* to meet a host, and a sporangial phase that results from the encystment of the zoospore into keratinizing tissue of a host, or those tissues fated to keratinize, where matures and produce new zoospores (Longcore et al., 1999; Fellers et al., 2001; Berger et al., 2005).

Bd infection is restricted to the oral region in tadpoles, the only keratinized part of this life cycle phase (Marantelli et al., 2004; Berger, 1998, 1999; Fellers et al., 2001). In adults, *Bd* colonize keratinized cells of the stratum corneum and granulosum of the epidermis, with higher concentrations in ventral and pelvic regions, hind limbs and feet (Longcore et al., 1999; Pessier et al., 1999). Superficial pathologies associated with the infection vary from swollen and dekeratinization of the oral disk (Berger et al., 1999; Fellers et al., 2001; Navarro-Lozano et al., 2018), to hyperkeratosis, hyperplasia, ulceration, depigmentation, slower rehydration and sloughing of metamorph skin (Berger et al., 1998; Voyles et al., 2009; Carver et al., 2010), as well as behavioral abnormalities such as inappetance, lethargy and loss of reflex and escape response in adults (Berger et al., 1998, 1999; Nichols et al., 2001; Pessier et al., 1999, Voyles et al., 2009). In has also been described pathophysiologies caused by the impaired performance of the amphibians skin such as significant reductions in osmolarity and concentrations of Na^+, K^+ and Cl^- in plasma, which may result in death by cardiac arrest (Voyles et al., 2009; Marcum et al., 2010). In addition, it has been demonstrated that *Bd* produces mycotoxins that block the amphibian immune response, inhibiting lymphocyte proliferation and causing cell apoptosis (Fites et al., 2013).

Not all species from affected regions have the same likelyhood to be infected (Blaustein et al., 2005; Searle et al., 2011; Lips 2006, Gahl 2012). Some species of salamanders show low susceptibility to *Bd* infection which has been linked to the presence of bacterial strains on their skin (Harris et al. 2006). Indeed, the bacteria *Janthinobacterium lividum*, found in the skin of some salamander species, produces lethal concentrations of antifungal metabolites for *Bd* (Brucker et al., 2008). Functional traits can also determine the likelyhood of species to be infected. Species inhabiting permanent waterbodies seem to be more infected by *Bd* than those inhabiting temporal waterbodies (Kriger & Hero, 2007). In turn, among species that share the same habitat, those occuping warmer and dryer microhabitats are also less likely to be infected (Rowley & Alford, 2013).

BOX 2

Despite *Bd* came into the spotlight for its detrimental effect on amphibians, this fungus has been found in other animals that share amphibian habitats as:

- **Lizards**: *Anolis humilis* and *Anolis lionotus* (Kilburn et al., 2011).
- **Snakes**: *Pliocercus euryzonus, Imantodes cenchoa* and *Nothopsis rugosus* (Kilburn et al., 2011).
- **Birds**: *Branta canadensis* and *Anser domesticus* (Garmyn et al., 2012); *Anas falvirostris, Anas georgica, Fulica ardesiaca, Fulica gigantean, Lophonetta specularioides, Plegadis ridgwayi, Rollandia Rolland* and *Spatula puna* (Burrowes & De la Riva, 2017a).
- **Arthropods**: *Procambarus* spp. and *Orconectes virilis* (McMahon et al. 2013).
- **Fishes**: *Danio rerio* (Liew et al., 2017).

Origin of *Batrachochytrium dendrobatidis*

Since chytridiomycosis was first reported, there has been a great debate to explain the origin of this disease. And although it has taken almost two decades of studies, a research with more than 200 *Bd* isolates, collected from various regions of the planet, has managed to point to East Asia as the geographical origin of *Bd* (Dr. Jaime Bosch's personal communication). This recent research by Dr. Jaime Bosch and other 52 authors, which will be published in Nature this year, highlights the presence of four divergent main lineages of Bd: Bd-Asia1, Bd-Asia2/Brazil, Bd-Cape and Bd-GPL (*Global Panzootic Lineage*). It is Bd-GPL who stands out over the rest of lineages for its hypervirulence and widely distribution, being associated with species extinction and mass mortality events of amphibians occurred in Central America, Australia and Spain (Farrer et al., 2011; Schloegel et al., 2012). From this research it is also concluded that *Bd*-GPL has a recent origin, and that its global expansion took place until the first half of the 20th century, coinciding in time with the commercialization of amphibians for scientific and feed purposes (Dr. Jaime Bosch's personal communication).

BOX 3

Until today, the international trade of amphibian species seems to be the main source of dispersion of the fungus around the world (Laurance et al., 1996; Fisher & Garner, 2007; Schloegel et al. 2012). ***Why?*** For example, *Xenopus laevis* and *Lithobates catesbeiana*, commonly commercialized for consumption, as pets or for laboratory use, can accommodate high loads of *Bd* without succumbing to the disease (Mazzoni et al., 2003; Weldon et al., 2004; Daszak et al., 2004). The lack of signs of disease and the ineffectiveness of transcontinental biosecurity have turned these species into perfect vectors for the global dissemination of *Bd*. Specimens of *L. catesbeiana* infected by the fungus been reported in markets in Asia, South and North America, and Europe (Garner et al., 2006; Bai et al., 2010).

Eighteen years after the discovery of *Bd,* many studies have been published. However, there is still much to be known, particularly regarding to its ecology. This doctoral dissertation presents a study of the distribution and ecology of *Bd* in the Brazilian Atlantic forest, which stands out as an ideal place for the study of *Bd* due to its importance as a global hotspot (Myers, 2000; Jenkins et al., 2015) and to the absence of harmful pathologies, despite of the large number of amphibian species Bd-infected. The dissertation includes four chapters: (1) ***Batrachochytrium dendrobatidis*** **infection of frogs is driven by different biotic and abiotic factors in ponds and streams of the megadiverse Atlantic Forest of Brazil**, in which biotic and abiotic environmental factors that influence the dynamic of *Bd* infection (prevalence and intensity) in a pristine area of the Brazilian Atlantic Areas are studied; (2) **Global warming could decrease potential geographic distributon of amphibian-killing fungus in Braziian Atlantic Forest**, in which we have analyzed the effect of climate change over *Bd* distribution in the Brazilian

Atlantic Forest; (3) ***Are oral deformities in tadpoles accurate indicators of anuran chytridiomycosis?***, in which the relationship between oral deformities and *Bd* infection in tadpoles of different species and is potential use as a proxy for the determination of *Bd* infection is studied; and (4) ***Batrachochytrium dendrobatidis in Brazil: www.quitribrasil.com***, in which we present the webpage www.quitribrasil.com, where information of all *Bd* cases reported in Brazil is provided. Finally, the conclusions section highlights the main findings that our studies have revealed.

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Chapter I

Batrachochytrium dendrobatidis infection of frogs is driven by different biotic and abiotic factors in ponds and streams of the megadiverse Atlantic Forest of Brazil

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Batrachochytrium dendrobatidis infection of frogs is driven by different biotic and abiotic factors in ponds and streams of the megadiverse Atlantic Forest of Brazil

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LRH: A. Navarro-Lozano et al.

RRH: *Bd* infection ecological driving factors

Abstract.—We assessed how abiotic and biotic factors regulate the prevalence and intensity of *Batrachochytrium dendrobatidis (Bd)* infection in tadpoles inhabiting ponds and streams in the Atlantic Forest of southeastern Brazil. The prevalence and intensity of *Bd* did not differ between ponds and streams, but were driven by different environmental factors in the two habitats. In streams, water velocity and depth explained variability in *Bd* prevalence, but *Bd* intensities remained unexplained. On the other hand, zooplankton density was the key factor in explaining *Bd* prevalence and intensity in ponds. Our study contributes to the ecological knowledge of *Bd* in tropical forests, which will be useful to researchers and wildlife managers in future scientific studies and conservation actions for amphibians.

Key-words: Amphibians; Atlantic Forest; Chytrid fungus; Tadpoles; Zooplankton.

Resumen.— Se evaluó cómo los factores abióticos y bióticos regulan la prevalencia e intensidad de la infección de *Batrachochytrium dendrobatidis* (*Bd*) en renacuajos que habitan charcas y arroyos en la Selva Atlántica del sureste de Brasil. Aunque la prevalencia e intensidad de *Bd* no difirió entre charcas y arroyos, se observó que estas estaban impulsadas por diferentes factores ambientales en los dos hábitats. En arroyos, la velocidad y la profundidad del agua explicaron la variabilidad en la prevalencia de *Bd*, sin embargo no fue posible explicar la variabilidad en la intensidad. Por otra parte, la densidad del zooplancton fue el factor clave para explicar la prevalencia e intensidad de *Bd* en estanques. Con este estudio contribuimos al entendimiento de la ecología de *Bd* en las selvas tropicales, lo cual será útil para investigadores y gestores en el desarrollo de futuros estudios y planes de conservación de anfibios.

Palabras clave: Anfibios; Hongo quitridio; Renacuajos; Selva Atlántica; Zooplancton.

INTRODUCTION

Parasite virulence, host susceptibility and environmental conditions are all regulatory factors of infection dynamics (Begon et al., 2007). *Batrachochytrium dendrobatidis* (*Bd*) is an aquatic parasitic fungus that infects amphibians worldwide and can lead to chytridiomycosis. Both hypo- and hypervirulent lineages of the fungus have been recorded, with the latter associated with the onset of epizootics in America, Australia and Europe (Farrer et al., 2011). However, the presence of this hypervirulent lineage is not always linked to amphibian population declines, with anuran species behavior and life history seemingly involved in regulating infection dynamics (Bielby et al., 2008; Rowley et al., 2013; Blooi et al., 2017).

Environmental characteristics, such as temperature, precipitation or moisture, are important regulators of amphibian-*Bd* interactions (Piotrowski et al., 2004; Fisher et al., 2009; Rohr et al., 2010). Temperature has been identified as one of the most important factors regulating *Bd* biology, being directly related to seasonality and the distribution of infections in various regions (Daszak et al., 2003; Pounds et al., 2006; Bosch et al., 2007; Kielgast et al., 2009). For instance, increases in seasonal temperature have been associated with decreased prevalence and intensity of *Bd* infections in amphibian populations (Berger et al., 2004; Phillott et al., 2013). In addition to abiotic factors, biotic interactions seem to regulate *Bd* infection. Low susceptibility to *Bd* infection of some species of amphibians has been linked to the presence of bacterial symbionts on their skin (Harris et al., 2006; Brucker et al., 2008). Zooplankton has also been shown to play a regulatory role in *Bd* infection.

Laboratory and mesocosm experiments have demonstrated that zooplankton regulates *Bd* prevalence by consuming its free-swimming infectious zoospores, thus drastically decreasing tadpole infection prevalence (Hamilton et al., 2012; Searle et al., 2013; Schmeller et al., 2014).

We aimed to identify how abiotic and biotic factors regulate the prevalence and intensity of *Bd* infection in streams and ponds in one of the most preserved and megadiverse regions of the Atlantic Forest in southeastern Brazil. We attempted to answer three main questions: (1) do abiotic characteristics of the environment influence the prevalence and intensity of *Bd* infection? (2) do communities of zooplankton regulate the prevalence and intensity of *Bd* infection? and (3) do these regulatory factors act differently in ponds versus streams?

MATERIAL AND METHODS

Study Site and Sampling.—We sampled 19 permanent water bodies (11 streams and 8 ponds; see figures 1 to 6 in Extra Supplementary Material) in Núcleo Curucutu, a poorly studied area of old growth forest with patches of grassland in Parque Estadual da Serra do Mar in the Atlantic Forest of southeastern Brazil (Garcia and Pirani, 2005). This area serves as a refuge for one of the most biodiverse amphibian faunas of any single locality in the Atlantic Forest, with 66 species from 12 families having already been recorded (Malagoli, 2013). Since *Bd* prevalence and intensity has been shown to be greater at higher elevations (Gründler et al., 2012), the selected water bodies were at higher elevations (750 to 880 m) of Núcleo Curucutu.

Tadpoles between Gosner stages 25 and 40 were sampled in February of 2016 by dip netting. We selected to use individuals within this range of stages because their oral discs are normally completely keratinized and *Bd* infection occurs only in keratinized tissues (Berger et al., 1998; McDiarmid and Altig, 1999; Berger et al., 1999; Nichols et al., 2001; Marantelli et al., 2004). Once sampled, individuals were euthanized by immersion in lidocaine solution and preserved in 90% alcohol solution for subsequent analyses. Species identification was made by direct comparison of external morphology and tooth row formulae with tadpole specimens deposited in the Amphibia - Tadpoles collection of the Department of Zoology and Botany of UNESP – São José do Rio Preto (DZSJRP-Amphibia-Tadpoles), and the use of dichotomous keys (Heyer et al., 1990; Frost, 2004). The tadpoles collected belonged to six species: *Crossodactylus caramaschii* (Hylodidae); *Physalaemus cuvieri* (Leptodactylidae); and *Aplastodiscus* aff *albosignatus*, *Boana albopunctata (=Hypsiboas albopunctatus)*, *B. faber (=H. faber)*, and *Scinax hayii* (Hylidae) (Table S1).

Prevalence and Intensity of Bd.—Oral discs of tadpoles were excised (Hyatt et al., 2007) and analyzed individually using real-time PCR following Boyle et al. (2004). DNA extraction was made using PrepMan Ultra (Applied Bioysistems) and amplified using a CFX96^{TM} Real-Time PCR Detection System (Bio-Rad) with a *Bd*-specific Taqman Assay (Boyle et al., 2004). Each 96-well assay plate included a negative control and four different standards containing DNA from 100, 10, 1 and 0.1 *Bd* genome equivalents. Samples, negative control and standards were run in duplicate. When both duplicate analyses revealed *Bd* zoospore genome equivalents >0.1 and amplification curves demonstrated the typical sigmoidal shape, *Bd* was considered present. When only one of the replicates met these criteria we re-ran the other replicate, and if it still did not comply we considered the sample negative for *Bd*. Samples that showed signs of inhibition (nonsigmoidal amplification) were further diluted to 1:100 and re-analyzed. The prevalence of *Bd* (hereafter *prevalence*) in each water body was determined by dividing the number of infected individuals by the total number of individuals analyzed, which was expressed as a percentage. Numbers of zoospore genomic equivalents recovered from the oral discs are reported as infection intensity. Infection intensity in different water bodies was estimated as mean zoospore load of infected tadpoles in each water body (hereafter *intensity*), excluding non-infected tadpoles.

Data Collection.—The abiotic variables measured in streams were: (1) velocity (average of three measures of water velocity; cm/seg), (2) depth (maximum depth; cm), (3) width (maximum width; m), and (4) vegetation cover (percentage canopy cover measured at five points – two points in each stream margin and one in its center – with a concave densiometer and expressed as the average of the measures). Abiotic variables were measured along 15-m river sections. The abiotic variables measured for ponds were: (1) depth (maximum depth; cm), (2) area (m^2), and (3) vegetation cover (both canopy cover and vegetation covering the surface of the water). In order to standardize measurements all abiotic variables were measured by the same person.

Zooplankton samples were collected in each water body by filtering (mesh size 45 μm) 20 liters of water to estimate population density. The samples were fixed in 4% formaldehyde and the density of zooplankton (sum of cladocerans, rotifers and copepods; ind/m^3) was determined at Laboratório de Ecologia de Zooplâncton da UNESP (São José do Rio Preto, São Paulo, Brazil). We used the sum of densities of these three zooplankton groups, and not the composition of the zooplankton community, because low *Bd* prevalence has been linked with total zooplankton density (Hite et al., 2016).

Data Analysis.—Differences in the prevalence and intensity of *Bd* infection between ponds and streams were assessed with one-way analysis of variance (ANOVA). Multiple linear regression analyses were used to evaluate the influence of abiotic and biotic parameters on the prevalence and intensity of *Bd* infection in both kinds of habitats. Prior to regression analyses, multicollinearity among independent variables was assessed using the Variance Inflation Factor (VIF) of the usdm package (Naimi, 2017) of R software (R Core Team, 2016), with a VIF > 3 indicating multicollinearity (Zuur et al., 2010). In addition, prevalence values were logit transformed (Warton and Hui, 2011) in the faraway package (Faraway, 2016) and the explanatory variables were standardized using the “decostand” function included in the vegan package (Oksanen et al., 2017). VIF analysis led to the exclusion of “maximum width” from the set of explanatory variables for streams, and “vegetation cover” from the set of explanatory variables for ponds. Thus, the global model for ponds comprised depth, area, and zooplankton; and the global model for streams comprised velocity, depth, vegetation cover, and zooplankton. For each response variable (prevalence and intensity), we generated all possible models, including a null model (without a predictor variable and considering only the intercept), global models, models with a single variable, and complex models including two or three variables. Sixteen models were thus generated for streams and eight for ponds. In order to better understand which variables best explained both prevalence and intensity, Akaike's Information Criterion corrected for small samples sizes (AICc) was used to rank all models, with only those with AICc of less than 2.0 being considered for interpretation (Burnham and Anderson, 2002). The likelihood of the models was also assessed using Akaike weights, which quantify the probability that a given model is the best among those selected (Burnham and Anderson, 2002). Normality and homoscedasticity of residuals were visually confirmed to meet the assumptions for parametric statistical testing. Analyses were performed in R-3.3.1 (R-Core Team, 2016).

RESULTS

Infected tadpoles were found in thirteen of the nineteen water bodies studied (eight streams and five ponds). Prevalence varied from 0% to 67% in ponds and from 0% to 84% in streams; however, one-way ANOVA revealed no significant differences between habits (mean ± standard deviation; 35.00 ± 26.90 in ponds and 48.64 ± 24.22 in streams; $F_{1,11} = 0.89$, $p = 0.36$) (Fig. 1). The intensity of infection also did not different between ponds and streams (127.87 ± 113.12 in streams and 151.86 ± 91.36 in ponds; $F_{1,11} = 0.16$, $p = 0.69$) (Fig. 1, Table S2).

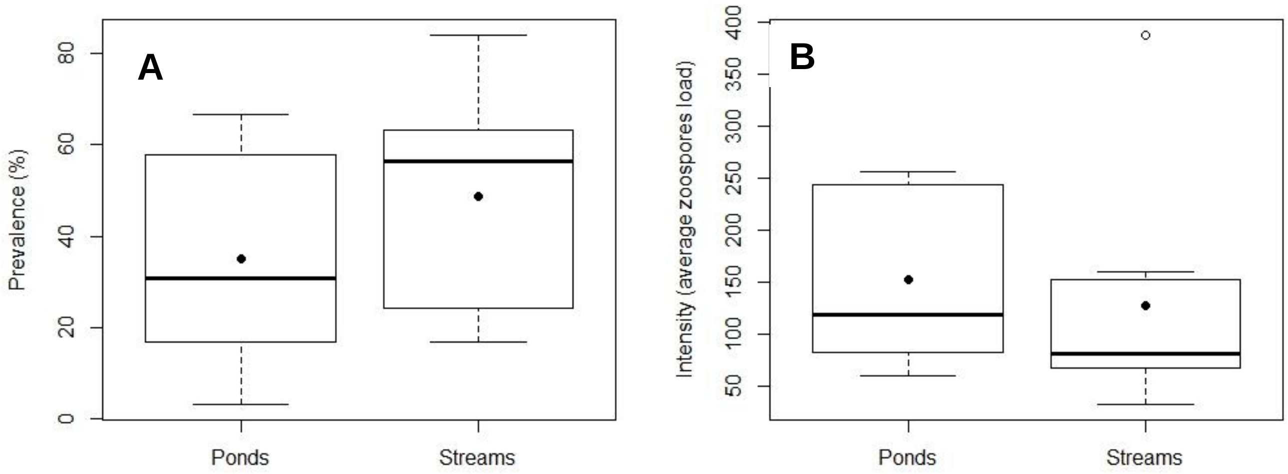

Figure 1. (A) Variation in the prevalence ($F_{1,11} = 0.89$, $p = 0.36$) and (B) intensity ($F_{1,11} = 0.16$, $p = 0.69$), of *Batrachochytrium dendrobatidis* infection in lentic and lotic habitats of Núcleo Curucutu in the Atlantic Forest of southeastern Brazil. Boxes correspond to the 25th to 75th percentiles; bold lines within boxes indicate medians while whiskers indicate minimum and maximum values; empty dots correspond to outliers; black dots are average values.

Among the models fitted to assess the influence of abiotic and biotic factors on prevalence of *Bd* infection (see supplementary Table S3), only one (zooplankton) was considered for ponds, and two (Velocity + Depth and Velocity) for streams (Table 1). Zooplankton density showed a negative relationship with *Bd* prevalence in ponds, and explained 85% of the variation in prevalence (Table 1). In turn, both velocity and depth explained 91% of the variation in *Bd* prevalence in streams (velocity in isolation explained up to 86%), with velocity showing a negative relationship and depth a positive relationship (Table 1). Regarding intensity, only the zooplankton model was considered for ponds, while none of the models were better than the null model for streams (Table 1 and Table S4). Again, zooplankton was highlighted as a regulatory factor for *Bd* infection in ponds, explaining 50% of the variation in intensity of infection.

Table 1. Models considered in examining the relationships between abiotic and biotic factors of ponds and streams, and the prevalence and intensity of *Batrachochytrium dendrobatidis* infection in Núcleo Curucutu in the Atlantic Forest of southeastern Brazil.

Models	**ΔAICc**	**df**	**wAICc**	R^2	p	β_0	β_1	β_2
STREAMS								
Prevalence								
Velocity + Depth	0	4	0.506	0.91	<0.001	-2.56	-4.71	1.17
Velocity	0.5	3	0.391	0.86	<0.001	-2.56	-4.08	-
NULL	19.4	2	<0.001	-	-	-	-	-
Intensity								
NULL	0	2	0.532	-	-	-	-	-
PONDS								
Prevalence								
Zooplankton	0	3	0.954	0.85	<0.001	-4.05	-4.16	-
NULL	11.1	2	0.004	-	-	-	-	-
Intensity								
Zooplankton	0	3	0.589	0.50	0.03	94.9	-79.03	-
NULL	1.2	2	0.329	-	-	-	-	-

DISCUSSION

Despite numerous studies reporting that amphibians inhabiting tropical streams are more severely impacted by *Bd* (Laurence, 1996; Berger et al., 1998, 1999; Lips, 1999; Kriger and Hero, 2007; Blooi et al., 2017), neither prevalence nor intensity of *Bd* infection differed between streams and ponds in the present study. This discrepancy could be due to the fact that, for the most part, these other studies were performed on adult individuals, while our study focused on tadpoles, and it is well known that *Bd* affects the different life phases of amphibians differentially (Berger et al., 2016). Therefore, studies of the adult amphibian community at Núcleo Curucutu are needed to confirm whether they have the same prevalence and intensity of *Bd* infection in streams and ponds, or whether they follow the pattern observed in adults studied at other locations.

The present study found that in streams, *Bd* prevalence was negatively related to water velocity. Piotrowski et al. (2004) reported that *Bd* zoospores loose their capacity for motility after 24 hours, and during that period the zoospores reached distances of less than 2 cm. Therefore, water velocity could be acting as a barrier to dispersion, and thus preventing encystment of zoospores in the oral discs of tadpoles. This possibility was also suggested by Medina et al. (2015), who pointed out that increased water flow could reduce the density of infectious particles, reducing the likelihood of successful *Bd* transmission. Since no data related to *Bd* zoospore resistance to water currents could be found, we encourage future research to study zoospores performance in order to better understanding their behavior in the field. Although the fastest streams exhibited a negative effect on the prevalence of *Bd*, those with greater depths seemed to favor the prevalence of *Bd*. The explanation for this may be that the deepest zones of streams possess lower temperatures and lower water velocity (Tarbuck and Lutgens, 2005), which could propitiate the presence of microhabitats that serve as refugia from the current for both *Bd* zoospores and tadpoles, and thus promote host-parasite interactions and increase *Bd* prevalence among tadpoles.

On the other hand, when zooplankton density was higher, the prevalence of *Bd* decreased. Indeed, the three ponds with the highest zooplankton density did not possess tadpoles infected with *Bd*. Furthermore, zooplankton density also explained a significant amount of the variation in infection intensity, with lower infection intensities occurring in tadpoles inhabiting ponds with higher densities of zooplankton. A similar trend was found in lentic habitats of Spain (high mountain ponds) and Honduras (bromeliads) (Schmeller et al., 2014; Hite et al., 2016; Blooi et al., 2017). These studies, and ours, demonstrate that biotic factors may be important regulators of *Bd* in the wild. Furthermore, they support the results of laboratory and mesocosm experiments that showed zooplankton to be predators of *Bd* zoospores [e.g. Hamilton et al., 2012; Searle et al., 2013; Schmeller et al., 2014).

None of the models assessed met our selection criteria for the analysis of the intensity of streams. We consider there to be three non-mutually exclusive explanatory hypotheses. First, we may have failed to select key explanatory variables to study. Second, infection intensity could be species dependent (host susceptibility), which, if true, would mean that environmental factors are acting as filters for the presence of *Bd* in habitats, but once an individual becomes infected, the susceptibility of the particular species would become an additional key factor for the intensity of infection. However, our ANOVA analyses that assessed differences in *Bd* intensity among the studied species do not support this proposal (see supplemental Table S5). Third, the intensity of infection is dependent on the particular *Bd* isolate present in each stream. Indeed, a high level of phenotypic diversity among isolates in proximate streams was documented in Serra do Mar (Lambertini et al., 2016). Thus, the confirmation of the existence of different isolates in our study area is the only way to test this hypothesis.

We conclude that, in a megadiverse region of the Brazilian Atlantic Forest, *Bd* infection is driven by biotic and abiotic factors, but in different ways in ponds and streams. We also identify zooplankton as an important factor in the ecology of *Bd* in lentic habitats, and so we encourage researchers to investigate in-depth which zooplankton species are potentially responsible for this *Bd* regulation. This study contributes to understanding the ecology of *Bd* in tropical forests, which has great significance for amphibian conservation actions.

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SUPPLEMENTARY MATERIAL

TABLE S1. Species sampled in Núcleo Curucutu. N = number of individuals; ST = streams; and PO = ponds.

	Habitat	**N**
Family Hylodidae		
Crossodactylus caramaschii	ST	34
Family Leptodactylidae		
Physalaemus cuvierii	PO	20
Family Hylidae		
Aplastodiscus aff. *albosignatus*	ST/PO	143/54
Boana albopunctata	PO	63
Boana faber	PO	33
Scinax hayii	PO	32

TABLE S2. Sample of tadpoles used to determine the prevalence and intensity of *Batrachochytrium dendrobatidis* infection at the study sites. N = number of tadpoles analyzed; Ni = number of tadpoles infected; Pv = prevalence per site; Int = intensity (average zoospores load per site).

	N	**Ni**	**Pv (%)**	**Int**
Pond_1	29	0	0.0	0
Pond_2	33	1	3.0	59.9
Pond_3	18	0	0.0	0
Pond_4	39	12	30.8	82.3
Pond_5	5	0	0.0	0
Pond_6	12	2	16.7	255.4
Pond_7	45	25	57.8	118.8
Pond_8	21	14	66.7	242.9
Stream_1	4	0	0.0	0
Stream_2	35	20	60.0	160.2
Stream_3	38	31	84.2	83.3
Stream_4	20	12	60.0	61.3
Stream_5	36	23	66.7	74
Stream_6	17	9	52.9	79
Stream_7	10	1	20.0	145.3
Stream_8	6	1	16.7	387.5
Stream_9	7	2	28.6	32.4
Stream_10	1	0	0.0	0
Stream_11	3	0	0.0	0

TABLE S3. Models fitted to examine relationships among biotic and abiotic factors and the prevalence of *Batrachochytrium dendrobatidis*. ΔAICc = difference in corrected Akaike's information criteria; df = degrees of freedom; wAICc = weights of corrected Akaike's information criteria.

Models	**ΔAICc**	**df**	**wAICc**
STREAMS			
Velocity + Depth	0	4	0.5062
Velocity	0.5	3	0.3910
Velocity + Zooplankton	5.1	4	0.0392
Velocity + Canopy Cover	5.4	4	0.0345
Velocity + Zooplankton + Depth	7.1	5	0.0142
Velocity + Depth + Canopy Cover	7.2	5	0.0135
Velocity + Zooplankton + Canopy Cover	12	5	0.0012
Canopy Cover + Velocity + Depth + Zooplankton	18	6	<0.001
NULL	19.4	2	<0.001
Canopy Cover	21.5	3	<0.001
Depth	22.2	3	<0.001
Zooplankton	23.2	3	<0.001
Canopy Cover + Depth	25.6	4	<0.001
Zooplankton + Canopy Cover	26.7	4	<0.001
Zooplankton + Depth	27.5	4	<0.001
Canopy Cover + Zooplankton + Depth	32.9	5	<0.001
PONDS			
Zooplankton	0	3	0.9544
Zooplankton + Depth	6.9	4	0.0308
Zooplankton + Area	9	4	0.0104
NULL	11.1	2	0.0038
Depth	15.8	3	<0.001
Area	16.6	3	<0.001
Area + Depth	25	4	<0.001
Area + Depth + Zooplankton	25.4	5	<0.001

TABLE S4. Model sets fitted to examine the relationship between biotic and abiotic factors and the intensity of *Batrachochytrium dendrobatidis* infections. ΔAICc = difference in corrected Akaike's information criteria; df = degrees of freedom; wAICc = weights of corrected Akaike's information criteria.

Models	**ΔAICc**	**df**	**wAICc**
STREAMS			
NULL	0	2	0.532
Velocity	2.8	3	0.128
Cover	3.4	3	0.096
Zooplankton	3.5	3	0.093
Depth	3.9	3	0.077
Cover + Velocity	6.6	4	0.019
Depth + Velocity	7	4	0.015
Zooplankton + Velocity	7.8	4	0.010
Cover + Zooplankton	8.1	4	0.009
Zooplankton + Depth	8.5	4	0.007
Cover + Depth	8.6	4	0.007
Cover + Depth + Velocity	12	5	0.001
Cover + Zooplankton + Velocity	13.6	5	<0.001
Zooplankton + Depth + Velocity	13.8	5	<0.001
Cover + Zooplankton + Depth	15.2	5	<0.001
Cover + Depth + Velocity + Zooplankton	22.1	6	<0.001
PONDS	**ΔAICc**	**df**	**wAICc**
Zooplankton	0	3	0.589
NULL	1.2	2	0.329
Area	6.1	3	0.027
Zooplankton + Area	6.2	4	0.026
Depth	6.6	3	0.022
Zooplankton + Depth	9.3	4	0.005
Area + Depth	15.4	4	<0.001
Zooplankton + Depth + Area	24.8	5	<0.001

TABLE S5. Intensity of *Batrachochytrium dendrobatidis* infection per species in streams and ponds (mean of zoospores and standard deviation), and the results of analyses of variance among species sharing each habitat.

	Aplastodiscus aff. *albosignatus*	*Boana albopunctata*	*Boana faber*	*Scinax hayii*	*Crossodactylus caramaschii*	*Physalaemus cuvieri*	ANOVA	
							p-value	*F*
Ponds	118.8 ± 88.8	76.5 ± 51.3	94.4 ± 44.9	244.5 ± 256.4	-	0	0.26	1.37
Streams	94 ± 88.1	-	-	-	161.1 ± 163.1	-	0.48	0.49

EXTRA SUPPLEMENTARY MATERIAL

Figures 1-3. Examples of lentic localities sampled in Núcleo Curucutu, Parque Estadual da Serra do Mar, São Paulo state, southeastern Brazil.

Figures 4-6. Examples of lotic localities sampled in Núcleo Curucutu, Parque Estadual da Serra do Mar, São Paulo state, southeastern Brazil.


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Chapter II

Global warming could decrease potential geographic distribution of amphibian-killing fungus in Brazilian Atlantic Forest

This chapter will be submitted to *Diversity and Distributions*, and will be exposed in the *IBS Climate Change Biogeography Meeting* (Évora – Portugal, March 2018)

This chapter has been formatted following the journal authors' guide.

Global warming could decrease potential geographic distribution of amphibian-killing fungus in Brazilian Atlantic Forest

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ABSTRACT

To better understand the possible effects of climate change in the potential geographic distribution in the Brazilian Atlantic Forest of *Batrachochytrium dendrobatidis* (*Bd*), we constructed species distribution models for *Bd* for the present and future climatic scenarios. For present time, 58.8% of the Brazilian Atlantic Forest present suitable areas for *Bd*. Future scenarios for year 2070 forecast a reduction of these areas by between 27.5% and 42.6%. Future projections also indicate that *Bd* suitable areas will remain mainly in highlands of the southern half of the biome, tending to disappear from lowlands and low latitudes. In the light of these results, we recommend monitoring amphibian populations inhabiting areas that could correspond as refuges for *Bd* in the near future, which would enable prompt responses from possible outbreak of the disease in those areas.

Key words

Batrachochytrium dendrobatidis, Brazilian Atlantic Forest, Conservation, Climate Change, Global Warming, Species Distribution Model

INTRODUCTION

The aquatic chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), described at the end of the last century (Berger et al., 1998; Longcore et al., 1999) has been identified as responsible for the decline and extinction of amphibian populations in different parts of the world (Stuart et al., 2004; Skerratt et al., 2007; Olson et al., 2013). Chytridiomycosis, name given to the infectious disease caused by this fungus, is considered the biggest disease-driven vertebrate biodiversity loss ever recorded (Berger et al, 1998, 1999; Lips, 1998, 1999; Carnaval et al., 2005; Skerratt et al., 2007; Bosch et al., 2001; Bai et al., 2010; Kielgast et al., 2009; http://www.bd-maps.net). Since the disease was highlighted as the main cause of global amphibian declines, *Bd* has become one of the best studied wildlife pathogens by the scientific community and has also meant an increase in the role given to infectious diseases in species conservation plans (Berger et al., 2016).

The increment in virulence of a local *Bd* isolates (Fisher et al., 2009, Phillips et al., 2013); the arrival of a novel *Bd* isolate from Asia, probably belonging to *Bd*-GPL (Global Panzootic Lineage), the most virulent *Bd* linage that has been associated with amphibian population declines worldwide (Farrer et al., 2011); and, collateral effects of climate change (Pounds et al., 1999, 2006; Bosch et al., 2007; Rohr et al., 2010), are the hypotheses that the scientific community proposed to explain these declines. Recents studies have pointed that the arrival of the *Bd*-GPL lineage, possibly by the trade of species, could be the begginig of the important population declines that occurred in Central America, Australia and Spain (Dr. Jaime Bosch personal communication, unpublished data), and that climate change could be responsible of increased chytridiomycosis epizootics in regions where the fungus is already present (Raffel et al., 2013, 2015). Therefore, understanding the effects of climatic change on *Bd* potential geographic distribution is essential for prediction of future chytridiomycosis epizootics. The use of Species Distribution Models (SDMs; see Franklin, 2010; Guisan et al, 2017) to predict potential geographic distribution of *Bd* has been applied in different regions affected by chytridiomycosis (*e.g.* Ron et al., 2005; Rödder et al., 2009; Liu et al., 2012; James et al., 2015). However, the knowledge about future potential distributions associated to climate change scenarios are still poorly explored.

The chytridiomycosis is affecting more severely pristine areas characterized by short-range temperatures, abundant rainfalls and high amphibian richness (Olson et al., 2013). Although the Brazilian Atlantic Forest (BAF) does not escape from *Bd* (Navarro-Lozano, 2016), amphibian population declines linked to chytridiomycosis have not been demonstrated for this bioma. Therefore, to better understand the potential implications of climate change over *Bd* presence in BAF, we build species distribution models to analyze current and future potential distribution of chytrid fungus in this high diverse area.

METHODS

Data collection

Our data correspond to positive cases of *Bd*-infection combining both published scientific reports (198 localities; see Navarro-Lozano, 2016) and our own field samples (five localities). Our field study were conducted in Nucleo Curucutu (NC), Parque Estadual da Serra do Mar (São Paulo state, southeastern Brazil). This old growth area shows low human activity and is one of the less studied regions in São Paulo state Atlantic Forest (Garcia & Pirani, 2005). Twenty-one adults were captured in nine different locations of NC during February 2016, and swabbed gently with sterile cotton swabs following Hyatt et al. (2007) procedure, and released back into their place of capture. Swabs preserved in alcohol 90% were analyzed in duplicate to detect presence of *Bd* following the methodology described by Boyle et al. (2004). We considered *Bd* presence when both analysis performed to each swab detected *Bd*, zoospore genome equivalents were >0.1, and the amplification curves have sigmoidal shape. The analyses determined *Bd* presence in six adults from five different locations, all of them with no apparent signs of chytridiomycosis (see Appendix S1 in Supporting Information).

Species Distribution Modeling

Our predictor variables correspond to four bioclimatic variables of present time with a spatial resolution of 2.5 min, downloaded from WorldClim databased (v1.4; Hijmans et al., 2005). This set of predictor variables, Mean diurnal range (Bio2; Mean of monthly (maximum temperature - minimum temperature)), Maximum temperature of warmest month (Bio5), Annual Precipitation (Bio 12), and Precipitation of warmest quarter (Bio18) were selected based on their relevance to *Bd* biology in both, field (Kriger & Hero, 2007; Kriger et al., 2007; Kriger, 2009) and laboratory (Piotrowski et al., 2004; Woodhams et al., 2008) studies. For future projections, we inlcuded the same set of predictor variables for the year 2070 by four different climatological research centers: The Beijing Climate Center Climate System Model (BCC-CSM1-1), The Hadley Centre Global Environment Model (HadGEM2-ES), The Institute Pierre Simon Laplace

(IPSL-CM5A), and the Community Climate System Model (CCSM4). All future climatic models were obtained from WorldClim database (v1.4; Hijmans et al., 2005). Each of the four Atmosphere-Ocean General Circulation Model (AOGCM) of future climatic models was composed by four different scenarios of Representative Concentration Pathways (RCPs) 2.6, 4.5, 6.0 and 8.5 (see Wayne, 2013 and Vuuren et al., 2011 for details).

To create the potential current distribution model of *Bd* in BAF, we implemented a multi-model ensemble strategy, including four different algorithms, Maxent, BRT, GAM and GLM, in the R package “sdm” (Naimi & Araújo, 2016). In turn, future potential projections were made by a consensus of the four different AOGCMs for each RCPs.

Previously to the modeling, we assessed spatial autocorrelation of the data by a Mantel test in package ecospat (Broennimann et al. 2016) in R (R Core Team, 2016). Distances equal or greater than to 55 km among localities did not present spatial autocorrelation; therefore to avoid spurious results, occurrence data was rarefied in spThin (Aiello-Lammens et al., 2014), considering only localities separated by at least 55 km. Then, from the 203 original records, a set of 60 localities remained for modeling procedures. Models were calibrated a random 75% of the final data set (training sample) and evaluated by the remaining 25% (test data). Performance of models were evaluated by the area under the Receiver Operating Characteristic curve (AUC), and the true skill statistic (TSS). AUC is a common method for evaluating the accuracy (ability of the model to classify correctly a specie as present or absent) of classification models (Fielding & Bell, 1997), and varies between 0 and 1, with 1 indicating a perfect prediction (model discriminates perfectly between presence and absence records) and values below 0.5 indicating random prediction (Hanley & McNeil, 1982). TSS is defined as sensitivity (correctly classified presences) + specificity (correctly classified absences) -1 (Allouche et al., 2006). TSS varies between -1 and +1, where -1 indicates a performance worse than those randomly expected and +1 indicates a perfect fit (Allouche et al. 2006). Models with AUC values over 0.80 and TSS values over 0.50 are considered high-accurate and with strong predictive power (Araujo et al., 2005; Allouche et al., 2006), only the algorithms with equal or higher of these values were included in the consensus model.

The background of the geographic studied area impacts on the appropriateness and accuracy of the model prediction when extrapolating the species distribution in space (VanDerWal et al., 2009; Elith et al., 2011). In order to check the effect of spatial extent in our models, four different geographic backgrounds where tested: BAF and BAF plus buffer areas of 100, 200 and 300 km (see Appendix S2). In turn, since there is no consensus in a number of pseudo-absences to be used in SDMs of presence-ony datasets, and given that the relationship between the amount of occurrence data and the absence data influence the accuracy of the model (McPherson et al., 2004; Barbet-Massin et al., 2012), we assessed four different amounts of pseudo-absences for each of the study backgrounds: same pseudo-absences than occurrences and five, ten and one hundred times more pseudo-absences than occurrences. Preliminary analyses made us to choose the universe BAF plus buffer area of 300 Km with five times more pseudo-absences than occurrences (see results of this preliminary analysis in Appendix S3).

All projections of potential *Bd* presence were reclassified in five classes for easier interpretation using ArcGis® 10.0 (ArcMap^{TM}) ESRI. The first of this five classes represents *Bd* absence in the maps. The *Bd* positive locality with the lower occurrence probability value was used as turning limit for *Bd* presence, that is, all areas with lower values were assumed as free of *Bd*. The remaining four classes represented different probability ranges of *Bd* occurrence (see Fig 1).

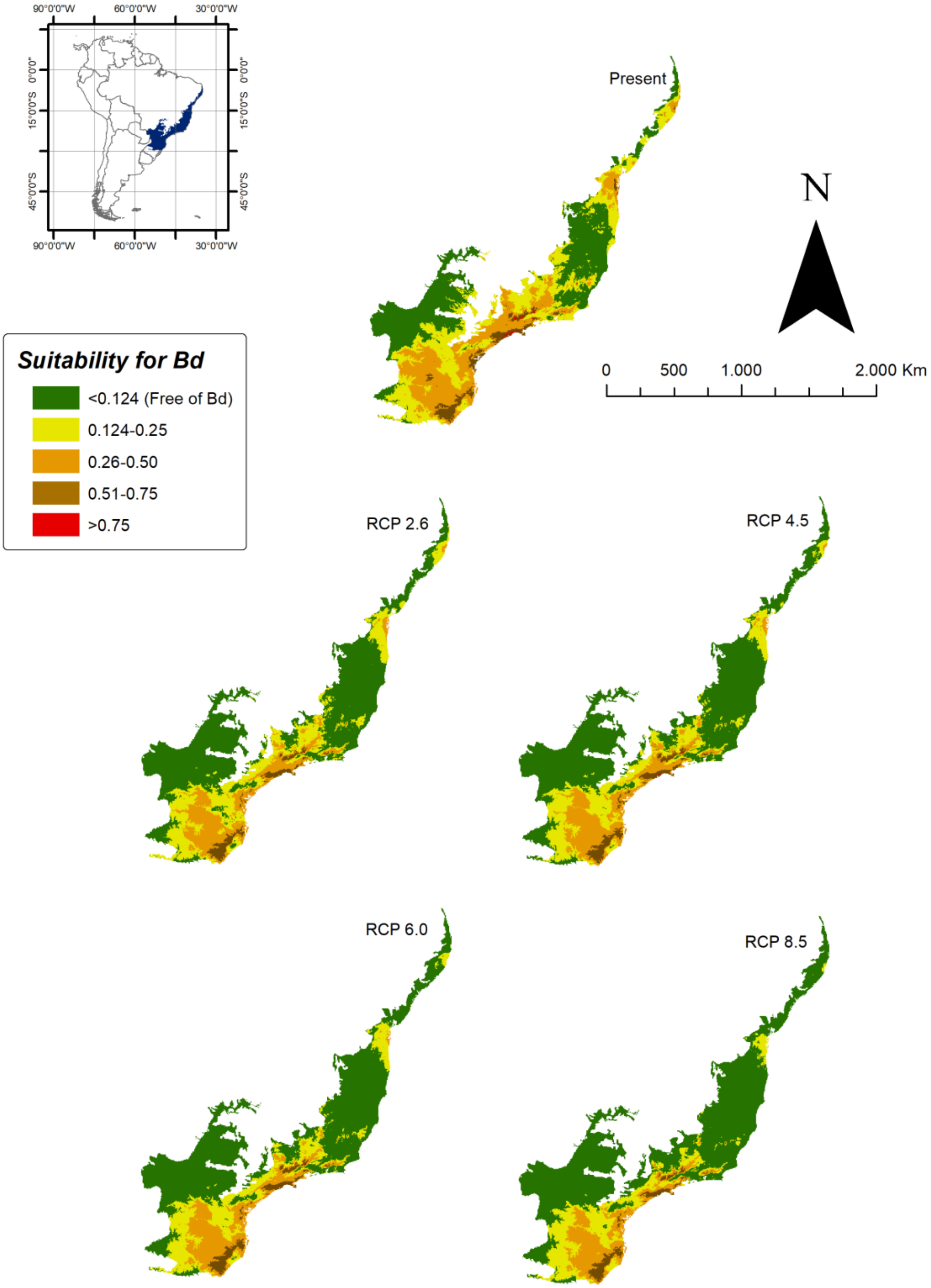
RESULTS

The *Bd* positive locality with lower value of suitability in models for present time had a value of 0.124, and was used as turning limit between presence and absence for current and future maps (first class in the maps of the Fig 1), with which were calculated the potential occurrence areas for *Bd*. Present time potential occurrence area for *Bd* in BAF varied from 617,787 Km^2 (GLM) to 717,579 Km^2 (GAM) (see details in Table 1). All SDMs algorithms presented high-accuracy and strong predictive power (AUC ≥ 0.84, TSS ≥ 0.6). Therefore, all of them were included in the consensus current potential distribution model which estimated an area of 665,769.2 km^2 of potential occurrence for *Bd* (58.8% of the BAF territory). The BAF areas with higher probabilities of *Bd* occurrence are largely concentrated in its southern half, although there is a smalle area with moderate probabilities of occurrence in northern areas of BAF, in coastal regions of Bahia and Pernambuco states (Fig 1 – Present time). Higher suitability areas for *Bd*, for which the consensus model predicts suitabilities higher than 0.75, occupy 2,296.4 Km^2 (0.2% of the BAF territory), and are mainly concentrated in states of Minas Gerais, São Paulo and Rio de Janeiro (Fig 1 – Present time).

Table 1 Predicted total suitable areas (Km^2) for *Batrachochytrium dendrobatidis* in the Brazilian Atlantic Forest for present time for each SDM algorithms and a consensus model, and for four future projection climatic scenarios of 2070 (BCC-CSM1-1, HadGEM2-ES, IPSL-CM5A, and CCSM4 atmosphere-ocean general circulation models). Estimated loss of suitability areas for the change scenarios are provided from the consensus model of present time.

		Km^2	**% Loss**
Present	MAXENT	686880	-
	GLM	617787	-
	GAM	717579	-
	BRT	694089	-
	Consensus	665769.2	-
2070	RCP 2.6	482780.3	↓27.5
	RCP 4.5	459978.8	↓30.9
	RCP 6.0	438351.8	↓34.1
	RCP 8.5	382340.3	↓42.6

Figure 1 Habitat suitability maps for *Batrachochytrium dendrobatidis* in the Brazilian Atlantic Forest in present time (consensus of Maxent, BRT, GAM and GLM algorithms of Species Distribution Models) and future projections (consensus of BCC-CSM1-1, HadGEM2-ES, IPSL-CM5A, and CCSM4 atmosphere-ocean general circulation models).



Potential suitability areas predicted for future scenarios of climate change in 2070 decreased in all four RCPs assessed. Climate change scenario with higher GHG emissions (RCP 8.5) corresponded to a decrease of 42.6% from the present time potential distribution area estimated for *Bd*, whereas the scenario that predicts lower GHG emissions (RCP 2.6) corresponded to a decrease of 27.5% (Table 1). In all future scenarios, the reductions in the potential *Bd* distribution are more accentuated in northern areas of the BAF (Fig 1). Areas with higher suitability (probabilities >0.75) are the most affected in all climate change scenarios, losing up to 99% in RCP 8.5 (Table 2). In turn, areas classified as free from *Bd* (<0.124) suffer a great gain (Table 2).

Table 2 Loss and gain of suitable area for predicted occurrence of *Batrachochytrium dendrobatidis* in the Brazilian Atlantic Forest in present time (consensus of Maxent, BRT, GAM and GLM algorithms of Species Distribution Models) and future projections for four different scenarios of representative concentration pathways (RCPs) 2.6, 4.5, 6.0 and 8.5 (consensus of climatic atmosphere-ocean general circulation models BCC-CSM1-1, HadGEM2-ES, IPSL-CM5A, and CCSM4).

Suitability for *Bd*	Present	2.6	% Loss or gain	4.5	% Loss or gain	6.0	% Loss or gain	8.5	% Loss or gain
<0.124	465982,8	648972	↑39.3	671773.5	↑44.2	693400.5	↑48.8	749412	↑60.8
0.124-0.25	346584,7	277951,5	↓19.8	243364.5	↓29.8	234879.8	↓32.2	206064	↓40.5
0.26-0.5	270998,7	172044	↓36.5	178584.8	↓34.1	166536	↓38.5	144099	↓46.8
0.51-0.75	45889,4	32602,5	↓28.9	37644.7	↓17.9	36753.7	↓19.9	32157	↓29.9
>0.75	2296,4	182,25	↓92	384.7	↓83.2	182.2	↓92	20.2	↓99.1

DISCUSSION

A wide range of suitable areas for *Bd* occurrence was estimated for the 60 localities where *Bd* has been detected in the BAF (varing from 0.124 to 0.784), being the highest suitability values in two of the most preserved areas of BAF, Serra do Mar and Serra da Mantiqueira ranges, which correspond to 12% of original BAF remnants that still persist nowadays (Ribeiro et al., 2009). Our results coincide with previous observations from Becker et al. (2011), who pointed higher prevalences and infection intensities of *Bd* in pristine areas of forests in Serra do Mar. Compared to other studies that modelled global *Bd* potential distribution for present time (e.g. Liu et al., 2012; Rödder et al., 2010), our results offer a better detail of suitable areas for *Bd* in BAF, which could help in the development of more precise management and conservation plans.

All Climate Change scenarios inlcuded in our models predict temperature increases in the BAF for the year 2070 (see Appendix S5). Temperature has proved as an important regulating factor of *Bd* occurrence, explaining the seasonality and distribution of chytridiomycosis infections (Berger et al, 2004; Bosch et al., 2007; Kielgast et al., 2010; Murray et al., 2013). Our results along others already reported, have been evidencing the impact that Climate Change could have on geographic ranges of species. Several studies have shown that species geographical range vary in response to climatic change, with movements toward high altitudes and latitudes as results of global warming (e.g. Parmesan, 2006; Pounds et al., 1999, 2006; Parmesan et al., 2003; Chen et al., 2011). Our results also reinforce this trend, predicting a decrease in *Bd* suitable areas in lowlands, and increases in highlands of southern BAF (São Paulo, Rio de Janeiro, Minas Gerais, Paraná, Santa Catarina and Rio Grande do Sul states). While it may seem beneficial for amphibians, it must have not been forgotten that this reduction on distributional ranges of species as a result of climate change may also affects amphibians (Pounds et al., 1999; Raxworthy et al., 2008; Vasconcelos & Nascimento, 2016). Indeed, species of amphibians in BAF are considered 'climate change susceptible' due mainly to their restricted distribution ranges (Foden, 2008). This joint reduction of geographic ranges could facilitate the contact between *Bd* and amphibians, and even lead to contacts with novel host species for which the infection effects are unknown.

Although there is no single study reporting amphibian populations declines attributed to *Bd* in the last decades in Brazil, we must continue to be vigilant against potential future epizootic outbreaks driven by global warming. Therefore, we recommend monitoring amphibian populations inhabiting areas that have been determined as refuges for *Bd* in the near future, especially highlands of southern Brazilian Atlantic Forest, which would enable a prompt response in the case that an outbreaks occurs.

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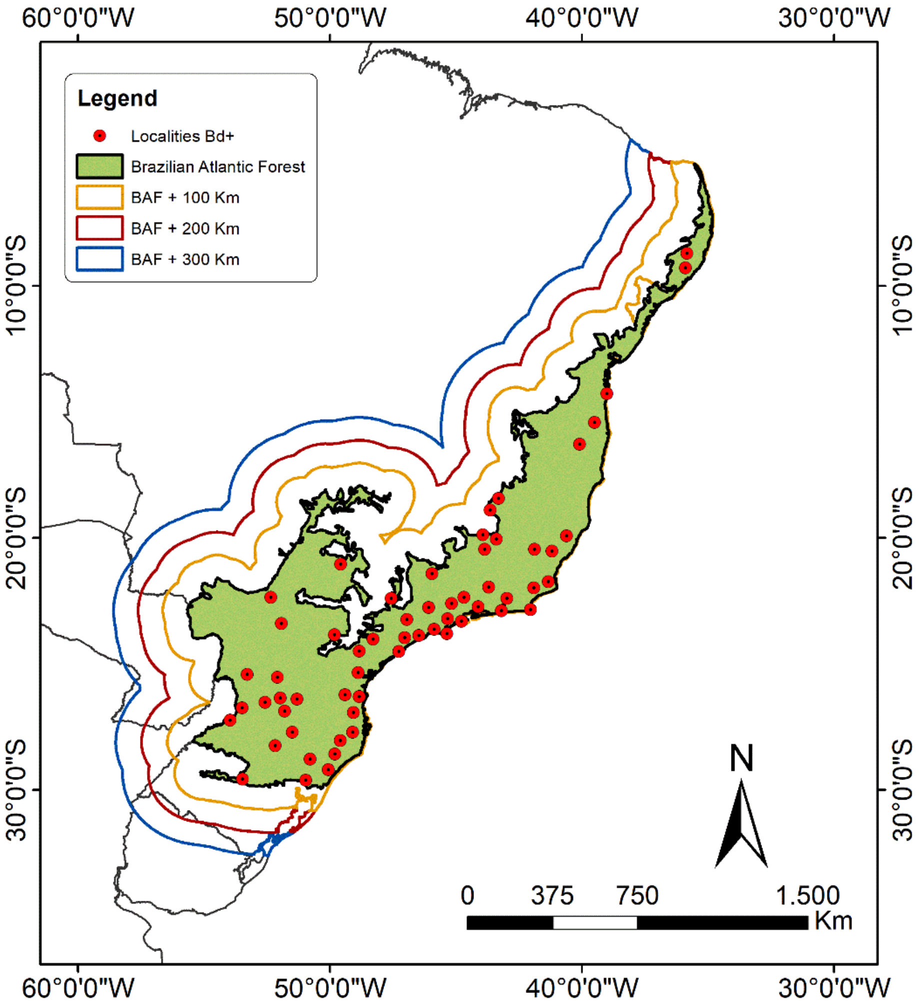
SUPPORTING INFORMATION

Appendix S1. Infection and location (in decimal degrees) of adult frogs swabbed at Nucleo Curucutu in southeastern Brazilian Atlantic Forest. *Bd* = *Batrachochytrium dendrobatidis* infection ('+' for positive infection and '-' for negative infection).

	Bd	**Latitude**	**Longitude**
Family Brachycephalus			
Ischnocnema guentheri	-	-23.9863	-46.7315
Family Bufonidae			
Rhinella ornata	+	-23.9708	-46.7338
Family Craugastoridae			
Haddadus binotatus	+	-23.9916	-46.7433
Family Cycloramphidae			
Cycloramphus sp.	-	-23.9991	-46.7376
Family Hylidae			
Bokermannohyla hylax	-	-23.9977	-46.7386
Boana albopunctata	-	-23.9854	-46.7429
Boana albopunctata	-	-23.9854	-46.7429
Boana bischoffi	-	-23.9991	-46.7376
Boana faber	-	-23.9854	-46.7429
Boana faber	-	-23.9854	-46.7429
Scinax hayii	+	-23.9916	-46.7433
Scinax hayii	-	-23.9916	-46.7433
Scinax hayii	-	-23.9916	-46.7433
Family Hylodidae			
Crossodactylus caramaschii	-	-23.9989	-46.7385
Hylodes phyllodes	+	-23.9977	-46.7386
Hylodes caete	+	-23.9965	-46.7458
Hylodes caete	-	-23.9977	-46.7386
Hylodes caete	-	-23.9989	-46.7385
Hylodes caete	-	-23.9965	-46.7458
Hylodes caete	-	-23.9968	-46.7373
Hylodes caete	-	-23.9977	-46.7386

Appendix S2. Background evaluated prior to species distribution modelling: Brazilian Atlantic Forest (BAF), and BAF plus buffer areas of 100, 200 and 300 km. Red dots correspond to 60 presence records of anuran amphibians infected with *Batrachochytrium dendrobatidis* used for modelling, after random selection from 203 original records. This selection was implemented in spThin R package (see methods) considering only localities separated by at least 55 km in order to avoid spatial autocorrelation and sampling bias.



Appendix S3. Evaluation of species distribution models performed for the selection of background and number of pseudo-absences. BAF = Brazilian Atlantic Forest; Ps = pseudo-absences; AUC = Area Under the receiver operating characteristic Curve; and TSS = True Skill Statistic. Selected combination applied in final modelling is denoted in green.

	BAF								**BAF + 100 Km**							
	AUC				**TSS**				**AUC**				**TSS**			
Ps	max	glm	gam	brt	max	glm	gam	brt	max	glm	gam	brt	max	glm	gam	brt
60	0.71	0.71	0.71	0.73	0.45	0.45	0.48	0.47	0.75	0.76	0.73	0.75	0.48	0.5	0.49	0.49
300	0.72	0.71	0.71	0.71	0.42	0.4	0.41	0.41	0.79	0.78	0.78	0.79	0.54	0.52	0.51	0.52
600	0.72	0.71	0.7	0.71	0.41	0.41	0.39	0.39	0.79	0.79	0.78	0.78	0.5	0.5	0.51	0.5
6000	0.71	0.71	0.7	0.7	0.4	0.4	0.38	0.37	0.77	0.77	0.77	0.75	0.49	0.5	0.49	0.46

	BAF + 200 Km								**BAF + 300 Km**							
	AUC				**TSS**				**AUC**				**TSS**			
Ps	max	glm	gam	brt	max	glm	gam	brt	max	glm	gam	brt	max	glm	gam	brt
60	0.79	0.78	0.79	0.8	0.54	0.56	0.59	0.56	0.86	0.84	0.82	0.83	0.64	0.64	0.59	0.61
300	0.8	0.79	0.79	0.79	0.54	0.56	0.53	0.53	**0.86**	**0.86**	**0.84**	**0.84**	**0.66**	**0.63**	**0.6**	**0.64**
600	0.8	0.79	0.8	0.8	0.56	0.56	0.54	0.55	0.83	0.83	0.82	0.82	0.58	0.6	0.57	0.58
6000	0.8	0.8	0.79	0.8	0.58	0.58	0.56	0.53	0.84	0.84	0.83	0.82	0.61	0.61	0.6	0.58

Appendix S4. Predicted suitable areas (in km^2) for *Batrachochytrium dendrobatidis* in Brazilian Atlantic Forest for present-time and year 2070, considering four different scenarios of Representative Concentration Pathways (RCPs) 2.6, 4.5, 6.0 and 8.5, and estimated loss of suitable areas.

	BCC-CSM1	% loss	CCSM4	% loss	HadGEM2-ES	% loss	IPSL-CM5A	% loss
Present	665769,24	-	665769,24	-	665769,24	-	665769,24	-
RCP 2.6	498939,75	25.1	522551,25	21.5	486870,75	26.8	403764,75	39.3
RCP 4.5	423954	36.3	435537	34.6	458237,25	31.2	496793,25	25.4
RCP 6.0	406539	38.9	462368,25	30.5	416745	37.4	446208,75	32.9
RCP 8.5	401699,25	39.7	321813	51.6	390197,25	41.4	388597,5	41.6

Appendix S5. Bioclimatic variables values predicted for 2070 by four climatic scenarios of Representative Concentration Pathways (RCPs) 2.6, 4.5, 6.0 and 8.5, in the Brazilian Atlantic Forest. Each value corresponds to the average of Atmosphere-Ocean General Circulation Models used in modelling.

	Annual Mean Temperature (ºC)		**Mean Diurnal Range (ºC)**		**Max Temperature of Warmest Month (ºC)**		**Annual Precipitation (mm)**		**Precipitation of Warmest Quarter (mm)**	
	Min	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**
Present	11	26.1	5.7	14.1	18.9	33.6	515	3537	73	1197
RCP 2.6	12,35	27,43	5,68	14,38	20,40	35,70	481,25	3556,00	68,25	1167,50
RCP 4.5	13,08	28,13	5,68	14,28	20,93	36,70	485,25	3602,00	71,75	1215,25
RCP 6.0	13,18	28,30	5,60	14,28	21,20	37,10	480,75	3656,00	76,75	1255,50
RCP 8.5	14,23	29,45	5,58	14,33	21,75	38,90	436,00	3715,25	77,50	1257,50

Chapter III

Are oral deformities in tadpoles accurate indicators of anuran chytridiomycosis?

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Are oral deformities in tadpoles accurate indicators of anuran chytridiomycosis?

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Abstract

We evaluated the use of oral deformities as reliable proxies for determining *Batrachochytrium dendrobatidis (Bd)* infection in tadpoles of six anuran species of the Atlantic Forest in southeastern Brazil. We examined oral discs of 2156 tadpoles of six species of anurans collected in 2016: *Aplastodiscus albosignatus, Boana albopunctata, Boana faber, Scinax hayii, Crossodactylus caramaschii*, and *Physalaemus cuvieri*. Three oral deformities were recognized: lack of keratinization only in upper and/or lower jaw sheaths, lack of keratinization only in upper or lower tooth rows, and both deformities together. A subsample composed of all the individuals possessing oral deformities (N = 195) plus randomly selected individuals without oral deformities (N = 184) were tested for *Bd* via qPCR. Oral deformities were observed in all six species, but only five were infected with *Bd*. Since we found that dekeratinization of tooth rows was not associated with the presence of *Bd* in any of studied species we used a new proxy (jaw sheaths dekeratinization with or without dekeratinization in tooth rows: JSD-proxy) for *Bd* detection. Our results showed a nonrandom relationship between *Bd* infection and JSD-proxy in three species of the family Hylidae. However, the use of JSD-proxy for *Bd* detection in these species resulted in up to 30.8% false positives and up to 29.3% false negatives. The use of the JSD-proxy in species for which no relationship was found reached 100% of false positives. We conclude that the use of oral dekeratinization as a generalized proxy for *Bd* detection in tadpoles should not be used as a single diagnosis technique.

Key-words

Amphibia, *Batrachochytrium dendrobatidis*, Conservation, Disease, Morphology, Oral deformities, Tadpoles

Introduction

Global loss of biodiversity is one of the most serious problems of our time, with Amphibia being the most affected vertebrate class [1, 2]. Habitat loss, introduction of exotic species, environmental contamination, increased UV radiation, climate change and infectious diseases have been identified as major causes of amphibian population declines [3-17]. Chytridiomycosis, an infectious fungal disease caused by *Batrachochytrium dendrobatidis* (*Bd*) [15], has been linked to many incidents of amphibian mass mortality worldwide [15,18,19], and is considered one of the greatest causes of global amphibian declines [20]. In amphibians, *Bd* infection occurs only in keratinized tissues, which are restricted to the oral region (jaw sheaths and teeth) of tadpoles, and the epidermis of metamorphs and adults [15, 21-24].

Several studies have established a relationship between *Bd* infection and the occurrence of anomalies in the oral region of tadpoles of a number of different amphibian species. Fellers et al. [25] found that 67% of *Rana muscosa* tadpoles with abnormally keratinized mouthparts were infected by *Bd*. In turn, Knapp and Morgan [26] found for the same species that 89% of tadpoles with less than 90% jaw sheath pigmentation were infected. This relationship was later reinforced by Drake et al. [27], who found clear nonrandom relationships between oral deformities and *Bd* presence in *Lithobates sphenocephalus* (=*Rana sphenocephala)* tadpoles. A similar result was reported for *Hylodes japi* tadpoles, for which 94.5% of infected individuals possessed depigmented mouthparts [28]. These results suggest the possibility of using tadpole oral deformities as a general proxy for *Bd* detection in tadpoles, as has been previously suggested by Fellers et al. [25] and recently applied by Carvalho et al. [29].

Despite this promising proxy for which only a quick visual inspection is required, there are many studies that have indicated that oral deformities are not always related to *Bd* infection. For example, no relationship was found between depigmentation of mouthparts and *Bd* infection of *Lithobates catesbeianus (=Rana catesbeiana)* and *Pseudacris regilla* [30]. Furthermore, a laboratory study found no differences in the proportions of tadpoles with mouthpart abnormalities between *Bd* infected and uninfected individuals of *Anaxyrus boreas* (=*Bufo boreas*) and *Pseudacris regilla (=Hyla regilla)* [31]. In addition, many other factors have been associated with oral deformities in tadpoles, including low temperatures [32], water contamination [33], nutrition [21], competition [34], and predation risk [35]. Herein we quantitatively evaluate, in detail, the use of oral deformities as a reliable proxy for determination of *Bd* infection in six anuran species from southeastern Brazil.

Material and methods

Ethics statement

Field studies did not involve endangered or protected species.

Tadpoles were captured in accordance with collection permits, and subsequently killed using lidocaine, and preserved in 90% alcohol for subsequent study of oral deformities. Collection permits were provided by Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) (#47148-2). All sampling procedures were reviewed and specifically approved by ICMBio and Comissão Técnico-Científica do Instituto Florestal (COTEC; a committee of

Instituto Florestal, the state research agency and responsible for the reserve) (Processo SMA #260108-001.809/2015).

Study animals

A total of 2156 tadpoles were studied. External morphology and tooth rows formulae were used to identify the species of tadpoles. Tadpoles were collected by dip netting during February (2016) in Núcleo Curucutu (23º 59' 08.52''S, 46º 44' 35.76'' W), Parque Estadual da Serra do Mar (São Paulo State, Brazil), an understudied old growth area of Atlantic Forest in southeastern Brazil [42].

Identification of oral deformities

It is important to emphasize that studies regarding the relationship between *Bd* infection and oral deformities have used different, and sometimes ambiguous terms for describing the deformities. For example, while some of studies describe oral deformities as depigmentation of mouthparts [e.g. 28, 30], others refer to them as dekeratinization [e.g. 27, 31]. This ambiguity was recognized by Altig [36], who pointed out that the "depigmentation" as described in some studies was, in fact, dekeratinization of mouthparts. Therefore, we have chosen to use the term dekeratinization of mouthparts.

All individuals studied were between Gosner stages 25 and 40, since the oral disc of tadpoles within these stages normally possesses completely keratinized tissues [24, 37]. Oral discs of tadpoles were examined in detail using a stereoscopic microscope (Leica 54 MZ75), and tadpoles were classified in accordance with dekeratinization (from partial to complete absence of keratinized structures) that presented in mouthparts: **JS** (tadpoles with lack of keratinization only in upper and/or lower jaw sheath), **TR** (tadpoles with lack of keratinization, with non-disrupted supporting tissue, only in upper and/or lower tooth rows), and **JT** (tadpoles with lack of keratinization in jaw sheaths and tooth rows) (Fig 1). All tadpole specimens were deposited in the Amphibia - Tadpoles collection of the Department of Zoology and Botany of UNESP – São José do Rio Preto (DZSJRP-Amphibia-Tadpoles).

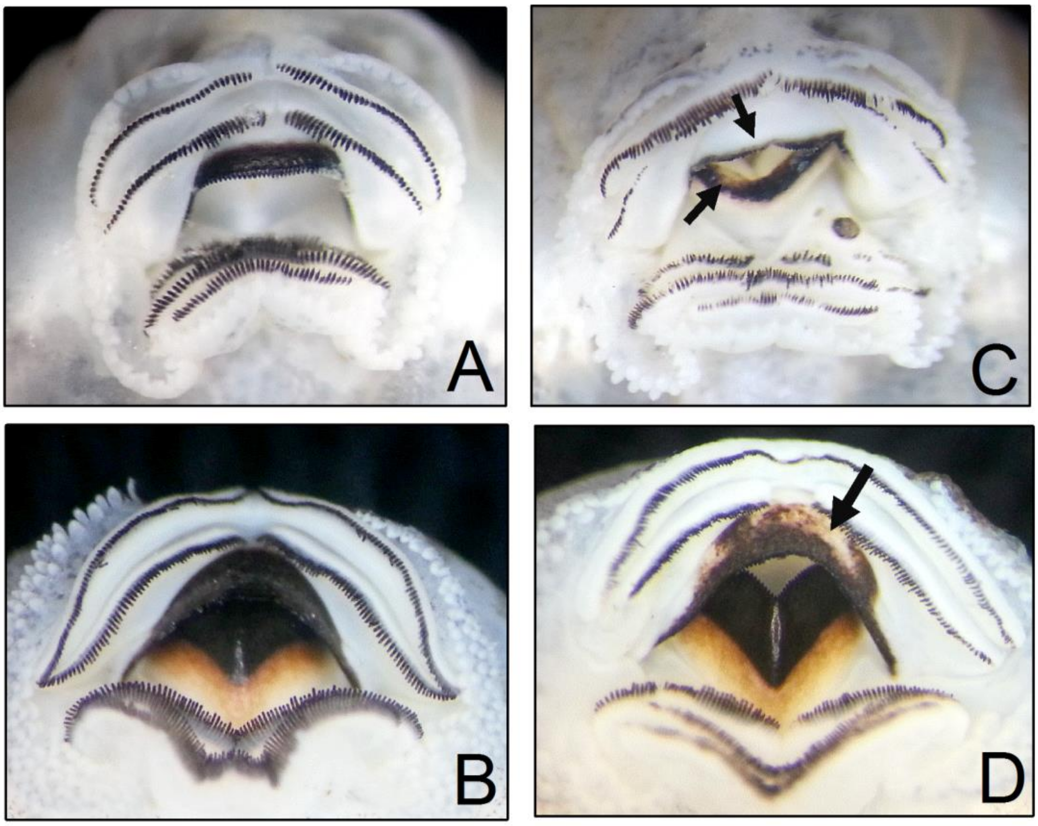

Fig 1. Example of oral deformities found among the studied tadpoles. (A) and (B) = normal tadpoles; (C) = tadpole with dekeratinized jaw sheaths (black arrows) and generalized dekeratinization in tooth rows; (D) = tadpole with dekeratinized upper jaw sheath (black arrow). Upper images *Boana albopunctata*, and lower images *Scinax hayii*.

Batrachochytrium dendrobatidis detection

After the oral discs of all tadpoles were inspected, a subsample, composed of all the individuals that possessed oral deformities (N = 195) and 184 randomly selected individuals without oral deformities, was analyzed for *Bd* detection. Oral discs of selected tadpoles were excised and air-dried on filter paper as described in Hyatt et al. [38]. DNA oral disc extraction was performed using PrepMan Ultra (Applied Bioysistems) and amplified using a CFX96^{TM} Real-Time PCR Detection System (Bio-Rad) with a *Bd*-specific Taqman Assay [39]. Each 96-well assay plate included a negative control and four different standards containing DNA from 100, 10, 1 and 0.1 *Bd* genome equivalents. For all samples, the negative control and standards were run in duplicate. Samples that showed signs of inhibition (nonsigmoidal amplification) were further diluted to 1:100 and re-analyzed. Only the presence or absence of *Bd* was determined, and samples were considered *Bd* positive when both of the two duplicate analyses revealed *Bd* zoospore genome equivalents >0.1, and the amplification curves have a sigmoidal shape. If not, the sample was re-run and considered positive only with another positive result recorded. DNA analyses were carried out at Museo Nacional de Ciencias Naturales, CSIC (Madrid, Spain).

Statistical analyses

Two-tailed Fisher's exact test was used to assess the association between individuals with oral deformities and infection with *Bd* for the total number of tadpoles analyzed for *Bd* presence of all six species sampled. We constructed contingency tables (2 x 2) with two binary variables: 1 = oral deformities present, 0 = oral deformities not present; and 1 = *Bd* infection, and 0 = no *Bd* infection.

Results

The tadpoles studied belonged to six different species: *Aplastodiscus albosignatus* (N = 721), *Boana albopunctata* (N = 793), *Boana faber* (N = 467), *Scinax hayii* (N = 80), *Crossodactylus caramaschii* (N = 34) and *Physalaemus cuvieri* (N = 61). At least one type of oral deformity was found in 9% (195) of the 2156 tadpoles inspected. Of the tadpoles that exhibited oral deformities, 51.3% had JT, while 29.2% had only TR, and 19.5% only JS.

There was no relationship between *Bd* infection and tooth rows dekeratinization (TR) in any of studied of species (Table 1). However, dekeratinization of the jaw sheath, accompanied or not with dekeratinization of teeth (JS or JT), was related to *Bd* infection in three of the species studied: *A. albosignatus* showed a nonrandom relationship with JS (Fisher's exact test; N = 197, two-tailed $p = 0.0023$) and JT (N = 197, two-tailed $p < 0.001$); *B. albopunctata* showed nonrandom relationship with JT (N = 63, two-tailed $p < 0.001$); and *S. hayii* showed nonrandom relationships with JS (N = 32, two-tailed $p = 0.0068$). By contrast, *B. faber*, *C. caramaschii* and *P. cuvieri* did not exhibit relationship between any of the oral deformities studied and *Bd* infection (Table 1).

Table 1. Oral deformities and related *Batrachochytrium dendrobatidis (Bd)* infections of tadpoles of six species of Atlantic Forest anurans in southeastern Brazil.

				JS			**TR**			**JT**			**JSD-Proxy**		
	Ns	**Na**	**N_{Bd+}**	**Nd**	**N_{Bd+}**	**P-value**	**Nd**	**N_{Bd+}**	**P-value**	**Nd**	**N_{Bd+}**	**P-value**	**Nd**	**N_{Bd+}**	**P-value**
Aplastodiscus albosignatus	721	197	124	27	24	**0.0023**	25	6	< 0.001[a]	78	73	**< 0.001**	105	97	**< 0.001**
Boana albopunctata	793	63	12	1	1	0.1904	20	0	0.0124[a]	12	8	**< 0.001**	13	9	**< 0.001**
Boana faber	467	33	8	2	2	0.0530	5	1	0.9999	0	0	0.9999	2	2	0.0530
Scinax hayii	80	32	16	7	7	**0.0068**	2	1	0.9999	4	4	0.1012	11	11	**< 0.001**
Crossodactylus caramaschii	34	34	5	1	0	0.9999	2	0	0.9999	5	2	0.1464	6	2	0.2053
Physalaemus cuvieri	61	20	1	0	0	0.9999	3	0	0.9999	1	0	0.9999	1	0	0.9999
TOTAL	2156	379	158	38	34	**< 0.001**	57	8	< 0.001[a]	100	87	**< 0.001**	138	121	**< 0.001**

Significant p-values of two tailed Fisher's exact tests (in bold) indicate a non-random relationship between presence of oral deformities and *Bd* infection. **Ns** = tadpoles studied; **Na** = tadpoles analyzed for *Bd* detection; **N_{Bd+}** = tadpoles infected by *Bd*; **Nd** = tadpoles with oral deformities corresponding to **JS** = lack of keratinization only in jaw sheaths, **TR** = lack of keratinization, with non-disrupted supporting tissue, only in tooth rows, **JT** = lack of keratinization in jaw sheaths and tooth rows, and **JSD-Proxy** = jaw sheath dekeratinization with or without dekeratinization in tooth rows.

[a] Significant relationship but in the opposite direction (absence of TR deformity related to presence of *Bd*).

In the light of these results, we decided to sum JS and JT into a single proxy, JSD-proxy (JSD = jaw sheaths dekeratinization with or without dekeratinization in tooth rows), in order to simplify the determination of oral dekeratinization of tadpoles in future studies. Same statistical analyses performed for TR, JS and JT were then done for this JSD-proxy. The results showed that JSD-proxy considerably improved the accuracy of identifying *Bd* infection. Our original proxies (JS, TR and JT) included 33.8% false positives and 20.1% false negatives for *Bd* infection; the JSD-proxy, on the other hand, included only 12.3% false positives and 18.7% false negatives (see details in Table 2). False positives corresponded to tadpoles that possessed oral deformities but were negative for *Bd* infection via qPCR analysis. False negatives, on the other hand, corresponded to tadpoles that possessed normal oral mouthparts but were positive for *Bd* infection via qPCR.

Table 2. Cases of false positives and false negatives for the detection of *Batrachochytrium dendrobatidis (Bd)* infection produced by the use of the three oral deformities studied as proxies (JS, TR and JT) and for the JSD-Proxy.

	JS, TR and JT						**JSD-Proxy**					
	Nd	**False positives**		**Nn**	**False negatives**		**Nd**	**False positives**		**Nn**	**False negatives**	
		n	**%**		**n**	**%**		**n**	**%**		**n**	**%**
Aplastodiscus albosignatus	130	27	20.8	67	21	31.3	105	8	7.6	92	27	29.3
Boana albopunctata	33	24	72.7	30	3	10.0	13	4	30.8	50	3	6.0
Boana faber	7	4	57.1	26	5	19.2	2	0	0.0	31	6	19.3
Scinax hayii	13	1	7.7	19	4	21.1	11	0	0.0	21	5	23.8
Crossodactylus caramaschii	8	6	75.0	26	3	11.5	6	4	66.7	28	3	10.7
Physalaemus cuvieri	4	4	100	16	1	6.25	1	1	100	19	1	5.26
TOTAL	195	66	**33.8**	184	37	**20.1**	138	17	**12.3**	241	45	**18.7**

Nd = tadpoles with oral deformities corresponding to **JS** = lack of keratinization only in jaw sheaths, **TR** = lack of keratinization, with non-disrupted supporting tissue, only in tooth rows, **JT** = lack of keratinization in jaw sheaths and tooth rows, and **JSD-Proxy** = jaw sheath dekeratinization with or without dekeratinization in tooth rows; **Nn** = normal tadpoles.

Although the use of JSD-Proxy was more accurate at identifying *Bd* infection in tadpoles than our original proxies, the analyses of the relationships between *Bd* infection and JSD-proxy found a nonrandom relationship only for three species of the family Hylidae: *A. albosignatus* (Fisher's exact test; N = 197, two-tailed p < 0.001), *B. albopunctata* (N=63, two-tailed p<0.001), and *S. hayii* (N = 32, two-tailed p < 0.001) (see details in Table 1).

Discussion

This is the first study to report the presence of *Bd* in Núcleo Curucutu, one of the largest and most preserved remnants of Atlantic Forest in São Paulo State, which is also a refuge to one of the most biodiverse amphibian faunas of a single locality [40]. Therefore, this virtually pristine area is added to the many others around the world that possess *Bd* [e.g. 18, 41].

Not all of the tadpoles that possessed oral deformities were infected by *Bd*, and so some of the factors that caused the deformities in our studied tadpoles remain unknown and deserve further investigation. Since our study area is a very well preserved remnant of Atlantic Forest, we considered that one might rule out chemical contamination as the cause of deformities. More importantly, our results showed that TR was the worst proxy for *Bd* infection; in fact, of the 57 tadpoles exhibited only TR deformities, only 8 (14%) were infected with *Bd*. On the other hand, of the 138 tadpoles possessing dekeratinization of the jaw sheaths, accompanied or not with dekeratinization of the teeth, 121 (87.7%) were infected. These results are similar to those obtained by Knapp and Morgan [26] and Vieira et al. [28], who also found a strong correlation between jaw sheaths dekeratinization and *Bd* infection. This finding is also consistent with Marantelli et al. [24], who found that the dekeratinization of jaw sheaths indicates a heavier infection than dekeratinization of tooth rows.

Despite the fact that the analyses of all studied tadpoles together found a strong relationship between tadpoles possessing dekeratinization of jaw sheaths and *Bd* infection, the results by species were less clear. Only three species of the family Hylidae exhibited a significant relationship between JSD-Proxy and *Bd* infection, which was not observed for *B. faber* (Hylidae), *C. caramaschii* (Hylodidae) and *P. cuvieri* (Leptodactylidae). These results agree with Blaustein et al. [31], who also detected varying relationships between *Bd* infection and oral deformities among species. Therefore, and in discordance with Padget-Flohr and Goble [30], who concluded that *Bd* infection and anuran larval mouthpart deformities are two separate processes, we found that a relationship between dekeratinization in jaw sheaths and *Bd* infection does indeed exist in three species of family Hylidae.

Although the relationship between jaw sheaths and *Bd* infection was found for some of our studied species, the use of JSD-proxy remains unreliable for purposes of assessing *Bd* prevalence in tadpole populations. Based on this proxy, *Bd* detection in species that exhibited a positive relationship with jaw sheaths dekeratinization resulted in up to 30.8% false positives in *B. albopunctata* and up to 29.3% false negatives in *A. albosignatus*. Furthermore, the use of the JSD-proxy in species for which no relationship was found, reached 100% of false positives. Further illustrating the unreliability of JSD-proxy, in an open area pond where we sampled 23 tadpoles of *B. albopunctata* (two of them with dekeratinized jaw sheaths), the use of JSD-proxy would have classified the pond as a *Bd* positive site although qPCR analyses did not confirm this assumption.

Thus, we conclude that the use of oral dekeratinization as a generalized proxy for *Bd* detection in tadpoles should not be used as a single diagnosis technique. We then recommend the use of more accurate techniques such as histology, histochemistry, or PCR analyses in order to obtain accurate diagnosis. However, it is worth noting the usefulness of using JSD-proxy as a screening tool in those species for which the relationship between *Bd*-infection and oral dekeratinization has been proven, since its use minimize the number of individuals evaluated by more costly or time-intensive methods.

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Chapter IV

Batrachochytrium dendrobatidis in Brazil: www.quitribrasil.com

Batrachochytrium dendrobatidis in Brazil: www.quitribrasil.com

Although *Bd* is one of the most studied pathogen of wildlife because its importance as a global diversity loss factor, it is still a stranger for the most of the society, including biologists.

While it is true that most researchers agree with the relevance of scientific popularization, the level of their participation remains relatively low when biologists are compared with other scientists (Jensen et al., 2008; Jensen, 2011). The popularization of science is even more important at a time of economic crisis, such as that Brazil is currently experiencing, since only the mobilization of civil society can avoid drastic cuts in funding for basic research (Varner, 2014).

With the goal of promote the knowledge of *Bd* among Brazilian society, including researchers, we have developed and launched the **Quitri Brasil Project - *www.quitribrasil.com*** (Fig 1).

Projeto Quitri Brasil
Batrachochytrium dendrobatidis

Descrito no final dos anos noventa, o fungo quitrídio *Batrachochytrium dendrobatidis* é considerado hoje uma das maiores ameaças para a biodiversidade de anfíbios. A doença causada pela infecção de *B. dendrobatidis* em anfíbios é conhecida mundialmente como quitridiomicose. Como a maioria das doenças infecciosas emergentes, a quitridiomicose é uma doença com grande número de hospedeiros, o que a torna uma ameaça significativa para a biodiversidade e funcionamento dos ecossistemas.

Em 2004 foi detectada pela primeira vez no Brasil uma espécie de anfíbio infectado com o fungo *B. dendrobatidis*. Análises histológicas mostraram infecção em girinos de *Hylodes magalhaesi* no sudeste do Brasil. Uma década depois, o número de espécies registradas com *B. dendrobatidis* no Brasil tem aumentado para mais de 150, abrangendo cinco dos seis biomas brasileiros, estando na Mata Atlântica o maior número de espécies infectadas.

O **Projeto Quitri Brasil** tem como objetivo fornecer um recurso on-line acessível para gestores, voluntários ambientais, pesquisadores e cidadãos em geral, com a intenção de facilitar a divulgação do conhecimento sobre o fungo *Batrachochytrium dendrobatidis* no Brasil.

Figure 1. Homepage of www.quitribrasil.com

Currently available only in Portuguese, this quarterly updated webpage offers essential information obtained from national and international scientific publications about *Bd* in Brazil. Visitors can access different tabs offering different informations such as: **(1)** ***'Bd'***, containing a detailed description of the biology of *Bd* in an easy language; **(2)** ***'Espécies de anfíbios infectadas'***, listing amphibian *Bd*-infected species reported in each of the Brazilian biomes (Fig 2); **(3)** ***'Mapas'***, offering maps created in ArcGis® 10.0 (ArcMap^{TM}), incluiding layers available by the *Instituto Brasileiro de Geografia e Estatística*, with location of sites where *Bd* has been registered, overlapped with different layers of Brazil, such as states, biomes, and different climatic descriptors (Fig 3); **(4)** ***'News'***, collecting most recent and salient papers focusing on *Bd*, which is more intended for researcher users; and finally **(5)** ***'Contact'***, allowing users to interact with the webpage developer, to send questions or suggestions.

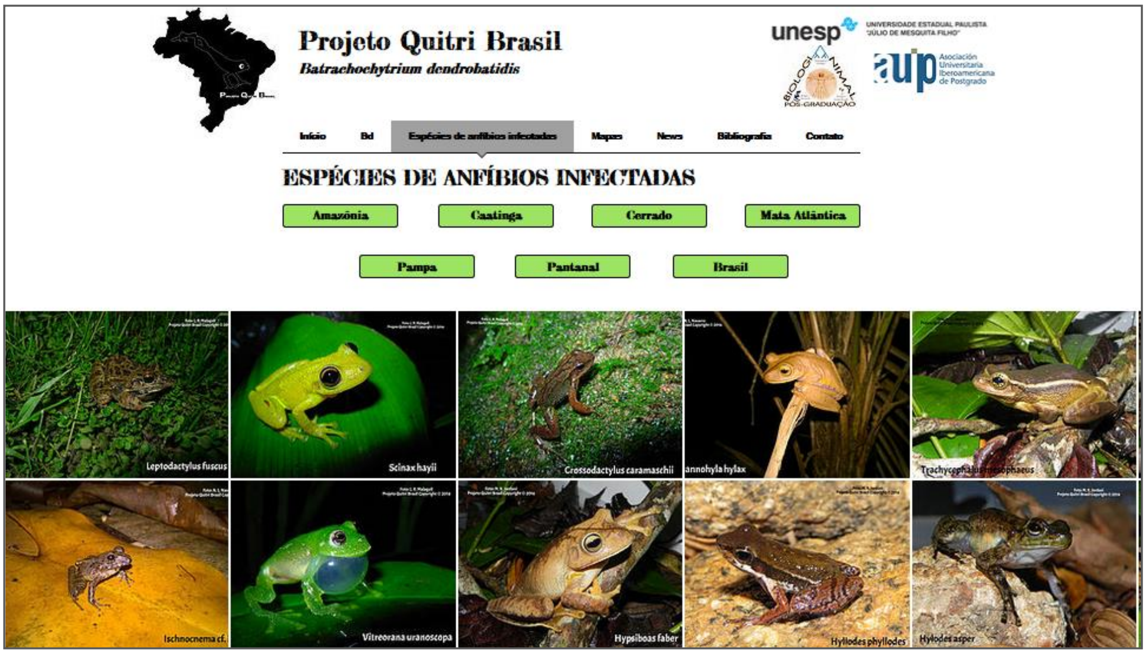
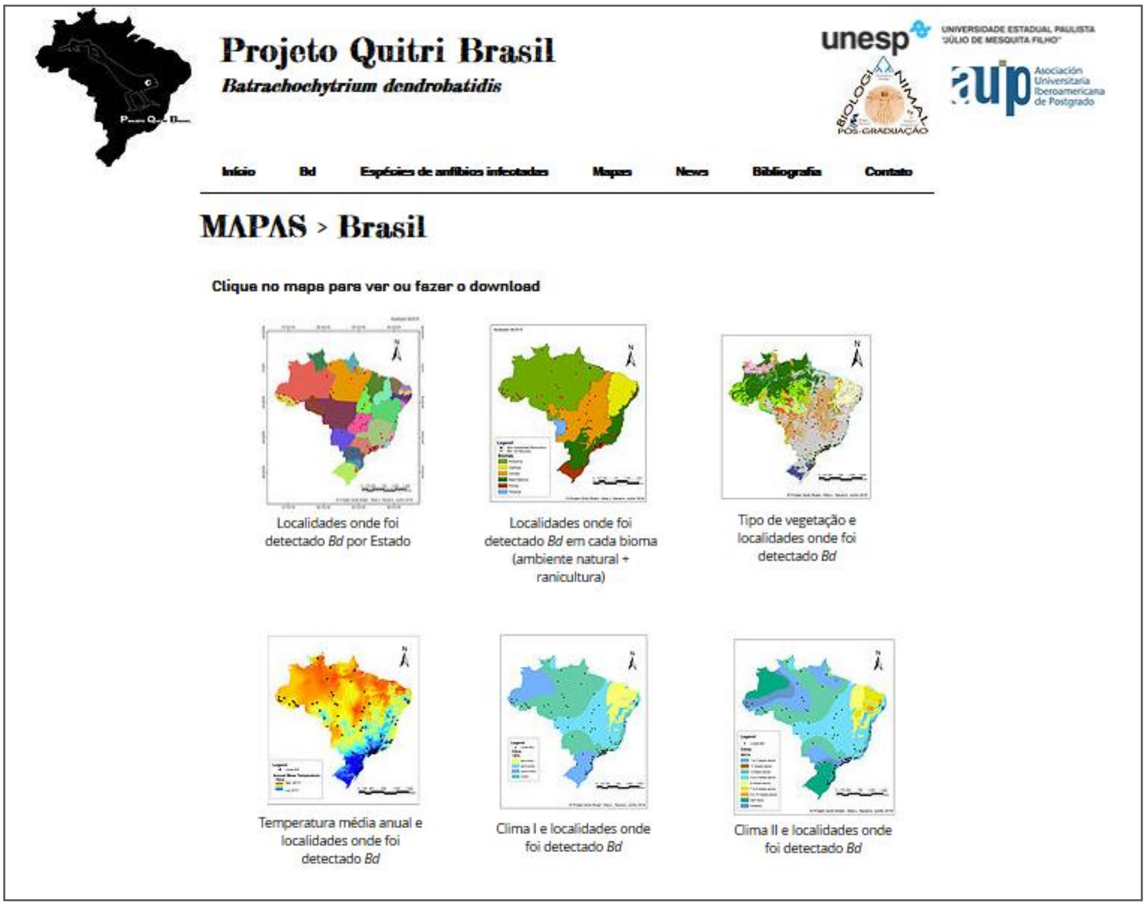


Figure 2. Tab "*Espécie de Anfíbios Infectadas*", listing all amphibian Bd-infected reported in each one of the Brazilian biomes to date. A link is also available for each species to access information offered by International Union for Conservation of Nature (IUCN) and its Red List of Threatened Species.



Projeto Quitri Brasil

Batrachochytrium dendrobatidis

MAPAS > Brasil

Clique no mapa para ver ou fazer o download

Localidades onde foi detectado *Bd* por Estado

Localidades onde foi detectado *Bd* em cada bioma (ambiente natural + ranicultura)

Tipo de vegetação e localidades onde foi detectado *Bd*

Temperatura média anual e localidades onde foi detectado *Bd*

Clima I e localidades onde foi detectado *Bd*

Clima II e localidades onde foi detectado *Bd*

Figure 3. Maps section from www.quitribrasil.com

Webpage impact

In their first 15 months online, from November 2016 to January 2018, ***quitribrasil.com*** has had a total of 983 visits from 13 countries: Australia, Brazil, Chile, Colombia, England, France, Germany, India, Italy, Perú, Russia, Spain and United States. Brazil has been the country with the highest number of visits, surpassing 700 (mostly from the states of São Paulo, Rio de Janeiro and Minas Gerais, but also from states such as Pará, Mato Grosso do Sul, Santa Catarina, Rio Grande do Sul, Goiás, Bahia, Ceará, Maranhão, Paraíba and Rondonia). These large number of visits received from various regions of Brazil and the various interactions with users of the webpage ocurred highlight the importance of this scientific outreach project, and show that the public investment of resources in this type of project is very valuable.

To better promote the homepage, we announced its launch in the media:

Jornal UNESP - Brazil

https://issuu.com/acireitoria/docs/ju332

4

Maio 2017 • Ciências Biológicas

Anfíbios sob ameaça

Site apresenta informações sobre fungo que ataca populações desses animais em todo o mundo

André Louzas

Mapa mostra locais onde doença já foi registrada no país

Alba: comércio de espécies promove disseminação do fungo

Proposta contra a extinção

Docente de Rio Claro é um dos autores de artigo na revista Science sobre preservação de espécies

SOS Anfibios Guadarrama - Spain

SOS Anfibios Guadarrama

El hongo asesino en el bosque atlántico brasileño

06 FEBRERO 2017 | ESCRITO POR ALBA NAVARRO LOZANO

El Bosque Atlántico (Mata Atlântica, en portugués) es el bioma de Brasil con el mayor número de especies infectadas con el hongo *Batrachochytrium dendrobatidis*, 140 especies por el momento. El Bosque Atlántico es uno de los biomas más ricos y amenazados del planeta, y aunque tan sólo sobrevive el 12% de su superficie original, contiene más biodiversidad que el Amazonas. En lo que a anfibios se refiere, de las 1080 especies registradas en Brasil, más de la mitad están presentes en el Bosque Atlántico, ¡y cerca del 90% de ellas son endémicas! Su elevada humedad, relieve accidentado y gran heterogeneidad ambiental, son factores que contribuyeron a procesos de especiación y la consecuente aparición de endemismos.

Dada su importancia y riqueza en especies de anfibios, el Bosque Atlántico se ha convertido en un escenario relevante para el estudio del hongo *B. dendrobatidis*. Por eso, en 2014 arrancábamos el proyecto "Novas abordagens de ecologia e conservação: diversidade filogenética e funcional de anfíbios e serpentes da Mata Atlântica brasileira" financiado por la Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). Este proyecto tiene entre sus objetivos principales la evaluación del estado de conservación de los anfibios anuros y serpientes del Bosque Atlántico, con especial interés en el estudio del impacto que tiene sobre las poblaciones de anfibios la pérdida de hábitat y el hongo parásito. El proyecto se está llevando a cabo principalmente en el sur del bioma, pues esta región cuenta con las áreas más extensas y mejor conservadas del mismo.

Aunque aún no tenemos resultados definitivos sobre el impacto de *B. dendrobatidis*, el número de individuos infectados aumenta con cada análisis realizado. Sin embargo, aún es pronto para conocer la situación real, y hasta la fecha aún no se ha registrado ninguna disminución dramática en las poblaciones de anfibios estudiadas.

Para divulgar el conocimiento del hongo patógeno a la sociedad brasileña, en junio del 2016 pusimos en marcha la web www.quitribrasil.com. A través de ella, los internautas pueden conocer las especies de anfibios que han sido diagnosticadas como infectadas por el hongo, las regiones del país donde ha sido detectado, así como información general sobre el hongo *B. dendrobatidis*.

Etiquetado como

Jornal Juca Post - Brazil

Anfíbios ameaçados

Aluna lança site para divulgar fungo que causa a quitridiomicose

A doutoranda Alba Navarro, aluna do Programa de Pós-Graduação em Biologia Animal do Instituto de Biociências, Letras e Ciências Exatas (Ibilce) da Unesp, Câmpus São josé do Rio Preto, com o apoio da Fundação de Amparo a Pesquisa do Estado de São Paulo - FAPESP, lançou um site de divulgação sobre o fungo Batrachochytrium dendrobatidis.

A doença produzida por esse fungo, a quitridiomicose, tem sido associada a declínios populacionais de anfíbios em várias regiões do planeta. A doença, por ter um grande número de hospedeiros, torna-se uma ameaça significativa para a biodiversidade e funcionamento dos ecossistemas, no entanto, poucas pessoas têm acesso a essa informação. "Embora hoje em dia seja um dos patógenos mais estudados da fauna, tal conhecimento parece limitado apenas aos pesquisadores envolvidos no assunto", afirmou a pesquisadora.

Dessa forma, surgiu a ideia de criar uma ferramenta on-line que facilitasse a difusão do conhecimento sobre esse fungo no Brasil. A ferramenta é destinada a pesquisadores de outras áreas, gestores, voluntários ambientais e cidadãos em geral. Conheça o projeto em: http://www.quitribrasil.com/

Descrito no final dos anos noventa, o fungo quitrídio Batrachochytrium dendrobatidis

TV Record - Brazil

http://www.recordtvriopreto.com.br/noticia/32307/estudo-busca-detectar-fungos-que-ameacam-anfibios.html

Diário da Região - Brazil

https://www.diariodaregiao.com.br/cidades/o-fungo-que-amea%C3%A7a-acabar-com-os-sapos-1.702156

14/12/2017 - 21h21min

BIOLOGIA

O fungo que ameaça acabar com os sapos

BIOLOGIA

Milena Grigoleti

Pesquisadora Alba Navarro, da Unesp, com girinos

Um fungo está ameaçando a sobrevivência de sapos, rãs, pererecas e todos os outros anfíbios brasileiros. O Batrachochytrium dendrobatidis (Bd) se alimenta das células queratinizadas do animal e causa uma infecção, conhecida mundialmente como quitridiomicose. As células são as epiteliais, da pele, que guardam a queratina, uma proteína. Detectado pela primeira vez no Brasil em 2004, o fungo ainda é um desconhecido até mesmo para biólogos e herpetologistas (estudiosos que se dedicam a anfíbios e répteis). Isso motivou a pesquisadora espanhola Alba Navarro, que é doutoranda do Programa e Pós-Graduação em Biologia Animal do Ibilce, campus da Unesp em Rio Preto.

Ela criou o projeto Quitri Brasil (www.quitribrasil.com), que fornece recurso para gestores, voluntários ambientais, pesquisadores e cidadãos, com a intenção de facilitar a divulgação de conhecimento sobre o fungo. "Um dia pensei: se para pesquisadores da própria área o fungo não é conhecido, imagina para o resto da sociedade. Além disso, sinto que a divulgação científica é essencial para que os projetos sobre conservação atinjam seus objetivos", conta. O site é atualizado com base em informações científicas sobre o fungo. Dentre outros dados, ele mostra os locais em que o Bd foi encontrado e a temperatura média de cada lugar.

Segundo Alba, a quitridiomicose tem se tornado uma das ameaças de maior impacto nas populações de anfíbios. "Ela está envolvida no declínio de muitas populações e na extinção de espécies em várias regiões do planeta", afirma. O fungo não tem preferência por um grupo de anfíbios específico. Já foram encontradas mais de 700 diferentes espécies de salamandras, sapos, pererecas, rãs e cecílias infectadas pelo fungo em várias regiões do planeta. Somente no Brasil, são pelo menos 160 espécies de

Acknowledgments

We are in debt to Leo R. Malagoli and Mainara X. Jordani for the photographies donated for the homepage, and to Denise C. Rossa-Feres and David Sánchez Domene for ideas that substantially improved the webpage. In turn, we would like to express our thanks for the support of Programa de Pos-Graduação em Biologia Animal offered from the very beggining of the project, especially to Eliane Gonçalves de Freitas, the former coordinator of the program.

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Only literature cited in this chapter; references used for the creation of the webpage are cited on the web, in the tab “Bibliografia”.

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Conclusions

The main conclusions obtained after the work carried out during this research are listed below.

FINAL CONCLUSIONS

1) Nucleo Curucutu becomes part of the list Brazilian Atlantic Forest areas in which anuran amphibian infected by *Batrachochytrium dendrobatidis* (*Bd*) has been detected.

2) Neither signs of chytridiomycosis nor dead or dying amphibian individuals were detected in Nucleo Curucutu.

3) The prevalence and intensity of *Bd* did not differ between ponds and streams in Nucleo Curucutu.

4) In ponds, zooplankton density explained variability of *Bd* prevalence and intensity.

5) In streams, water velocity and depth were the key factors that explained *Bd* prevalence, however, *Bd* intensity remains unexplained.

6) The potential geographic distribution of *Bd* in the Brazilian Atlantic Forest reaches 665,769.2 km^2, corresponding to 58.8% of the total area of this domain.

7) Greater climatic habitat suitability for *Bd* is located in southeastern Brazil, in the states of São Paulo, Minas Gerais and Rio de Janeiro.

8) All global warming scenarios for year 2070 predict a decrease of the potential distribution of *Bd* in the Atlantic Forest.

9) All global warming scenarios predict occurrence of *Bd* in higher altitudes and latitudes of the Brazilian Atlantic Forest.

10) The dekeratinization of tooth rows themselves was not necessarily associated with presence of *Bd* in any of the anuran species studied (*Aplastodiscus albosignatus, Boana albopunctata, B. faber, Scinax hayii, Crossodactylus caramaschii*, and *Physalaemus cuvieri*).

11) Dekeratinization of jaw sheaths showed a nonrandom relationship with *Bd* infection in A*. albosignatus, B. albopunctata* and *S. hayii,* but its use as a proxy for *Bd* detection resulted in up to 30.8% false positives and up to 29.3% false negatives.

12) Dekeratinization of jaw sheaths is even worse as a proxy for *Bd* detection in species that do not show relationship between dekeratinization and *Bd* infection, generating up to 100% false positives.

13) Oral deformities in tadpoles are not an accurate indicator for chytridiomycosis in frogs.

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São José do Rio Preto, 23 / 4 / 2018

Alba Navarro

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