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High Prevalence and Low Intensity of Infection by *Batrachochytrium dendrobatidis* in Rainforest Bullfrog Populations in Southern Brazil

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Abstract.—**American Bullfrogs (*Lithobates catesbeianus*) are considered important reservoirs and vectors of the fungus *Batrachochytrium dendrobatidis* (*Bd*), which can cause the disease chytridiomycosis in many amphibian species. In this study, we assessed the potential of bullfrog farms as centers for *Bd* dispersion. Specifically, we compared the incidence of *Bd* in captive and wild populations of bullfrogs by sampling six frog farms and eight natural ponds located at different distances from frog farms in southern Brazil. All sampled frog farms and natural ponds had infected individuals, but frog farms had a higher prevalence of *Bd* than natural ponds. While prevalence was high, infection intensity was low. In contrast, infection intensity was similar across natural ponds and frog farms. The distance of natural ponds to frog farms had no influence on *Bd* infection prevalence or load among bullfrogs; however, considering the higher prevalence of *Bd* in frog farms, the data suggest that frog farms might act as a constant source of this pathogen to the natural environment via escape and releases of frogs and through the release of contaminated water. Therefore, we emphasize the need for additional studies to assess the effect of the different types of frog farm management on the incidence of *Bd* in natural environments and encourage efforts to monitor free-living populations in surrounding areas. We also highlight the need for urgent measures to tighten the control and regulation of frog farms throughout Brazil to reduce their impact on native amphibians.**

Key Words.—biological conservation; chytridiomycosis; invasive species; *Lithobates catesbeianus*; wildlife diseases

***Resumo.*—Rãs-touro (*Lithobates catesbeianus*) são consideradas importantes reservatórios e vetores do fungo *Batrachochytrium dendrobatidis* (*Bd*), que pode causar quitridiomicose em anfíbios. Neste estudo, avaliamos ranários como possíveis centros de dispersão de *Bd*. Comparamos a carga de infecção de populações selvagens e cativas de rãs-touro em seis ranários e oito lagoas naturais no sul do Brasil. Em geral, todos os ranários e lagoas naturais tiveram indivíduos infectados por *Bd* e, enquanto a prevalência foi alta, a carga de infecção foi baixa. Os ranários apresentaram maior prevalência de *Bd* do que lagoas naturais. No entanto, os indivíduos das lagoas naturais e ranários apresentaram cargas similares de zoósporos. A distância entre ranários e lagoas naturais não interferiu na prevalência e carga de infecção de *Bd* nas lagoas naturais. Devido a elevada incidência de *Bd* em ranários, nós sugerimos que estes estabelecimentos podem agir como fontes constantes de *Bd* para o ambiente natural, devido a possíveis escapes, solturas e/ou eliminação de efluentes contaminados. Além disso, nós sugerimos estudos que considerem como as distintas formas de manejo adotadas nos ranários influenciam na incidência de**

***Bd* e presença de rãs-touro em ambientes naturais, encorajando esforços de monitoramento das populações de vida livre em áreas próximas aos criadouros. Nós também, sugerimos a adoção de medidas de controle e regulamentação de ranários no Brasil, a fim de evitar impactos negativos à fauna de anfíbios.**

Palavras Chave.—conservação biológica; quitridiomicose; espécies invasoras; *Lithobates catesbeianus*; doenças de animais silvestres

Introduction

The American Bullfrog, *Lithobates catesbeianus*, is on the list of the 100 most environmentally impactful invasive species in the world and, among amphibians, is the most impactful (Lowe et al. 2000; http://193.206.192.138/gisd/search.php). In Brazil, American Bullfrog (hereafter, bullfrog) populations occur mainly in the southern and southeastern regions (Loyola et al. 2012), particularly in areas of the Pampa and Atlantic Forest (Giovanelli et al. 2008; Both et al. 2011). The Atlantic Forest, considered a biodiversity hotspot (Myers et al. 2000; Mittermeier et al. 2005), has a high richness of amphibian species and endemism (Haddad et al. 2013); however, studies indicate that climatic changes in the next few years may provide favorable conditions in the Atlantic Forest that will allow the colonization of new invasive species (Forti et al. 2017; Toledo and Measey 2018), such as the bullfrog (Ficetola et al. 2007; Giovanelli et al. 2008), via the expansion of their distribution range. Hence, bullfrogs represent an important threat to Brazilian amphibian species, along with habitat fragmentation (Myers et al. 2000; Becker et al. 2007; Ribeiro et al. 2009), climate change (Bellard et al. 2012, 2014; Oliveira et al. 2016a, b), and emergent infectious diseases (Carvalho et al. 2017).

The negative effects to natural environments caused by an invasion of bullfrogs can be direct or indirect via predation and/or competition with native amphibian species (Kiesecker et al. 1997; Hanselmann et al. 2004; Kraus 2015). These effects may result in changes to the amphibian community structure and can be aggravated by the high density and dispersion capacity of bullfrogs (Quiroga et al. 2015). Early sexual maturation, high fecundity, and, in southern Brazil, a year-long breeding period (Kaefer et al. 2007; Medeiros et al. 2016), facilitate the capacity of bullfrogs to reach high population density and disperse widely. Moreover, bullfrogs can be important propagators of infectious agents, such as ranavirus and the fungus *Batrachochytrium dendrobatidis* (*Bd*), which causes the disease chytridiomycosis and is associated with amphibian population declines globally (Skerratt et al. 2007; Olson et al. 2013; O'Hanlon et al. 2018). Thus, because they are highly invasive and carriers of pathogens, bullfrogs represent a double threat to native amphibian populations (Kats and Ferrer 2003; Hanselmann et al. 2004). Bullfrogs have been associated with *Bd* invasions to native amphibians in north America and Europe (Miaud et al. 2016; O'Hanlon et al. 2018; Yap et al. 2018).

Bullfrogs can serve as both a reservoir and important vectors of *Bd* (Garner et al. 2006; Schloegel et al. 2009; Adams et al. 2017). Within this context, studies conducted in bullfrog farms determined a high *Bd* prevalence and an absence of morbidity, reinforcing the hypothesis that they are efficient carriers (Schloegel et al. 2010; Greenspan et al. 2012), and capable of maintaining low levels of *Bd* infection (Daszak et al. 2004). Moreover, the high density of individuals in bullfrog farms and their generalist behavior might enhance *Bd* proliferation via the spread of individuals into adjacent areas, potentially resulting in a distribution range expansion (Rödder et al. 2013).

The potential expansion of the bullfrog distribution range in southern Brazil (Giovanelli et al. 2008; Both et al. 2011; Loyola et al. 2012), makes the conservation scenario in this region worrisome due to a potential increase in the probability of infection of native species by *Bd*. Considering that frog escapes and releases are relatively common in frog farms, invasive populations might establish (Both et al. 2011; Adams et al. 2017; Marhanka et al. 2017), and bullfrogs from farms may be important disseminators of *Bd* to natural environments (Ribeiro et al. 2019). There is a lack of studies assessing bullfrogs as effective dispersers of *Bd*, however, particularly from frog farms that have no control of escapes, which makes them a potential constant source of individuals to natural environments (Ribeiro et al. 2019).

To assess the relative risk of frog farms to act as potential dispersal points of *Bd*, we compared the prevalence of *Bd* in bullfrog populations from frog farms and natural ponds in southern Brazil. We also examined whether distance to bullfrog farms influences the prevalence of *Bd* on bullfrogs occurring in natural ponds. We hypothesized that distance from bullfrog farms affects the prevalence of *Bd* in nearby natural ponds. We predict that the prevalence of *Bd* in nearby natural ponds would be comparable to that of bullfrog farms, in contrast to those natural ponds located farther away from farms.

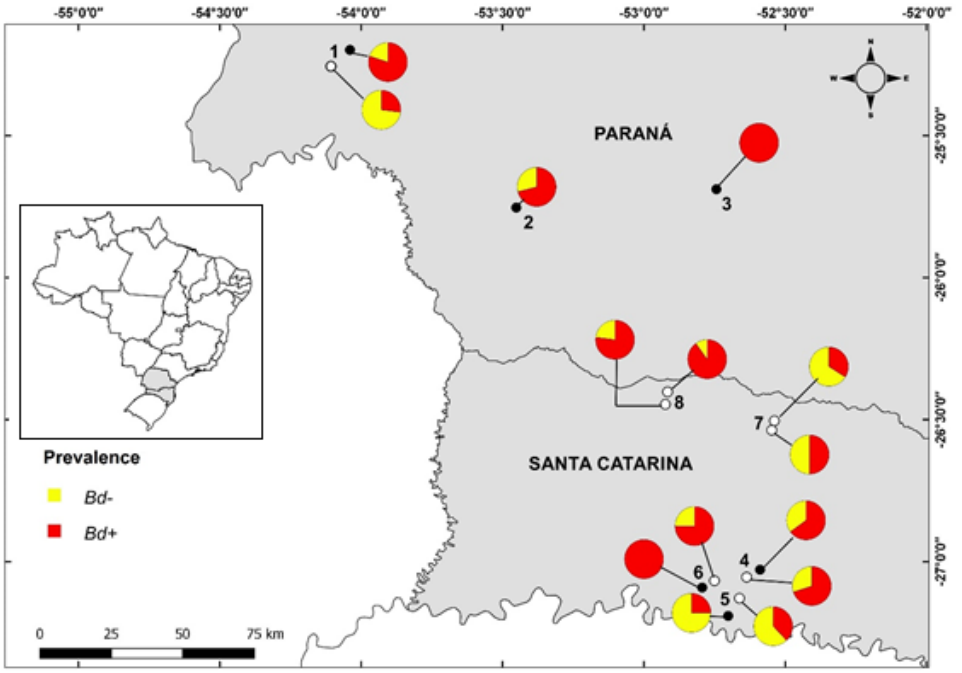

Figure 1. Sampling locations in southern Brazil of natural ponds (NP; white) and frog farms (FF; black), and *Bd* prevalence at the site-level. Municipalities: 1 (FF1 and NP1) = Medianeira; 2 (FF2) = Santa Izabel do Oeste; 3 (FF3) = Sulina; 4 (FF4 and NP2) and 5 (FF5 and NP3) = Chapecó; 6 (FF6 and NP4) = Guatambu; 7 (NP5 and NP6) = São Domingos; and 8 (NP7 and NP8) = São Lourenço do Oeste.

Materials and Methods

Study area.—We conducted surveys in the western and southwestern regions of Paraná (municipalities of Medianeira, Santa Izabel do Oeste, and Sulina) and western Santa Catarina (municipalities of Chapecó, Guatambu, São Domingos, and São Lourenço do Oeste), both located in southern Brazil (Fig. 1). The study area is in the Atlantic Forest biome, in mixed ombrophilous forest and deciduous seasonal forests (Veloso et al. 1991; Vibrans et al. 2008). The study area includes two types of climate: Cfa (humid subtropical mesothermic, with hot summer) and Cfb (humid subtropical mesothermic, with temperate summer), according to Köppen's classification (Alvares et al. 2013). In the state of Paraná, Cfa climate occurs along the coast and in the western region, in the valley of the Iguaçu River below elevations of about 750 or 800 m (Alvares et al. 2013). In Santa Catarina, the Cfa climate covers the western region at elevations below 700 m, and Cfb covers the northwest region, following the elevational gradient of relief, in the border between the states of Paraná and Santa Catarina, at elevations between 800 and 1000 m (Alvares et al. 2013).

We conducted surveys at 14 sampling sites (Appendix Table; Fig. 1). The sites included natural ponds (NP1, NP2, NP3 and NP4) close to frog farms (FF1, FF4, FF5 and FF6, distance range = 0.1 to 10 km) and far from frog farms (NP5, NP6, NP7, NP8, FF2 and FF3, distance range = 40 to 80 km). All frog farms had an intensive breeding system and consisted of masonry facilities with concrete floors. Each stall had a linear trough, a shelter and a pool with water depth 10–15 cm. A screen or canvas that surrounded the walls acted as a barrier to prevent escapes (Appendix Figure). Importantly, during surveys, we witnessed escapes of juveniles through the water outlet channel, and we observed that the water supplying the frog farms came from nearby springs, and effluents were released untreated back into the environment.

One of the six frog farms (FF5; Appendix Table) had tadpoles or juveniles usually being imported from other frog farms, defined as closed management. The other farms (FF1, FF2, FF3, FF4 and FF6; Appendix Table) used open management, where the amplexus of adults and development of tadpoles occurs in adjacent ponds within the same property, and, right before metamorphosis, the tadpoles are taken to the frog farms to improve the recruitment and development of the frogs. The frog farms used in this study have the capacity to house between 500 and 5,000 frogs, except for FF5 (Appendix Table), which can house approximately 50,000 individuals. The number of individuals in a sampling stall ranged from 200 to 440. The straight-line distance between frog farms varied from nine to 160 km.

Regarding the natural ponds, we considered a group of adjacent ponds as a single sampling unit. All sampled

ponds were in open areas or on forest edges, usually with predominant vegetation composed of grasses on the banks (Appendix Figure). We considered all ponds to be natural environments, even those located in grazing or non-intensive fish farming areas, because they are in rural areas with no frog farms in the vicinity.

Data collection.—We collected skin swabs from bullfrogs to test for *Bd* infection from September 2016 to May 2017. We captured each individual by hand using a new pair of sterile disposable gloves. We performed five strokes with a sterile swab in the inguinal regions, and ventral surface of hands and feet, for a total of 30 strokes per individual (Boyle et al. 2004; Lambertini et al. 2013). We placed swabs in individual 1.5 ml Eppendorf tubes, kept vials in an ice-containing thermal box during fieldwork, and subsequently stored them in a freezer at -4º C in the laboratory.

We collected 121 samples in frog farms (20–21 individuals/farm). We randomly selected enclosures to sample at each frog farm. After swabbing an individual, we released it in a different enclosure to avoid recaptures. At each frog farm, we recorded the area of the enclosure, depth of water, number of individuals in the enclosure, and management type (i.e., closed management or open management). In addition, we measured the area of the enclosure where frogs were sampled by calculating the product of the measurements of length × width. We calculated absolute density (AD) as the number of individuals per unit area. We calculated natural pond area using the polygon area with Google Earth (version 7.1.7.2606).

In natural ponds, we collected skin swabs from 11–22 individuals per pond, totaling 155 samples. We located the individuals by active search, using visual and acoustic cues, from 2000 to 0000. We recorded abundance of individuals and depth of each pond. We estimated abundance of individuals at each pond by the breeding site survey method (*sensu* Scott Junior and Woodward 1994), which considers all individuals vocalizing along the perimeter of the ponds. We considered abundance as the maximum number of individuals counted (Gottsberger and Gruber 2004) by location at each sampling night. We euthanized individuals using Lidocaine 2% according to Brazilian regulations (National Council for Animal Experimentation Control 2018) and transported them to the laboratory. We deposited specimens in the Amphibian Collection of Universidade Comunitária da Região de Chapecó (UNOCHAPECÓ).

We extracted DNA from skin swabs using the protocol developed by Boyle et al. (2004), including the changes made by Lambertini et al. (2013). Specifically, for each Eppendorf tube containing the swab, we added 50 µL of PrepMan™ ULTRA Sample Preparation Reagent (Applied Biosystems® by Life Technologies, Warrington, UK). Then, we vortexed tubes for 45 s and centrifuged for 30 s at 12,000 rpm. We heated tubes in a boiling water bath for 10 min, cooled at room temperature for 2 min, and centrifuged again for 1 min at 12,000 rpm. We then inverted the swabs in the Eppendorf tube using sterile flanged tweezers (i.e., by applying a flame sterilization technique between samples) and centrifuged the tubes for 5 min at 12,000 rpm. Lastly, we discarded swabs, briefly centrifuged (for a few seconds) the tubes, then transferred approximately 45 µL of solution to new tubes and stored in a freezer at -22º C.

Prior to performing real time PCR reactions (qPCRs) to detect and quantify *Bd* infections, we diluted the extracted DNA in a 1:10 dilution (Lambertini et al. 2013). To prepare the qPCR reactions, we made a master mix, which is also based on the protocol developed by Boyle et al. (2004), and contains: 1250 µL of Taqman Master Mix (Applied Biosystems®), 125 µL of the primer ITS1-3 Chytr (5'-CCTTGATATAATACAGTGTGCCATATGTC-3') at 18 µM, 125 µL of the primer 5.8S Chytr (5'-AGCCAAGAGATCCGTTGTCAAA-3') at 18 µM, 125 µl of ChytrMGB2 probe (5'-6FAM CGAGTCGAACAAAAT MGBNFQ-3') at 5 µM, 275 µL of distilled water, and 100 µL of bovine serum albumin (BSA). To prepare the qPCR 96-well plate, we added 20 µL of the mix to each well and 5 µL of the extracted DNA dilution. We ran samples in singlicate. To make the standard curve, we used the Bd isolate CLFT 159, an isolate associated with a genotype from the Bd lineage GPL, from a frog of the genus *Hylodes* from the Atlantic Forest (e. g., Greenspan et al. 2018). To make the standard curve, we diluted the *Bd* isolate to the concentrations 10^3, 10^2, 10^1, 10^0 and 10^{-1} zoospores and we ran the standards 10^3, 10^2, 10^1 in duplicates and the standards 10^0 and 10^{-1} in quadruplicates. We considered an individual infected (Bd^+) when we detected at least one *Bd* genomic equivalent (≥ 1 g. e.; Kriger et al. 2007). We rounded g. e. values to integers. We calculated intensity of infection by multiplying the values resulting from the qPCR by the dilution factor (1:10) used in the DNA extraction.

Data analysis.—We determined prevalence of infection by *Bd* by calculating the proportion of infected individuals from the total number of frogs sampled, and from those sampled in each management type (i.e., frog farms and natural ponds). We calculated Wilson 95% confidence intervals for binomial distributions for prevalence of infection using the function binconf from the package Hmisc (Harrel et al. 2019) in R v.3.6.0 (R Core Team 2019). We calculated mean infection intensity values for the total frogs sampled and per management type based only on the infected frogs. Because we have only one sampled frog farm with closed management,

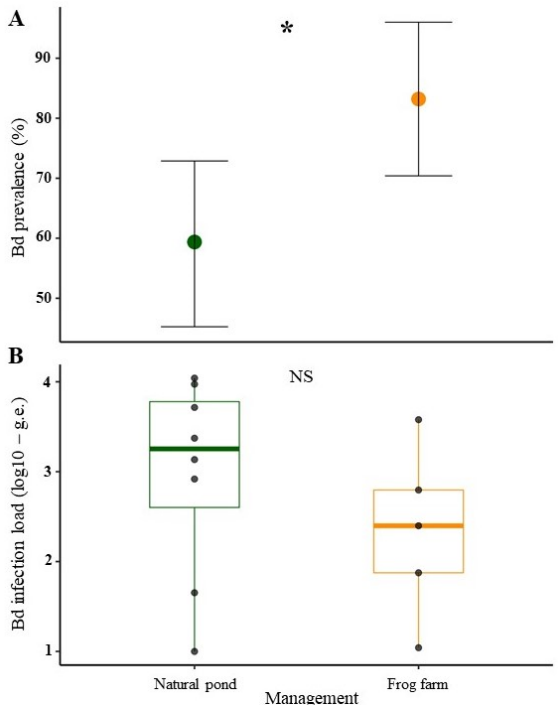

Figure 2. *Batrachochytrium dendrobatidis* mean prevalence with 95% confidence intervals (A) and average infection load (in log10) at the site-level (B) across management types. Estimates of average infection load do not include *Bd* negative individuals. Boxplots represent the median, upper and lower quartile, and maximum and minimum values. An asterisk (*) represents significant difference ($P \leq 0.05$) and NS = non-significant differences based on the Generalized Linear Mixed-effects Models.

we excluded it from the analysis, which resulted in the removal of 20 bullfrogs.

We built Generalized Linear Mixed-effects Model (GLMMs) to test the effect of source (frog farms vs. natural ponds) and distance to farms on *Bd* prevalence and infection load (i.e., number of g.e.). Specifically, the GLMMs included source type and distance to farm as fixed factors, and the locality as a random factor (i.e., random intercept). To test for effects on infection prevalence, we used a binomial error distribution with a logit link function to build the GLMM, and we fit the model using the function glmer of the package lme4 (Bates et al. 2015). For infection intensity, we fit the model to infection loads using a negative binomial error distribution and a log link function to account for overdispersion, and we built the model using the function glmer.nb, also from the package lme4. We calculated *P*-values of fixed factors using a likelihood ratio test comparing progressively simplified nested models (Zuur et al. 2009). We do not report degrees of freedom from the mixed models because there is no formula to calculate the correct degrees of freedom (Wiley and Wiley 2019), and it is unknown whether the null distribution of the calculated ratio of sums of squares follows an *F* distribution.

Table 1. Zoospore load and prevalence of *Batrachochytrium dendrobatidis* in American Bullfrogs (*Lithobates catesbeianus*) from frog farms and natural ponds in southern Brazil from September 2016 to May 2017. Abbreviations for sites are FF = frog farm and NP = natural pond. Zoospore loads are the mean ± standard deviation (range) of genome equivalents. Prevalence of infection is represented by the percentage of infected individuals among the total number sampled.

Site	Zoospore load of *Bd*$^+$ frogs	Prevalence
FF1	625 ± 1,070 (6–3,029)	80% (16/20)
FF2	11 ± 9 (3–40)	71% (15/21)
FF3	250 ± 378 (4–1,359)	100% (20/20)
FF4	75 ± 122 (3–391)	65% (13/20)
FF5	4 ± 2 (3–7)	25% (5/20)
FF6	3,788 ± 9,292 (19–39,738)	100% (20/20)
NP1	10,979 ± 18,389 (4–32,209)	27% (3/11)
NP2	2,358 ± 5,924 (3–21,875)	70% (14/20)
NP3	828 ± 2193 (2–6,249)	38% (8/21)
NP4	1,363 ± 3,566 (1–14,395)	70% (16/20)
NP5	45 ± 65 (2–175)	38% (8/21)
NP6	11 ± 14 (1–40)	50% (10/20)
NP7	5,181 ± 10,971 (3–35,038)	90% (18/20)
NP8	9,401 ± 37,826 (3–156,176)	77% (17/22)
All sites		66.3% (183/276)

Results

We found bullfrogs infected with *Bd* in all frog farms and natural ponds sampled in this study (Fig. 1). Of the 276 frogs sampled (natural ponds: n = 155; frog farms: n = 121), 183 (66.3%) were positive for *Bd* (Table 1). Zoospore load of *Bd*$^+$ individuals ranged from 1 to 156,176 g.e. (mean = 2,457 ± 12,902 standard deviation). Most *Bd*$^+$ frogs (n = 109; 59%) had a load below 100 g.e. and only one individual (0.6%) had a load above 100,000 (Table 2).

We found that human cultivation of bullfrogs (farms vs. natural ponds) had a significant effect on the prevalence of *Bd* infection (back-transformed parameter estimate = 0.866, $X^2 = 5.811$, $P = 0.016$; Fig. 2), with

Table 2. Number (n) of *Bd*$^+$ American Bullfrogs (*Lithobates catesbeianus*) within each zoospore load range category and respective percentage.

Zoospore load	Individual n	%
< 100	109	59.5
101–1000	46	25.1
1,001–10,000	20	11.0
10,001–100,000	7	3.8
> 100,000	1	0.6
Total	183	100.0

higher prevalence in frog farms (83.2%; 95% confidence interval [CI] = 75–89%) compared to natural ponds (61.3%; 95% CI = 53–69%). We did not determine a significant effect of distance to farms on the prevalence of *Bd* in natural ponds (back-transformed parameter estimate = 0.504, X^2 = 1.457, P = 0.227; Fig. 2). We did not find an effect of management (back-transformed parameter estimate = 0.319, X^2 = 0.541, P = 0.462; mean *Bd* load in natural pond = 3,663 ± 1,710 g.e.; mean *Bd* load in frog farms 1,094 ± 4,727) or distance to farms (back-transformed parameter estimate = 0.998, X^2 = 0.008, P = 0.929) on the observed variation in *Bd* load.

Discussion

We detected *Bd* across all sampled sites (natural ponds and frog farms), which suggests that this pathogenic fungus might be consistently present across the Atlantic Forest at sites where bullfrogs occur. This is relevant considering that, in southern Brazil, bullfrog populations are widespread due, in part, to releases and escapes from frog farms (Both et al. 2011) to natural environments with favorable climatic conditions that enable the establishment of invasive populations and subsequent dispersal of individuals (Giovanelli et al. 2008). We found a relatively high occurrence of *Bd* in our surveys, however, most infection loads were low (< 100 g.e.), with only 4% of individuals having more than 10,000 zoospores. Zoospore loads below 10,000 are considered infectious with low risk of death (Vredenburg et al. 2010; Kinney et al. 2011), but this pattern does not apply to all reported cases (Preuss et al. 2016; Horner et al. 2017). The low zoospore load in bullfrogs seems to be common in both natural and laboratory settings (Schloegel et al. 2010; Greenspan et al. 2012; Gervasi et al. 2013). This characteristic might be due to defensive mechanisms, which prevent advanced stages of infection, facilitating the permanence of the fungus on the host (Eskew et al. 2015).

We show that bullfrogs from frog farms harbor a higher prevalence of *Bd* compared to those from natural ponds. This higher prevalence among frogs sharing the same stall might result from the high density of individuals (Piovia-Scott et al. 2015), which also allows for the maintenance of the pathogen in the host population (Rödder et al. 2013). Considering the potential for escapes of individuals from frog farms, these facilities might act as a constant source of *Bd*-infected individuals to natural environments (Mazzoni et al. 2003). Given the presence of *Bd* across all sampled natural ponds and similar infection loads to those from frog farms, however, it was not possible to accurately determine the role of frog farms as a source of *Bd* to the natural environment. Determining whether the flow of *Bd* zoospores goes either one way (out of frog farm to the natural environment) or two ways (frog farm to the natural environment and natural environment to frog farm) is the critical next step to elucidate the role of frog farms in the dispersion of *Bd* in the region.

A recent study by Ribeiro et al. (2019) determined that tadpoles from frog farms harbor virulent lineages of *Bd* (*Bd*GPL and *Bd*ASIA-2/*Bd*BRAZIL) and found high concentrations of *Bd* zoospores (of undetermined lineages) in water released by frog farms to natural environments. Therefore, there could be a potential constant exchange of *Bd* strains/lineages between frog farms and natural environments, either via wastewater from frog farms, water collected for breeding (Mazzoni et al. 2003), or uncontrolled transit of individuals. Furthermore, the amphibian trade can facilitate the introduction of *Bd* zoospores/strains from distant regions, overcoming natural barriers (Kolby and Daszak 2016; O'Hanlon et al. 2018). Hence, frog farms can facilitate the circulation of distinct strains/lineages of *Bd* across different environments, which can produce hybrid strains (Schloegel et al. 2012; Jenkinson et al. 2016; O'Hanlon et al. 2018) that may be highly virulent to native species (Greenspan et al. 2018).

We determined that distance to frog farms was not correlated with both prevalence and load of *Bd* in bullfrogs from natural ponds, which reflects the broad occurrence of *Bd* in the region. Other studies have shown that bullfrog populations can have a high prevalence of *Bd*, and, in some cases, can be higher than that of native species (Beyer et al. 2015; Marhanka et al. 2017; Yap et al. 2018). Thus, the widespread occurrence and rapid expansion of bullfrogs in the study area reinforces the possibility that bullfrogs might act as a potential reservoir and vector of *Bd* or Ranavirus in southern Brazil (Ruggeri et al. 2019).

Our study indicates that frog farms may facilitate the dispersion and maintenance of *Bd* in natural environments, rather than act as an amplifier of infection loads, due to their role as a constant source of infected bullfrogs. Thus, native species coexisting with bullfrogs are likely to be exposed to *Bd*, as well as increased competition and predation (Oliveira et al. 2016c; Adams et al. 2017). The management type adopted by frog farms in the region is a critical factor because open management does not control for escapes of animals to the natural environment. Open management uses adjacent ponds for the reproduction and development of tadpoles, thus facilitating the establishment of this invasive species in new areas. Also, in closed management systems, the release of water from the farm into the natural environment can play an important role in the release of zoospores (Ribeiro et al. 2019). Therefore, we highlight the need for future studies assessing the effect of management type (closed vs. open) on *Bd* prevalence and genetic structure. These

effects are critical to improving conservation initiatives and farming regulations.

From a conservation perspective, we suggest the adoption of monitoring programs for *Bd*, among other pathogens, in frog farms (Winters et al. 2014). In the case of *Bd*, monitoring programs can be implemented by visually inspecting tadpole mouthparts (Lambertini et al. 2013; Carvalho et al. 2017; Ribeiro et al. 2019), analyzing skin swabs with molecular techniques (Boyle et al. 2004), and/or histology (Lambertini et al. 2013). Monitoring programs can promote collaborative work among academic institutions, producers and government agencies. Also, the creation of a national biological invasion monitoring program would be a key element for management at national and regional scales (Latombe et al. 2017).

We suggest urgent measures be adopted to tighten the control and regulation of frog farms throughout Brazil, making frog farming less harmful to native fauna. Further studies on the direct and indirect impact of bullfrog invasion on native populations, considering the spread of diseases, predation, and competition (Kraus 2015; Adams et al. 2017; Ruggeri et al. 2019) are required. Studies that identify different strains of *Bd* (e.g., Jenkinson et al. 2016; Ribeiro et al. 2019) will also help to explain the role of frog farms in the dispersal of highly virulent strains, which might occur within a region. Thus, understanding host-pathogen dynamics, even within frog farms and their surroundings, is essential to minimize the deleterious effects of emerging infectious diseases on susceptible native species.

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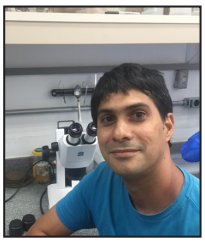

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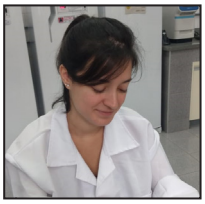

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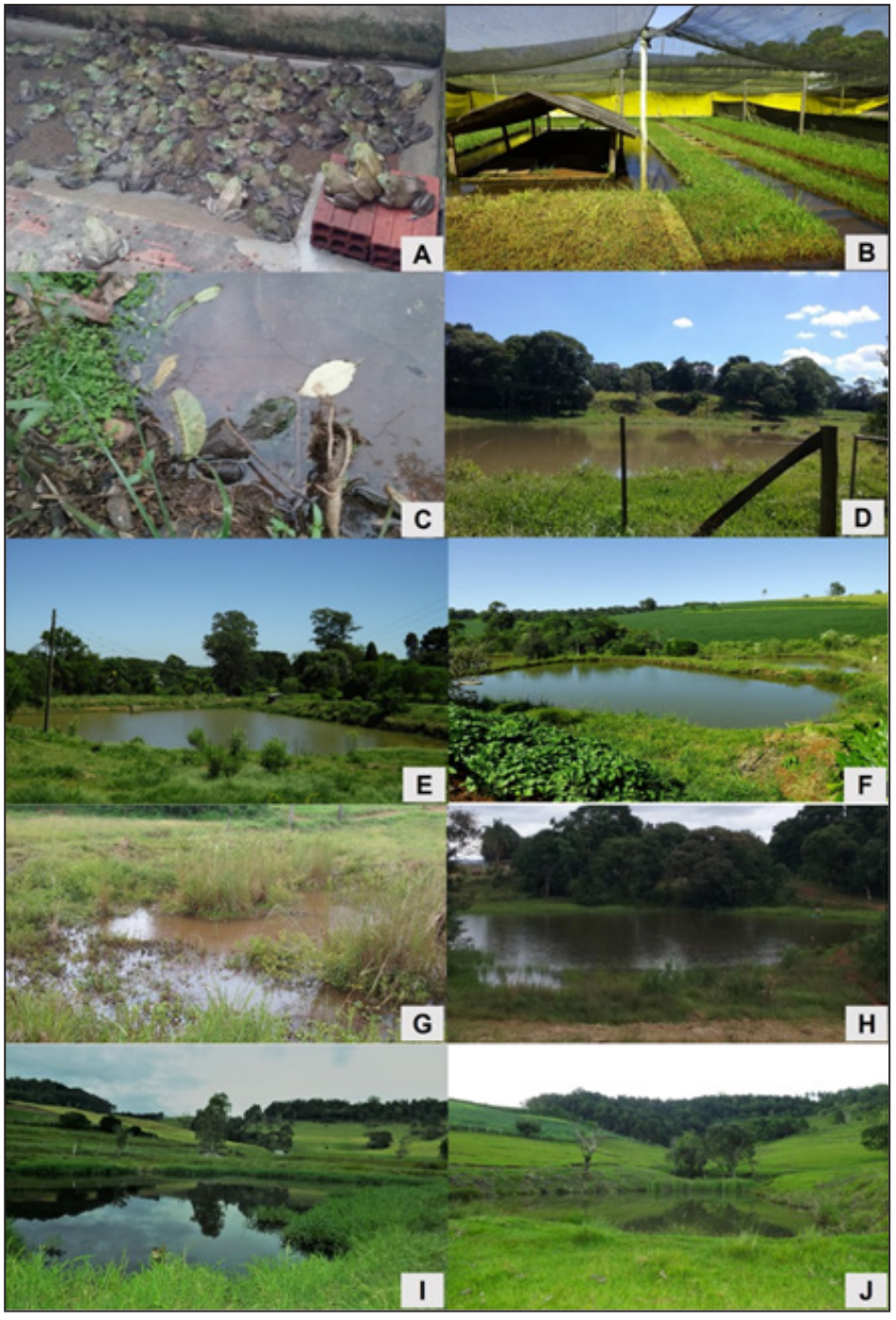
Appendix Table. Sampling sites characteristics taken during the sampling period (September 2016 to May 2017). Sites located in the states of Paraná (PR) and Santa Catarina (CR) in southern Brazil. Sampling sites = frog farm (FF) or natural ponds (NP); Locality = municipality/state; Elevation = meters above sea level; Area = superficial area in m^2; Depth = vertical mean distance (in meters) from the surface to the bottom in stalls and ponds; Density = maximum number of males calling in natural ponds or in sampled stalls in frog farms, divided by area, in m^2; Type of vegetation = predominant vegetation on pond margins.

Sampling units	Locality	Elevation	Area	Depth	Density	Minimum distance between frog farms (km)	Minimum distance between pond sampled and frog farm (km)	Management	Type of vegetation
Frog farms									
FF1	Medianeira/PR	383	12.5	0.11 ± 0.01 (0.10–0.12)	24	160	--	Open	--
FF2	Santa Izabel do Oeste/PR	414	10.5	0.12 ± 0.02 (0.10–0.15)	38.1	83	--	Open	--
FF3	Sulina/PR	482	28	0.12 ± 0.02 (0.10–0.15)	17.8	83	--	Open	--
FF4	Chapecó/SC	649	12.2	0.12 ± 0.02 (0.10–0.15)	28.7	9	--	Open	--
FF5	Chapecó/SC	649	14	0.12 ± 0.02 (0.10–0.15)	21.4	10	--	Closed	--
FF6	Guatambu/SC	589	150	0.15 ± 0.03 (0.15–0.20)	1.3	9	--	Open	--
Ponds									
NP1	Medianeira/PR	380	824	1.16 ± 1.11 (1.00–1.30)	0.02	--	0.1	--	Trees
NP2	Chapecó/SC	659	4661	1.37 ± 0.27 (1.00–1.80)	0.003	--	1.5	--	Grasses
NP3	Chapecó/SC	645	2758	1.64 ± 0.48 (1.00–2.50)	0.006	--	0.9	--	Grasses
NP4	Guatambu/SC	579	2046	1.25 ± 0.15 (1.00–1.50)	0.006	--	2.5	--	Grasses
NP5	São Domingos/SC	564	131	1.22 ± 0.12 (1.00–1.40)	0.10	--	46	--	Shrubs
NP6	São Domingos/SC	579	5600	0.90 ± 0.20 (0.50–1.20)	0.002	--	47	--	Grasses
NP7	São Lourenço do Oeste/SC	815	1076	0.88 ± 0.35 (0.30–1.50)	0.008	--	72	--	Grasses
NP8	São Lourenço do Oeste/SC	832	1329	0.87 ± 0.27 (0.50–1.30)	0.024	--	71	--	Grasses

New York, USA.



Appendix Figure. Images of frog farms and natural ponds sample for this study. Located in the states of Paraná and Santa Catarina in southern Brazil. A and C = municipality of Medianeira in the state of Paraná; D and E = municipality of Chapecó in the state of Santa Catarina; B and F = municipality of Guatambu in Santa Catarina; G and H = municipality of São Domingos in Santa Catarina; and I and J = municipality of São Lourenço do Oeste in Santa Catarina. (Photographed by Roseli Coelho dos Santos).