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MARIANA RETUCI PONTES

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VARIAÇÃO TEMPORAL DE *Batrachochytrium dendrobatidis* EM UMA ESPÉCIE DE ANURO AMEAÇADA DE EXTINÇÃO NO URUGUAI

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Orientador: Luis Felipe de Toledo Ramos Pereira

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If you do it right, you'll love where you are

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RESUMO

Os anfíbios constituem o grupo de vertebrados mais ameaçado do mundo, e uma das principais causas dos declínios populacionais é a quitridiomicose. Esta doença infecciosa é causada pelo fungo aquático *Batrachochytrium dendrobatidis* (Bd) e está levando a uma perda catastrófica na biodiversidade. Fatores ambientais e bióticos são importantes na dinâmica de infecção deste patógeno na natureza. A compreensão sobre quais e como estes fatores interferem na dinâmica do Bd em anuros é crucial para a conservação das espécies, em especial as ameaçadas de extinção. Assim, os objetivos deste trabalho foram (i) caracterizar a variação temporal da prevalência e carga de infecção por Bd no Sapinho-de barriga-vermelha, *Melanophryniscus montevidensis* (Bufonidae), espécie ameaçada da América do Sul, (ii) avaliar a importância de fatores ambientais e bióticos nas taxas de infecção e (iii) avaliar se a carga de infecção influencia na condição corporal dos indivíduos infectados desta espécie. Nós coletamos 266 *swabs* de pele entre 2011 e 2015, em Laguna de Rocha, Departamento de Rocha, Uruguai. Quarenta e dois porcento dos indivíduos estavam infectados e registramos variação sazonal para carga de infecção. A abundância de *M. montevidensis* foi preditora positiva da prevalência e da carga de infecção de Bd, e a carga afetou negativamente a condição corporal dos indivíduos. A temperatura teve um efeito positivo na carga de infecção, enquanto a precipitação influenciou negativamente. Este é o primeiro registro de Bd em uma espécie ameaçada de extinção do Uruguai. A abundância da espécie foi um importante fator na intensidade de infecção por Bd, possivelmente devido à relação entre abundância e atividade de forrageamento deste anuro. Portanto, fatores ambientais estão relacionados à intensidade de infecção por Bd nos hospedeiros e desta forma, essas interações devem ser consideradas para elaboração de medidas de conservação para esta espécie, que é considerada como alta prioridade para conservação. Este estudo contribui para o conhecimento de como os fatores ambientais influenciam na infecção por Bd. Finalmente, destacamos a necessidade do monitoramento de Bd em populações de anfíbios ameaçados, especialmente as espécies do gênero *Melanophryniscus*, um gênero com várias espécies ameaçadas de extinção ou com deficiência de dados em diferentes países da América do Sul.

Palavras-chave: Conservação, *Melanophryniscus montevidensis,* quitridiomicose.

ABSTRACT

Amphibians constitute the most threatened vertebrate group in the world, and one of the main causes is of population declines is chytridiomycosis. This infectious disease is caused by the waterborne chytrid fungus *Batrachochytrium dendrobatidis* (Bd) and is leading to a catastrophic loss of biodiversity. Environmental and biotic factors are important in the infection dynamics of this pathogen in the wild. Understanding what and how these factors interfere in Bd dynamics in anurans is crucial for the conservation of species, especially the threatened ones. Thus, the goals of this study were (i) to characterize the temporal variation of the Bd prevalence and infection intensity in Montevideo Redbelly Toad, *Melanophryniscus montevidensis* (Bufonidae), an endangered species from South America, (ii) evaluate the importance of environmental factors in infection rates and (iii) evaluate if Bd infection intensity influences the body condition of the infected individuals of this species. We collected 266 skin swabs between 2011 to 2015, in Laguna de Rocha, Rocha Department, Uruguay. Forty-two percent of the individuals were infected, and we report a seasonal variation in Bd intensity infection. The abundance of *M. montevidensis* was a positive predictor for Bd prevalence and infection intensity, and the Bd infection intensity negatively affected the body condition of individuals. Temperature had a positive effect on the infection intensity whereas precipitation influenced it negatively. This is the first record of Bd in an endangered species of Uruguay. The species abundance was an important factor on Bd infection intensity, possibly due to the relationship between abundance and activity of this anuran. Therefore, environmental factors influence Bd infection intensity on hosts, and these interactions should be considered for the elaboration of conservation measures for this species, which is considered a high conservation priority. This study contributes to the knowledge of how environmental factors influence Bd infection. Finally, we highlight the need for monitoring Bd in populations of threatened amphibians, especially species of the genus *Melanophryniscus*, a genus with several endangered or data deficient in different countries of South America.

Keywords: Chytridiomycosis, conservation, *Melanophryniscus montevidensis.*

SUMÁRIO

INTRODUÇÃO GERAL

Os anfíbios são o grupo de vertebrados mais ameaçados do mundo (Stuart et al. 2004, Hoffmann et al. 2010, Monastersky 2014). Estes animais estão sofrendo uma crise de grandes proporções desde a década de 1980 (Young et al. 2001, Collins & Crump 2009, Verdade et al. 2010, Carvalho et al. 2017). Cerca de um terço das espécies são consideradas ameaçadas (Stuart et al. 2004), e inúmeros fatores contribuem para o declínio das populações, como as mudanças climáticas, destruição de hábitats, introdução de espécies exóticas, doenças e poluição (Lannoo 2005, Collins 2010).

A quitridiomicose é um dos fatores que contribui para declínios populacionais de anuros em todo o mundo (Scheele et al. 2019) e esta doença de pele, é causada pelo fungo *Batrachochytrium dendrobatidis* (Bd) (Longcore et al. 1999). O leste da Ásia é o ponto original das linhagens deste patógeno, que se encontra amplamente disseminado e com registros em todos os continentes (Olson et al. 2013). Esta doença preocupa os conservacionistas principalmente devido ao seu potencial de rápida disseminação (Olson et al. 2013, James et al. 2015) e está associada a declínios de pelo menos 501 espécies de anfíbios (Scheele et al. 2019).

Este patógeno tem um ciclo de vida com dois estágios, a fase infectante e a fase fixa. A fase infectante é constituída pelos zoósporos flagelados livre-natante, que dependem da presença de água ou do contato entre indivíduos para sua transmissão (Berger et al. 2005). Ao entrar em contato com as células epidérmicas do hospedeiro, o zoósporo forma um tubo germinativo, e injeta seu material nuclear dentro da célula da pele do anfíbio. O estágio fixo é caracterizado pelo zoosporângio com desenvolvimento intracelular, que descarrega os zoósporos na superfície da pele do anuro, e consequentemente em corpos d'água (Rosenblum et al. 2010, Greenspan et al. 2012). Ao serem descarregados, os zoósporos podem infectar outros hospedeiros ou até mesmo reinfectar o indivíduo do qual foram liberados (Berger et al. 2005, DiRenzo et al. 2018). Como a transmissão desse fungo pode ocorrer tanto pela água como pelo contato direto entre indivíduos (Berger et al. 2005), sua dispersão é bastante rápida e eficaz (Lips et al. 2008, Cheng et al. 2011). Além disso, apesar de não sobreviver à dessecação (Johnson et al. 2003), pode ser transportado por outros animais além dos anfíbios (Brannelly et al. 2015, Pontes et al. 2018).

Em anuros adultos, o desenvolvimento da quitridiomicose é caracterizado pela colonização do fungo na camada superficial da epiderme, causando hiperqueratose, ou seja, aumentando o número de camadas de queratina que formam a pele (Pessier et al. 1999). Já nos girinos, o Bd infecta principalmente o aparelho bucal, afetando os dentículos e bico córneo, levando a uma despigmentação dessas estruturas (Pessier et al. 1999, Knapp & Morgan 2006, Vieira et al. 2013). Ao infectar a epiderme dos anfíbios, o Bd pode alterar processos fisiológicos essenciais, como a osmorregulação (Berger et al. 2005, Van Rooij et al. 2015). Essas alterações podem causar efeitos subletais como perda de peso (Retallic et al. 2007), diminuição na perda de água evaporativa e desequilíbrio osmótico (Voyles et al. 2007, Bovo et al. 2016), diminuição na força de contração do coração e alterações na osmolalidade plasmática (Voyles et al. 2009, Salla et al. 2018). Ainda, a infecção por Bd pode levar a morte dos animais devido a uma parada cardíaca assistólica, causada por um desequilíbrio osmótico (redução nas concentrações de sódio e potássio no plasma sanguíneo) (Voyles et al. 2009).

Além disso, o Bd produz fatores tóxicos que interferem na proliferação e atuação de linfócitos, reduzindo parte da resposta imune do hospedeiro contra a infecção (Fites et al. 2013, Young et al. 2014). Embora haja sinais clínicos da quitridiomicose, diferentes hospedeiros apresentam diferentes respostas imunológicas e fisiológicas, que variam de acordo com seu hábito de vida, tempo de exposição ao patógeno e condições ambientais (Gervasi et al. 2013, Savage & Zamudio 2011, Mesquita et al. 2017).

A dinâmica de infecção por Bd é influenciada por muitos fatores, como a genética do hospedeiro (Savage & Zamudio 2011, Ellison et al. 2014), genótipo e fenótipo da linhagem do fungo (Berger et al. 2005, Becker et al. 2017), tipos de peptídeos antimicrobianos e diversidade do microbioma na pele (Woodhams et al. 2007, Burkart et al. 2017), defesas do sistema imune do hospedeiro e ontogenia (Rollins-Smith, 2017). Fatores ambientais e que variam sazonalmente tais como temperatura, cobertura vegetal e disponibilidade de água também interferem na dinâmica desta doença (Kriger et al. 2007, Longo et al. 2010, Becker & Zamudio 2011, Stevenson et al. 2013, Ruggeri et al. 2015). Na natureza, a prevalência e a intensidade de infecção estão relacionadas com as condições ambientais, sendo que em meses mais frios e em maiores elevações essas taxas são maiores (Woodhams & Ross 2005, Kriger et al. 2007a). A temperatura do ambiente é importante para as infecções causadas por Bd, e temperaturas baixas ou com alta variabilidade podem diminuir o crescimento deste patógeno (Piotrowski et al. 2004). Em relação aos hospedeiros, a temperatura também é importante para combater a quitridiomicose, já que em temperaturas quentes os anfíbios podem melhorar a resposta imunológica contra o Bd (Ribas et al. 2009) e temperaturas corporais elevadas do hospedeiro podem eliminar a infecção por este patógeno (Woodhams et al. 2003). Assim como a maioria das espécies de anuros, o Bd depende de ambientes úmidos para completar seu ciclo de vida (Wells 2007, Longcore et al. 1999), e uma maior densidade de corpos d'água em uma área pode aumentar a prevalência de infecção por Bd e sua transmissão (Ruggeri et al. 2018). Assim, a compreensão sobre como a precipitação influencia na dinâmica do Bd é importante para o entendimento dos padrões de ocorrência de quitridiomicose.

Fatores bióticos, como por exemplo, diversidade de hospedeiros (Becker et al. 2014), variações de história de vida (Mesquita et al. 2017), fase de desenvolvimento (Woolhouse et al. 2001), tamanho corporal (Valencia-Aguilar et al. 2016) densidade de hospedeiros (Rachowicz et al. 2007), também são importantes para a dinâmica de infecção por Bd em anfíbios. Assim, o entendimento sobre os fatores bióticos e abióticos que interferem na dinâmica deste fungo em anuros é crucial para a conservação das espécies, em especial as ameaçadas de extinção. Este é o caso de diversas espécies do gênero *Melanophryniscus,* gênero pertencente à família Bufonidae, que apresenta o maior número de declínios causados pela quitridiomicose (Scheele et al. 2019). Este gênero é composto por 29 espécies com distribuição limitada ao sul da América do Sul (Frost, 2019). Seis ocorrem no Uruguai (Maneyro & Carreira 2012), sendo que cinco apresentam algum grau de ameaça em escala global e/ou nacional (IUCN 2019, Carreira & Maneyro 2015). Dentre elas, está o anuro *M. montevidensis* (Philippi, 1902) (Sapinho-de-barriga-vermelha) (Figura S1), classificado como Vulnerável (VU) na lista da IUCN (2019), e que apresenta populações extintas no Uruguai (Carreira & Maneyro 2015). Na lista de espécies ameaçadas do Uruguai este anuro é classificado como Criticamente Em Perigo (CR) (Carreira & Maneyro 2015) e no Brasil, esta espécie está categorizada como Em Perigo (EN) (Fundação Zoobotânica, 2014).

No Uruguai, *M. montevidensis* está associado a solos arenosos da planície litorânea no sul do país, e ocorre nas regiões de Canelones, Maldonado, Montevidéu e Rocha. Esta espécie tem reprodução explosiva, que ocorre em poças temporárias (Langone et al. 2004, Maneyro & Carreira 2012, Pereira & Maneyro 2016). Populações de *M. montevidensis* apresentam uma tendência populacional negativa (IUCN 2019), sendo que a agriculta e desenvolvimento urbano na região costeira do Uruguai nos últimos 30 anos, tem sido atribuído como responsáveis pelos declínios desta espécie (Langone et al. 2004, Achaval & Olmos 2007, Maneyro & Carreira 2012). Além disso, o Bd já foi detectado em outras espécies de anfíbios em regiões costeiras do Uruguai, inclusive onde *M. montevidensis* ocorre (Borteiro et al. 2009). Assim, a presença do Bd em populações de *M. montevidensis* é uma potencial ameaça para as populações deste anuro. Em Laguna de

Rocha, Departamento de Rocha, Uruguai (Figura S2), *M. montevidensis* apresenta um padrão de atividade sazonal, com maior atividade durante a primavera e menor atividade no outono (Bardier et al. 2019). Dado que a infecção pelo fungo Bd já foi relatada em Laguna de Rocha em outras espécies de anuros (Borteiro et al. 2009), torna-se fundamental compreender os fatores que influenciam na dinâmica de infecção na população de *M. montevidensis*.

Desta forma, o objetivo deste trabalho foi caracterizar a variação temporal da prevalência e intensidade de infecção por Bd em *Melanophryniscus montevidensis* em uma população do Uruguai. Devido à importância do ambiente para a relação patógeno-hospedeiro, hipotetizamos que variáveis ambientais locais desempenham um papel importante na regulação da dinâmica do Bd, e que essas taxas apresentam um padrão sazonal. Outra hipótese é que a abundância da população influencia nas taxas de infecção e que a condição corporal é também influenciada negativamente pela infecção por Bd.

ARTIGO

Temporal variation of *Batrachochytrium dendrobatidis* in a threatened anuran species of Uruguay

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ABSTRACT

Chytridiomycosis, an amphibian infectious disease caused by the fungus *Batrachochytrium dendrobatidis* (Bd), is leading to a catastrophic loss of biodiversity. Environmental and biotics factors are known to have a role in the dynamics of this fungus in the wild. Understanding how these factors interfere with the fungal dynamics in endangered species is crucial for the conservation of species. Thus, in this study, we aimed to characterize the temporal variation of Bd prevalence and infection intensity in individuals of a population of *Melanophryniscus montevidensis* (Bufonidae), an endangered anuran from South America. We also evaluated the importance of environmental factors in infection rates and whether the infection intensity influences the body condition in infected specimens of this species. Therefore, we collected 266 samples by swabbing the skin from individuals of *M. montevidensis* between 2011 to 2015, in Laguna de Rocha, Rocha Department, Uruguay. Forty-two percent of the individuals were infected, and we report a seasonal variation in the Bd infection intensity in infected specimens from this population. The abundance of *M. montevidensis* was a predictor for both Bd prevalence and infection intensity; however, the infection intensity negatively affected the body condition of individuals of *M. montevidensis*. Temperature had a positive effect on the infection intensity whereas precipitation influenced it negatively. This is the first record of Bd in an endangered species in Uruguay. This is the first report on abundance of specimens as an important factor on Bd infection, and such correlation might be explained by the species activity as during the period when the individuals of *M. montevidensis* are more active is when they are more susceptible of getting infected with Bd. Our results also corroborate that environmental factors indeed influence Bd infection intensity on hosts, and these interactions should be contemplated when considering conservation measures for this species. Finally, we highlight the need for monitoring Bd in populations of threatened amphibians, especially species of *Melanophryniscus*, a genus with several endangered and data deficient species throughout South America.

Keywords: Chytridiomycosis, conservation, Montevideo Redbelly Toad, *Melanophryniscus montevidensis.*

Introduction

Chytridiomycosis is an amphibian infectious disease caused by the waterborne fungus *Batrachochytrium dendrobatidis* (hereafter Bd) (Longcore et al. 1999) responsible for worldwide amphibian populations declines (Scheele et al. 2019). This disease is causing a significant and continuous loss in amphibian biodiversity (Scheele et al. 2019), and the effects are a major contributor the so-called 6^{th} mass extinction event (Wake & Vredenburg 2008, Fisher et al. 2012) ranking amphibians as the most threatened vertebrate group (Stuart et al. 2004, Hoffmann et al. 2010, Monastersky 2014). The transmission of Bd can occur both by water and by direct contact between individuals (Berger et al. 2005). Its dispersion is fairly fast and effective (Lips et al. 2008, Cheng et al. 2011), and could even be enhanced by the transport of non-amphibian carriers (McMahon et al. 2013, Brannelly et al. 2015, Pontes et al. 2018). This pathogen infects epidermal tissues of amphibian hosts, altering essential physiological processes as osmoregulation (Berger et al. 2005, Van Rooij et al. 2015), which may cause sub-lethal effects, such as weight loss, increase in skin resistance, and decrease in heart contraction force values (e.g. Retalick et al. 2007, Bovo et al. 2016, Salla et al. 2018). *Batrachochytrium dendrobatidis* also produces mycotoxins that interfere with lymphocytes proliferation and performance, reducing the host's immune response (Fites et al. 2013, Young et al. 2014). In addition, the infected hosts may die, through asystolic cardiac arrest, as a consequence of reductions in blood plasma sodium and potassium concentrations (Voyles et al. 2009).

Environmental factors subjected to seasonality, such as temperature and water availability, interfere in Bd dynamics (Kriger et al. 2007a, Stevenson et al. 2013, Ruggeri et al. 2015). For example, Bd prevalence and infection intensity are correlated with environmental conditions, wherein higher elevations and cooler months these rates are higher (Woodhams & Ross 2005, Kriger et al. 2007a, Olson et al. 2013). The environmental temperature influences Bd infections; low temperatures or temperatures with high variability decrease growth rates of this pathogen (Piotrowski et al. 2004). Temperature is also important for hosts to combat Bd infection, as temperatures higher than 26 ºC can improve immune response against Bd (Ribas et al. 2009) and reduce or eliminate the infection (Woodhams et al. 2003). Besides temperature, water availability is also important in chytrid ecology (Ruggeri et al. 2018). A higher density of water bodies in an area can increase Bd prevalence and its transmission (Ruggeri et al. 2018). Thus, precipitation is also important to understand the patterns of occurrence of the chytrid in natural amphibian populations (Ruggeri et al. 2018).

Biotic factors, such as host diversity (Becker et al. 2014), variation in life natural history (Mesquita et al. 2017), development phase (Woolhouse et al. 2001), body size (Valencia-Aguilar et al. 2016), density of host (Rachowicz et al. 2007), are also important for the dynamics of Bd infection in amphibians. Understanding the factors that interfere in the Bd ecology in the wild is particularly valuable for the elaboration of strategies for the conservation of threatened species. The Bufonidae family is the one with the most severe declines caused by chytridiomycosis, thus it is important to understand the dynamics of Bd infection in Bufonidae species threatened. This rationale could be applied to the Montevideo Redbelly Toad, *Melanophryniscus montevidensis* (Philippi, 1902) (Anura, Bufonidae), a small coastal and endangered species, endemic to Brazil and Uruguay (Zank et al. 2014, Frost, 2019). It is categorized as Vulnerable (VU) in the global IUCN red list of threatened species (2019). In Brazil, this species is categorized as Endangered (EN) (Fundação Zoobotânica, 2014).

In Uruguay, there are records of extinctions in Montevideo Redbelly Toad populations (Carreira & Maneyro 2015), and the agriculture and urban development have been identified as responsible for these declines (Langone et al. 2004, Achaval & Olmos 2007, Maneyro and Carreira 2012). *Melanophryniscus montevidensis* is associated with psammophilic vegetation (vegetation from sandy soils) and have an explosive breeding pattern in ephemeral ponds after heavy rains (Langone et al. 2004, Maneyro and Carreira 2012, Pereira and Maneyro 2016). *Melanophryniscus montevidensis* has a seasonal activity pattern (Bardier et al. 2019), and Bd has already been detected in sites where this species occurs (Borteiro et al. 2009), thus it is essential to evaluate the seasonal interaction between Bd and individuals from *M. montevidensis* in this population. Hence, we characterized the temporal variation of Bd prevalence and infection intensity in *M. montevidensis.* We expect that local temperature and precipitation will be related to Bd prevalence and infection intensity. Furthermore, as Bd may cause sublethal effects, we also expect that Bd would affect negatively individuals body condition and we predict that the abundance of hosts will be important in Bd prevalence and infection intensity.

Methods

Sample collection

We collected individuals of *Melanophryniscus montevidensis* in four ephemeral ponds between 2011 and 2015, located in Laguna de Rocha, Rocha Department, Uruguay, an area included in the National System of Protected Areas (34°39'51" S; 54°13'31" W; 0 a.s.l). The climate is humid subtropical, and the four seasons (summer, autumn, winter and spring) are clearly defined based on temperature variation (Fernández 2011), in spite of the low variation in precipitation (Figure 2A).

We captured toads and placed them individually in plastic bags in order to avoid Bd cross-contamination. We sampled Bd using sterile swabs, which were passed 5 times in the right inguinal region, 5 times in the left inguinal region, 5 times in the interdigital region of the right and left lower limbs, and 5 times in the interdigital region of the right and left upper limbs of each toad (Hyatt et al. 2007, Lambertini et al. 2013). Each swab was kept in an individual cryotube, at -20 °C until DNA extraction.

We obtained temperature and precipitation data from the meteorological station located in Rocha, belonging to the *Instituto Uruguayo de Meteorología* (Inumet). The total precipitation and average temperature of the 15 days prior to the toad sampling were considered to evaluate the influence of precipitation and temperature in Bd incidence. The choice of 15 days prior to sampling is based on Bd life cycle; i.e., this period is enough to allow at least one generation of the fungus (Berger et al. 2005). We related samples to each season based on the mean monthly temperature (Figure 2A).

We measured the snout-vent length (SVL) of each individual using a digital caliper of 0.01 mm of precision and obtained the body mass with a field scale (Pesola) of precision of 0.1 g. Then, we obtained the body condition of each individual through the Scalar Mass Index (SMI) (Peig & Green 2009). The SMI was calculated using the following formula:

$$SMI = M_i \left[\frac{L_0}{L_i}\right]^{b_{SMA}}$$

SMI = Scalar Mass Index; M_i = individual body mass; L_0 = mean value of SVL of the population; L_i = individual SVL; bSMA = slope value from an ordinary least squares (OLS) regression divided by the coefficient r of the Pearson correlation between mass and RCL. We calculated the bSMA using online software (Bohonak & van der Linde 2004). We extracted abundance (total number of individuals sampled) and capture, marking and recapture (CMR) data of *M. montevidensis* in these ponds from Bardier et al. (2019). Then, we used this data to test whether the abundance of *M. montevidensis* was a predictor of Bd prevalence and infection intensity, and to evaluate the difference in Bd infection intensity and SMI in the capture and recapture of the same individuals.

DNA extraction and molecular analysis

For DNA extraction, we add 100 μL of the Prepman ULTRA® reagent (Applied Biosystems) to each cryotube containing the swab, and subsequently, we shook the tubes in the vortex for 45 seconds and then centrifuged for 30 seconds at 12.000 RPM (revolutions per minute). After, we shake the tubes again in the vortex for 45 seconds, and then we centrifuged for 30 seconds at 12.000 RPM. After that, we placed the tubes in a bath with boiling water for 10 minutes, then we let the tubes at room temperature for 2 minutes and centrifuged them again for 1 minute at 12.000 RPM. We inverted the swab inside the cryotube with the aid of sterile forceps and centrifuged the tubes for 5 minutes again, and then the swabs were discarded (Lambertini et al. 2013). We deposited each sample of extracted DNA in the SFLT collection at Unicamp and kept the samples in -20 ° C.

We ran a qPCR to diagnose the presence or absence of the fungus in each sample and quantified the Bd infection intensity in each individual. We prepared a mix containing 1250 μL of TaqMan Master Mix (Applied Biosystems®), 125 μL of the primer ITS1-3 Chytr (5 'CCTTGATATAATACAGTGTGCCATATGTC 3') concentrated at 18 μM, 125 μL of the primer 5.8S Chytr (5 'GCCAAGAGATCCGTTGTCAAA 3') concentrated at 18 μM, 125 μl of the probe ChytrMGB2 (5 '6 FAM CGAGTCGAACAAAAT MGBNFQ 3') concentrated at 5 μM, 275 μL distilled water, and 100 μL BSA (Bovine Albumin Serum), and the extract was added into this mix. For the quantification of target DNA, we constructed a standard curve with serial dilutions of 10^3, 10^2, 10^1, 10 and 0.1 zoospore genomic equivalents (g.e) obtained from the isolated strain CLFT 123 and we used distilled water for the negative control. On a plate of 96 wells, we added 20 μL of the mix in all wells and we used the concentrations of 10^3, 10^2, 10^1 in duplicates, and 10^0 and 10^{-1} in quadruplicates. We diluted the extracted DNA at the concentration of 1:10 and added 5 μL to each plate well. We considered Bd positive samples when g.e was > 1 (Kriger et al. 2007b).

Statistical analyses

We used the Kruskal-Wallis test to determine if there were differences in Bd prevalence (numbers of animals infected/animals examined) and Bd infection intensity (g.e.) between seasons. We used this non-parametric test due to the reduced sample size for prevalence and the lack of normality for infection intensity. We used a multiple comparison Dunn *post hoc* test available in the R package PMCMR, which applies the Holm *P*-value adjustment method (Pohlert 2016) to find which seasons had significant differences.

We also grouped seasons into hot and cold periods (summer and spring *vs.* autumn and winter) and tested the differences between them in Bd prevalence and infection intensity. For this, we used the Mann-Whitney U test, as the normality of each data set was rejected by the Shapiro-Wilk test. We performed a Pearson correlation between prevalence and Bd infection intensity to evaluate if there was a relationship between these two variables. For each value of Bd infection intensity, we used the Bd prevalence of the sampled period.

We fitted a General Linear Model (GLM) in order to determine the predictive capacity of abiotic and biotic variables on the Bd prevalence. In this model, the prevalence was the response variable and we used the *logit* link function and a quasi-binomial error distribution family (due to data overdispersion). We fitted a Multiple Linear Model (MLM) in order to determine the predictive capacity of abiotic and biotic variables over the Bd infection intensity, as this variable was continuous. For these models (GLM and MLM), the variables included as predictors were: temperature (15 days prior to sampling), precipitation (cumulative rainfall of the 15 days prior to sampling) and abundance (number of individuals collected). We searched for highly correlated variables based on pairwise Pearson's correlations matrix with $|\rho| > 0.7$ to avoid multicollinearity between predictors. For each highly correlated pair, we retained the variable with the higher explanatory power on the prevalence and Bd infection intensity, according to a univariate GLM or a linear model (depending on the case). The correlation between abundance and activity (non-breeding activity) was also tested using the Pearson correlation coefficient. We ran a Mann-Whitney U test to determine differences in SMI values between infected and non-infected individuals, due to the lack of normality of the data. We used SMI data only from adult males; females were excluded from this analysis as the presence or absence of eggs might bias the SMI values. A linear regression was performed to test whether Bd infection intensity was related to SMI values.

We performed a Wilcoxon test (due to the lack of normality of the data) to establish whether the SMI values of the infected individuals in the capture differed from the recapture values. We used the Wilcoxon test to test whether the Bd infection intensity of the recaptured individuals was different from its capture data. All statistical analyses were performed in R (R Core Team 2018).

Results

We made 266 swabs from 247 *Melanophryniscus montevidensis* individuals, including females, males, juveniles and undefined (Table 1). The exceeding 19 swabs were made on 17 recaptured individuals, being two recaptured two times. Of these 17 individuals recaptured, 11 were infected in the first capture and were clear of infection or had less infection in its recapture, 5 individuals were without infection in the capture and in the recapture, and only one was without infection in the capture and infected in the recapture.

Table 1. Bd Prevalence and number of *swabs* sampled from *Melanophryniscus montevidensis* by sex and life stage.

Category	Bd prevalence (Bd^+/sampled)
Males	43 % (70/162)
Females	36 % (21/58)
Juveniles	47 % (9/19)
Undefined	44 % (12/27)
Total	42 % (112/266)

Overall Bd prevalence was 42% and the mean infection intensity was 14 g.e., varying from 1 to 195 g.e. (only considering Bd^+ individuals). This is the first record of Bd in *M. montevidensis*. The Bd prevalence did not differ among seasons (Table 2; Figure 1A; Kruskal-Wallis $\chi^2 = 4.1523$; $P = 0.2455$). We found differences in Bd infection intensity between seasons (Table 2; Figure 1B; Kruskal-Wallis $\chi^2 = 12.012$; $P = 0.0073$), with Bd infection intensity being higher in the spring than in autumn (Dunn $P = 0.0057$). There was a correlation between Bd prevalence and infection intensity (Figure 1C; Pearson's $r = 0.6831$; $P = 0.0144$), as well between abundance and activity (Pearson's $r = 0.71$; $P = 0.0450$).

Table 2. Temporal variation of Bd prevalence and mean infection intensity (given in genomic equivalent of zoospores) in *Melanophryniscus montevidensis* between 2011 and 2015. Prevalence is the amount of Bd positive in total sampled.

Year	Season	Bd Prevalence (Bd^{+}/sampled)	Mean of g.e.
2011	Spring	33.33 (2/6)	1.24 (1.02 – 1.45)
2012	Autumn	52.63 (10/19)	2.31 (1.00 – 8.76)
2013	Summer	27.27 (6/22)	2.99 (1.41 – 7.19)
2013	Autumn	35 (14/40)	3.24 (1.13 – 17.02)
2013	Winter	12.5 (2/16)	2.76 (1.87 – 3.65)
2013	Spring	80.43 (37/46)	18.68 (1.13 – 194.86)
2014	Summer	10 (2/20)	2.48 (1.42 – 3.53)
2014	Winter	9.5 (2/21)	2.93 (1.28 – 4.57)
2014	Spring	37.5 (12/32)	29.37 (1.07 – 139.96)
2015	Summer	83.33 (5/6)	27.76 (8.55 – 60.50)
2015	Autumn	43.75 (7/16)	10.74 (1.18 - 28-37)
2015	Spring	59.09 (13/22)	14.05 (1.07 – 57.52)

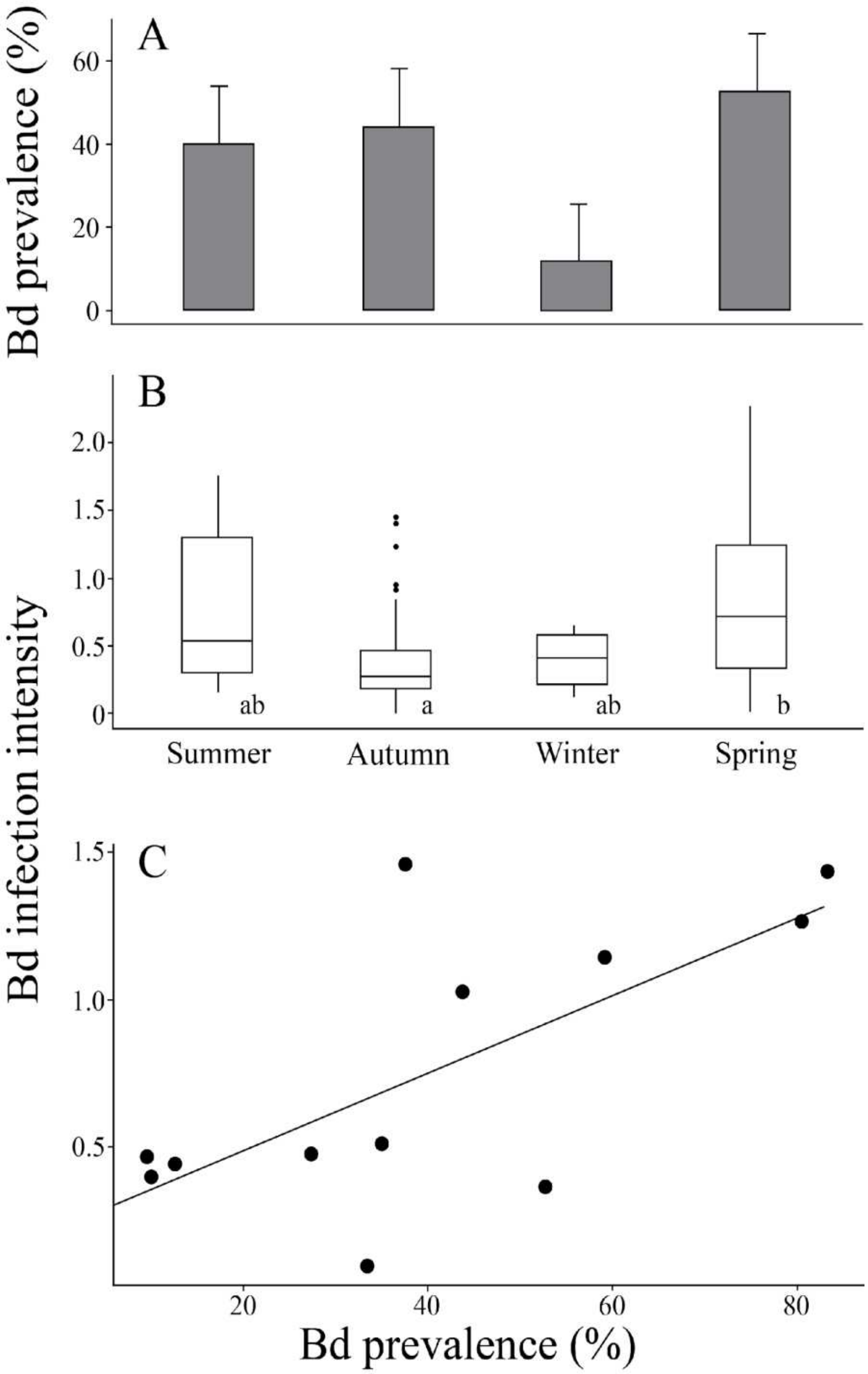

Figure 1. Bd prevalence in *M. montevidensis* for each season. The error bar is the standard error (A). Bd infection intensity for each season (B). The upper and lower part of the boxplot represents the Percentiles 75 and 25, respectively; the horizontal lines in the center of the box indicate the values of the median; the limits of the standard error bar connect the maximum and minimum values within the interquartile range; and the isolated dots are outliers. Relationship between Bd prevalence and infection intensity (C). The line was only generated for visualization, does not indicate a linear regression.

When we considered warm (spring and summer) *vs.* cold months (autumn and winter) there was no difference in the Bd prevalence (Figure 2B; Mann-Whitney U test = 22.5; P = 0.4641). There was a difference in Bd infection intensity of these two periods (Figure 2C; Mann-Whitney U test = 1898; P = 0.0006).

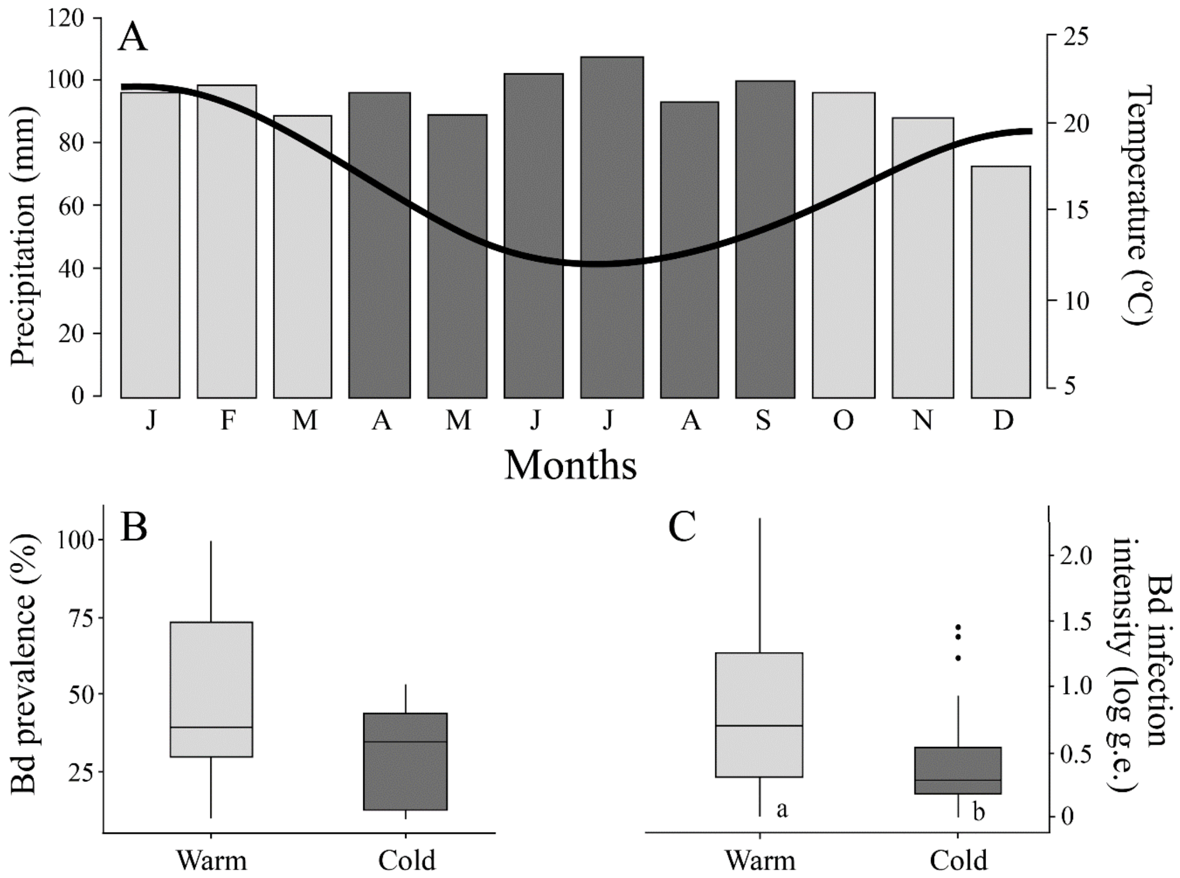

Figure 2. Mean monthly precipitation (bars) and mean monthly temperature (line) in Laguna de Rocha region. Dark gray bars represent the cold period and light gray bars represent warm period (A0.). BoxPlot of the Bd prevalence during the warm and cold periods (B). BoxPlot of the Bd infection intensity during the warm and cold periods (C).

Out of the three variables analyzed (temperature, precipitation and abundance), only abundance had an effect on Bd prevalence (Table 3; GLM P = 0.0028). All variables had effects on Bd infection intensity (Table 4; MLM P = < 0.05). There was a difference in SMI values between Bd^+ and Bd^- males (Figure 3A; Mann-Whitney U test = 2554.5; P = 0.0296). Bd infection intensity had a negative effect on the SMI values, and the infection intensity explains 33.7 % reduction of SMI (Figure 3B; b = -0.83; P = 0.005; r^2 = -0.3369). Out of the 11 animals captured with Bd and recaptured, 9 were recaptured without infection and two with lower infection values. Thus, there was a difference in Bd infection intensity between animals in the capture and recapture moments (Figure 3C; Wilcoxon V-statistic =

66; P = 0.003). SMI values in the capture did not different these values at the recapture (Figure 3D; Wilcoxon V-statistic = 27; P = 0.6247).

Table 3. Estimates of the Generalized Linear Model (GLM) showing the predictive effect of each parameter on the prevalence of Bd infection in *Melanophryniscus montevidensis*. For a model in the form $y = a + b_1x_1 + ... + b_nx_n$; y is the logit link of prevalence, "intercept" is the coefficient *a* of the model (intercept of the model with the y axis); "*b*" are the estimates of the coefficients of each variable *x*; "SE" are the standard errors of the estimates *b*; "*t*" value are the t-statistic values of each coefficient *b*; "*P*" are the significance values of the t-test of each coefficient *b*. In bold are the predictive variables with a significant effect.

	B	SE	*t*	*P*
Intercept	***-0.543***	***0.278***	***-1.95***	***0.052***
Temperature	-0.010	0.015	-0.68	0.491
Precipitation	-0.014	0.009	-1.43	0.152
Abundance	**0.006**	**0.002**	**3.02**	**0.002**

Null deviance: 1010.20 on 179 degrees of freedom
R2=NA, AIC=NA

Table 4. Estimates of the Multiple Linear Regression Model (MLM) showing the predictive effect of each parameter on Bd infection intensity in *Melanophryniscus montevidensis*. For a model in the form $y = a + b_1x_1 + ... + b_nx_n$; "intercept" is the coefficient a of the model (intercept of the model with the y axis); "*b*" are the estimates of the coefficients of each variable *x*; "SE" are the standard errors of the estimates *b*; "*t*" value are the t-statistic values of each coefficient *b*; "*P*" are the significance values of the t-test of each coefficient *b*. In bold are the predictive variables with a significant effect.

	B	SE	*T*	*P*
Intercept	*0.409*	*0.227*	*1.798*	*0.075*
Temperature	**0.058**	**0.014**	**3.892**	**<0.05**
Precipitation	**-0.006**	**0.001**	**-4.111**	**<0.05**
Abundance	**-0.006**	**0.001**	**-4.264**	**<0.05**

Residual standard error: 0.497 on 108 degrees of freedom
Multiple R-squared: 0.2351, Adjusted R-squared: 0.2139
F-statistic: 11.07 on 3 and 108 DF, p-value: 2.156e-06

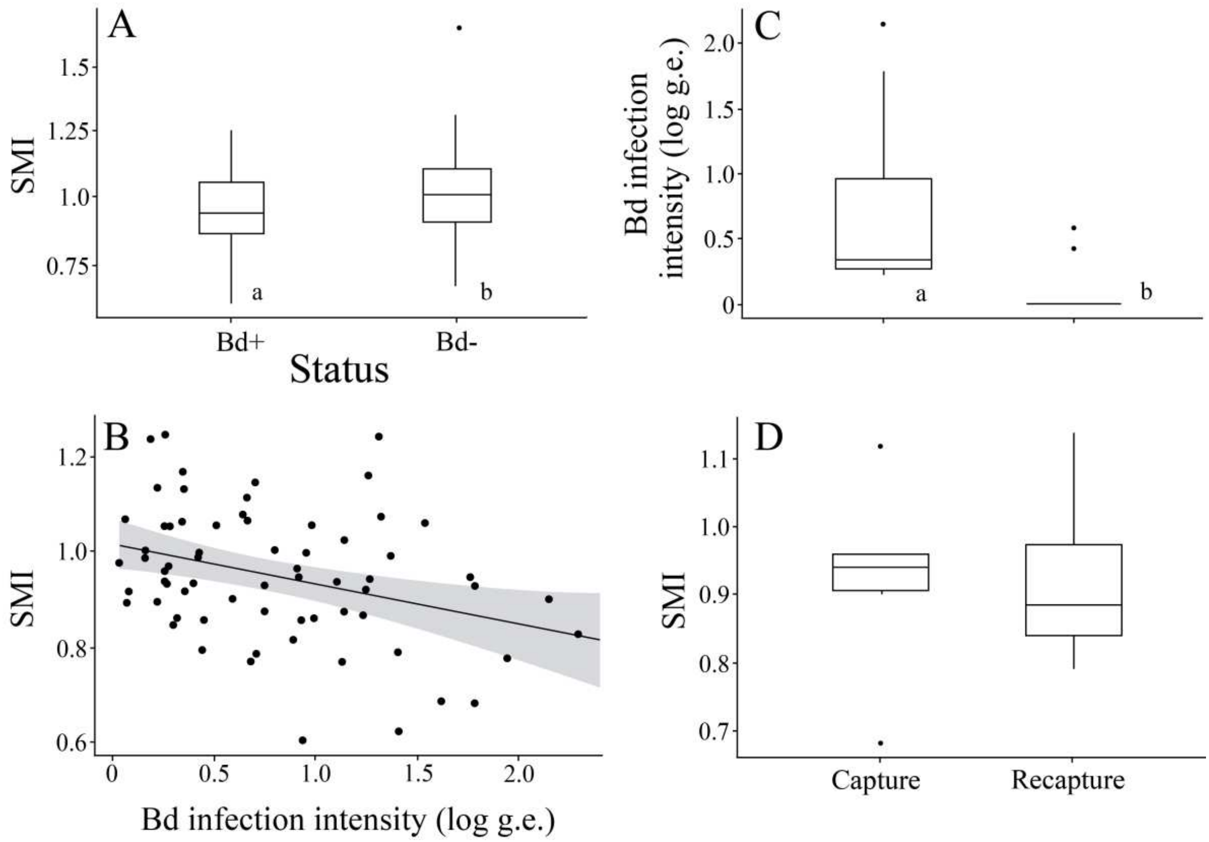

Figure 3. Boxplot of the SMI values of infected and uninfected individuals of *Melanophryniscus montevidensis* (A). Linear Regression between Bd infection intensity in *M. montevidensis* and the individuals SMI values, with a 95% confidence interval (B). Boxplot of Bd infection intensity on capture and recapture (C). Boxplot of the SMI values for captured individuals and recaptured individuals (D).

Discussion

We identified that the Bd infection negatively affects *Melanophryniscus montevidensis* individuals' body condition in the wild. Chytrid infection can cause sublethal physiological alterations, such as weight loss (Retallick et al. 2007, Campbell et al. 2019), decreased evaporative water loss and osmotic imbalance (Voyles et al. 2007, Bovo et al. 2016), decrease in the contraction force of heart and alterations in plasma osmolality (Voyles et al. 2009, Salla et al. 2018). Thus, these alterations may have contributed to a lower SMI in infected animals, either by increasing their energy expenditure (Salla et al. 2015, Brannely et al. 2015, Campbell et al. 2019), or by reducing their energy consumption (Parris et al. 2004, Venesky et al. 2009). However, other factors not included in the model, for example, the reproductive period or food availability, may help explaining such relationship. Body condition decrease may jeopardize the reproductive success of infected individuals and possibly affect the survivorship of a population (Grooms et al. 2004, Chatfield et al. 2013). Although these could happen, we have also shown that individuals were mostly recaptured without infection or with lower infection values. Therefore, despite having their body condition affected, after getting rid of the infection, these toads could have their body condition improved again.

Melanophryniscus montevidensis abundance predicted Bd prevalence in this population and was positively correlated with individuals activity. Thus, when the abundance of hosts is higher, the transmission rate of Bd can also increase (Anderson & May 1992, Rachowicz & Briggs 2007). Hence, Bd transmission may have been optimized due to greater physical contact between individuals (Rowley & Alford 2007) in periods where they are more active, either by fights between males (Alonzo et al. 2002, Pereira & Maneyro 2016), the contact between males and females during amplexus, or random encounters in the wild. Abundance has negatively influenced Bd infection intensity. It may also be linked to the activity of this species. Bd transmission occurs during physical contact (Rowley & Alford 2007, Becker et al. 2014), and when hosts are more active, there will be a greater chance that zoospores find new hosts. Thus, Bd infection intensity in periods of higher activity should decrease. In addition, in periods of greater activity, individuals of the Montevideo Redbelly toad, that habit coastal dunes (Langone, 1994), maybe more exposed to sunlight, which may increase their body temperature and possibly reducing their infection (Chatfield et al. 2011, Rowley & Alford 2013).

Bd infection seasonality between warm and cold periods could be related to mean temperatures of these periods. The fact that temperature has a positive effect on Bd infection intensity may be related to Bd optimal growth temperature, between 17 and 25 ºC (Piotrowski et al. 2004, Raffel et al. 2013). The region where Laguna de Rocha in inserted has a mean temperature under 17 ºC in colder months (Figure 2A), a mean temperature of 18.3 ºC in spring and 21.0 ºC in summer (warms months) (Figure 2A). Therefore, the optimal for Bd in this region occurs in the warmer months. Previous studies reported a negative relationship between precipitation and Bd infection intensity (Ruggeri et al. 2018), just as we observed. Bd infection intensity can be influenced mainly by growth and reinfection in the individual (DiRenzo et al. 2018). Amphibians with less contact with water have higher infection intensity when exposed to Bd infection, as in the case of direct developing species (Mesquita et al. 2017). As Bd is an aquatic fungus, when the amount of water in the environment is reduced, there is a greater chance of zoospores reinfecting the skin of the host that released them, increasing their infection intensity.

Individuals that were sampled with Bd were subsequently recaptured without or with lower infection intensity. This could be related to behavioral fever, when amphibians increase their body temperature to clean up infections (Kluger 1977, 1991), including Bd (Woodhams et al. 2003). Alternatively, infected individuals may have acquired immunity and resistance against Bd (e.g., Voyles et al. 2011, Richmond et al. 2009, McMahon et al. 2014), as they did not present high Bd infection intensities. Resistant hosts have a higher risk of infection, but low levels of infection (Greenberg et al. 2017), which may be a characteristic of the studied *M. montevidensis* population. These two hypotheses are not mutually exclusive and if they are corroborated they may be useful for the conservation of this species because it would be possible to heal animals with thermal treatments (Moreno et al. 2015) or even previous exposures to Bd, i.e., immunotherapy (McMahon et al. 2014). Individuals that cleaned up Bd infection or had the infection decreased did not present a higher SMI at the moment of recapture. Among the factors that can help frogs to get rid of Bd infection, are adaptive defenses (Ramsey et al. 2010), via gene expression of the major histocompatibility complex (MHC class 2) (Savage & Zamudio 2011, Bataille et al. 2015), microbiome selection (Harris et al. 2006, Bell et al. 2013, Kueneman et al. 2016), or even elevation of body temperature (Woodhams et al. 2003). There is a metabolic cost associated with increasing body temperature (Kluger 1991) and if individuals get rid of Bd, they might not have had enough time to regain their initial body condition.

The population studied has a seasonal dynamic (Bardier et al. 2019) and in this work, we also detected seasonality for Bd infection intensity, being the spring the period with the highest values and with less survival (Bardier et al. 2019). As this species is considered a "high conservation priority" (Zank et al. 2014), efforts to conserve *M. montevidensis* should consider the fact that in the spring we found the highest Bd infection intensities. Bd prevalence was relatively high (more than 40 %). A previous study showed that populations with high prevalence may suffer some kind of population decline due to chytridiomycosis in some stochastic event, for example, severe droughts (Longo et al. 2010). Thus, the high Bd prevalence throughout the year should be also considered as alarming for this population endurance.

Out of the 29 species described for the genus *Melanophryniscus*, nine, including *M. montevidensis*, are threatened (vulnerable [VU], endangered [EN] or critically endangered [CR]). Nine other species are classified as data deficient (DD) or were not evaluated (NE) (Motte et al. 2000, Canavero et al. 2010, Haddad et al. 2013, Fundação Zoobotânica 2014, IUCN 2019). The genus is consolidated as an important group for conservation efforts (Vaira et al. 2012, Zank et al. 2014) and consequently, studies that increase our knowledge about the effect of Bd infection on demographic and physiological parameters of *Melanophryniscus* spp. are important for the elaboration of effective conservation strategies. This is the first record of Bd infecting an endangered anuran of Uruguay (Borteiro et al. 2009, Bardier et al. 2011), a country that still has little information about the real impact of chytridiomycosis on its Anuran fauna (Scheele et al. 2019). Given the high prevalence of the fungus found and its constant incidence throughout the seasons and years, we suggest conducting Bd monitoring in these populations and also an effort to immunize or eradicate Bd from these individuals. This is necessary, since the decline of amphibian populations may occur due to chytridiomycosis, even in areas already historically occupied by Bd (Carvalho et al. 2017, Toledo 2017).

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MATERIAL SUPLEMENTAR

Figura S1. Indivíduo adulto de *Melanophryniscus montevidendis* em Cerro Verde, Uruguai. Foto: Cecília Bardier e Nicolás Martínez-Latorraca.

Figura S2. Localização das poças amostradas (34°39'51"S; 54°13'31"W). Laguna de Rocha, Departamento de Rocha, Uruguai.
Foto: Google, Acessado em 01 de Abril de 2019.

CONSIDERAÇÕES FINAIS

Neste trabalho, evidenciamos uma variação sazonal para a intensidade de infecção por Bd em uma população de *Melanophryniscus motevidensis* no Uruguai. Ainda, mostramos que temperatura e precipitação são importantes para determinar a carga de infecção por Bd em *M. montevidensis*, e que a abundância de indivíduos desta espécie é um fator biótico preditor de prevalência e carga de infecção por Bd. Os indivíduos infectados pelo patógeno tiveram uma menor condição corporal quando comparados com os indivíduos não infectados, e os efeitos subletais da infecção podem ser um dos dos fatores que contribuíram para esta diferença.

Este trabalho é o primeiro registro de infecção de Bd em uma espécie ameaçada de extinção no Uruguai. Assim, nós sugerimos a continuidade do monitoramento da prevalência e da intensidade de infecção por Bd nesta população, e indicamos a necessidade de novos trabalhos que busquem compreender a variação sazonal deste patógeno nas populações do gênero *Melanophryniscus.* Uma vez que este gênero pertence a família mais afetada pela quitrídiomicose e é considerado um importante grupo para esforços conservacionistas, entender a dinâmica dos patógenos de anfíbios em populações deste grupo torna-se fundamental para evitar futuros declínios e extinções.

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ANEXOS

Campinas, 19 de julho de 2019

Eu, Luis Felipe Toledo, atesto para os devidos fins que o projeto da aluna de mestrado do PPG Ecologia, Mariana Retuci Pontes, cujo título é "Variação temporal de *Batrachochytrium dendrobatidis* em uma espécie de anuro ameaçada de extinção no Uruguai" não necessita de autorização do CEUA. Nesse projeto, a aluna trabalhou com bancos de dados de análises moleculares (qPCR) já realizadas. Esses dados moleculares são referentes a swabs realizados durante o período de 2011 a 2015 na espécie de anuro *Melanophryniscus montevidensis*, em regiões do Uruguai. As análises moleculares já foram realizadas pela Dr. Cecília Bardier, minha ex-aluna de doutorado (Facultad de Ciencias - Universidad de la Republica Uruguai), cuja tese foi "Análisis de la viabilidad poblacional de dos poblaciones de *Melanophryniscus montevidensis*"

Sem mais,

permaneço à disposição para maiores esclarecimentos,

Prof. Dr. Luis Felipe Toledo
LaHNAB, Depto. Biologia Animal
Instituto de Biologia, Unicamp

Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **TEMPORAL VARIATION OF Batrachochytrium dendrobatidis IN A THREATENED ANURAN SPECIES OF URUGUAY**, não infringem os dispositivos da Lei n.º 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 22 de agosto de 2019

Assinatura: ______________________________
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