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Orientador: PROF. DR. LUÍS FELIPE DE TOLEDO RAMOS PEREIRA
Co-orientadora: DRA. JOICE RUGGERI GOMES

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RESUMO

Os anfíbios se destacam na atual crise da biodiversidade como o grupo de vertebrados mais ameaçado do mundo. Entre as principais ameaças estão as doenças infecciosas emergentes. A doença que mais afeta os anfíbios é a quitridiomicose, causada principalmente pelo fungo quitrídio *Batrachochytrium dendrobatidis* (Bd), responsável por declínios globais e suspeita de causar nove a cada onze extinções. Além deste patógeno, outro desafio para os anfíbios são os vírus do gênero *Ranavirus* (Rv), também relacionados a declínios populacionais. A presença de ambos patógenos é especialmente preocupante em populações de espécies em risco de extinção, pois a combinação de diferentes ameaças pode acelerar o declínio destes animais. A presente tese lança um olhar para a presença dos patógenos Bd e Rv no gênero *Melanophryniscus* (Sapinhos-de-barriga-vermelha), um grupo de particular importância para conservação devido ao alto número de espécies ameaçadas. Desta forma, no capítulo I, buscamos entender a distribuição do Bd em populações de espécies do gênero *Melanophryniscus* no Brasil, e os fatores ambientais que influenciam na dinâmica de infecção. Verificamos que as populações deste gênero apresentaram uma baixa prevalência do fungo, com baixas cargas de infecção. Além disso, encontramos que a temperatura ambiental estava negativamente associada à prevalência de Bd, enquanto a elevação estava positivamente associada. No entanto, apenas a elevação foi relacionada às cargas de infecção. Adicionalmente, as populações de espécies ameaçadas (cinco populações, quatro espécies) apresentaram maior prevalência do fungo, e somente o ameaçado *M. biancae* exibiu alta prevalência e altas cargas de Bd. Entre as espécies ameaçadas do gênero, destaca-se o Sapinho-admirável-de-barriga-vermelha (*M. admirabilis*), classificado como criticamente ameaçado pela Lista Nacional de Espécies Ameaçadas de Extinção. Assim, considerando a vulnerabilidade de *M. admirabilis*, no capítulo II, monitoramos o fungo Bd durante 3,3 anos na única população conhecida. Além disso, utilizamos modelos estatísticos para avaliar como os extremos climáticos de temperatura e pluviosidade, causados pelas mudanças climáticas, influenciaram na dinâmica de infecção do Bd nesta população. Identificamos que a prevalência do Bd estava ligada à baixa precipitação e a temperaturas mais elevadas, sugerindo que as anomalias climáticas desempenham um papel crucial na dinâmica do Bd nessa espécie. No capítulo III, considerando a presença do Bd em espécies de *Melanophryniscus* brasileiras do sul do Brasil e a diversidade de linhagens de Bd nessa região, identificamos os genótipos Bd-GPL e o genótipo híbrido em três espécies. Além disso, identificamos a infecção por Rv em indivíduos de duas espécies do gênero que estão ameaçadas de extinção. As populações dos Sapinhos-de-barriga-vermelha que ocorrem no

Brasil estão majoritariamente na região sul, onde também existem populações de *Aquarana catesbeiana*, espécie invasora e tolerante às infecções por Bd, que atua como vetor de patógenos. Portanto, no capítulo IV, utilizamos uma abordagem experimental para avaliar o processo de infecção intracelular de diferentes cepas de Bd isoladas de *A. catesbeiana.* Uma das cepas apresentou tubos germinativos mais longos, o que possivelmente acelera o processo de infecção. A presente tese elucida a distribuição de patógenos emergentes em espécies de *Melanophryniscus* e buscou contribuir para a conservação destes anfíbios.

Palavras-chave: Anfíbios, *Batrachochytrium dendrobatidis*, conservação, doenças emergentes, espécies ameaçadas, Mata Atlântica, sapinhos-de-barriga-vermelha, quitridiomicose, ranavirose.

ABSTRACT

Amphibians stand out in the current biodiversity crisis as the most threatened group of vertebrates in the world. Among the main threats to amphibians are emerging infectious diseases. The disease that has most affected the amphibians is chytridiomycosis, caused mainly by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), responsible for global declines and suspected of causing nine out of eleven extinctions. In addition to this pathogen, another challenge for amphibians are the viruses of the genus *Ranavirus* (Rv), which are also related to populations declines. The presence of both pathogens is especially concerning in populations of species at risk of extinction, as the combination of different threats can accelerate the decline of these animals. This thesis focuses on the presence of the pathogens Bd and Rv in the genus *Melanophryniscus* (Redbelly toads), a group of particular importance for conservation due to the high number of threatened species. Thus, in chapter I, we aimed to understand the distribution of Bd in populations of *Melanophryniscus* species in Brazil, and the environmental factors influencing infection dynamics. We found that populations of this genus had a low prevalence of the fungus, with low infection loads. Furthermore, we found that environmental temperature was positively associated with Bd prevalence, while elevation was positively associated. However, only elevation was related to infection loads. Furthermore, populations of threatened species (five populations, four species) have a higher prevalence of the fungus, and only the threatened *M. biancae* showed high prevalence and high Bd loads. Among the threatened species of the genus, the Admirable Redbelly Toad (*M. admirabilis*) is classified as critically endangered by the Lista Nacional de Espécies Ameaçadas de Extinção. Thus, considering the vulnerability of *M. admirabilis*, in chapter II, we monitored the fungus Bd over 3.3 years in the only known population of the species. Moreover, we used statistical models to assess how extreme climate conditions of temperature and precipitation, caused by climate change, influenced Bd infection dynamics in this population. We identified that Bd prevalence was linked to low precipitation and higher temperatures, suggesting that climate anomalies play a crucial role in Bd dynamics in this species. In chapter III, considering the presence of the Bd pathogen in *Melanophryniscus* species from southern Brazil, and the diversity of Bd lineages in this region, we identified the Bd-GPL genotype and the hybrid genotype in three species. Additionally, we identified Rv infection in individuals of two species of the genus that are threatened with extinction. Populations of Redbelly toads in Brazil are mostly found in the southern region, where there are also populations of *Aquarana catesbeiana*, an invasive species tolerant to Bd infection, which that acts as a vector of pathogens. Therefore, in chapter IV, we

used an experimental approach to evaluate the intracellular infection process of different Bd strains isolated from *A. catesbeiana*, a species tolerant to Bd infections. One of the strains showed longer germ tubes, which possibly accelerates the infection process. This thesis elucidates the distribution of emerging pathogens in *Melanophryniscus* species and seeks to contribute to the conservation of these amphibians.

Key-words: Amphibian, Atlantic Forest, *Batrachochytrium dendrobatidis*, chytridiomycosis, conservation, emerging diseases, ranaviruses, redbelly toads, threatened species.

SUMÁRIO

INTRODUÇÃO GERAL

A era do Antropoceno, marcada pelo impacto do ser humano no planeta, é caracterizada pelas crises interligadas do clima e da biodiversidade (Crutzen, 2006). A combinação de diferentes atividades de mais de 8 bilhões de seres humanos impõe pressões evolutivas que a maioria dos táxons é incapaz de superar, fazendo com que a perda de biodiversidade contemporânea esteja relacionada diretamente às ações de uma única espécie (Palumbi 2001, Bull & Maron 2016). Apesar de sua importância ecológica e seu papel como indicador do estado da biodiversidade do nosso planeta, os anfíbios estão sofrendo com declínios e extinções a nível global. De acordo com a mais recente Avaliação Global de Anfíbios (GAA2; Re:wild 2023) realizada pela União Internacional para a Conservação da Natureza (IUCN), 41 % das espécies de anfíbios estão ameaçadas de extinção (IUCN 2023). Desta forma, os anfíbios são considerados o grupo de vertebrados mais ameaçado do mundo, sendo a perda e degradação de habitat, mudanças climáticas, doenças infecciosas, queimadas e espécies invasoras as principais causas que ameaçam a existência e persistência destes animais (GAA2; Re:wild 2023).

Entre as doenças que ameaçam os anfíbios destaca-se a quitridiomicose. Um dos agentes etiológicos causadores desta doença é o fungo *Batrachochytrium dendrobatidis* (Bd) (Berger et al. 1998, Longcore et al. 1999), que afeta principalmente os anfíbios anuros. Já os anfíbios caudatos são afetados pelo *B. salamandrivorans* (Bsal) (Martel et al. 2013). Até o momento, a doença foi associada ao declínio de mais de 500 espécies de anfíbios, incluindo 90 extinções (Scheele et al. 2019). Segundo a GAA2, a quitridiomicose é uma ameaça contínua para 600 espécies ameaçadas de extinção, sendo responsável por nove das 11 extinções registradas desde a década de 1980 (Re:wild 2023).

O fungo Bd é um patógeno que possui um ciclo de vida com dois estágios principais: o móvel e o séssil (Berger et al. 2005, Figura 1). O zoósporo flagelado representa o estágio móvel, de vida curta, transmitido principalmente pela água, e é considerado a fase infectante do Bd (Longcore et al. 1999, Berger et al. 2005). A fase séssil é representada pelo zoosporângio, onde o conteúdo celular se divide em novos zoósporos (Berger et al. 2005). Este fungo infecta a pele dos anfíbios, e as primeiras interações do Bd com a pele têm início com a adesão dos zoósporos, seguida pela formação do tubo germinativo que penetra nas células do estrato córneo (Berger et al. 2005, Figura 1 A-C). Posteriormente, ocorre a maturação dos zoosporângios, seguido pela formação das papilas de descargas, que atravessam a epiderme e liberam os zoósporos recém-formados na superfície das células epidérmicas (Berger et al. 2005,

Figura 1 D-F). Na superfície da pele, os zoósporos infectantes entram em contato com o ambiente, geralmente através da água. Este processo resulta na perda do citoplasma da célula hospedeira (Berger et al. 2005). Como a pele dos anfíbios é fundamental para regulação de água e dos eletrólitos (Wells 2007, Voyles et al. 2011), as infecções por Bd perturbam essa regulação, levando ao desequilíbrio osmótico dos animais infectados (Voyles et al. 2011). A gravidade das infecções depende de diversas condições, e pode evoluir para casos graves de quitridiomicose. No entanto, mesmo em casos mais leves, as infecções por este fungo provocam pequenas alterações na osmorregulação do hospedeiro (Voyles et al. 2011, Wardziak et al. 2013), o que pode resultar em diminuições na aptidão da população a longo prazo.

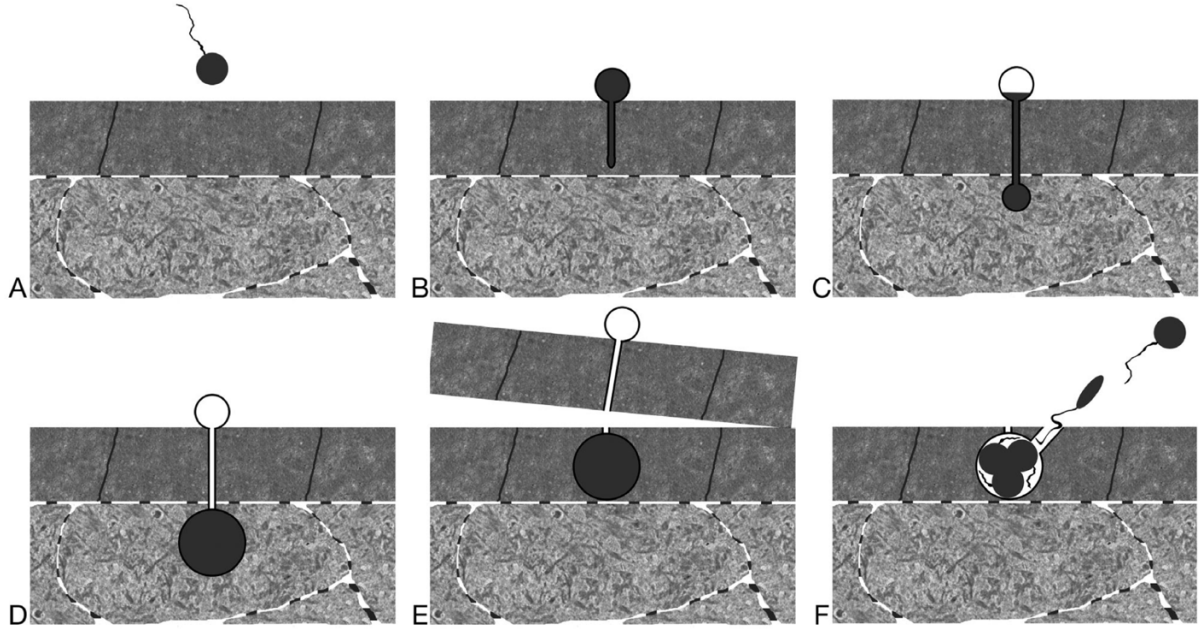

Figura 1. Esquema retirado de Greenspan et al. (2012). Ciclo de vida do fungo *Batrachochytrium dendrobatidis* na pele de um hospedeiro anuro. Zoósporo flagelado e móvel (A) se adere à superfície da pele e produz um tubo germinativo que penetra no hospedeiro (B), e geralmente se estendendo através do estrato córneo (C). O conteúdo migra através do tubo germinativo, e um zoosporângio surge na porção do tubo da qual o conteúdo celular do Bd se acumulou (D). Forma-se um septo para segregar o zoosporângio do tubo (E). As células hospedeiras sofrem maturação e movimenta-se para fora à medida que o zoosporângio se desenvolve. (F) A maturação do zoosporângio resulta na formação de novos zoósporos, que são liberados através de uma papila de descarga (Berger et al. 2005).

A virulência (capacidade de causar danos ao hospedeiro) do Bd depende de diversos fatores, incluindo a genética das cepas e linhagens do patógeno, além de outros fatores bióticos e abióticos (Fisher & Garner 2020). Cepas e linhagens de Bd isoladas de diferentes localidades apresentam variações genotípicas e de virulência. Atualmente, existem seis linhagens de Bd (O'Hanlon et al. 2018, Byrne et al. 2019, Carvalho et al. 2024) (Figura 2), e a linhagem

panzoótica global (Bd-GPL) é considerada hipervirulenta e está associada a declínios e extinções em todo o mundo (Lips 2016, Scheele et al. 2019). Adicionalmente, genótipos híbridos foram identificados como resultado da coocorrência da linhagem Bd-GPL com linhagens pré-existentes (Schloegel et al. 2012, Jenkinson et al. 2016, O'Hanlon et al. 2018, Byrne et al. 2019), e podem apresentar alta virulência para espécies nativas da América do Sul (Greenspan et al. 2018).

O Bd é um patógeno cosmopolita e generalista capaz de infectar centenas de espécies de anfíbios, interagindo intimamente com os diversos ambientes que ocupa (Bower et al. 2017, Scheele et al. 2019). O meio abiótico desempenha um papel importante na dinâmica das infecções por Bd (Fisher & Garner 2020). Fatores ecológicos como temperatura, precipitação, sazonalidade e elevação são identificados como determinantes na dinâmica das infecções (Fisher & Garner 2020). Desta forma, devido à importância das características ambientais para as interações do Bd e seus hospedeiros, o entendimento sobre como os fatores abióticos interferem na dinâmica da quitridiomicose é crucial para a conservação das espécies, pois fornece informações importantes sobre os riscos de doenças em populações. Isso se torna especialmente relevante no atual cenário de variabilidade climática acelerada, causada pelas mudanças climáticas.

Além da quitridiomicose, outra ameaça global à existência e persistência das populações de anfíbios é a Ranavirose. A ranavirose é uma doença infecciosa causada por agentes patogênicos virais do gênero *Ranavirus* (Rv), da família Iridoviridae (Chinchar 2002). Este grupo de patógenos infecta vertebrados ectotérmicos como peixes, anfíbios e répteis (Duffus et al. 2015). Assim, o Rv é capaz de infectar múltiplas espécies em um local e pode ser transmitido entre classes taxonômicas de vertebrados ectotérmicos (Duffus et al. 2015). Com distribuição global (Figura 3), o Rv foi identificado como ameaça significativa às populações de anfíbios (por exemplo, Duffus & Cunningham 2010, Teacher et al. 2010, Earl & Gray 2014, Price et al. 2014).

Atualmente, os Rv associados aos anfíbios (AARVs – Amphibian-associated ranaviruses), incluem as linhagens *Frog virus* 3 (FV3-like), *Ambystoma tigrinum virus* (ATV-like) e *Common midwife toad virus* (CMTV-like) (Duffus et al. 2015). A linhagem FV3-like apresenta uma distribuição global e já foi responsável por declínios de peixes, anfíbios e répteis, tanto em cativeiro quanto em ambientes naturais (Duffus et al. 2015). A distribuição geográfica deste patógeno se concentra em regiões da América do Norte, Europa e Sudeste Asiático (Brunner et al. 2021; Figura 3). Embora a maioria dos casos de Rv não ocorra em *hotspots* de biodiversidade, a detecção desse vírus é esporádica em algumas regiões, como na América do

Sul, o que está mais relacionado à escassez de estudos e amostragens adequadas do que à ausência real do vírus (Brunner et al. 2021; Figura 3). Assim, apesar da importância das doenças infecciosas na perda da biodiversidade de anuros, ainda existem grandes lacunas de conhecimento, principalmente ligadas às infecções por Rv.

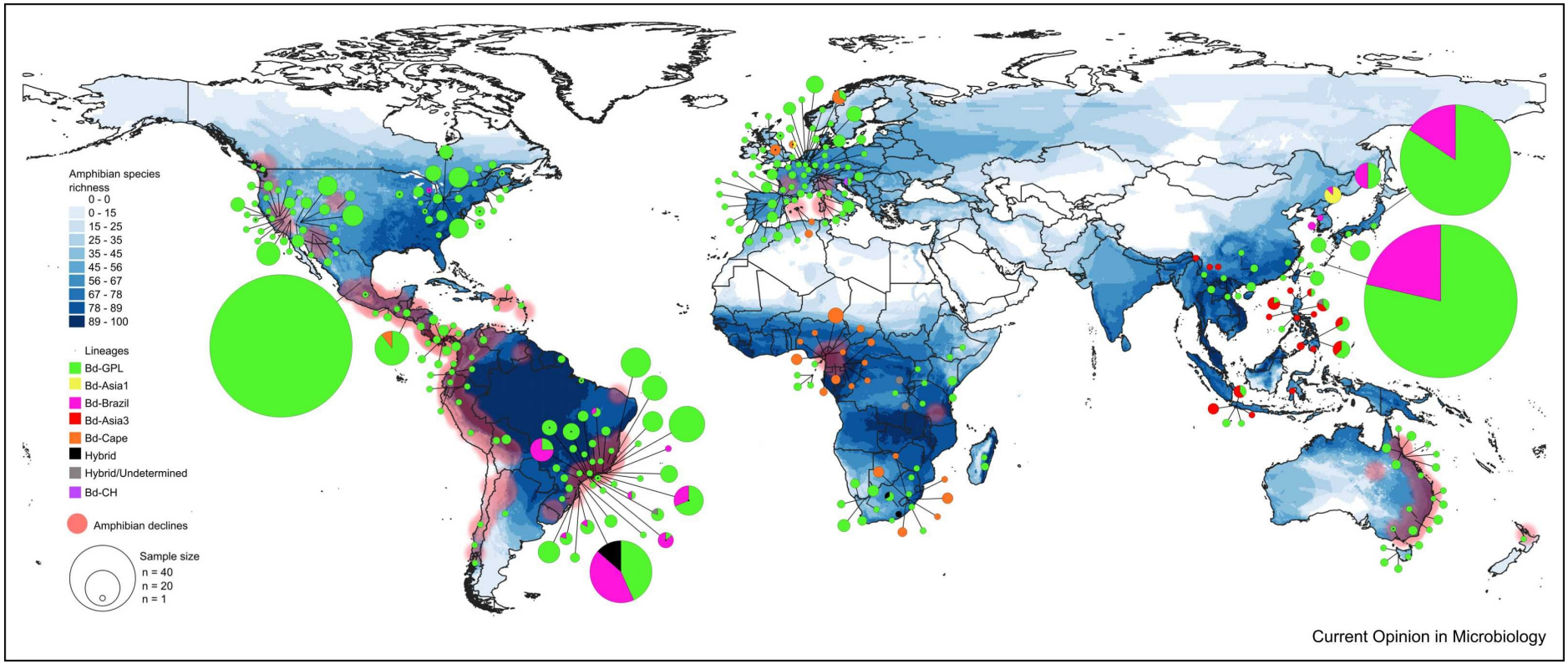

Figura 2. Distribuição das linhagens do fungo *Batrachochytrium dendrobatidis*. Fonte: Carvalho et al. (2024).

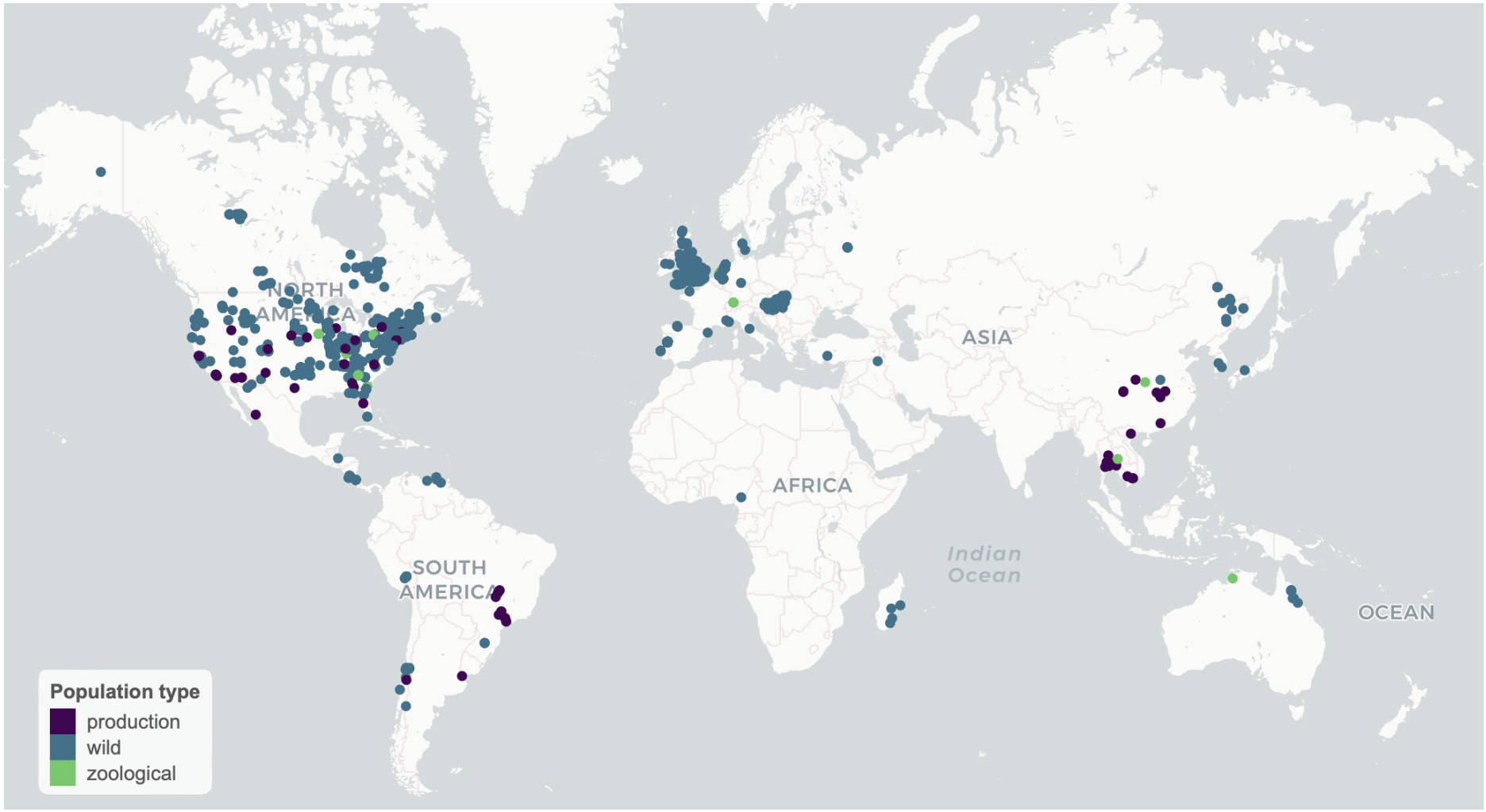

Figura 3. Distribuição mundial de registros do patógenos *Ranavirus* em anfíbios nativos, ranários e em zoológicos. Acesso em 25 de maio de 2024. Global Ranavirus Reporting System (GRRS). Fonte: Brunner et al. (2021).

A América do Sul sofreu uma perda devastadora de biodiversidade de anfíbios devido às doenças infecciosas (Carvalho et al. 2017, Scheele et al. 2019). As consequências das infecções por Bd e Rv se tornam ainda mais preocupantes em locais com altas taxas de endemismo. Com a maior diversidade de anfíbios no mundo, o Brasil se destaca com mais de mil espécies de anuros descritas (Segalla et al. 2021). Declínios e extinções históricas de populações de anfíbios brasileiros foram ligados à quitridiomicose (Carvalho et al. 2017). No entanto, os estudos sobre a presença e as consequências do Rv em anfíbios nativos são incipientes (Ruggeri et al. 2019, 2023). Desta forma, estudos que detectem e avaliem os fatores que influenciam na dinâmica desses patógenos em espécies nativas são valiosos, principalmente para contribuir com elaboração de estratégias de conservação para as espécies mais vulneráveis, como as espécies ameaçadas de extinção.

Na América do Sul, um grupo de anuros que chama atenção do ponto de vista da conservação é o gênero *Melanophryniscus* Gallardo, 1961. Este gênero inclui 31 espécies restritas à América do Sul (Frost 2024) e 12 estão ameaçadas de extinção (IUCN 2023). A distribuição deste gênero se estende pelos vales interandinos da Bolívia até a costa atlântica do sul do Brasil, abrangendo também Paraguai, Uruguai e centro e norte da Argentina (Frost 2024). Popularmente conhecidos como Sapinhos-de-barriga-vermelha (Figura 4), as espécies de *Melanophryniscus* destacam-se como alguns dos anuros neotropicais mais conspícuos e distintos, devido aos seus padrões de coloração apostemáticos (Figura 4). O gênero apresenta diversos modos reprodutivos que incluem oviposição e desenvolvimento larval em ambientes lóticos como riachos temporários e em ambientes lênticos, como poças temporárias (Nunes-de-Almeida et al. 2021). Além disso, algumas espécies estão associadas a fitotelmata (Langone et al. 2008, Steinbach-Padilha et al. 2008, Bornschein et al. 2015), que são pequenos corpos d'água que se acumulam em cavidades de estruturas vegetais terrestres (Varga 1928).

No Brasil ocorrem 22 espécies do gênero *Melanophryniscus* (Segalla et al. 2021), e segundo a Lista Nacional de Espécies da Fauna Brasileira Ameaçadas de Extinção (Brasil 2022), oito espécies do gênero estão classificadas como ameaçadas. Isso representa 13,55 % das espécies de anfíbios ameaçados no país, que atualmente conta com 59 espécies ameaçadas de extinção (Brasil 2022). O gênero *Melanophryniscus* é considerado o mais ameaçado entre os anfíbios brasileiros, com 36 % das espécies em algum grau de ameaça (Brasil 2022, Anunciação et al. 2024), sendo assim, um importante grupo para planejar e executar esforços de conservação (Zank et al. 2014, Anunciação et al. 2024). No Brasil, todas as espécies de *Melanophryniscus* ameaçadas de extinção foram incluídas nesta categoria com base em critérios de distribuição geográfica (Critério B e D1; Brasil 2022, Salve 2023), principalmente

relacionados à extensão de ocorrência (B1; Brasil 2022). Espécies e populações deste gênero têm distribuições restritas ou são endêmicas, tornando este grupo particularmente suscetível à extinção (Brasil 2022, IUCN 2023). Além disso, as populações de *Melanophryniscus* podem ser significativamente afetadas pela redução de áreas adequadas devido às mudanças climáticas (Zank et al. 2014), o que destaca ainda mais a necessidade de esforços de conservação para este grupo.

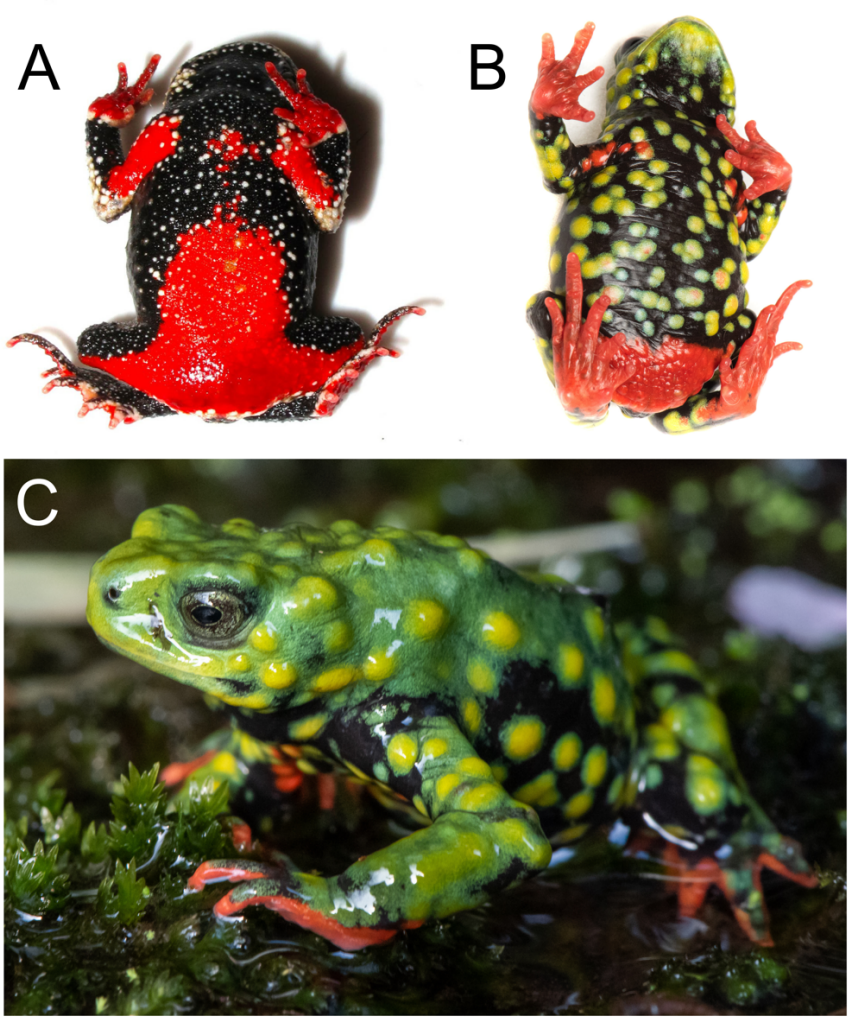

Figura 4. Sapinhos-de-barriga-vermelha. Região ventral de um indivíduo da espécie *Melanophryniscus devincenzii* (A), foto: Luís Felipe Toledo; região ventral de um indivíduo da espécie *M. admirabilis*, foto: Pedro Peloso. *Melanophryniscus admirabilis* em ambiente natural (C), foto: Pedro Peloso.

Apesar da ligação entre declínios de anfíbios no Brasil nas décadas de 1970 e 1980 com a quitridiomicose (Carvalho et al. 2017), ainda existem lacunas de conhecimento sobre a dinâmica e os fatores ambientais que influenciam o fungo Bd em muitas espécies brasileiras. As espécies do gênero *Melanophryniscus*, apesar de serem chaves para esforços de conservação, não foram amplamente estudadas em relação a presença de agentes patogênicos.

Existem poucos trabalhos no Brasil que diagnosticaram a presença de Bd em espécies de Sapinhos-de-barriga-vermelha (Slys et al. 2007, Rodriguez et al. 2014, Preuss et al. 2016). Assim, estudos com Bd focados em espécies deste gênero se tornam uma importante ferramenta para o planejamento e elaboração de ações de conservação eficazes.

Em relação ao Rv, esta lacuna se estende ainda mais, pois a detecção desse patógeno em anfíbios nativos é recente (Ruggeri et al. 2019, 2023). Até o momento, no Brasil o Rv foi relacionado com eventos de mortalidade em massa de girinos de rã-touro (*Aquarana catesbeiana*) em ambientes de produção (ranários) (Galli et al. 2006, Mazzoni et al. 2009, Candido et al. 2019). Recentemente, este vírus foi diagnosticado em populações selvagens de rã touro (Ash et al. 2024, Campião et al. 2024), e em altas prevalências em anfíbios nativos no sul da Mata da Atlântica (Ruggeri et al. 2023). Portanto, pesquisas focadas em detectar Rv em anuros nativos e ameaçados de extinção no Brasil são essenciais para compreender a distribuição deste vírus e as relações entre o patógeno e os hospedeiros.

Assim, este trabalho buscou compreender a distribuição do fungo Bd em populações do gênero *Melanophryniscus* e os fatores que influenciam na dinâmica de infecção por Bd em seus hospedeiros. Com a alta prevalência de infecção por Rv detectada recentemente em anfíbios nativos (Ruggeri et al. 2023), buscamos compreender se as espécies de Sapinhos-de-barriga-vermelha estão infectadas por este vírus.

CHAPTER I

CLIMATIC AND CONSERVATION STATUS INFLUENCE ON CHYTRID INFECTION IN REDBELLY TOADS

Influência do clima e categoria de ameaça na infecção por quitrídio nos Sapinhos-de-barriga-vermelha

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Climatic and conservation status influence on chytrid infection in Redbelly toads

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Abstract

Amphibian population declines across the Americas have been linked to chytridiomycosis, a disease caused by *Batrachochytrium dendrobatidis* (*Bd*). The anuran bufonid toads stand out as the family most affected by the disease, however, there is a notable knowledge gap regarding *Bd* in the neotropical genus *Melanophryniscus*. Commonly known as Redbelly toads, this genus is of great conservation concern in Brazil, where 36 % of the congeneric species are classified as threatened with extinction. *Melanophryniscus* spp. inhabit a wide range of environments, from coastal lowlands to mountaintops, and their reproductive microhabitat varies significantly. We therefore investigated the spatial distribution of *Bd* in populations of *Melanophryniscus* spp. and factors affecting the pathogen infection in threatened and non-threatened species. Our study revealed that most of the populations of *Melanophryniscus* spp. exhibited low *Bd* prevalence and infection loads. We found that temperature and elevation were associated with *Bd* prevalence, and populations of threatened species were more likely to have higher chytrid prevalence. Interestingly, elevation was the unique variable linked with *Bd* infection load, while species associated with phytotelmata showed higher *Bd* prevalence and infection load. Additionally, the endangered *M. biancae* exhibited an unexpectedly high *Bd* prevalence and infection load. These findings are of conservation concern for *M. biancae,* given its limited geographic distribution and other threats faced by this species. The overall low pathogen prevalence and infection load in *Melanophryniscus* spp. populations suggest that these toads may have mechanisms to limit *Bd* infection. However, as threatened South American Redbelly toads have limited distributions, populations may be driven to extinction by chytridiomycosis even in the absence of mass mortalities. Thus, the long-term monitoring of emerging infectious pathogens is crucial to inform conservation efforts targeted at highly threatened amphibian genus.

Keys-words: Amphibian, *Batrachochytrium dendrobatidis*, emerging infectious pathogen, *Melanophryniscus*, threatened species.

Introduction

South America has experienced a devastating amphibian diversity loss due to the chytridiomycosis disease, caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) (Scheele et al., 2019). Among all anuran clades experiencing population declines, bufonids are particularly affected by the disease, figuring as the most impacted family, and the cause of this disproportionate impact remains unclear (Scheele et al., 2019; Azat et al., 2022). Within bufonids, the genus *Melanophryniscus* (Redbelly toads) includes 31 species restricted to South America (Frost 2024). The primarily known threat to the Redbelly toads is loss of habitat quality (Brasil 2022; IUCN 2023) and many species exhibit restricted distributions or are microendemic, rendering them particularly susceptible to extinction (Brasil 2022; IUCN 2023). Additionally, populations of *Melanophryniscus* spp. can be significantly affected by the alterations of suitable climatic areas due to climate change (Zank et al., 2014; Oliveira et al., 2016), with projections indicating a reduction of more than 50% in suitable habitats for some species (Zank et al., 2014). Despite the significance of chytridiomycosis to amphibian declines, there is a knowledge gap concerning chytrid infection as a threat to the Redbelly toads. Consequently, the combined effects of climate change, the chytrid fungus, and other threats could worsen their survivorship on a short-term scale.

Historical amphibian declines in the southern Atlantic Forest (Toledo et al., 2023) were linked to chytridiomycosis (Carvalho et al., 2017; Scheele et al., 2019) and it is worth noting that most of the threatened Redbelly toad populations in Brazil are concentrated in this region (Toledo et al., 2021). Moreover, this area currently exhibits a widespread occurrence of *Bd* (James et al., 2015; Jenkinson et al., 2016; Preuss et al., 2016) and a predominant prevalence of Ranavirus (Ruggeri et al., 2019, 2023), another amphibian emerging infectious pathogen. Besides these, southern Brazil is also a region with a widespread occurrence of bullfrog farms (Ribeiro & Toledo, 2022), and the production of this alien species could have played an important role in increasing the spread of infectious pathogens to native hosts (Carvalho et al., 2017; Ribeiro et al., 2019; Ruggeri et al., 2019, 2023), due to their escapes and invasion into natural environments (Both et al., 2011; Forti et al., 2017). Consequently, there are numerous conservation concerns regarding the role of infectious diseases affecting threatened species within the southern Atlantic Forest.

Despite the link between chytrid infection and amphibians declines in Brazil (Carvalho et al., 2017), there remains a gap in studies concerning *Bd* presence in the threatened species and populations of *Melanophryniscus.* Emerging infectious pathogens dynamics are influenced by numerous environmental conditions. *Bd* is a waterborne fungal pathogen that

depends on water for its growth, survival, and dispersion (Berger et al., 2005), thus, being influenced by rainfall seasonality (Ruggeri et al., 2018). Also, environmental temperature plays a crucial role in *Bd* lifecycle, which usually prefers cooler temperatures (Piotrowski et al., 2004). Consequently, *Bd* exhibits higher prevalence in mountainous regions, where the prevalence is generally positively associated with elevation (Brem & Lips, 2008; Gründler et al., 2012), and where most of the population declines due to chytridiomycosis has been observed (Brem & Lips, 2008; Carvalho et al., 2017; Scheele et al., 2019).

The populations of *Melanophryniscus* spp. in Brazil display a distribution that spans diverse environments, ranging from coastal lowland to high-elevation environments encompassing various ecoregions (Griffith et al., 1998; Frost 2024). Furthermore, their reproductive microhabitat (*sensu* Nunes-de-Almeida et al., 2021) vary significantly. Some species lay eggs on phytotelmata (water-filled reservoirs in terrestrial or epiphytic plants) (e.g., Bornschein et al., 2015; Steinbach-Padilha, 2008), while others utilize temporary ponds or even temporary streams (e.g., Baldo et al., 2014; Toledo et al., 2021) as breeding site. In this context, *Melanophryniscus* spp. toads face a diverse range of microclimatic conditions, including variations in seasonal temperature and rainfall patterns. These traits make the species of this genus ideal models to understand how environmental factors may influence chytrid infection and its impacts on Redbelly toads populations.

In Brazil, out of the 22 congeneric species of *Melanophryniscus* (Segalla et al., 2021), eight are classified as threatened by the Brazilian red list (Brasil 2022), making this genus the one with the highest number of threatened species in the country (Brasil 2022; Anunciação et al., 2024). This underscores the urgency of understanding and addressing the consequences of pathogens in these bufonids, and the investigation of chytrid infection in *Melanophryniscus* spp. populations can guide the prioritization of effective conservation actions in South America (Azat et al., 2022). Although there are no records of mass mortalities in *Melanophryniscus* spp. populations, *Bd* infection has been associated with population declines without obvious mortalities in South America and Europe, through sublethal effects that diminish host fitness (Valenzuela-Sánchez et al., 2017; Palomar et al., 2023). These consequences extend beyond the immediate impacts and can affect long-term population dynamics and persistence (Valenzuela-Sánchez et al., 2017; Palomar et al., 2023). For this reason, studies assessing *Bd* infection in populations of this genus are essential to inform about populations health.

Given the importance of climatic variables in forecasting amphibian host-pathogen dynamics, we aimed to test whether local temperature, rainfall, elevation and ecoregions are

linked with *Bd* infection in *Melanophryniscus* spp. populations. Considering the climatic role in *Bd* infection, we hypothesized that temperature, rainfall and ecoregion will drive *Bd* prevalence and infection loads in Redbelly toads. Specifically, we expect that populations experiencing cooler temperatures (or ecoregions), higher rainfall levels, and situated in highlands will have higher *Bd* prevalence and infection loads. Additionally, some studies have shown that threatened amphibian species commonly experience stress-inducing conditions, such as lower bacteriome diversity (Greenspan et al., 2022), which can exacerbate *Bd* infection. Thus, as 8 out of 22 Redbelly Toads are threatened with extinction, we hypothesized that threatened *Melanophryniscus* species would exhibit higher *Bd* prevalence and infection loads.

Finally, we expect different *Bd* infection rates throughout *Melanophryniscus* spp. populations. Specifically, as phytotelmata provide a consistent water resource, enabling *Bd* to persist, we expect higher *Bd* rates in Redbelly toads associated with such breeding sites, potentially leading to diminished body condition due to *Bd* sublethal effects. Conversely, temporary ponds and streams may dry up, resulting in a scarcity of water to support viable *Bd* zoospores. Consequently, we expect lower *Bd* infection rates in *Melanophryniscus* associated with these environments.

Methods

Study populations and sites

We conducted field surveys in the southern portion of Brazil's Atlantic Forest, in the states of Paraná, Santa Catarina and Rio Grande do Sul, and in the southern portion of the midwest region, in the state of Mato Grosso do Sul, between September 2019 and November 2022. We collected skin swabs from post-metamorphic toads across eight *Melanophryniscus* species (4 threatened; 4 least concern; Table S1) from 10 localities, totaling 10 populations (Table 1; Figure 1). We captured each toad using new disposable latex gloves or plastic bags to avoid *Bd* cross-contamination. We applied skin swabs following standard swabbing protocols (Hyat el al., 2007). Each swab was stored in sterile plastic tubes at −20 °C until further processing.

Pathogen detection and quantification

To quantify the presence and infection loads of *Bd* in each sample, we extracted DNA from all skin swabs using PrepMan ULTRA® (Life Technologies) and then quantified infection loads using a Taqman® qPCR Assay (Life Technologies) with standards ranging from 10^{-1} to 10^{3} genomic equivalents of zoospores, hereafter referred as GE (Boyle et al., 2004).

Samples were run individually; negative controls and standards from 10^3 to 10^1 in duplicate; and the standards 10^0 to 10^{-1} in quadruplicate. The negative control consisted of distilled water. The qPCR was carried out using a final volume of 25 µL, containing 5 µl of the extracted DNA diluted in DNA- free water (1:10), 12.5 µl of TaqMan Master Mix (Applied Biosystems®), 3.75 µl of DNA free water, 1.25 µl of the probe ChytrMGB2 (5′-6FAM CGA GTCGAACAAAAT MGBNFQ-3′) (5 µm), 1.25 µl of the primer ITS1-3 Chytr (5′-CCTTGATATAATACA GTGTGCCATATGTC-3′) (18 µm), and 1.25 µl of the primer 5.8S Chytr (5′-AGCCAAGAGATCCGTTGT CAAA-3′) (18 µm). We considered samples to be *Bd*-positive (*Bd*$^+$) when the infection loads were ≥ 0.1 GE (Pontes et al., *in prep.*).

Abiotics factors and conservation status

We obtained data on maximum, minimum and mean temperature for the 15 days leading up to the sampling, and accumulated rainfall for 30 days prior to the sampling for each sampled site using the Visual Crossing Weather dataset. We used 15 d prior to sampling based on the Bd life cycle (i.e. this period is enough to allow at least 1 generation of *Bd*; Berger et al. 2005). We accessed the level III ecoregion of each site from Griffith et al. (1998) classification.

We obtained species conservation status using the Brazilian red list (Brasil 2022; Salve 2023). We classified conservation status as a binary variable with Least Concern species assigned a value of 0 and threatened (Vulnerable, Endangered, or Critically Endangered) species assigned a value of 1 (Table 1).

Statistical analysis

To explore the temperature data (mean maximum, mean minimum and mean temperature) that strongly predicted *Bd* prevalence and infection load, and reduce potential multicollinearity bias in downstream pruned models, we employed a model selection approach. For *Bd* prevalence, we fitted a Generalized Linear Mixed Models (GLMM) with binomial distribution and logit link function (lme4::glmer), and based on the Akaike Information Criterion (AIC) (Mazerolle, 2006), the mean maximum temperature was the best temperature predictor of *Bd* prevalence (Table S2). Additionally, we employed the same approach to access the temperature data that strongly predicted *Bd* infection load. For this, we fitted a Gaussian Generalized Linear Model (GLM) with a log link function, using *Bd* infection load (log10-transformed GE; only *Bd*$^+$ samples) as the response variable. Based on the AIC, mean minimum temperature was the best temperature predictor. A detailed description of these models can be found in Table S2.

To test for the potential effect of temperature, rainfall, elevation, ecoregion, conservation status, and species explaining *Bd* prevalence among the Redbelly toads, we fitted a GLMM with binomial distribution and logit link. We tested multicollinearity among predictor variables (car::vif) and included in the global model those variables presenting a low variance inflation factor (VIF < 4). Each logistic GLMM included the binary conservation status as a fixed factor and the year of sampling as a random factor. We included the mean maximum temperature of the 15 days prior to sampling date, accumulated monthly rainfall from the month prior to sampling date, elevation, ecoregion and conservation status as explanatory variables in each GLMM, and trimmed each model using a backward stepwise approach based on AIC. For model reductions, we ran models with all possible combinations of explanatory variables, and we sequentially removed non-significant variables with the highest P-values until delta AICc stopped dropping by the order of AICc = 2. For analysis of infection load, we fitted a Gaussian GLM with log link function, with *Bd* infection load (log10-transformed GE; only Bd^+ samples) as the response variable. We included the mean minimum temperatures of the 15 days prior to sampling date, accumulated monthly rainfall from the month prior to sampling date, elevation, ecoregion and conservation status as explanatory variables in each GLM. In this case, we used the function step (package 'stats') to find the model with the best AIC. A detailed description of these models can be found in Table S3.

We evaluated the significant association between the presence of *Bd* in each toad and the type of its breeding site (phytotelma, pond or stream, Figure 2) using a Pearson's chi-square test, followed by pairwise comparisons of proportions (chi-square tests with Holm correction). We also performed a Kruskal-Wallis test (due to the lack of normality of the data) followed by Dunn's post-hoc test with Bonferroni correction to assess variations in *Bd* infection loads across different breeding sites types.

Given the high *Bd* prevalence and infection loads observed in the endangered *M. biancae* (Figure S1), we conducted a Kruskal-Wallis test to assess whether toad-level infection loads were related to a specific *Melanophryniscus* spp. population. We further assessed the body condition of *M. biancae* toads by employing the scaled mass index (SMI) approach, based on standardized major axis regression between mass and snout−vent length (Peig & Green, 2009). To explore any significant differences in body condition between Bd^+ and Bd^- toads (explanatory categorical variable), we conducted a Student's t-test to establish whether the SMI of the Bd^+ toads differed from Bd^- toads. Finally, we performed a linear regression to test whether *Bd* infection loads are related to the SMI of Bd^+ adult males (n = 28). Females were

excluded from this analysis to minimize potential biases stemming from egg presence. All statistical analyses were performed using R version 2023.6.2 (R Core Team 2023).

Results

We sampled 658 swabs from post metamorphs toads, and the overall *Bd* prevalence in *Melanophryniscus* spp. populations was 17.93 % (118/658), and the mean infection loads in Bd^+ toads was 413 GE ± 1,341 sd, ranging from 0.11 to 9262 GE (Table 1). *Melanophryniscus biancae* exhibited the highest prevalence (Figure 1) and infection load, with a prevalence of 69 % (36/52), and the mean infection load of 1120 GE ± 2232 sd. (Table 1, Figure S1). Additionally, we did not detect *Bd* in four populations of different species (Table 1, Figure 1, Figure S1).

Our AIC logistic model-averaging revealed that 'threatened' conservation status (z = 1.845, $P < 0.001$; Figure 3A), cooler temperatures (z = -3.238, $P = 0.001$; Figure 3B), and higher elevation (z = 5.762, $P < 0.001$; Figure 3C), predicted higher *Bd* prevalence in *Melanophryniscus* spp. (Figure 3, Table 2). The most parsimonious GLM model explaining *Bd* infection load only included conservation status and elevation. However, only elevation had a positive effect on *Bd* infection load in *Melanophryniscus* spp. (z = 4.479, $P = < 0.001$; Figure 3D).

The chytrid prevalence significantly differed by the type of breeding site (χ^2 = 15.117, $P < 0.001$; Figure 4A, Table S4), with phytotelma having the higher prevalence compared to both ponds and streams breeders (pairwise chi-square test: $P = 0.001$ and $P = 0.005$, respectively; Figure 4A), which did not differ between each other (pairwise chi-square test, $P = 0.795$; Figure 4A). Additionally, the mean ranks of *Bd* infection loads significantly differed among breeding site types (χ^2 = 15.989, $P < 0.001$; Figure 4B, Table S4) and phytotelma-related individuals had higher *Bd* infection load when compared to pond (z = 3.954, $P < 0.001$; Figure 4B) and stream breeders (z = 2.403, $P = 0.024$; Figure 4B). No difference was found between pond and stream breeders (z = -0.616, $P = 0.807$, Figure 4B).

Batrachochytrium dendrobatidis infection loads differed among *Melanophryniscus* spp. populations (χ^2 = 20.362, $P = 0.001$, Figure S1), and *M. biancae* infection loads were different only from *M. admirabilis* (z = - 4.173, $P < 0.001$, Figure S1).

No significant differences were observed in SMI between Bd^+ and Bd^- toads of *M. biancae* (*t-value* = -1.101, $P = 0.289$), and we did not find correlation between *Bd* infection loads and toad's SMI (*t-value* = -0.75, $P = 0.46$; Figure 5).

Discussion

For the first time, we detected *Bd* infection in threatened Brazilian Redbelly toads, showcasing varying levels of prevalence and infection loads. The Redbelly toads exhibited lower *Bd* prevalence compared to the general pattern in the Atlantic Forest (Azat et al., 2022; Carvalho et al., 2017). Notably, the endangered *M. biancae* from Serra do Araçatuba showed a high unexpected *Bd* prevalence of 69.23 %.

Threatened Redbelly toads populations were more likely to exhibit higher *Bd* prevalence than non-threatened ones. *Melanophryniscus* spp. populations more vulnerable to extinction share certain biological traits, including limited geographic distributions (Di-Bernardo et al., 2006; Peloso et al., 2012), poor dispersal ability (Santos et al., 2010; Pereira & Maneyro, 2016) and high site fidelity (Di-Bernardo et al., 2006; Pereira & Maneyro, 2016). These traits related to site fidelity along with other stressors such as environmental disturbances, climate change, and pollution, may collectively influence Bd persistence in the environment and difficult amphibians' ability to avoid infection. Additionally, an important defense mechanism is through the skin bacteriome, which can produce secondary metabolites with antifungal properties (Heard et al. 2013; Woodhams et al., 2018). However, a recent study revealed that threatened amphibians exhibit lower skin bacteriome diversity, and the microbiome diversity was negatively correlated with host conservation status (Greenspan et al., 2022). Hence, all these traits may influence how threatened Redbelly toads cope with *Bd*.

It is important to note that we found an overall low infection load throughout the genus, and we did not find host conservation status as an important factor influencing *Bd* infection loads. This pattern of consistently low *Bd* infection loads in Redbelly toads (Slys et al. 2007; Agostini et al., 2015; Pontes et al., 2021), suggests that these toads may possess mechanisms to limit the intensity of *Bd* infection, a property of amphibians resistant to chytrid (Grogan et al., 2023). Nevertheless, accurately assessing toads levels of *Bd* resistance solely based on field data remains challenging in the absence of controlled laboratory experiments (Grogan et al., 2023).

Our results showed that temperature and elevation were positively associated with *Bd* prevalence in Redbelly toads. Cooler temperatures together with higher tropical elevation can provide better conditions for *Bd* growth (Piotrowski et al., 2004; Voyles et al., 2017; Muletz-Wolz et al., 2019). Elevation consistently correlates positively with *Bd* prevalence in Brazil (Gründler et al., 2012; Carvalho et al., 2017, 2024), and as we predicted, it was positively associated with *Bd* prevalence in *Melanophryniscus* spp. However, our findings revealed no

correlation between temperature and *Bd* infection loads, suggesting that in this case, temperature is more likely associated with the spread of *Bd* rather than its infection loads.

Although in our analysis temperature was not a significant predictor of *Bd* infection loads in *Melanophryniscus* spp., *Bd* infection load was positively associated with sites at higher elevations. The observed positive correlation may be attributed to the ability of *Bd* to lower the upper thermal tolerance of hosts (Greenspan et al., 2017; Neely et al., 2020). Consequently, cool-adapted Redbelly toads could be more sensitive to *Bd* under warming periods, and despite we did not find a correlation between temperature and infection loads, this could be attributed to potential sampling biases, as toads were not uniformly sampled across seasons. Notably, high- elevation environments are experiencing more rapid changes in temperature than environments at lower elevations, with projections indicating an increase in frequency and intensity of climatic anomaly in response to global warming (Sillmann et al., 2013). Temperature anomaly can affect *Bd* prevalence and increase the infection in high-altitude cool-adapted hosts from the Atlantic Forest during warmer years (Carvalho et al., 2024). Therefore, climate change poses a significant threat to *Melanophryniscus* spp., not only because it can cause a reduction in habitat suitability area (Zank et al., 2014; Oliveira et al., 2016) but also because can exacerbate the susceptibility of cool-adapted toads to *Bd* infection during warming periods. The interplay between *Bd* infection and warming may lead to mortality in cool-adapted toads, even at lower infection loads (Neely et al., 2020). For this reason, even in the absence of mortality, it is important to monitor chytrid infection in threatened populations closely.

The high *Bd* prevalence and infection loads observed in *M. biancae*, specie associated with phytotelma, may be linked to the small volume of water within the plant reservoir. Phytotelma systems provide suitable environmental conditions for long-term persistence *Bd* due to the humid and buffered microhabitat. This restricted water volume can amplify the frequency of contact rate among toads (Ruano-Fajardo et al., 2016), and the interaction between *Bd* zoospores and hosts. Chytrid was more prevalent in amphibians associated with phytotelmata in Neotropical regions (Rodriguez et al., 2014; Ruano-Fajardo et al., 2016; Zumbaldo-Ulate et al., 2019), conversely, in this study, *Bd* was not detected in the other two phytotelma-associated *Melanophryniscus* spp.. This suggests that other factors, such as water pH, temperature, or even arthropods diversity (Greenspan et al., 2019) can regulate the environmental zoospore source in the phytotelma microenvironment and influence *Bd* presence in these species.

The high *Bd* prevalence and infection loads found in the endangered *M. biancae* are of special concern given the multiple threats confronting the Serra do Araçatuba region. Among

these threats are the fast and intense invasion of exotic *Pinus* spp., and the frequent burning for livestock grazing, which poses a direct risk to the plants of the Eriocaulaceae family, the one used as breeding site by *M. biancae* (Bornschein et al., 2015; Nadaline et al., 2019). Consequently, these threats, combined with emerging infectious pathogens, pose an eminent risk to the survival of this population. Only two populations of *M. biancae* are known, and considering the threats faced by this toad, we advocate monitoring chytrid and other pathogens in both populations. Therefore, even in the absence of obvious body detriments, efforts to monitor *Bd* in amphibian hosts at the brink of extinction, such as *M. biancae* are recommended, primarily to assess the potential impacts of the chytrid on this species. However, it is important not to overlook species or populations showing low prevalence or those not yet diagnosed with *Bd*, as some may be driven to extinction by chytridiomycosis even in the absence of mass mortalities (Valenzuela-Sánchez et al., 2017; Azat et al., 2022).

Amphibian populations can be affected differently by the same combination of stressors, and susceptibility to these stressors depends on numerous factors. Populations of Redbelly toads may be affected by climate changes in different ways (Zank et al., 2014), and planning conservation efforts such as monitoring emerging infectious pathogens should be conducted on the populational level (Zank et al., 2014). Chytridiomycosis is indeed an important disease linked with amphibian population declines in Brazil (Carvalho et al., 2017). Besides this, many *Melanophryniscus* species are known by one or a few populations only. Thus, identifying *Bd* positive populations, toads' health, and monitoring the dynamic of this pathogen in threatened species remains important. Finally, we believe that conservation strategies for the Redbelly toads should not only focus on protecting their environment but also address specific threats and vulnerabilities faced by populations and their reproductive microhabitats.

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Ethics approval

All applicable institutional and national guidelines for the care and use of animals were followed. This work was conducted under permits by Instituto Chico Mendes de Conservação da Biodiversidade (SISBio #72718, # 79490/4, #79836/3, #79836/4, #49080-5), Instituto Água e Terra (#51.19, #32.21), Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SisGen #A3D44D1). This work was approved by Unicamp Animal Care and Ethics Committee (CEUA #5581-1/2020).

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Tables

Table 1. *Batrachochytrium dendrobatidis* (*Bd*) prevalence and infection load in *Melanophryniscus* spp. populations. *Bd* prevalence is the proportion of infected toads out of the total number of toads sampled. *Bd* infection load is represented by the mean and standard deviation (sd) of genomic equivalents of zoospores (GE) (Bd^+ toads). Total refers to the general *Bd* prevalence and mean of infection load and sd.

Species	Conservation status	*Bd* prevalence (Bd^+/n)	*Bd* infection load	Site (city, state)
M. admirabilis	CR	15.92 % (54/339)	73.58 ± 293.36	Arvorezinha, RS
M. biancae	EN	69.23 % (36/52)	1120.63 ± 2232.34	Tijucas do Sul, PR
M. macrogranulosus	EN	5.71 % (2/35)	2.16 ± 1.02	Dom Pedro de Alcântara, RS
M. dorsalis	VU	38.09 % (8/21)	63.07 ± 145.83	Imbituba, SC
		0 % (0/20)	-	Rio Grande, RS
M. alipioi	LC	0 % (0/36)	-	Campina Grande do Sul, PR
M. devincenzii	LC	22.80 % (13/57)	290.77 ± 631.87	Arvorezinha, RS
		10.20 % (5/49)	15.07 ± 29.67	Sertão, RS
M. fulvoguttatus	LC	0 % (0/18)	-	Bela Vista, MS
M. vilavelhensis	LC	0 % (0/31)	-	Ponta Grossa, PR
	Total	**17.93 % (118/658)**	**412.55 ± 1341.23**	

Table 2. Outcomes from linear models testing the effects of each predictor variable on *Batrachochytrium dendrobatidis* (*Bd*) (GLMM) and infection load (GLMM) in *Melanophryniscus* spp. Statistically significant predictor variables are highlighted in bold.

Predictors	Estimate	*SE*	*z*	*P*
Bd prevalence				
Intercept	**-1.434**	**0.777**	**-1.845**	**0.065**
Status	**1.398**	**0.301**	**4.638**	**< 0.001**
Temperature	**-0.098**	**0.030**	**-3.238**	**0.001**
Elevation	**0.001**	**0.000**	**5.762**	**< 0.001**
Infection load				
Intercept	**1.418**	**0.231**	**6.132**	**<0.001**
Status	-0.154	0.252	-0.613	0.540
Elevation	**0.407**	**0.090**	**4.479**	**<0.001**

The *Bd* prevalence model included host conservation status (threatened or non-threatened) (Status), mean maximum temperatures of the 15 days prior to sampling date (Temperature), elevation (Elevation), and year as fixed factor. The *Bd* infection load model included host conservation status (threatened or non-threatened) (Status) and elevation (Elevation).

Figures captions

Figure 1. Spatial distribution of *Batrachochytrium dendrobatidis* (*Bd*) in *Melanophryniscus* spp. populations along Brazilian midwest and southern region. Pie graphs represent *Bd* prevalence in each infected population. An insert map of South America highlights (shaded in green), from north to south, the states of Mato Grosso do Sul (MS), Paraná (PR), Santa Catarina (SC) and Rio Grande do Sul (RS) where samples were collected. The populations sampled were: *Melanophryniscus fulvoguttatus* from Bela Vista (1), MS; *M. vilavelhensis* from Ponta Grossa (2), *M. alipioi* from Campina Grande do Sul (3), and *M. biancae* from Tijucas do Sul (4), PR; *M. dorsalis* from Imbituba (5), SC; *M. macrogranulosus* from Dom Pedro de Alcântara (6), *M. devincenzii* from Sertão (7) and from Arvorezinha (8), *M. admirabilis* from Arvorezinha (9), and *M. dorsalis* from Rio Grande (10), RS.

Figure 2. Breeding sites used by *Melanophryniscus* spp. *Melanophryniscus cambaraensis* in amplexus within a temporary stream (A). *Melanophryniscus biancae* in amplexus within a phytotelma plant (B). *Melanophryniscus admirabilis* within a temporary pond (C). Temporary stream used by *M. macrogranulosus* as a breeding site, formed after rainfall (D). *Paepalanthus planifolius* (Eriocaulaceae) plant used by *M. biancae* as a breeding site (E). Temporary pond used by *M. admirabilis* as a breeding site (F). An overview of the high-altitude grassland environment of *M. biancae* (G), and overview of temporary ponds forming on flat rock outcrops environment (H).

Figure 3. Proportion of non-threatened and threatened *Melanophryniscus* spp. infected with *Batrachochytrium dendrobatidis* (*Bd*) and proportion of genomic equivalents of zoospores (GE) (a). Visualization of the Generalized Linear Mixed Model (GLMM) fit for *Bd* prevalence and mean maximum temperature (ºC) (b), and elevation (m) (c). Visualization of the Generalized Linear Model (GLM) fit for *Bd* infection loads and elevation (m) (d).

Figure 4. *Batrachochytrium dendrobatidis* (*Bd*) prevalence in *Melanophryniscus* spp. by types of breeding site (a). Boxplot of *Bd* infection loads (log of genomic equivalents of zoospores; GE) between types of breeding sites (b). An adult of *M. macrogranulosus* was inserted in the graph. Upper and lower parts of the boxplot represent the 75th and 25th percentiles, respectively; the horizontal line in the center of the box is the median; the limits of the standard error bar connect the maximum and minimum values within the interquartile range.

Figure 5. *Batrachochytrium dendrobatidis* (*Bd*) infection loads (genomic equivalents of zoospores; GE) in males of the endangered *Melanophryniscus biancae* and the respective individuals body condition. SMI: scaled mass index, *t-value* = -0.75, $P = 0.46$. An adult of *M. biancae* was inserted in the graph.

Figures

Figure 1

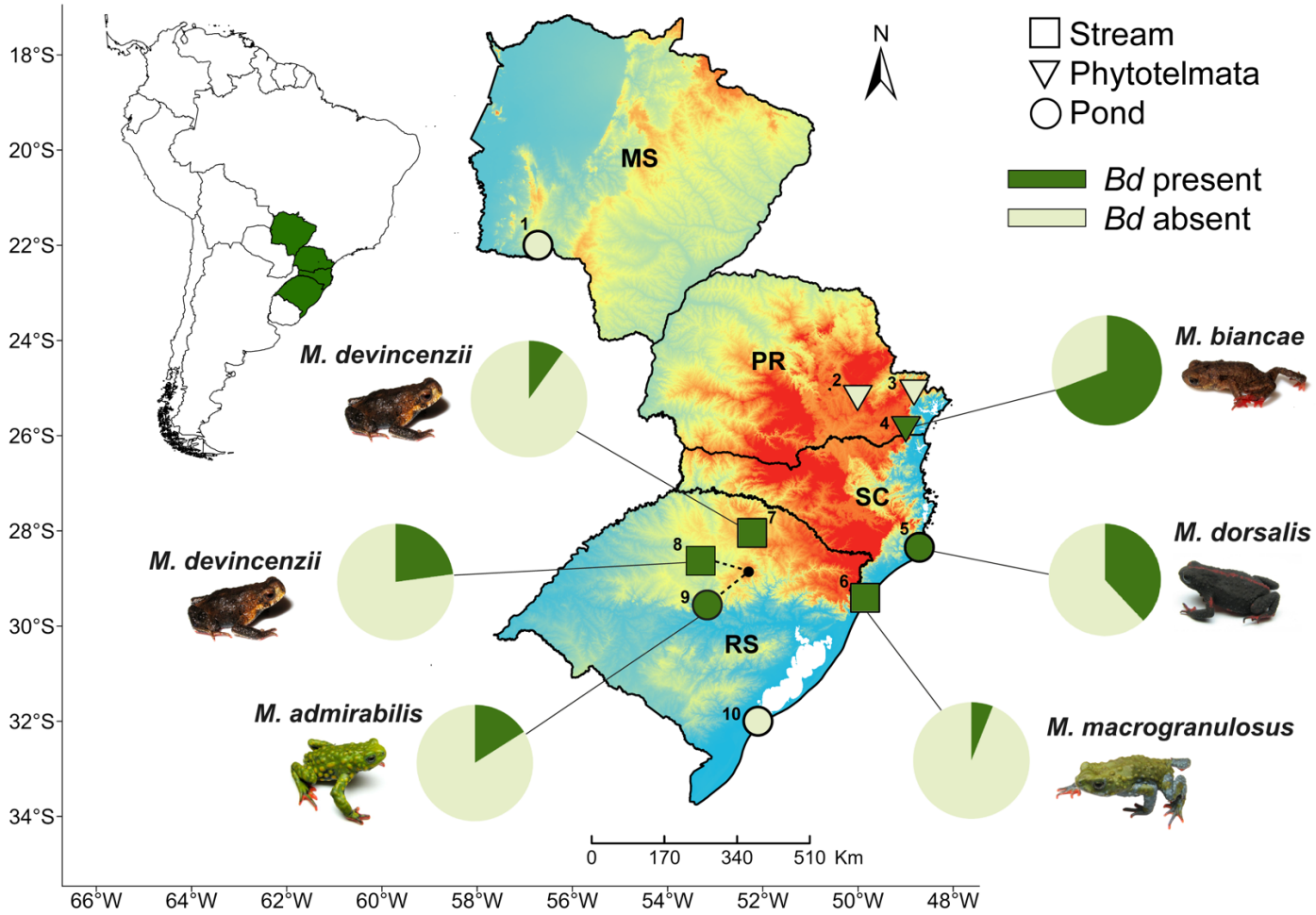


Figure 2

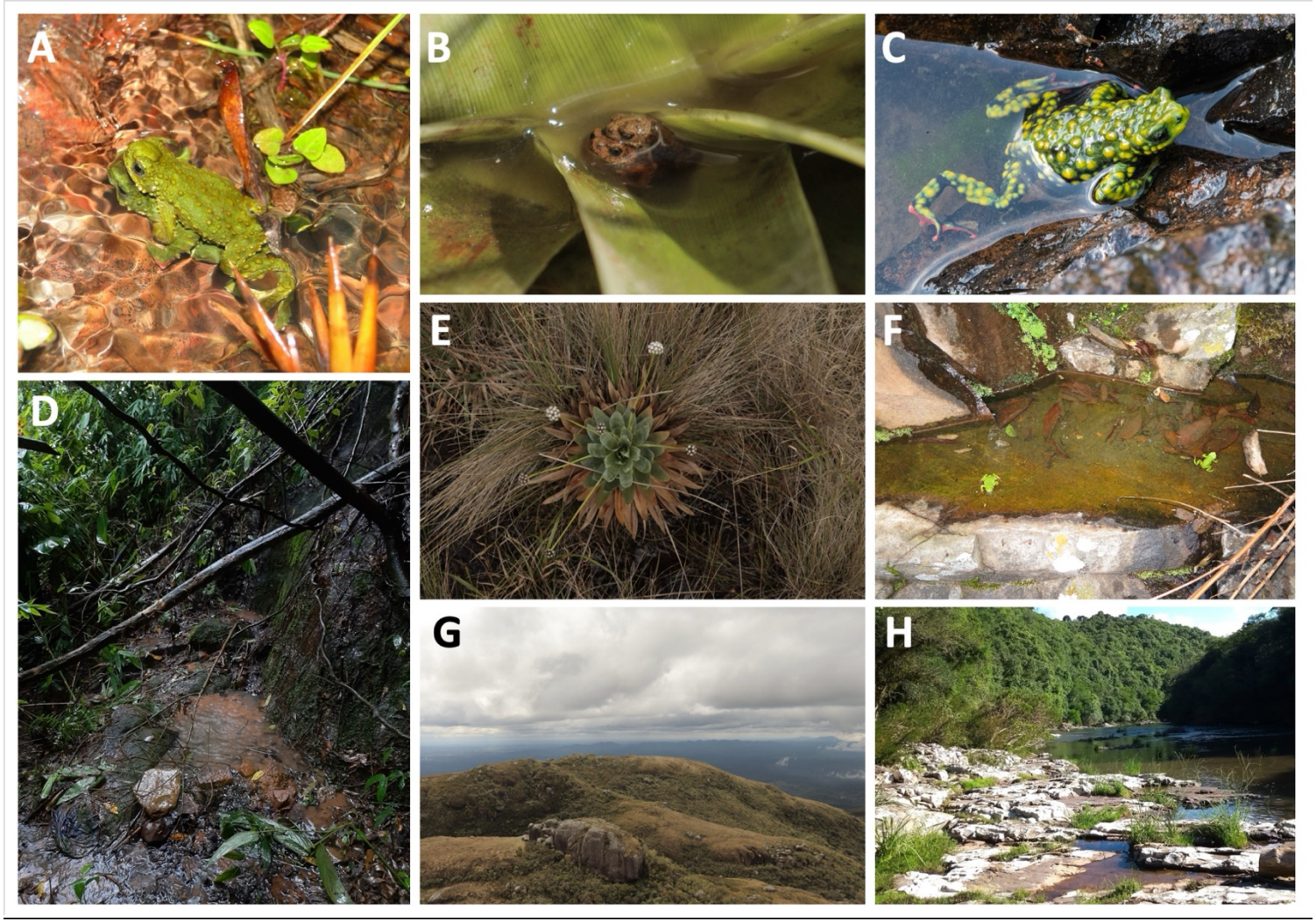


Figure 3

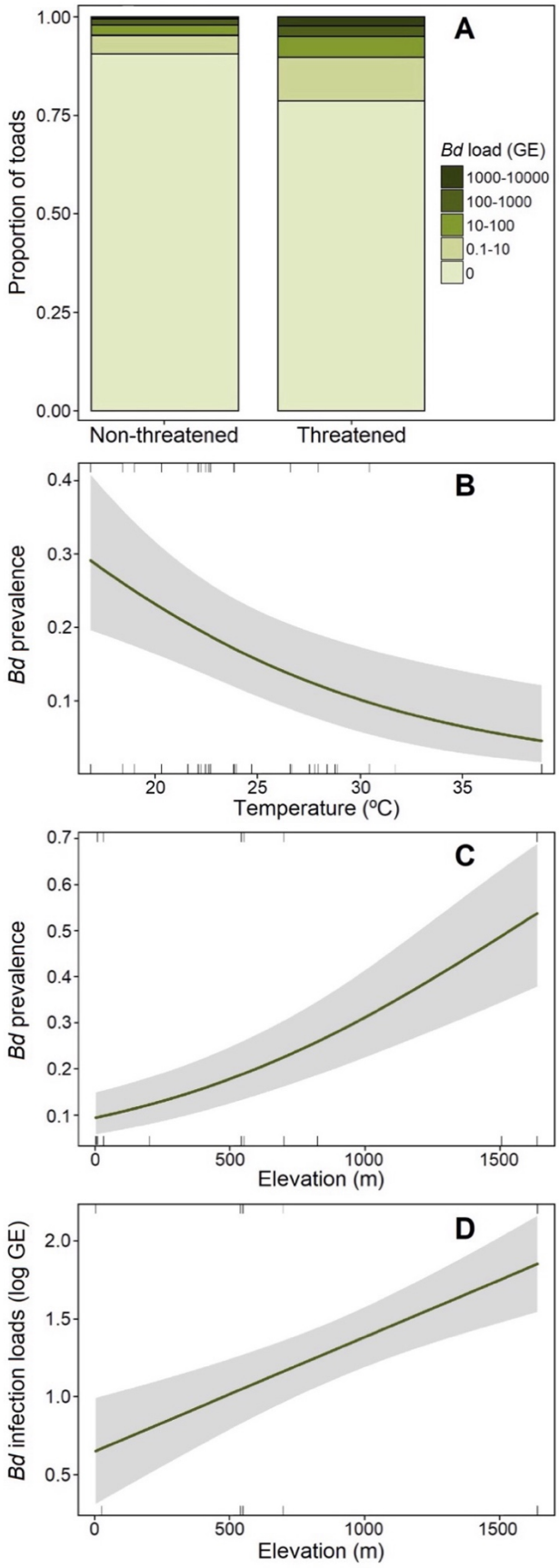

A
Proportion of toads
1.00
0.75
0.50
0.25
0.00
Bd load (GE)
1000-10000
100-1000
10-100
0.1-10
0
Non-threatened
Threatened
B
Bd prevalence
0.4
0.3
0.2
0.1
20
25
30
35
Temperature (ºC)
C
Bd prevalence
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
500
1000
1500
Elevation (m)
D
Bd infection loads (log GE)
2.0
1.5
1.0
0.5
0
500
1000
1500
Elevation (m)

Figure 4

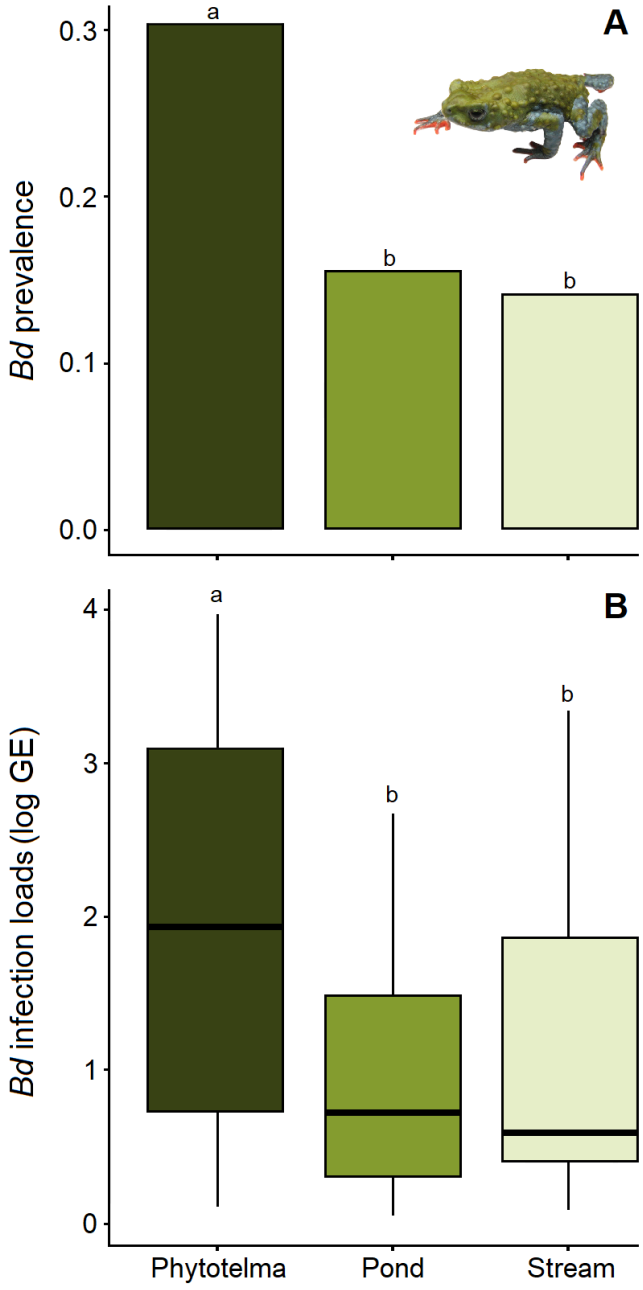
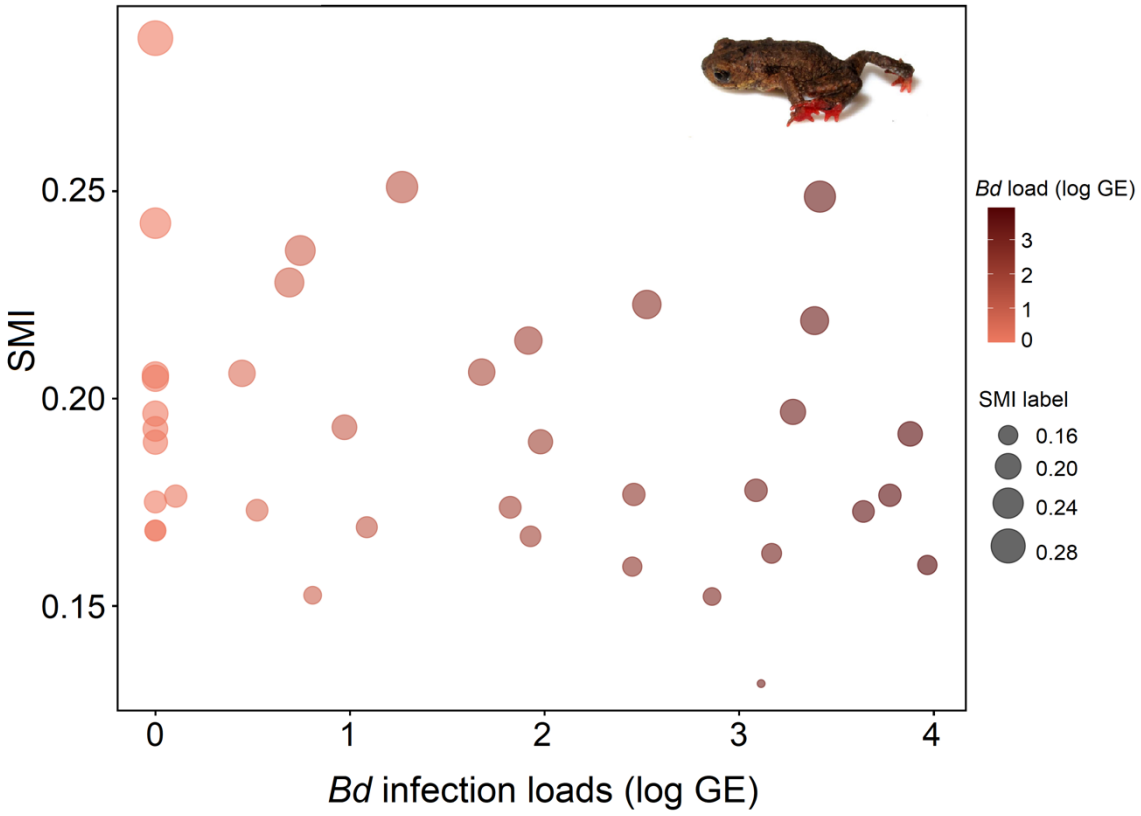

A
B
Bd prevalence
Bd infection loads (log GE)
0.0
0.1
0.2
0.3
0
1
2
3
4
a
b
b
a
b
b
Phytotelma
Pond
Stream

Figure 5


SMI
0.25
0.20
0.15
0
1
2
3
4
Bd infection loads (log GE)
Bd load (log GE)
3
2
1
0
SMI label
0.16
0.20
0.24
0.28

Supplementary material

Climatic and conservation status influence on chytrid infection in Redbelly toads

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Table S1. Classification of *Melanophryniscus* species according to the Brazilian red list (Salve 2023). Threatened species were categorized based on their geographic distribution and categorized under criteria B1, related to Extent of occurrence (EOO). All EOO were considered severely fragmented or less than 10, 5 or 1 number of locations (a). Additionally, they were considered with a continuing decline observed, estimated, inferred or projected (b) in area, extent and/or quality of habitat (iii). Only *M. admirabilis* was also classified into criteria related to area of occupancy (AOO) (criteria B2). Abbreviations for categories are CR = Critically endangered; EN = Endangered; VU =Vulnerable; LC = Least concern. Data on EOO not available on Salve was accesses from ICMBio (ICMBio 2024).

Species	Conservation status	Criteria	EOO	AOO	Number of locations
M. admirabilis	CR	B1ab(iii)+2ab(iii)	≤ 1 km^2	≤ 1 km^2	= 1
M. biancae	EN	B1ab(iii)	≤ 16 km^2	-	≤ 5
M. macrogranulosus	EN	B1ab(iii)	≤ 670 km^2	-	≤ 5
M. dorsalis	VU	B1ab(iii)	≤ 16 km^2	-	≤ 10
M. alipioi	LC	-	≤ 8 km^2	-	-
M. devincenzii	LC	-	≤ 80.000 km^2	-	-
M. fulvoguttatus	LC	-	-	-	≤ 10
M. vilavelhensis	LC	-	≤ 80 km^2	-	≤ 5

Salve. (2023). Sistema de Avaliação do Risco de Extinção da Biodiversidade. Retrieved from https://salve.icmbio.gov.br/#/. Accessed February 20, 2024.
ICMBio, 2024. Sistema de Avaliação do Risco de Extinção da Biodiversidade - SALVE. Data not published. Accessed May 07, 2024.

Table S2. Model selection for minimum, maximum and mean temperature of the 15 days prior to sampling date influencing *Batrachochytrium dendrobatidis* (*Bd*) prevalence and infection load in *Melanophryniscus* spp. populations in Brazil.

Model	AIC
Prevalence	
MaxTemp	558.5
MinTemp	561.8
MeanTemp	613.7
Infection load	
MinTemp	340.4
MeanTemp	344.5
MaxTemp	349.4

To screen for the strongest temperature that potentially predict *Bd* prevalence, we employed a model selection approach based on Generalized Linear Mixed Models (GLMM) with binomial distribution and logit link function. We included year as random factor and tested three temperatures: mean maximum (MaxTemp), mean minimum (MinTemp) and mean temperature (MeanTemp) of the 15 days prior to sampling date as fixed variables. We additionally employed Gaussian GLMs with a log link function (using log10-transformed GE of Bd^{+} samples) as the response variable to find the strongest temperature that potentially predict *Bd* infection loads. The fixed variables included the mean maximum, mean minimum, and mean temperature of the 15 days prior to sampling date. In the *Bd* prevalence model, the best data for temperature was the maximum temperature. For the *Bd* infection load model, the best temperature dataset was the mean minimum temperature.

Table S3. Model selection for variables influencing *Batrachochytrium dendrobatidis* (*Bd*) prevalence and infection load in *Melanophryniscus* spp. populations.

Model	AIC	No. of variables	X^2
Prevalence			
Status, MaxTemp, Ele	555.8	3	7.295
Status, MaxTemp, Rain, Ele	557.5	4	7.0432
Status, Eco, MaxTemp, Rain, Ele	558.5	5	7.0432
Infection load			
Status, Ecoregion, Ele	333.1	3	0.018
Status, Ele	334.8	2	5.724
Status, Eco, MinTemp, Ele	335	4	0.004
Status, Eco, MinTemp, Rain, Ele	337.0	5	0.004

We compared all possible models using Akaike Information Criterion (AIC); we are reporting the three best models for *Bd* prevalence and the four best models for infection loads. The best predictors of *Bd* prevalence models were: host conservation status (Status), mean maximum temperatures of the 15 days prior to sampling date (MaxTemp), and elevation (Ele). The best predictors for *Bd* infection loads models were: host conservation status (Status) and elevation (Ele).

Table S4. *Batrachochytrium dendrobatidis* (*Bd*) prevalence and infection load in *Melanophryniscus* spp. individuals for in each breeding site category. *Bd* prevalence is the proportion of infected toads out of the total number of toads sampled. *Bd* infection load is represented by the mean ± standard deviation (sd) of genomic equivalents of zoospore. Only Bd^+ toads were considered.

***Bd* prevalence (Bd^+/n)** *Chi-square: $\chi^2 = 15.117$; $P < 0.001$*		***Bd* infection load** *Kruskal-Wallis: $\chi^2 = 15.989$; $P < 0.001$*
Breeding site	**Prevalence**	**Infection load**
Phytotelma	30.25 % (36/119)	1120.63 ± 372.05
Pond	15.57 % (62/398)	72.23 ± 35.29
Stream	14.18 % (20/141)	192.99 ± 116.41

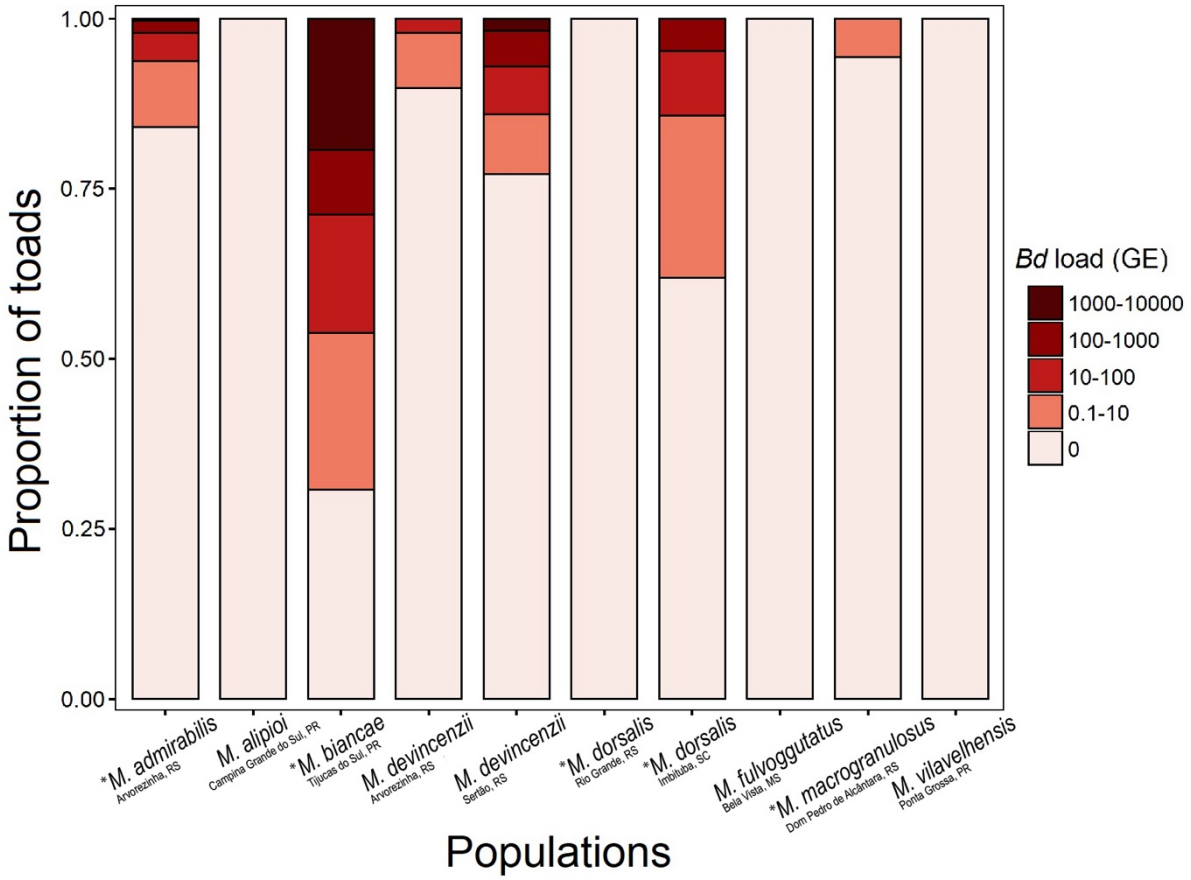

Figure S1. *Batrachochytrium dendrobatidis* (*Bd*) prevalence in *Melanophryniscus* spp. populations and proportion of genomic equivalents of zoospores (GE). Municipally of each sampled population and Brazilian state abbreviation (RS = Rio Grande do Sul; SC = Santa Catarina; PR = Paraná) is presented. Asterisk indicates threatened species.

CHAPTER II

CLIMATIC DRIVERS OF CHYTRID PREVALENCE IN THE CRITICALLY ENDANGERED ADMIRABLE REDBELLY TOAD

Determinantes climáticos da prevalência de quitrídio no Sapinho-admirável-de-barriga-vermelha

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This chapter has been submitted to the journal *Biodiversity and Conservation* and adheres to its guidelines.

Climatic drivers of chytrid prevalence in the critically endangered Admirable Redbelly Toad

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Abstract

Global warming is driving shifts in rainfall and temperature patterns, and projections indicate an increase in frequency and intensity of climate anomalies. These changes influence wildlife disease dynamics, affecting pathogen development, host behavior, physiology, and disease susceptibility. Understanding the intricate interplay between climatic anomalies and emerging pathogens in amphibian is essential to inform conservation efforts targeted towards this highly threatened vertebrate group. In-situ research is recommended as a conservation action by the *International Union for Conservation of Nature* (IUCN 2023*)* for the microendemic and Critically Endangered amphibian *Melanophryniscus admirabilis* (Admirable Redbelly Toad). We therefore investigated the seasonal climatic fluctuations and climatic anomalies affecting infections by the waterborne chytrid *Batrachochytrium dendrobatidis* (*Bd*) in this sole surviving population, which holds significant conservation concern. We found links between high *Bd* prevalence, monthly low rainfall and rainfall deficit. Additionally, an increase in *Bd* prevalence was associated with temperatures exceeding historical averages. These findings suggests that climatic anomalies play a crucial role in *Bd* transmission and infection status among toads, probably due to their aggregation behavior in few available pools during drier and warmer periods. Despite the current low prevalence of *Bd* and infection loads, the projected escalation of climatic anomalies might render *M. admirabilis* uniquely susceptible to synergistic interactions between *Bd* and extreme climatic conditions. The insights gained from this study can improve the conservation efforts and underscore the intricate relationship between climatic anomalies and chytrid infection, shedding light on potential vulnerabilities within threatened amphibian populations.

Keys-words: Atlantic Forest, *Batrachochytrium dendrobatidis*, Brazil, Climate change, Conservation, *Melanophryniscus admirabilis*.

Introduction

The modifications resulting from the expansion of human populations have led to an increased frequency and intensity of climatic extremes (Seneviratne et al. 2021). Rainfall and temperature patterns have globally shifted in response to global warming (Seneviratne et al. 2021), and climatic anomalies are projected to increase in frequency and intensity (Sillmann et al. 2013). Climatic anomalies can alter entire ecosystems (Frank et al. 2015; Bardgett and Caruso, 2020), extending their impact also over wildlife disease dynamics (Cohen et al. 2020; Greenspan et al. 2020; Rohr and Cohen, 2020). This intricate interplay occurs through a wide range of mechanisms, encompassing shifts in pathogen development (Nnadi and Carter, 2021), and alteration in host behavior and physiological traits (Staudinger et al. 2013; Bozinovic and Pörtner, 2015) that often lead to increased susceptibility to diseases (Bradley et al. 2019).

Wildlife disease dynamics are influenced by micro and macro-climatic variables (Harvell et al. 2003; Boser et al. 2021), often involving synergistic interactions between pathogens and their hosts (Rohr and Raffel, 2010; Altizer et al. 2013). The waterborne fungal pathogen *Batrachochytrium dendrobatidis* (hereafter *Bd*) causes the disease chytridiomycosis in amphibians and has been implicated in population declines and extinctions globally (Scheele et al. 2019). Moreover, alterations in temperature and rainfall patterns are known to impact the onset of breeding phenology in amphibians (Dalpasso et al. 2023). Amphibian *Bd* infection could be also influenced by seasonal temperature and rainfall patterns (Ruggeri et al. 2018; Turner et al. 2021). Consequently, accelerated climate variability could synergistically drive amphibians to the extinction path, as amphibians are already the most endangered vertebrate taxon according to the recent assessment led by the International Union for Conservation of Nature (IUCN 2023; Luedtke et al. 2023). The *Bd*-amphibian system thus provides an opportunity to investigate the impact of climate anomalies on disease dynamics in formally endangered species, such as the Admirable Redbelly Toad, *Melanophryniscus admirabilis* (Bufonidae), from southern Brazil, a region with widespread occurrence of *Bd*. This microendemic Neotropical species is classified as Critically Endangered (CR) by the IUCN redlist of threatened species (IUCN 2023) and by the Brazilian redlist (Brasil 2022). This species is included into public conservation policies of Brazil and has become a global success story and symbol of hope for amphibian conservation (Fonte et al. 2022). Additionally, chytridiomycosis outbreaks were classified as the greatest concern among the ten direct threats to the species (Abadie et al. unpublished results), further highlighting the urgency of understanding and addressing the intricate interplay between climate, disease, and the conservation of *M. admirabilis* (IUCN 2023).

Melanophryniscus admirabilis uses temporary small pools filled by rainfall as vocalization and breeding site (Di-Bernardo et al. 2006). Similarly, *Bd* depends on water for its growth, survival, and dispersion (Berger et al. 2005), although it can be transported by other non-amphibian vectors (*e.g.*, Pontes et al. 2018; Toledo et al. 2021; Prado et al. 2023). Therefore, rainfall and their anomalies could significantly impact both the reproductive biology of *M. admirabilis* and pathogen infection dynamic (Ruggeri et al. 2018; Moura-Campos et al. 2021). Temperature also plays an important role in successful reproduction of the Admirable Redbelly Toad, since this species depends on the duration of shallow pools, which can quickly evaporate under high temperatures (Abadie et al. unpublished results). Additionally, temperature can also influence *Bd* lifecycle, and despite *Bd* exhibiting varying thermal tolerance (Voyles et al. 2017), temperature can induce alterations in *Bd* life history (Woodhams et al. 2008; Muletz-Wolz et al. 2019) and play a major role in determining the *Bd* infection loads in hosts (Bielby et al. 2022). Amphibians demonstrate increased susceptibility to chytridiomycosis when experiencing anomalies in environmental temperatures (Clare et al. 2016; Cohen et al. 2019a; Serrano et al. 2022). Furthermore, the ability of hosts to mount innate immune responses against *Bd* can be considerably impaired by ambient temperature modification (Grogan et al. 2018). Hence, seasonal and historical variation in temperature and rainfall are recognized as relevant environmental variables likely influencing *Bd* infection dynamics in *M. admirabilis.*

Alternatively, the environmental context can benefit amphibians in mitigating *Bd* infections (Karavlan and Venesky, 2016). For instance, *Bd* growth is significantly suppressed at temperatures above 25 °C (Piotrowski et al. 2004; Stevenson et al. 2013), thus, predicted increases in average global temperatures could suppress pathogen growth in amphibians' skin. However, the interaction of *Bd* infection and warming could lead to an increase in mortality of cool-adapted host species at higher temperatures, despite lower infection loads (Neely et al. 2020). This suggests that even when temperatures approach the upper thermal limit of the pathogen, *Bd* infection may cause declines in cool-adapted montane frogs due to the combined pressures of pathogen infection and warming-related stress (Neely et al. 2020). Specifically, *Bd* infection can lead to host stress (Bielby et al. 2015), with downstream impacts on host body condition and fitness (Campbell et al. 2019; Pontes et al. 2021; Bosch et al. 2023). *Bd* infection has also been linked to population declines in wild amphibians through sublethal effects that reduce host fitness (Chatfield et al. 2013; Valenzuela-Sánchez et al. 2017; Brannelly et al. 2018; Palomar et al. 2023), extending beyond immediate consequences to affect long-term population dynamics and persistence (Valenzuela-Sánchez et al. 2017; Palomar et al. 2023). Understanding these broader consequences and *Bd* infection dynamics is vital for effective conservation

strategies for the Critically Endangered Admirable Redbelly Toad, particularly in light of its higher vulnerability to extinction due the stochastic events (Fonte et al. 2022).

Considering the pivotal role of climatic variables in predicting amphibian host-pathogen dynamics, we aimed to tested whether the local monthly climatic fluctuations and climatic anomalies would induce changes in *Bd* dynamics in *M. admirabilis*. As this toad depends on temporary pools to breed, we hypothesized that seasonal fluctuations in temperature and rainfall impact the *Bd* dynamics, and we expect that periods with higher temperatures and low rainfall will result in higher *Bd* prevalence and infection loads. Additionally, we hypothesized that the *M. admirabilis* population will experience higher *Bd* prevalence in periods with rainfall deficit, as the scarcity of pools would lead them to aggregate. Warming can reduce host immune capacity due to heat-induced stress (Cohen et al. 2019b; Neely et al. 2020), and may also contribute to the drying up of pools. Consequently, we expect higher *Bd* prevalence and infection loads in period when temperature exceeds the historical average. Finally, considering sublethal effects caused by *Bd* infection (Valenzuela-Sánchez et al. 2017; Palomar et al. 2023; Wu, 2023), we expect that *Bd* infection status has consequences on host body condition. Combined, our goals will allow us to elucidate the disease dynamics of a critically endangered micro-endemic tropical species.

Methods

Study site and field sampling

The only know site for *M. admirabilis* is located in municipality of Arvorezinha, State of Rio Grande do Sul, Brazil (52°18'W, 28°51'S), in the Southern portion of the Atlantic Forest (Di-Bernardo et al. 2006). The climate is humid subtropical, and seasons (autumn, winter, spring, and summer) are differentiated by temperature (Zepner et al. 2021). We conducted our study along ~ 400 m on the Forqueta river's bank, where most individuals of this microendemic species (range size of 1.6 km^2, IUCN, 2023) concentrate to breed on temporary pools formed on flat rock outcrops. We surveyed the site at least twice a year, from September 2019 to December 2022 (exclusively between August and December), totaling nine field campaigns (Table 1). We captured each toad using new disposable latex gloves and swabbed the skin using MedicalWire MW113 swabs, following standard swabbing protocols (Hyatt et al. 2007). To calculate the body condition of each toad, we measured the snout-vent length (SVL) of each individual using a digital caliper with a precision of 0.01 mm and the body mass using a field scale with precision of 0.1 g. After sampling, we immediately released all toads at the exact point of capture.

We applied a photo-identification standardized procedure (Bardier et al. 2019; Caorsi et al. 2012) as a mark-recapture method. The ventral region of each toad was photographed (Figure S1), and we used the Wild-ID open-source software (Bolger et al. 2012) to identify recaptures. The software compares all images for pairwise similarity and returns the 20 top-ranked potential matches for each focal image; all recaptures were visually confirmed afterwards. This mark-recapture method has been successfully applied to *M. admirabilis* (Fonte et al. 2022) and other species of *Melanophryniscus* (Caorsi et al. 2012; Bardier et al. 2019) due to their black, brown, or green background with red, yellow, white, green, or orange spots on their belly (Figure S1).

Pathogen detection and quantification

To assess a sensitivity performance parameter for *Bd* diagnostic in our QuantStudio™ 6 Real-Time PCR equipment, we performed a series of replicate standard curves, totaling 20 replicates per standard concentration to calculate the limit of detection "LoD". LoD is defined as the lowest amount of target DNA sequence that can be detected with 95 % probability. We ran a dilution series of a known amount of total *Bd* DNA, ranging from 1.83×10^5 GE/µL to 10^{-3} GE/µL. Each plate included 4 technical replicates of each standard concentration, resulting in a total of 32 standards samples per plate, as well as 4 negative

controls consisted of DNA-free water. *Bd* DNA was extracted from a culture (isolate CLFT 159, global panzootic lineage) using PrepMan ULTRA® (Life Technologies).

For the qPCR assay, we utilized a final volume of 25 µL, containing 5 µL of template DNA, 12.5 µL of TaqMan Fast Master Mix (Applied Biosystem), 3.75 µL of ddH2O, 1.25 µL of forward primer (ITS-1 Chytr CCTTGATATAATACAGTGTGCCATATGTC, 18 µM), 1.25 µL of reverse primer (5.8S Chytr AGCCAAGAGATCCGTTGTCAAA, 18 µM), and 1.25 µL of probe (Chytr MGB2 GCAGTCGAACAAAAT, 5 µM). We used thermal cycling at 50 °C for 2 minutes and 95 ºC for 20 seconds, followed by 50 cycles at 95 ºC for 1 second and 60 °C for 20 seconds. The outcome of our analysis indicated that the lowest standard concentration with detection rate of 95 % or greater detection was 0.1 copies per reaction (1.83 x 10^{-1} GE/µL) (Table S1, Figure S2).

To determine the presence and infection loads of *Bd* in each swab sample, we extracted DNA from skin swabs using PrepMan ULTRA® (Life Technologies). To quantify *Bd* infection loads, we used a Taqman® qPCR Assay (Life Technologies) with standards ranging from 10^{-1} to 10^{3} genomic equivalents of zoospores, hereafter referred as GE (Boyle et al. 2004; Lambertini et al. 2013). We considered samples to be *Bd*-positive (Bd^{+}) when the infection loads were ≥ 0.1 GE.

Abiotic data

We obtained the mean temperature for the 15 days leading up to the sampling from two automated weather station located approximately 23 and 37 km from the study site (*Instituto Nacional de Meteorologia do Brasil*). We used 15 d prior to sampling based on the Bd life cycle (i.e. this period is enough to allow at least 1 generation of *Bd*; Berger et al. 2005). To assess temperature anomaly metrics, we used the TerraClimate dataset (Abatzoglou et al. 2018). We calculated the deviation from the historical temperatures by subtracting the historical monthly mean temperature over the past 50 years from the mean temperature of the target periods. These temperature deviations (ºC) were calculated for one, two, and three months preceding our sampling, incorporating one-month lagged deviations. The resulting deviation values could be negative for colder-than-average months, and positive for warmer-than-average months. Additionally, we recorded the accumulated rainfall for each month prior to sampling using data from a neighboring hydrometeorological station located at a similar altitude, 5.5 km from the study site (*Agência Nacional de Águas*). To assess rainfall anomaly metrics, we calculated the deviation from historical rainfall by subtracting the historical monthly mean of accumulated rainfall over the past 50 years from the mean accumulated rainfall of the target

periods (Abatzoglou et al. 2018). Like our temperature anomaly metrics, we extracted rainfall deviations (mm) for one, two, and three months prior to the sampled month. This analysis provided us with negative values for dryer-than-average months and positive values for wetter-than-average months.

Data analyses and modelling

For statistical analysis, we employed two model selection approach. Firstly, to screen for important climatic anomaly metrics explaining *Bd* prevalence and infections loads, we employed a model selection approach based on Generalized Linear Models (GLM), thus reducing potential multicollinearity bias in downstream pruned models. Based on the Akaike Information Criterion (AIC) (Mazerolle, 2006), the three-month temperature deviation, and two-month rainfall deviation were the best predictor explaining *Bd* prevalence. Additionally, two-month temperature deviation was the best predictor explaining *Bd* infection load, while three-month rainfall deviation was the best predictor variable. A detailed description of these models can be found in the supplementary information (Table S2).

Secondly, to test for the potential effect of monthly climatic fluctuations and climatic anomalies explaining *Bd* prevalence and infection loads, we employed a model selection approach based on GLMs. For *Bd* prevalence, we fit a GLM with binomial distribution and logit link function. We also fit a Gaussian GLM with log link function, with *Bd* infection loads (log10-transformed GE; only Bd^+ samples) as the response variable. The explanatory variables in the global models were: year, month, mean temperature of the 15 days prior to sampling date, accumulated monthly rainfall from the month prior to sampling date, and climatic anomaly metrics depicting temperature deviation and rainfall deviation. For each GLM, we included year, month (*Bd* prevalence) and season (warm or cold, *Bd* infection load model) and as fixed effects. A detailed description of these models can be found in the supplementary information (Table S3). We ran models with all possible combination of explanatory variables, and we ranked the most parsimonious models by employing a backward stepwise procedure based on the Akaike Information Criterion (AIC) (Mazerolle, 2006; Table S3).

Finally, to investigate any significant differences in body condition between *Bd* infected and uninfected toads (explanatory categorical variable), we performed a Student's *t*-test, including data of body condition from adult males sampled until August 2021 (n = 155). We performed a linear regression analysis to test for the potential effect of *Bd* infection loads on body condition of Bd^+ males (n = 33). The body condition metric utilized the Scaled Mass

Index (SMI) approach, based on standardized major axis regression between mass and snout−vent length (Peig and Green, 2009). We excluded females from this analysis because the presence of eggs could bias our results. All statistical analyses were performed using R version 2023.3.1 (R Core Team 2022).

Results

We took 339 skin swab samples from 305 individual toads, and overall *Bd* prevalence was 15.9 % (54 Bd^+; n = 339; Table 1 for the prevalence data at each sampling event), and the mean infection loads in Bd^+ toads was 73.58 GE, ranging from 0.1 – 2,112.45 GE ± 293.36 SD (Table 1 for infection loads data at each sampling event). *Bd* prevalence and infection loads were higher in August (Figure 1), with a prevalence of 22.14 % (31 Bd^+; n = 140) and a mean *Bd* infection loads of 103.50 GE ± 383.14 SD.

Our most parsimonious GLM model explaining *Bd* prevalence in the Admirable Redbelly Toads showed that month ($z = 4.084$, $P < 0.01$) and increased temperature deviation ($z = 4.555$, $P < 0.001$; Figure 2A) predicted higher *Bd* prevalence. Conversely, rainfall ($z = -4.241$, $P < 0.001$; Figure 2B) and rainfall deviation ($z = -4.735$, $P < 0.001$; Figure 2C) negatively affected *Bd* prevalence during our sampling timeframe (Table 2). Our best-fit model explaining *Bd* infection loads included month, year, temperature, and temperature deviation (Table S3). However, we did not find statistically significant effect of climatic seasonal and climatic anomaly metrics in *Bd* infection loads.

We did not observe significant difference in SMI between infected and uninfected toads (*t-value* = -0.64, *df* = 47.114, $P = 0.519$). Additionally, we did not find a significant correlation between SMI and infection loads of Bd^+ individuals ($P = 0.394$; Figure S3). A total of 305 toads were captured once, 29 captured twice, five captured three times, and one was captured four times. Regarding *Bd* infection status over time, a total of seven recaptured toads were found infected at least once. They gained infection seven times, while they cleared *Bd* infection only three times (Figure 3).

Discussion

Our study revealed that seasonal climatic fluctuations and climatic anomaly metrics are linked with *Bd* prevalence in *M. admirabilis*. Anomalies such as rainfall deficit, combined with temperatures that exceed the historical average, were key in predicting *Bd* infection risk in this amphibian species. Consequently, our findings suggest that with the projected increase

of climatic anomalies over the next decades (Sillmann et al. 2013), the only known Admirable Redbelly Toad population may face a scenario of *Bd* fluctuation.

In agreement with our predictions, the Admirable Redbelly Toads were more likely to have higher *Bd* prevalence when rainfall in the previous month was low, as well as in periods of rainfall deficit. Strikingly, this population also have higher *Bd* prevalence when the temperatures of the preceding three month exceeded the historical average. This species relies on small temporary pools for reproduction (Di-Bernardo et al. 2006, Bordignon et al. unpublished results) that depend on rainfall and evaporation. Hence, dry and warmer periods can rapidly lead to the drying up of shallow pools, directly or indirectly imposing physiological stress and forcing toads to aggregate in the fewer deeper pools that remain available over longer periods of time (Rohr and Palmer, 2013; Moura-Campos et al. 2021). These aggregations serve as a significant reservoir of infective *Bd* zoospores (Longo et al. 2010; Becker et al. 2016), increasing the likelihood of *Bd* transmission when toads interact (Malagon et al. 2020; Moura-Campos et al. 2021). Additionally, population size and contact rates among toads were also linked with higher *Bd* prevalence in other *Melanophryniscus* species (Pontes et al. 2021). Although not measured in this study, the seasonal demography of *M. admirabilis* may also play a role in *Bd* infections dynamic in the only known population of this species. Thus, our findings suggest that *M. admirabilis* may be particularly vulnerable to synergistic interactions between *Bd* and climatic anomalies, as rainfall deficit and warming not only facilitate the spread of *Bd,* but also reduce the availability of pools for reproduction.

Climatic variables were not correlated with *Bd* infection loads in *M. admirabilis* population, which experiences low *Bd* infection loads. As *Bd* infection depends on interaction between the thermal performance of the pathogen and the host (Cohen et al. 2017), even at low infection level, the synergistic effects of warming and *Bd* could result in host mortality in cool-adapted anurans (Neely et al. 2020). This implies that the combined effects of *Bd* infection and climate change, mainly temperature anomalies, might be underestimated, as *Bd* affect the amphibian persistence even with low prevalence and infection loads (Valenzuela-Sánchez et al. 2017; Palomar et al. 2023). This highlights the significance of population-level impacts of *Bd* (Valenzuela-Sánchez et al. 2017) that may potentially lead to slow and steady long-term declines. Consequently, in light of the projected increase in temperature anomalies (Sillmann et al. 2013), future studies on the thermal tolerance of the Admirable Redbelly Toads and its interaction with *Bd* infection become essential for a comprehensive understanding of the consequences of accelerated climate change on this species.

Contrary to our predictions, our findings suggest that body condition was not linked with *Bd*. Evidence of sublethal effects of *Bd* on body condition are rarely observed in wild populations that do not experience chytridiomycosis-related mortality. However, it is worth noting that even at low infection levels, *Bd* could lead to impaired skin physiology (*i.e.,* skin integrity, osmoregulation, and hormone production) (Wu, 2023). Additionally, *Bd* is known to dramatically alter the amphibian skin microbiome (Becker et al. 2017; Becker et al. 2019) and it is known that the presence of the bacteria *Serratia marcensis* could also be playing an important structural role in skin microbiome health in our focal amphibian species (Ienes-Lima et al. 2023a, 2023b; Woodhams et al. 2023). Furthermore, the capture-mark-recapture analysis showed that although the Admirable Redbelly Toads demonstrated the ability to clear *Bd* infection, they still gained more infection than managed to clear over time. The probability of becoming Bd^+ higher than the probability of clearance is a characteristic of population on a path of slow decline (Palomar et al. 2023). Therefore, tracking individual *Bd* infection status over time is crucial for future assessments of the population heath.

Melanophryniscus species could be affected by climate change in different ways, including the reduction of suitable habitat (Zank et al. 2014). This study represents the first long-term investigation of *Bd* prevalence and infection loads and its climatic drivers in the Admirable Redbelly Toad sole population. With trends in global warming, higher disease risk is expected in cool-adapted organisms (Rohr and Cohen, 2020), as the case of various *Melanophryniscus* populations. Therefore, studies that investigate the interactive effects of disease and climate change must be conducted to safeguard endangered amphibian species, as interactions between warming and infection could still lead to population declines (Neely et al. 2020). While chytridiomycosis may not pose an immediate threat to the microendemic Admirable Redbelly Toads, common stressors such as herbicides (Da-Silva et al. 2023) and anthropic impact (Ienes-Lima et al. 2023a) could amplify the fitness impacts of climate change (Greenspan et al. 2017; Rohr and Palmer, 2013). We strongly advocate for the continuation of research with *Bd* and its interaction with other prevalent pathogens in the southern Brazil, such as *Ranavirus* (Ruggeri et al. 2023), on *M. admirabilis* sole population. Surveying both pathogens mainly in dryer periods is especially crucial for its conservation, considering their vulnerability to declines resulting from stochastic events.

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Statements & declarations

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Ethics approval

All applicable institutional and national guidelines for the care and use of animals were followed. This work was conducted under permits by Instituto Chico Mendes de Conservação da Biodiversidade (SISBio #72718), Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SisGen #A3D44D1). This work was approved by Unicamp Animal Care and Ethics Committee (CEUA #5581-1/2020).

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

M.R.P. and L.F.T. designed the study. M.R.P, M.A., G.A.A. and M.B.M. carried out the fieldwork. M.R.P and L.P.R. carried out the molecular analysis. M.R.P and G.C.B analyzed the data. MRP drafted the manuscript. All authors critically revised the manuscript and approved the final manuscript.

Figures captions

Fig. 1 Proportion of *Melanophryniscus admirabilis* toads infected with *Batrachochytrium dendrobatidis* (*Bd*) and proportion of genomic equivalents of zoospores (GE) by months.

Fig. 2 Visualization of the Generalized Linear Model (GLM) fit for *Batrachochytrium dendrobatidis* (*Bd*) prevalence. Temperature deviation (ºC) (a), accumulated monthly rainfall (mm) (b) and two-month rainfall deviation (mm) (c).

Fig. 3 Individual variation in *Batrachochytrium dendrobatidis* (*Bd*) infection loads (log genomic equivalents of zoospores, GE) in *Melanophryniscus admirabilis* toads (lines connect the same individual). Values below zero indicate an uninfected sample.

Figures

Fig. 1

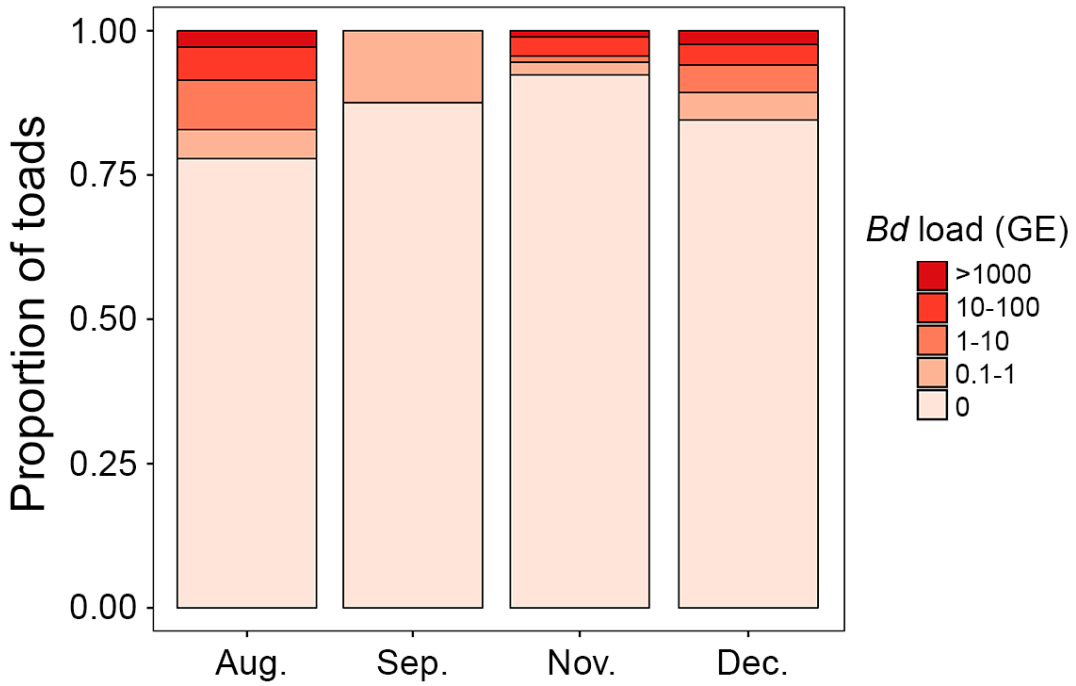


Fig. 2

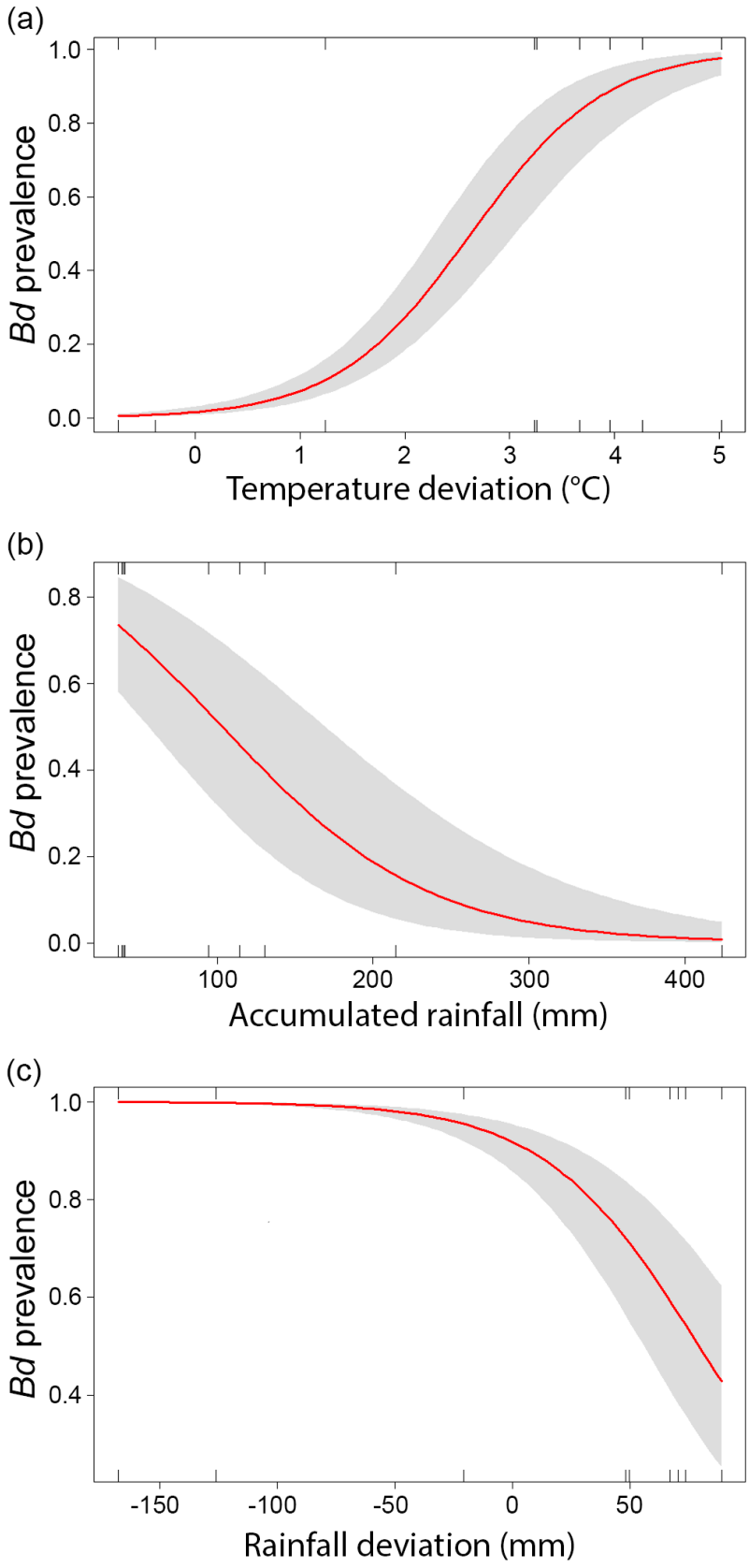
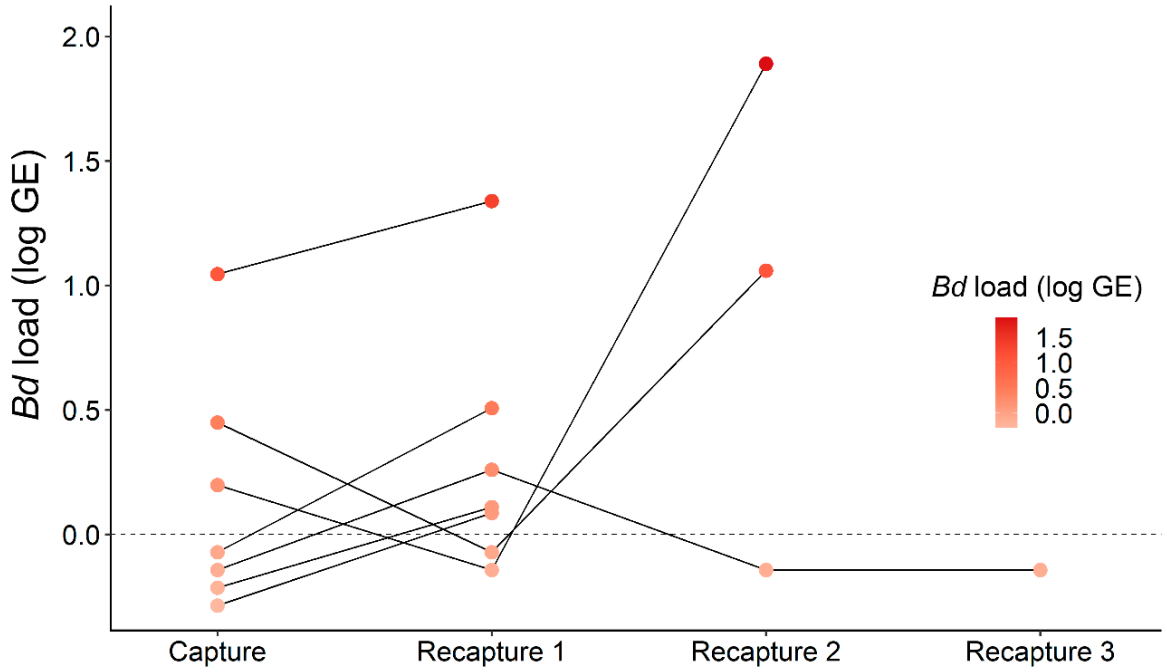

(a)
Bd prevalence
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Temperature deviation (°C)
(b)
Bd prevalence
0.8
0.6
0.4
0.2
0.0
100
200
300
400
Accumulated rainfall (mm)
(c)
Bd prevalence
1.0
0.8
0.6
0.4
-150
-100
-50
0
50
Rainfall deviation (mm)

Fig. 3


2.0
1.5
1.0
0.5
0.0
Bd load (log GE)
Capture
Recapture 1
Recapture 2
Recapture 3
Bd load (log GE)
1.5
1.0
0.5
0.0

Tables

Table 1. Variation of *Batrachochytrium dendrobatidis* (*Bd*) prevalence and infection loads in *Melanophryniscus admirabilis* between 2019 and 2022. *Bd* prevalence is the proportion of infected toads out of the total number of toads sampled. *Bd* infection load is represented by the mean and standard deviation (SD) of zoospore genomic equivalents (GE) by year and month (only Bd^+ toads). Total refers to the general *Bd* prevalence and mean of infection load and SD.

Year	Month	*Bd* prevalence (Bd^+ / n)	Mean *Bd* infection load
2019	September	13.33 % (2/15)	0.35 ± 0.33
	December	26.28 % (11/41)	23.20 ± 48.47
2020	August	13.63 % (6/44)	3.19 ± 3.84
	November	1.40 % (1/71)	16.67
2021	August	34.78 % (16/46)	155.05 ± 523.53
	September	11.10 % (1/9)	0.78
	December	4.65 % (2/43)	81.88 ± 114.69
2022	August	18 % (9/50)	78.74 ± 151.76
	November	30 % (6/20)	54.64 ± 93.24
	Total	15.92 % (54/339)	73.58 ± 293.36

Table 2. Estimates from Generalized Linear Models testing the effects of each predictor variable on *Batrachochytrium dendrobatidis* (*Bd*) prevalence in *Melanophryniscus admirabilis*. Statistically significant predictor variables are shown in bold.

Predictors	Estimate	*Std. Error*	*z*	*P*
Bd prevalence				
Intercept	329.046	431.644	0.762	0.446
Year	-0.171	0.213	-0.802	0.432
Month	1.404	0.343	4.084	**< 0.001**
Temperature deviation	1.557	0.341	4.555	**< 0.001**
Rainfall	-0.015	0.003	-4.241	**< 0.001**
Rainfall deviation	-0.030	0.066	-4.735	**< 0.001**

Supplementary information
Biodiversity and Conservation

Climatic drivers of chytrid prevalence in the critically endangered Admirable Redbelly Toad

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Table S1. *Batrachochytrium dendrobatidis* TaqMan Fast Master Mix Assays results. The lowest standard concentration with 95 % or greater detection is: 0.1 copies per reaction (1.83 x 10^{-1} GE/µL). Ct = cycle threshold.

Standard concentration	Replicates	Detected	Ct mean ± SD	Detection rate (%)
0.001	20	6	41.078 ± 1.196	30
0.01	20	17	40.191 ± 1.287	85
0.1	**20**	**20**	**37.649 ± 1.519**	**100**
1	20	20	33.950 ± 0.942	100
10	20	20	30.703 ± 0.831	100
100	20	20	27.316 ± 0.753	100
1000	20	20	23.974 ± 0.647	100
10000	20	20	20.566 ± 0.395	100
100000	20	20	18.068 ± 0.110	100

Table S2. Model selection for accumulated monthly rainfall, mean temperature of the 15 days prior to sampling date, and temperature and rainfall deviations metrics influencing *Batrachochytrium dendrobatidis* (*Bd*) prevalence and infection load in *Melanophryniscus admirabilis* in Brazil.

Model	AIC
Prevalence	
Temperature deviation	
YEAR, MONTH, TEMP, DEVTE3	277.4
YEAR, MONTH, TEMP, DEVTE1	278.6
YEAR, MONTH, TEMP, DEVTE2	279.8
Rainfall deviation	
YEAR, MONTH, RAIN, DEVRF2	293.8
YEAR, MONTH, RAIN, DEVRF1	294.2
YEAR, MONTH, RAIN, DEVRF3	298.2
Infection load	
Temperature deviation	
YEAR, SEASON, TEMP, DEVTE2	133.7
YEAR, SEASON, TEMP, DEVTE3	133.9
YEAR, SEASON, TEMP, DEVTE1	136.2
Rainfall deviation	
YEAR, SEASON, RAIN, DEVRF3	133.8
YEAR, SEASON, RAIN, DEVRF2	134.1
YEAR, SEASON, RAIN, DEVRF1	134.3

We employed a model selection approach based on Generalized Linear Models (GLMs) with binomial distribution and logit link function to screen for the strongest climatic anomaly metrics that potentially predict *Bd* prevalence, thereby reducing multicollinearity. Specifically, we included year, month, and mean temperature of the 15 days prior to sampling date (TEMP) as fixed variables in the temperature-models. Furthermore, we included temperature deviations of one, two, and three months prior to the sampling month. Additionally, to screen for the best rainfall anomaly metrics, we used year, month, and accumulated monthly rainfall from the month prior to sampling date (RAIN) as fixed effects, and we tested rainfall deviations of one, two, and three months prior to sampling. We additionally employed Gaussian GLMs with a log link function (using log10-transformed GE of Bd^{+} samples) as the response variable to screen for the strongest climatic anomaly variables of temperature and rainfall that potentially predict *Bd* infection loads. Specifically, the fixed variables included year, season (warm or cold), TEMP and RAIN, while we tested temperature and rainfall deviations of different periods, as described in the logistic model above. In the *Bd* prevalence model, best climatic anomaly metrics for temperature were temperature deviation subtracting the mean of the three months prior to sampling date from the historical temperature monthly mean (including 1-month lagged deviations) (DEVTE3), and rainfall deviation subtracting the mean of the two months prior to sampling date from the historical monthly rainfall mean (including 1-month lagged deviations (DEVRF2). For the *Bd* infection load model, the best climatic anomaly metrics were temperature deviation subtracting the mean of the two months prior to sampling date from the historical temperature monthly mean (including 1-month lagged deviations) (DEVTE2) and rainfall deviation subtracting the mean of the three months prior to sampling date from the historical monthly rainfall mean (including 1-month lagged deviations) (RAINRF3).

Table S3. Model selection for climatic variables influencing *Batrachochytrium dendrobatidis* (*Bd*) prevalence and infection load in *Melanophryniscus admirabilis* in Brazil.

Model	AIC	No. of variables	X^2
Prevalence			
YEAR, MONTH, DEVTE3, RAIN, DEVRF2	272.4	5	0.013
YEAR, MONTH, TEMP, DEVRF2	272.6	4	2.191
YEAR, MONTH, TEMP, RAIN, DEVRF2	273.5	5	1.158
YEAR, MONTH, TEMP, DEVTE3, DEVRF2	274.1	5	1.769
YEAR, MONTH, TEMP, DEVTE3, RAIN, DEVRF2	274.4	6	0.013
YEAR, MONTH, TEMP, DEVTE3, RAIN	275.2	5	2.796
Infection load			
YEAR, SEASON, TEMP, DEVTE2	133.7	4	0.234
YEAR, SEASON, DEVTE2	133.8	3	2.285
YEAR, SEASON, DEVRF2	133.8	3	2.358
YEAR, SEASON, RAIN, DEVRF3	133.8	4	0.274
YEAR, SEASON, RAIN	134.2	3	2.730
YEAR, SEASON, TEMP	134.5	3	2.777

We compared all possible models using Akaike Information Criterion (AIC); six best models are reported for *Bd* load and prevalence. Best predictors are: year of sampling date (YEAR), month of sampling date (MONTH), season of sampling (SEASON), mean temperature of the 15 days prior to sampling date (TEMP), accumulated monthly rainfall from the month prior to sampling date (RAIN), temperature deviation subtracting the mean of the three months prior to sampling date from the historical temperature monthly mean (including 1-month lagged deviations) (DEVTE3), temperature deviation subtracting the mean of the two months prior to sampling date from the historical temperature monthly mean (including 1-month lagged deviations) (DEVTE2), rainfall deviation subtracting the mean of the three months prior to sampling date from the rainfall historical monthly mean (including 1-month lagged deviations), (DEVRF3), rainfall deviation subtracting the mean of the three months prior to sampling date from the historical rainfall monthly mean (including 1-month lagged deviations) (DEVRF2)

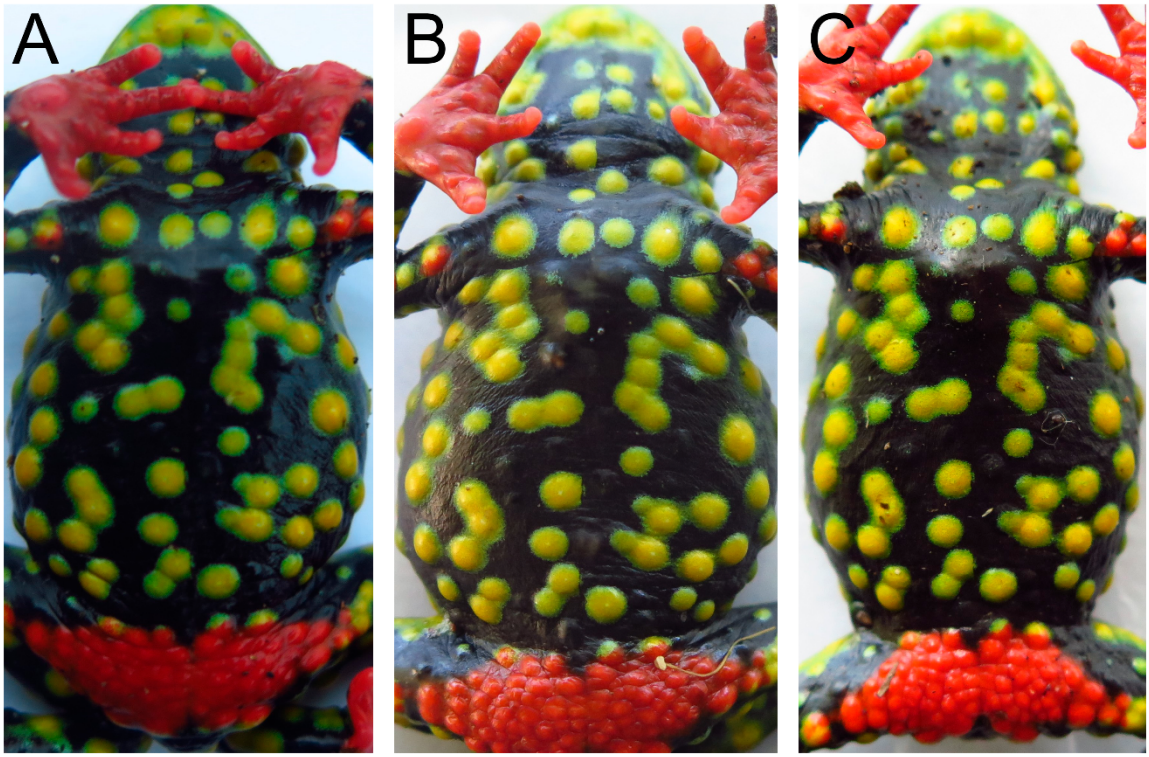

Figure S1. Female of *Melanophryniscus admirabilis* captured three times. We used a photo-identification as a mark recapture method, based on the natural spotted patterns of the toad's belly. These photographs depict the same individual captured during three separate field expeditions: A) September 2019; B) November 2020; C) November 2022.

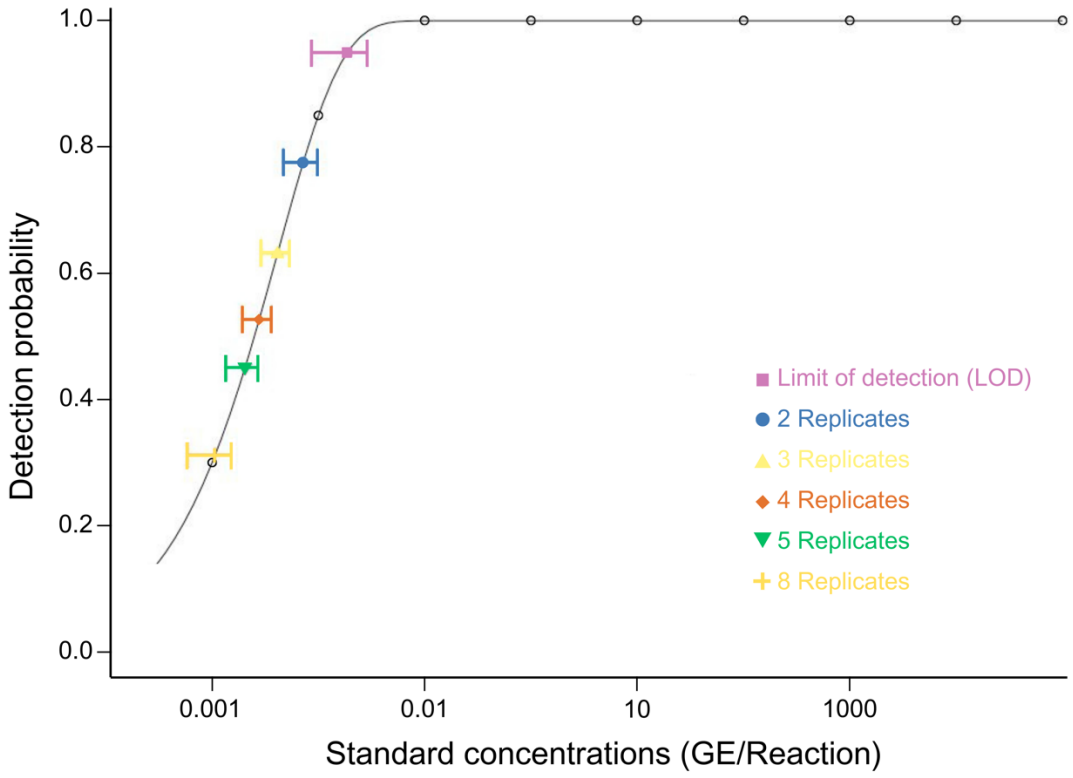

Figure S2. Limit of detection (LOD) plot for *Batrachochytrium dendrobatidis* TaqMan Fast Master Mix Assays. Detection probability is on the y-axis and standard concentrations are on the x-axis. The lowest standard concentration with 95 % or greater detection is: 0.1 copies per reaction (1.83×10^{-1} GE/µL). Points are drawn with open circles for the detection rates of each standard tested, and the line represents the LOD model. Colored points with 95 % confidence intervals are drawn to represent the LOD for multiple replicate analyses. We used a Weibull type II function to fit out data. Lack of fit test: p= 1.

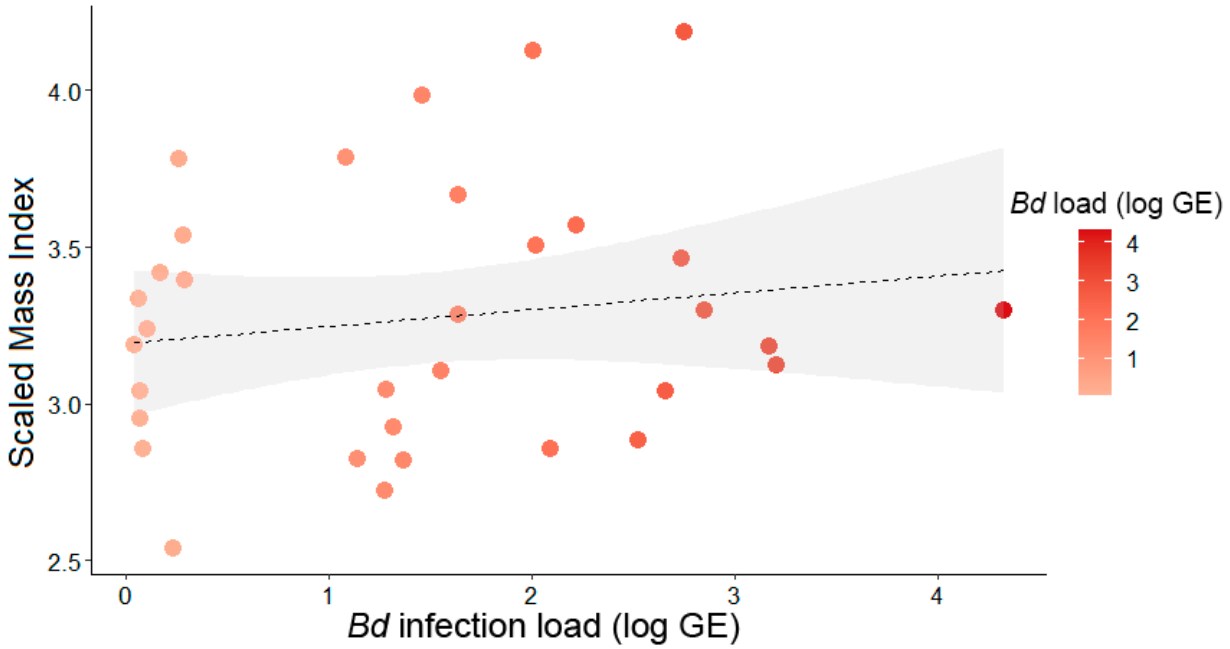

Figure S3. Linear regression between *Batrachochytrium dendrobatidis* infection load (log GE) in infected males of *Melanophryniscus admirabilis* and the individual scaled mass index (n = 33, with a 95 % confidence interval. Dashed line indicates the linear regression fit of a positive, non-significant trend.

CHAPTER III

Ranavirus INFECTION AND CHYTRID FUNGUS LINEAGES IN SPECIES OF THE GENUS *Melanophryniscus*

Infecção por *Ranavirus* e linhagens do fungo quitrídio em espécies do gênero *Melanophryniscus*

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This chapter will be submitted to the *Journal of Wildlife Diseases* and adheres to its guidelines.

Ranavirus infection and chytrid fungus lineages in species of the genus *Melanophryniscus*

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Abstract

Emerging infectious diseases are one of the most important causes of amphibian declines worldwide. Chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (Bd), along with ranavirosis, caused by viruses of the genus *Ranavirus* (Rv) have significantly impacted amphibian populations globally. In South America, the Atlantic Forest stands out as a region that has experienced historical amphibian declines linked to chytridiomycosis, with the southern region harboring a great genetic diversity of Bd. This region is also home to numerous threatened amphibian species, and the recent diagnostic of a high Rv prevalence in native amphibians underscored the importance of screening and monitoring infectious pathogens throughout the biome. We therefore investigated the presence of Rv and identified Bd genotypes in *Melanophryniscus* spp. from southern Atlantic Forest in Brazil. For the first time, we detected Rv infection in two amphibian toad species threatened with extinction, the Critically Endangered *M. admirabilis* and the Endangered *M. biancae.* We also identified the Bd Global Panzootic Lineage (Bd-GPL) in three species. Additionally, we detected the Bd hybrid strain in the same Rv infected *M. biancae* population, representing the first occurrence of this genotype outside the natural hybrid zone. The genus *Melanophryniscus* is the amphibian clade with the greatest number of threatened species in Brazil, and the presence of Rv and a Bd hybrid genotype pose additional threats for these species. The insights gained from this study on infectious pathogens can enhance the conservation efforts for Redbelly toads by identifying potential vulnerabilities within threatened amphibian species.

Key words: Atlantic Forest, *Batrachochytrium dendrobatidis*, Conservation, Infectious pathogens, Redbelly toads.

Introduction

Amphibians stand out as the most threatened class of vertebrates worldwide, and their status continue to deteriorate globally (Re:wild 2023). They are affected by multiple threats, including habitat loss, climate change, fires, overexploitation, invasive species, and infectious diseases (Re:wild 2023). Diseases can have a dramatic impact on entire communities and stand out as a critical threat to the amphibian extinction crisis (Scheele et al. 2019; Luedtke et al. 2023; Re:wild 2023). Among the emerging infectious diseases implicated in amphibian declines worldwide are chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd) (Berger et al. 2005), and a viral disease caused by iridoviruses of the genus *Ranavirus* (Rv) (Duffus et al. 2015). Both diseases pose significant challenges to amphibian conservation worldwide and efforts to understand the dynamics of these infectious pathogens must be prioritized to prevent further declines (Luedtke et al. 2023).

The Atlantic Forest is a hotspot for amphibian biodiversity, harboring more than 500 amphibian species (Toledo et al. 2021). Concurrently, this biome harbors one of the greatest genetic diversities of Bd (Jenkinson et al. 2016; Carvalho et al. 2024), and historical amphibian declines and extinctions were linked to chytridiomycosis (Carvalho et al. 2017). Furthermore, within this biome, three Bd lineages occur, the Bd Global Panzootic Lineage (Bd-GPL), the enzootic lineage (Bd-ASIA-2/BRAZIL), and hybrid genotypes formed between them (Jenkinson et al. 2016; Carvalho et al. 2024). The hybrid genotype can be highly virulent in native hosts (Greenspan et al. 2018), highlighting the importance of this area for amphibian conservation efforts.

Another infectious pathogen affecting native amphibians in the Atlantic Forest is Rv (Ruggeri et al. 2019, 2023). While numerous studies explored the dynamics of Bd in native Brazilian amphibians, research on Rv has focused on farmed North American bullfrogs (*Aquarana catesbeiana*) (Galli et al. 2006; Mazzoni et al. 2009; Candido et al. 2019). Escape of this exotic amphibian species from farms has resulted in the establishment of invasive populations in Brazil (Both et al. 2011; Garner et al. 2016), and only recently Rv was detected in these wild bullfrog populations (Ruggeri et al. 2019, 2023; Ash et al. 2024; Campião et al. 2024). Additionally, only two studies thus far have presented data on the presence of Rv in native Brazilian amphibians (Ruggeri et al. 2019; 2023), showing the presence of Rv in species from the southern Atlantic Forest, with cases of coinfection with Bd (Ruggeri et al. 2023). These new findings underscore the importance of screening and monitoring both pathogens for conservation amphibian research (Ruggeri et al. 2023).

The southern portion of the Atlantic Forest also harbors numerous amphibian species threatened with extinction (Brasil 2022), such as anurans in the genus *Melanophryniscus*. Popularly known as Redbelly toads, *Melanophryniscus* is the genus of amphibians with the greatest number of threatened species in Brazil (Brasil 2022; Anunciação et al. 2024), with eight out of 22 species classified as threatened by the Brazilian red list (Brasil 2022), six of which occurs in the southern region of the Atlantic Forest. Although all *Melanophryniscus* spp. have been classified as threatened according to distribution-related criteria (Brasil 2022, Salve 2023), there are few studies about infectious pathogens in this genus. Despite the Bd records in two Brazilian *Melanophryniscus* species (Slys et al. 2007; Rodriguez et al. 2014; Preuss et al. 2016), efforts to genotype Bd strains in these threatened amphibians are lacking (Rodriguez et al. 2014; James et al. 2015). Additionally, research on Rv in native amphibians is recent and incipient in Brazil (Ruggeri et al. 2019, 2023), and there are no specific studies on Rv infection in *Melanophryniscus* species. Thus, in this study, we aimed to evaluate the presence of Rv and identify Bd genotypes within *Melanophryniscus* species, thereby enhancing our understanding of the infectious pathogens in Brazilian Redbelly toads. Consequently, information about the occurrence of infectious pathogens in species at high risk of extinction will contribute to the development of strategies for mitigating infectious diseases affecting threatened groups and directing future conservation efforts.

Methods

Ranavirus sampling

Our Rv sampling comprised a total of 16 adults individuals of *Melanophryniscus* spp., one juvenile, four metamorphs and 205 tadpoles. We considered as tadpoles the ones in developmental stages from 26 to 41, and metamorphs from 42 to 45 (Gosner 1960). The species sampled were: the Critically Endangered (CR) *M. admirabilis* (n = 141), the Least Concern (LC) *M. alipioi* (n = 8), the Endangered (EN) *M. macrogranulosus* (n = 43), and the EN *M. biancae* (n = 34) (Brasil 2022, Table 1). We searched for *Melanophryniscus* spp. specimens via visual encounters between December 2019 and November 2021 (Table 1). We sampled Redbelly toads in the municipalities of Maquiné, Dom Pedro de Alcântara, and Arvorezinha, in the state of Rio Grande do Sul (RS), as well as in the municipalities of Campina Grande do Sul and Tijucas do Sul, state of Paraná (PR). We captured each adult individual using an individual pair of gloves. For *M. admirabilis* and *M. alipioi*, we sampled tadpoles using spoons or nets. Spoons and nets were cleaned between different sites by immersion in 2.5 % sodium hypochlorite. Additionally, we washed and rinsed nets and spoons with the water from each site before use. For *M. biancae* tadpoles, which inhabit phytotelmata plants, we used different sterile plastic pipettes between plants, but not between individuals from the same plant. For Rv detection, we extracted liver tissue from adults and internal organs (liver, kidneys, spleens and intestine) from tadpoles immediately after collection. To avoid cross-contamination, individual gloves, flame-sterilized scalpels, and scissors were utilized for handling each specimen individually. All samples were stored in individual cryotubes with 250 µL of DNAzol reagent (Invitrogen).

Batrachochytrium dendrobatidis sampling

For Bd genotyping, we used skin swabs from nine adults of *Melanophryniscus* spp.; this included one *M. admirabilis* (CR), six *M. biancae* (EN), and one *M. devincenzii* (LC) (Table 2). We conducted field surveys between September 2021 and August 2022 in the municipalities of Arvorezinha, RS, and Tijucas do Sul, PR. We captured each individual using new disposable latex gloves to avoid *Bd* cross-contamination. We applied skin swabs following standard swabbing protocols (Hyatt et al. 2007). We used PrepMan ULTRA® (Life Technologies) to extract DNA from swabs. We obtained data on the Bd infection load (genomic equivalents; GE) of each swab from another study (Pontes et al. *in prep.*). We used samples containing at least 81.54 GE and those with sufficient content for genotyping analysis.

DNA extraction and Ranavirus molecular analyses

We extracted DNA from all the samples (liver or pools of organs) using the DNAzol reagent, following the manufacturer's protocol. The samples were stored at -20 °C until use. First, we analyzed the presence or absence of viral DNA in all samples (n = 222) utilizing a TaqMan qPCR assay, employing a pooled sample approach consisting of three samples. We used a double-stranded DNA fragment (gBlocks) from the viral major capsid protein (MCP) gene as an Rv standard. Second, for Rv quantification, we conducted an additional TaqMan qPCR assay only with the samples from positive pools (n = 60). In this case, each sample was run individually and in quadruplicate (Brunner et al. 2004). We considered a sample as Rv-positive (Rv^+) when at least two wells amplified. For amplification in two wells, we considered positive when amplification was observed with cycle threshold (CT) values falling within or below the standard deviation calculated from the CT values of the lower concentration of the Rv standard.

Batrachochytrium dendrobatidis genotyping

We genotyped Bd samples using a sequence of 5 SNP markers as described by Schloegel et al. (2012). We amplified these five loci sequences (Table S1) with reaction conditions consisting of: 2 µL template DNA, 0.75 µL H_2O, 6.25 µL 2x Xtreme Buffer, 2.5 µL of dNTPs (2mM each), 0.375 µl each primer (forward and reverse – 10 µM) and 0.25 µL KOD Xtreme™ Hot Start DNA Polymerase (Applied Biosystems) in a 12.5 µL reaction. The thermal cycling parameters were as follows: an initial denaturation step at 94 °C for 2 min followed by 45 cycles of denaturation at 98 °C for 10 s, annealing at 54 °C for 30 s, and extension at 68 °C for 1 min, and a final extension at 68 °C for 7 min. We included a negative control and a positive control in each PCR assay. All amplification processes were conducted using the Mastercycler® (Eppendorf). The validation of DNA amplification was subsequently verified by gel electrophoresis on a 1 % agarose gel (2.5 g of agar, 250 ml of TAE buffer, and 0.125 ml of GelRed). We mixed 2 µL of a loading dye with 2 µL of DNA template and placed this mixture in each well of the electrophoresis gel. A GeneRuler 1kb DNA ladder was used to determine the band size of each sample. The gel electrophoresis program was run at 100 V for 40 min and the gel was analyzed with Quantity One software.

Subsequently, we used ExoSAP-IT *Express* to to purify the PCR product. The thermal cycling parameters included a step at 37 °C for 30 min, followed by 80 °C for 15 min, and a final cycle of 10 °C for 4 min. We sent the samples for Sanger sequencing to GENEWIZ (Azenta Life Sciences), employing forward primers exclusively, except in the case of

BdSC6.15, for which we utilized a reverse primer. We assigned genotypes to each Bd sample by comparing nucleotide sequences to reference sequences Schloegel et al. (2012) with the software Geneious Prime® 2023.2.1 (Kearse et al. 2012).

Results

We detected for the first time Rv in species of the genus *Melanophryniscus*. Specifically, we found Rv infection in individuals of two threatened species: *M. admirabilis* (CR) from Arvorezinha, RS, and *M. biancae* (EN) from Tijucas do Sul, PR (Figure 1). The overall Rv prevalence in *Melanophryniscus admirabilis* was 1.41 % (2 Rv^+; n = 141), and the mean infection load was 1.94 viral copies/µL in Rv^+ individuals (Table 1). For *M. biancae* species, the overall Rv prevalence was 23.52 % (8 Rv^+; n = 34), and Rv^+ individuals showed a mean infection load of 714.61 viral copies/µL, ranging from 1.37 – 5,682.63 viral copies/µL (Table 1). We detected Rv infection in all developmental stages of this species (tadpoles, metamorphs, juveniles and adults), with a high viral load of 5,682.63 viral copies/µL found in the only juvenile sampled. We did not detect the presence of Rv in individuals of *M. alipioi* or *M. macrogranulosus* (Figure 1).

We identified the Bd Global Panzootic Lineage (Bd-GPL) in all three species of the genus *Melanophryniscus*, *M. admirabilis* and *M. devincenzii*, and *M. biancae* (Figure 1). We also detected a hybrid Bd strain in one sample from the same population of *M. biancae.*

Discussion

Our Rv screening in four *Melanophryniscus* species of the southern Atlantic Forest revealed pathogen infections in two threatened species, the CR *M. admirabilis*, and the EN *M. biancae*. Both infected species have a restricted distribution with a small extent of occurrence and few known populations (Brasil, 2022, Salve 2023), thus highlighting the conservation concern about the presence of Rv pathogen in the Redbelly toads and in their habitats.

Despite the low Rv pathogen prevalence of 1.41 % found in *M. admirabilis*, we observed tadpoles with bubbles and swimming upside down in the same temporary pool containing the two Rv^+ tadpoles (M. R. Pontes, personal observation). The presence of the Rv in this population is especially concerning from a conservation standpoint given the single-location nature of this species, rendering them to decline due to stochastic threats (Reed 2008). For instance, a disease outbreak could result in massive mortality events and lead to a rapid decline or even extinction of *M. admirabilis* (Abadie et al. 2021). Additionally, tadpoles of this

species develop in temporary pools along the Forqueta river's bank, which are susceptible to inundation by river water following heavy rains (Figure 2A). Approximately 650 m downstream from this site, wild bullfrogs inhabit a pond (M. R. Pontes, personal observation; IUCN 2023, Figure 2B), which may overflow and connect to the river during heavy rainfall, possibly serving as a pathogen source. Thus, considering that Rv is a generalist pathogen that can be transmitted through water, the Rv presence in *M. admirabilis* could be linked to contamination from the river water. Additionally, Rv has also been detected in Brazilian fish species (Ruggeri et al. 2023), suggesting a broader ecological context for pathogen transmission in this region. Therefore, to better understand Rv source and the dynamic in this microendemic species, we recommend studies that assess pathogens not only in *M. admirabilis* population, but also in the amphibian and fish communities and environments along the margins and surroundings of the Forqueta river.

In *Melanophryniscus biancae* the overall Rv prevalence was about 24 %. Interestingly, the highest infection load was detected in a metamorphic individual, a stage where amphibian undergo natural immunosuppression (Rollins-Smith, 1998), and frequently associated with die-off events from Rv infections (Green et al. 2005). However, we did not find a considerable number of individuals in this stage and in different periods, making our sampling limited. Therefore, substantial efforts are needed to sample this pathogen at all developmental stages and to conduct new seasonal sampling to assess the prevalence and impact of Rv infection in *M. biancae.* The source of Rv in this population seems to not be related to wild bullfrogs. In contrast to *M. admirabilis*, the *M. biancae* population inhabits an environment unlike for the presence of invasive bullfrogs. Specifically, this population inhabits the grassland of the Serra do Araçatuba mountain range (Figure 3A), where individuals are associated with phytotelmata. Thus, Rv infection in this native amphibian is likely associated with factors other than bullfrog proximity. Ruggeri et al. (2023) suggested a link between the pet trade (Maximo et al. 2021) and Rv dispersion in Brazil, highlighting that Rv strains from native amphibians formed a clade with Rv isolated from animals imported to Europe (Ruggeri et al. 2023) via the live pet trade (Stöhr, 2015; Saucedo et al. 2017). However, amphibian community in Serra do Araçatuba does not appear to attract attention for the illegal pet trade, suggesting that while the pet trade may be a factor in the dispersion of Rv at other sites, it may not be the primary driver in this site. Further genetic research assessing Rv strains from these native amphibians can contribute to elucidating the specific mechanisms underlying the presence of Rv in this ecosystem.

Bd-GPL genotype was found in three *Melanophryniscus* species. The hybrid genotype found in this study was sampled from the same Rv^{+} *M. biancae* population and represent the first occurrence of this genotype outside the natural hybrid zone (Schloegel et al. 2012; Jenkinson et al. 2016, Carvalho et al. 2024). The hybrid zone in Brazil is confined to a narrow range of less than 1 km within the Serra da Graciosa mountain range, state of Paraná, situated approximately 60 km from the Serra do Araçatuba. Although hybrid genotypes are rare within this zone (Jenkinson et. al. 2016), this area overlaps the region with noteworthy evidence of disease-related mortality in Brazil (Carvalho et al. 2017). Additionally, hybrid genotypes can be more virulent than the parental lineages, potentially amplifying the disease risk in native hosts (Greenspan et al. 2018). This population also exhibited a high Bd prevalence and infection load compared with those of other *Melanophryniscus* species (Pontes et al. *in prep.*), suggesting possible vulnerability to infectious pathogens. Interestingly, the hybrid genotype was identified in the sample with higher Bd load. Considering that some host species are especially vulnerable to new genotypes (Greenspan et al. 2018), the role of hybrids in population declines needs to be explored.

The co-occurrence of two emerging infectious pathogens alongside a high Bd prevalence (Pontes et al. *in prep*.) in *M. biancae* suggested a possible interaction between these pathogens and their impact on the host response mechanism (Cox, 2001). Both Bd and Rv pathogens pose significant concerns for this endangered species, which faces other threats. Finally, we strongly advocate for ongoing monitoring efforts to assess the emerging infectious pathogens not only in this population, but also in the other population from Serra do Quiriri, state of Santa Catarina. Only comprehensive surveillance of both pathogens will provide an efficient amphibian conservation action plan for this species (Ruggeri et al. 2023).

In conclusion, for the first time, we report the presence of Rv in threatened Brazilian amphibians, and the presence of a Bd hybrid lineage infecting the Redbelly toads outside the known hybrid zone. Although our results can contribute to a better understanding and planning for amphibian conservation, it is important to integrate emerging infectious pathogens in conservation-oriented monitoring programs, especially among species facing heightened risks of extinction as some species of the genus *Melanophryniscus*.

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Tables

Table 1. *Ranavirus* (Rv) prevalence and infection load in *Melanophryniscus* spp. populations by sample date and developmental stage. Abbreviations for threatened IUCN categories are CR = critically endangered; EN = endangered; LC = least concern (Brasil 2022). Rv prevalence is the proportion of infected individuals out of the total number sampled. Rv infection load is represented by the mean Rv viral copies/µL (only for Rv^+ individuals). Abbreviations for Brazilian states are PR = Paraná; RS = Rio Grande do Sul.

Species	Date	Rv prevalence (Rv^+ / n)	Rv infection load	Life stage	Site (city, state)
M. admirabilis (CR)	Dec 2019	0 % (33)	-	Tadpole	Arvorezinha, RS
	Aug 2020	6% (2/33)	1.94	Tadpole	
	Nov 2020	0% (43)	-	Tadpole	
	Aug 2021	0% (32)	-	Tadpole	
		1.41 % (2/141)	**1.94 (1.49 – 2.39)**		
M. alipioi (LC)	Nov 2021	0 % (0/8)	0	Adult	Campina Grande do Sul, PR
M. biancae (EN)	Jan 2020	40 % (2/5)	2.56	Adult	Tijucas do Sul, PR
		100 % (1/1)	1.37	Juvenile	
		25 % (1/4)	5,682.63	Metamorph	
		16.66 % (4/24)	6.94	Tadpole	
		23.52 (8/34)	**714.61 (1.37 - 5,682.63)**		
M. macrogranulosus (EN)	Nov 2020	0 % (0/1)	-	Adult	Maquiné, RS
		0 % (0/2)	-	Adult	Dom Pedro de Alcântara, RS
		0 % (0/40)	-	Tadpole	
		0 % (0/43)	-		

Table 2 – Skin swabs from individuals of *Melanophryniscus* spp. used to genotype the *Batrachochytrium dendrobatidis* (Bd). Abbreviations for IUCN threatened categories are CR = critically endangered; EN = endangered; LC = least concern (Brasil, 2022). Bd infection load is the zoospore genomic equivalents (GE) of each swab. The data on Bd infection load were obtained from another study (Pontes et al. *in prep.*). Abbreviations for Brazilian states are PR = Paraná; SC = Santa Catarina; RS = Rio Grande do Sul.

Species	Date	Sample ID	Lineage	Load (GE)	Site (city, state)
M. admirabilis (CR)	Aug 2022	SLFT 20319	Bd-GPL	469.29	Arvorezinha, RS
M. biancae (EN)	Oct 2021	SLFT 18739	Hybrid	9,262.26	Tijucas do Sul, PR
		SLFT 18742	Bd-GPL	1,218.93	
		SLFT 18745	Bd-GPL	334.15	
		SFLT 18749	Bd-GPL	81.54	
		SFLT 18757	Bd-GPL	2,439.72	
		SLFT 18760	Bd-GPL	1,888.19	
M. devincenzii (LC)	Sep 2021	SLFT 20235	Bd-GPL	2,191.29	Arvorezinha, RS

Figures captions

Figure 1. Distribution of *Ranavirus* (Rv) and *Batrachochytrium dendrobatidis* (Bd) genotypes in species of *Melanophryniscus*. The rectangle shown in the inset map of Brazil highlights the states of Paraná (PR), Santa Catarina (SC) and Rio Grande do Sul (RS). Sites (dots) within the southern of Atlantic Forest (shaded in green) sampled for Rv detection. Rv sampling included: *Melanophryniscus alipioi* from Campina Grande do Sul (01) and *M. biancae* from Tijucas do Sul (02), PR; *M. macrogranulosus* from Dom Pedro de Alcântara (03) and Maquiné (04); and *M. admirabilis* from Arvorezinha (05), RS. Yellow stars indicate the presence of the Bd Global Panzootic Lineage (Bd-GPL) in *M. biancae* from Tijucas do Sul (02), PR, *M. admirabilis* (05) and *M. devincenzii* (06), both from Arvorezinha, RS. Blue star indicates Bd hybrid genotype in *M. biancae* from Tijucas do Sul (02).

Figure 2. Temporary pools on flat rock outcrops along the banks of the Forqueta's river used by *Melanophryniscus admirabilis* as breeding site (A). Site of a wild bullfrog population near the only known population of *M. admirabilis* (B).

Figure 3. Grassland environment of the Serra do Araçatuba mountain range, Tijucas do Sul, PR (A). Phytotelma plant used by *Melanophryniscus biancae* as breeding site (B).

Figures

Figure 1.

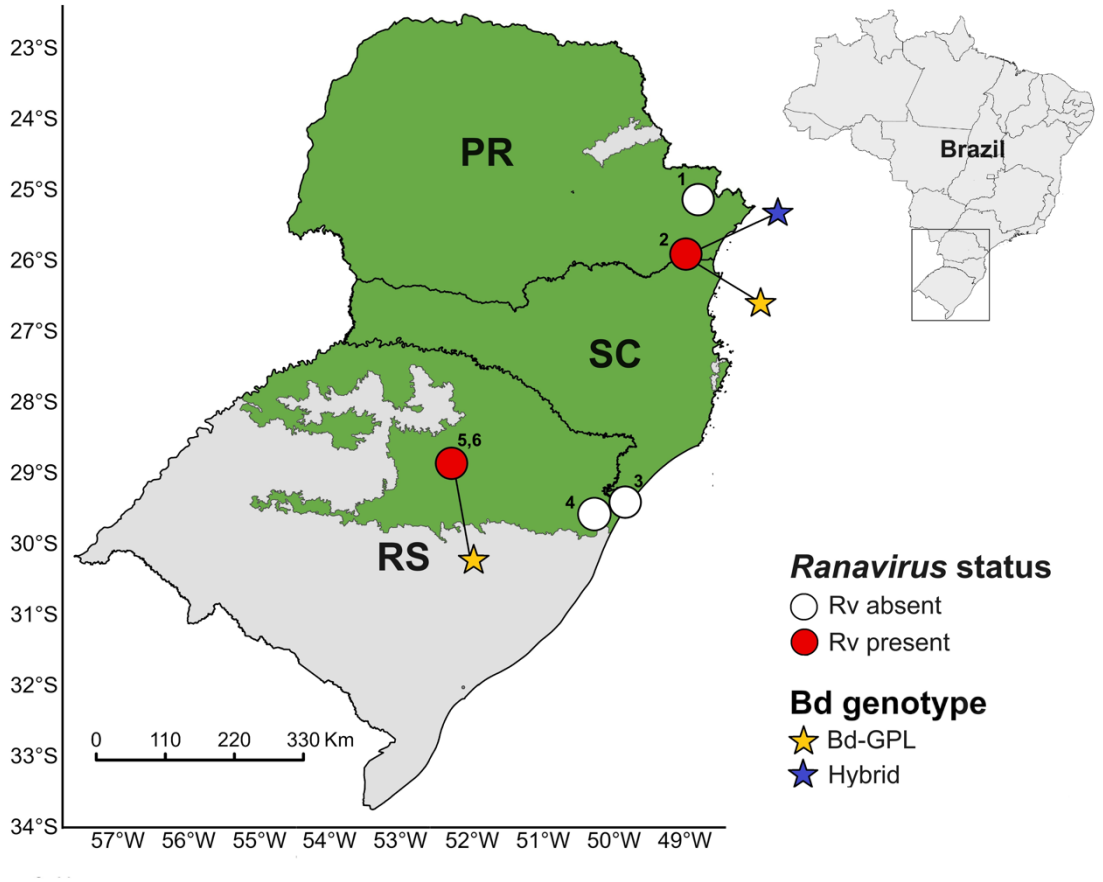


Figure 2.

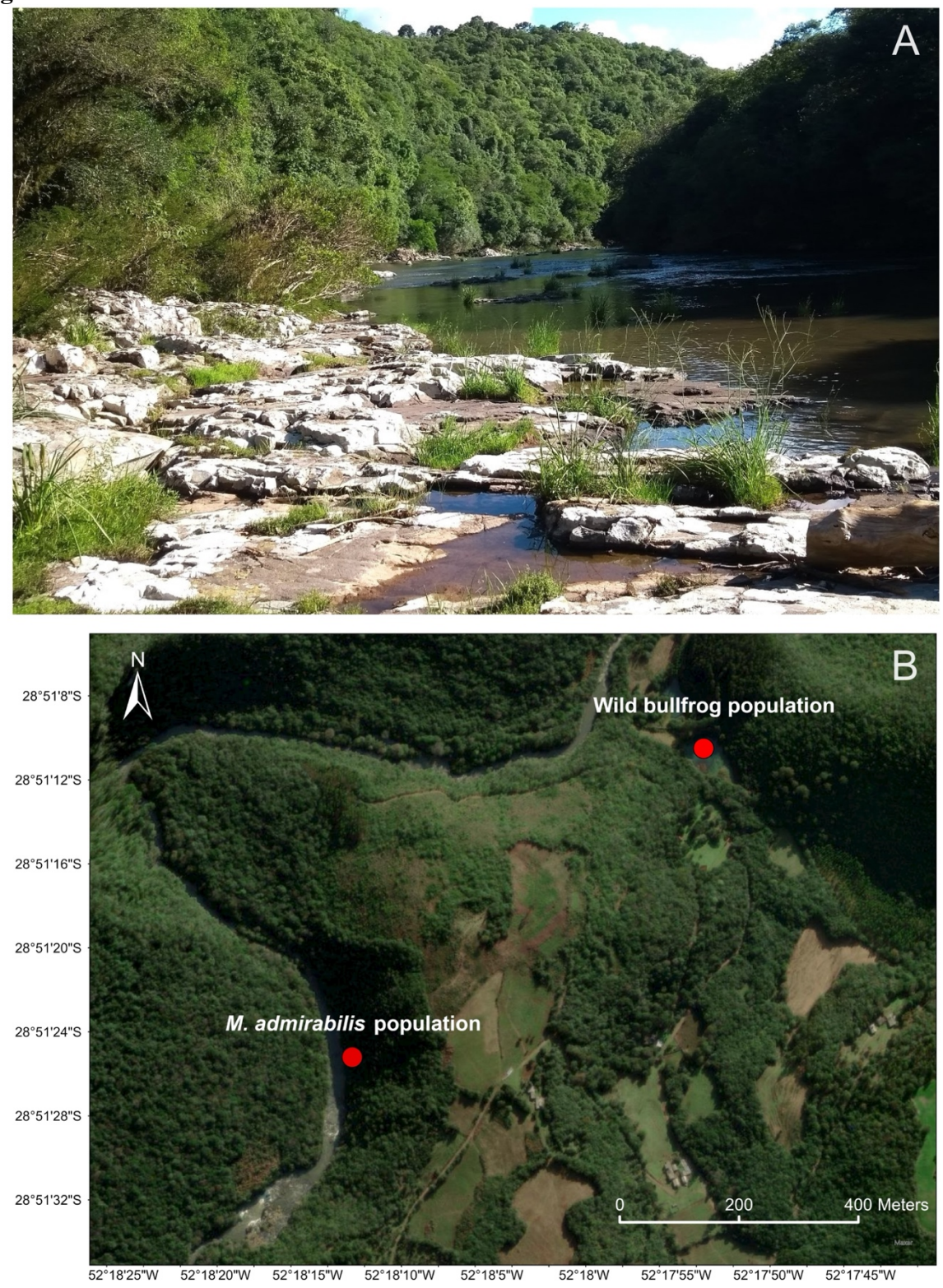
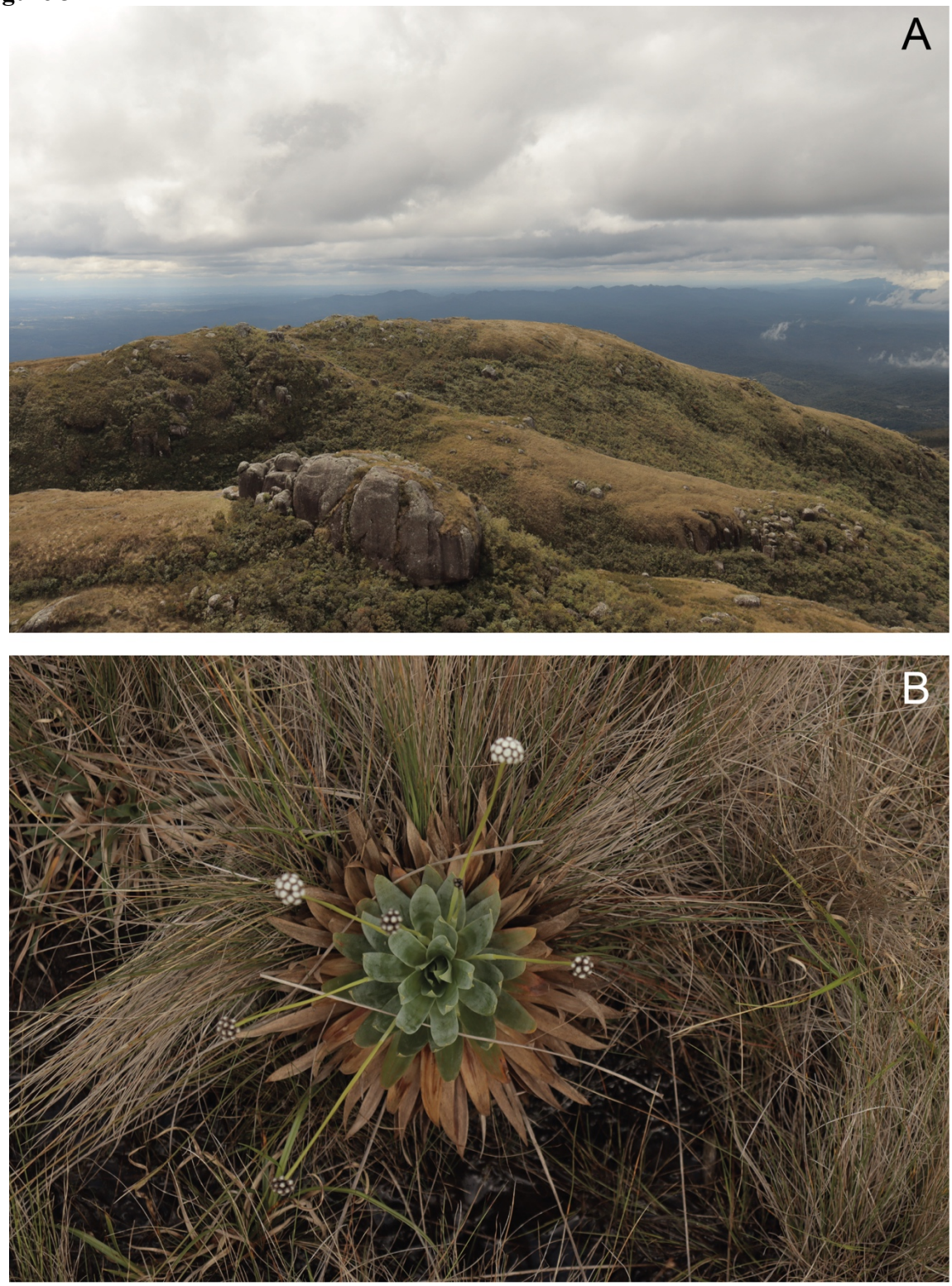

A
B
N
Wild bullfrog population
M. admirabilis population
0
200
400 Meters
28°51'8"S
28°51'12"S
28°51'16"S
28°51'20"S
28°51'24"S
28°51'28"S
28°51'32"S
52°18'25"W
52°18'20"W
52°18'15"W
52°18'10"W
52°18'5"W
52°18'W
52°17'55"W
52°17'50"W
52°17'45"W

Figure 3



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Supplementary material

Ranavirus infection and chytrid fungus lineages in species of the genus *Melanophryniscus*

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Table S1. Five locus sequence typing (MLST) markers genotyped used in this study to identify *Batrachochytrium dendrobatidis* lineages.

Locus	PCR primers
8009X2	F: 5'-TCGTGAAGAGCTTGGAAAGTCG-3'
	R: 5'-AGTTCTGTCGTCAATGCTGTAGGG-3'
BdC24-F	F: 5'-GACAATGTGCTCAC- GGCTTA-3'
	R: 5'-CTCTCCAAGGCTGAATCTGG- 3'
R6046	F: 5'-CTATCTGCGCTCCCGTGTCAA-3'
	R: 5'-AGGGCTGCAACAACTGGATTT-3'
BdSC6.15	F: 5'-GACGATAAAACGACAACAATCG-3'
	R: 5'-CCCTTTTTAGGTTGGCTTGC-3'
BsSC8.10	F: 5'-TGACAAAGTGCCGAGTGTTT-3'
	R:5'-TTGGCTATACCCGACTACGC-3'

CHAPTER IV

INSIGHTS INTO *Batrachochytrium dendrobatidis* DYNAMICS: *IN VITRO* MODELS WITH BRAZILIAN ISOLATES

Insights sobre a dinâmica do *Batrachochytrium dendrobatidis*: modelos *in vitro* com isolados brasileiros

Mariana Retuci Pontes, Elin Verbrugghe, Luís Felipe Toledo, Frank Pasmans & An Martel

Insights into *Batrachochytrium dendrobatidis* dynamics: *in vitro* models with Brazilian isolates

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Abstract

Chytridiomycosis stands as one of the major drivers of the current amphibian diversity loss and has been implicated in population declines and extinctions globally. The etiological agent of this disease in anuran is the waterborne fungus *Batrachochytrium dendrobatidis* (*Bd*). *Bd* infection can lead to antagonistic effects on the host due to the induction of epidermal hyperplasia, hyperkeratosis, and increased sloughing rates, ultimately disrupting essential homeostatic functions. Additionally, the invasive North American bullfrog (*Aquarana catesbeiana*), which is tolerant to *Bd*, represents a significant threat to native amphibian populations. In Brazil, the co-occurrence of feral bullfrog populations and the high prevalence of *Bd* in the southern Atlantic Forest raise concerns for native and threatened species. While *in vivo* studies are not recommended for assessing the consequences of *Bd* in threatened species, *in vitro* models are currently underexplored. Despite the numerous *in vivo* studies on *Bd* infection in Brazilian native hosts, our understanding of the early infection process of Brazilian *Bd* isolates at a cellular level remains limited. Therefore, we analyzed the *Bd* colonization cycle of the enzootic lineage Bd-Asia-2/Brazil and the Global Panzootic Lineage (Bd-GPL), both isolated from captives Brazilian bullfrogs, by modeling the *in vitro* infection in the continuous epithelial cell line A6 from *Xenopus laevis*. We successfully visualized the Bd-cells interactions of Brazilians isolates and observed the formation and growth of germ tubes, as well epibiotic and endobiotic growth in both isolates. However, the germ tubes formed by the Bd-Asia-2/Brazil isolate were significant longer than our reference isolate. The elongated germ tubes and higher occurrence of new thalli during the early stages of infection by Bd-Asia-2/Brazil lineage suggest a rapid invasion process of this isolate in amphibian's skin. This is the first *in vitro* trials with Brazilian isolates cultured from an invasive species. *In vitro* models can contribute to the knowledge of *Bd*-host dynamics and facilitate future studies addressing *Bd* virulence in Brazilian threatened amphibians.

Key-words: Atlantic Forest, amphibian disease, *Aquarana catesbeiana,* cell-based models, chytridiomycosis.

Introduction

Amphibians are an iconic example of the ongoing biodiversity crisis and a part of the 6th mass extinction event (Wake & Vredenburg 2008), with documented extinctions continuing to increase (IUCN 2023). Over the past few decades, the emergent fungal disease chytridiomycosis had a devastating impact on amphibian populations, emerging as one of the major drivers of amphibian diversity loss (Scheele et al. 2019). The chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is the etiological agent of chytridiomycosis in anurans. *Bd* colonizes the keratinized layers of amphibian's skin and induces epidermal hyperplasia, hyperkeratosis, and increased sloughing rates (Berger et al. 2005). *Bd* infection can lead to disruptions of essential homeostatic functions and severe infections can result in death (Berger et al. 2005, Voyles et al. 2007, Bovo et al. 2016, Salla et al. 2018).

In addition to infectious diseases, invasive species pose a significant threat to amphibians through direct predation (Leivas et al. 2013), competition for resources (Kiesecker et al. 2001, Boone et al. 2004), habitat degradation, and disease transmission (Rödder et al. 2013). The international wildlife trade exacerbates these dangers by facilitating the introduction of invasive species and pathogens. The North-American bullfrog *Aquarana catesbeiana* is farmed on a large scale to supply the international frog leg trade (Ribeiro & Toledo 2022, Salla et al. 2022, Auliya et al. 2023). Considered as one of the hundred worst Invasive Alien Species, bullfrog has invaded more than 40 countries (IUCN 2024), and unintentional escapes from farm have led to the establishment of invasive populations worldwide (Both et al. 2011, Garner et al. 2016, IUCN, 2024). Additionally, this species plays a crucial role in the dynamics of chytridiomycosis. Bullfrog farms release virulent zoospores into natural environments (Ribeiro et al. 2019) and their high tolerance to Bd (Eskew et al. 2015) enables individuals to harbor high *Bd* infection loads without developing the disease. Consequently, bullfrogs act as *Bd* reservoirs that could infect native amphibian species (Ribeiro et al. 2019, Santos et al. 2020), underscoring their ecological importance within amphibian conservation efforts, particularly in biodiversity hotspots.

In Brazil, the world's hotspot for amphibian diversity (Segalla et al. 2021, Frost 2024), feral populations of invasive bullfrogs are widespread across the southern region (Both et al. 2011). Moreover, *Bd* is highly prevalent in the southern Atlantic Forest (Carvalho et al. 2017, Lambertini et al. 2021), and a high genetic diversity of *Bd* lineages has already been reported (Jenkinson et al. 2016). While the enzootic lineage of Bd-Asia-2/Brazil occurs in a specific mountain in the southern Atlantic Forest, the Global Panzootic Lineage (Bd-GPL) is spread throughout the region (Jenkinson et al. 2016). The Bd-Asia-2/Brazil lineage, cultured

from southern native species showed hypovirulence in endemic hosts during *in vivo* trials, probably due to the long co-evolutionary history with endemic Brazilian amphibians (Greenspan et al. 2018). However, the impact of this lineage isolated from tolerant bullfrogs on native hosts is still unknown.

Southern Brazil encompasses numerous amphibians facing extinction risks, including species classified as Critically Endangered (CR) and even those classified as possibly extinct (CR-PEX) (Brasil 2022, Salve 2023). Among them, microendemic species or with limited distribution are of particularly concern (Anunciação et al. 2024), as they are more prone to extinction by stochastic events. Thus, the combination of bullfrog presence and *Bd* has the potential to increase disease risk to native and threatened species. Despite ecological studies investigating the *Bd* dynamics in wild Brazilian Redbelly Toads (Pontes et al. *in prep*.), given their high extinction risk, *in vivo* trials to evaluate *Bd*-host interaction is generally not recommended. Consequently, *in vitro* models can provide an alternative approach to accessing the stages of *Bd* infection without using wild amphibians. Thus, assessing the aspect of host-*Bd* interaction without the use of animals can be a promising alternative for further studies about chytridiomycosis in threatened species.

The early interactions of *Bd* with amphibian skin initiate with the adhesion of zoospores (motile flagellated stage of *Bd*) to skin, succeeded by zoospore germination, and germ tube development, followed by the penetration into cells (Berger et al. 2005). Subsequently, the thalli are formed (immature fungal bodies), and finally, mature zoospores are discharged on surface of epidermal cells of the stratum corneum, resulting in the loss of host cell cytoplasm (Berger et al. 2005). *In vitro* infection models using amphibian cells offer an excellent tool to gaining insight into amphibian-chytrid interactions, thereby reducing the need of live animals (Verbrugghe et al. 2019). Using the *Xenopus laevis* kidney epithelial cell line A6, Verbrugghe et al. (2019) developed a fluorescent cell-based *in vitro* model that mimics the complete colonization cycle of *Bd*. This model facilitates a rapid and efficient screening of host-*Bd* interactions, and enables the access of the differences in adhesion, invasion, and maturation interactions of distinct *Bd* isolates and lineages (Greener et al. 2020). Notably, this approach eliminates the need for live amphibians, offering a significant advantage, as the techniques are standardized.

Given the different *Bd* lineages present in southern Brazil (Jenkinson et al. 2016, Ribeiro et al. 2019) and the numerous threatened species, coupled with bullfrog spread in the southern region, this *in vitro* model developed by Verbrugghe et al. (2019) stands out as an useful tool for understanding the amphibian chytrid dynamic. Therefore, using a cell-based *in*

vitro model, we aimed to analyze the *Bd* colonization cycle of two lineages isolated from bullfrogs from Brazil.

Methods

Batrachochytrium dendrobatidis and A6 cells culture

We developed the study following Verbrugghe et al. (2019) protocols. We carried out the inoculations with a Bd-GPL CLFT 290 and with the enzootic lineage Bd-Asia-2/Brazil CLFT 301. Both isolates were obtained from tadpoles of captive bullfrogs (*Aquarana catesbeiana*) in southeastern Brazil. We used as isolate control/reference the hypervirulent Bd-JEL 423, isolated from a *Phyllomedusa lemur* treefrog in Panamá, and a representative of the Bd-GPL (Farrer et al. 2011). We routinely cultured *Bd* isolates in TGhL broth (1.6 % tryptone, 0.4 % gelatin hydrolysate and 0.2 % lactose in H_2O) in 75 cm^2 cell culture flasks at 20 ˚C for 5 days (Figure S1). To ensure that the zoospores were newly released, we washed the bottles with 10 mL of sterile distilled water to removed old zoospores and zoosporangia. We add 10 mL of sterilized distilled water and waited until the zoospores were released (2.5 hours). Once the zoospores were released, we carefully collected them and passed over a sterile mesh filter with pore size 10 μm (PluriSelect, Leipzig, Germany). The spore suspension was centrifuged for 5 min at 3000 rpm, the supernatant was removed, and the zoospores were resuspended/diluted in 30 % L15 medium (30 % L-15 medium, 60 % distilled water and 10 % fetal bovine serum [FBS]). Using a Neubauer chamber, we counted the zoospores and prepared a concentration of 10^6 spores/mL.

We grew the epithelial cell line A6 from *Xenopus laevis* kidney (ATCC-CCL 102) in 75 cm^2 cell culture flasks, and we maintained these cells in a complete growth medium (74 % NCTC 109 medium (Fisher Scientific), 15 % distilled water, 10 % FBS, 1 % of a 10,000 U/mL penicillin-streptomycin solution (Fisher Scientific), and 1 % of Kanamycin). We incubated the cells at 26 °C and 5 % CO_2 until they were ready to use (Figure S2). We then washed the A6 cells with 70 % Hanks' Balanced Salt Solution without Ca^{2}+, Mg^{2}+ (HBSS+), and detached them from the flasks using trypsin. We centrifuged them for 5 min at 1500 rpm, and we resuspended them in complete growth medium. The cells were counted using a Neubauer chamber and we seeded 10^5 cells per well in 24-well tissue culture plates containing collagen-coated glass coverslips for 24 hours at 26 ºC and 5 % CO_2.

Fluorescent in vitro model

Inoculation process

We analyzed the infected cells at three time points: 24 hours (adhesion/invasion), 72 (intracellular maturation) and 120 hours (release of intracellular *Bd* zoospores and cell death induction) post-infection. At each time point, two coverslips with cells were inoculated with zoospores of CLFT 290 (Bd-GPL), CLFT 301 (Bd-Asia-2/Brazil), and Bd-JEL 423 (Bd-GPL) isolates, totaling 18 coverslips. We use A6 cells without zoospores as cell control to check the cell morphology over different time points (3 controls; Figure S3 A–C). Additionally, we included one control of each *Bd* isolate (coverslips with *Bd* zoospores without cells; 9 controls; (Figure S3 D–K).

To start the inoculation process, we washed the cells already seeded in collagen-coated glass coverslips three times with 70 % HBSS+. We inoculated the cells with *Bd* zoospores of each isolate in 40 % L-15 medium (40 % L-15 medium, 55 % distilled water and 5 % FBS), at a multiplicity of infection (MOI) of 1:10. Two hours post-infection, we washed the infected cells three times with 70 % HBSS+ to remove non-adherent zoospores and we replaced cell medium by 70 % L-15 medium (70 % L-15 medium, 20 % distilled water and 10 % FBS). We maintained the plates at 20 ˚C, 5 % CO_2.

Staining process and analyses of the results

Before starting the staining process, we washed the infected cells two times with 70 % HBSS+. From 24, 72, and 120 hours post-infection, the infected cells were stained with 9 µM CellTracker™ Green for 45 min, at 15 ºC and 5 % CO_2. We removed the CellTracker™ Green and incubated them with Calcofluor White stain (10 µg/mL in 70 % HBSS+) for 10 min, at 15 ºC and 5 % CO_2. After washing three times with 70 % HBSS+, the cells were fixed using 3.7 % paraformaldehyde and we permeabilized them using 0.1 % Triton X-100, for 2 min. We incubated the plates for one hour with a polyclonal antibody against *Bd* raised in rabbit (1/1000) (Thomas et al. 2018). We washed them two times with 70 % HBSS+, and incubated with a monoclonal goat anti-rabbit Alexa Fluor 568 (1/500) antibody (Fisher Scientific; A11011). We incubated again for one hour, and we washed the cells two times with 70 % HBSS+, and finally, mounted the coverslips using ProLong™ Gold antifade mountant (Fisher Scientific). For CLFT 290 isolate, we analyzed only 24 and 72 hours post infection.

Using a combination of Alexa Fluor 568 targeting *Bd* and a Calcofluor White staining, we observed the intracellular and extracellular localization of *Bd*. We observed the epibiotic (development outside the host cells) and endobiotic (intracellular *Bd* colonization) *Bd* growth. We analyzed the germ tubes penetrating into A6 cells, maturation of the intracellular thalli, and possible formation of large intracellular zoosporangia. We also observe the formation

of a discharge tube (which eventually leads to the induction of cell death). We used fluorescence microscopy and Leica Application Suite (LAS) software with appropriate filter sets. We overlayed pictures using ImageJ 1.52d software.

Results

We successfully visualized the Bd-cells interactions of Brazilians isolates using fluorescent microscopy of the *Xenopus laevis* kidney epithelial cell line A6. We compared these isolates with the infection stages of the hypervirulent JEL 423 reference. From 24 hours post-infection, we observed the formation and growth of germ tubes, and their penetration into the A6 cells for all isolates (Figure 1). The germ tubes formed by the CLFT 301 isolate were longer compared with JEL 423, approximately 75 μm (Figure 1 A–B). The germ tubes of CLFT 290 isolate developed similar to the control JEL 423 (Figure 1C). We also observed the long germ tubes of CLFT 301 compared with JEL 423 even in the absence of cells (Figure 2 A–B; isolates control). Notably, there was a higher occurrence of intracellular swelling formation at the end of the germ tube, giving rise to a new *Bd* thallus (Figure 3).

From 24-72 hours post infection, we observed both epibiotic and endobiotic growth following germ tube protrusion into the cells across all isolates. We also observed a lot of infected cells containing *Bd* contents that were released into the cell (e.g., Figure 4), causing eventual induction of host cell death.

At 120 hours post-infection, we observed less A6 cells on the coverslips, probably due to apoptosis caused by the zoospores discharged into the cell by intracellular zoosporangia (Verbrugghe et al. 2019). The CLFT 290 interaction at 120 hours post-infection was not possible due a contamination of wells.

Discussion

Endobiotic development of *Bd* on susceptible hosts are crucial for successful colonization of the fungus into amphibian skin. By using the epithelial cell line A6 from *X. laevis* we observed the complete infection cycle of two Brazilian lineages isolated from bullfrogs. In our *in vitro* models we found similarities in *Bd* adhesion in A6 cells by CLFT 301 (Bd-BRAZIL) and the hypervirulent JEL 423 (Bd-GPL) isolates. However, we observe a significant difference in the germ tube development between these two isolates, with CLFT 301 showing a strong epibiotic elongation of the germ tube. Via germ tubes, the zoospores inject their nucleus and cytoplasm into the host cell, contents undergo mitosis, and zoospores are formed and released into the environment through discharge papillae (Longcore et al. 1999). In

susceptible hosts, germ tube development mediate the invasion of amphibian skin (van Rooij et al. 2012). Thus, the observed epibiotic long germ tube of the CLFT 301 could facilitate a rapid invasion process of *Bd* in amphibians skin. Additionally, the higher occurrence of *Bd* thalli formation at 24 hours post-infection by the CLFT 301 also suggests rapid development of mature zoosporangium (container for zoospores). The development of intracellular chytrid thalli at the end of a germ tube is important in the *Bd* infection process, as it transfers *Bd* cell contents into the newly formed thalli. As we used uniform *in vitro* conditions across all isolates, it is possible that the Bd-Asia-2/Brazil from a Brazilian bullfrog might exhibit hypervirulence such as JEL 463. Despite the possibility of have a faster early infection process, the colonization strategy of *Bd* is also influenced by inherent characteristics of the host epidermis.

While our *in vitro* models showed possibly higher virulence of Bd-Asia-2/Brazil CLFT 301 isolate, for Bd-GPL CLFT 290 the development was dominated by epibiotic growth, which can be linked to reduced virulence (Greener et al. 2020). Ribeiro et al (2019) showed that Bd-GPL isolates from bullfrogs can exhibit higher or lower virulence for Brazilian native amphibians. Controlled environments with high density and low diversity of hosts can favor the emergence of highly virulent genotypes by increasing transmission potential (Frank 1996). However, conditions associated with farms can also select for less virulent genotypes, possibly due to the constant availability of hosts (Carvalho et al. 2023) and our results are according to these previous findings. The implications of the enzootic lineage Bd-Asia-2/Brazil isolated from captive Brazilian bullfrogs for native and non-tolerant species are still unknown.

In a recent *in vivo* study, a Bd-Asia-2/Brazil lineage, isolated from a Brazilian bullfrog, exhibited lower virulence and competitiveness compared to a Bd-GPL (Carvalho et al. 2023). However, it is important to note that the study utilized another invasive species, *Eleutherodactylus johnstonei*, as model, known to tolerate *Bd* infections (Hudson et al. 2019). Hence, further research using *in vitro* models of *Bd* lineages is essential to fully understand the potential impacts of enzootic lineages isolated from tolerant hosts on native amphibian populations.

As the evolution of *Bd* virulence under farm conditions are complex and difficult to predict (Ribeiro et al. 2019), *in vitro* models can contribute to the knowledge into *Bd*-host dynamics. There are a currently a gap in studies regarding the interaction and consequences of Bd-Asia-2/Brazil isolate cultured from bullfrog for native Brazilian hosts. Our study represents the first cell-based *in vitro* trials with the Bd-Asia-2/Brazil isolated from a captive bullfrog of Brazil.

Bullfrogs are widespread in the southern Brazil and play an important role in the dynamics of infectious pathogens (Both et al. 2011, Ribeiro et al. 2019, Santos et al. 2020, Ribeiro & Toledo 2022, Ruggeri et al. 2019, 2013). Considering that chytridiomycosis is one of the major drivers of anuran diversity loss (Scheele et al. 2019), *in vitro* models could provide an important tool to understand and screen the Bd-host interaction, avoiding the overuse of animals (Verbrugghe et al. 2019). In Brazil, new studies using the epithelial cell line A6 from *X. laevis* to mimic the complete infection cycle of *Bd* can be useful, mainly in studies with questions related to endangered species and virulent *Bd* lineages and isolates. Cell-based *in vitro* models can contribute to new insights into the interaction between *Bd*-host skin of threatened species, such as the case of the Endangered (EN) *Melanophryniscus biancae* (Brasil 2022, Salve 2023), species exhibiting high Bd prevalence and infection loads (Pontes et al. *in prep.*).

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Figures

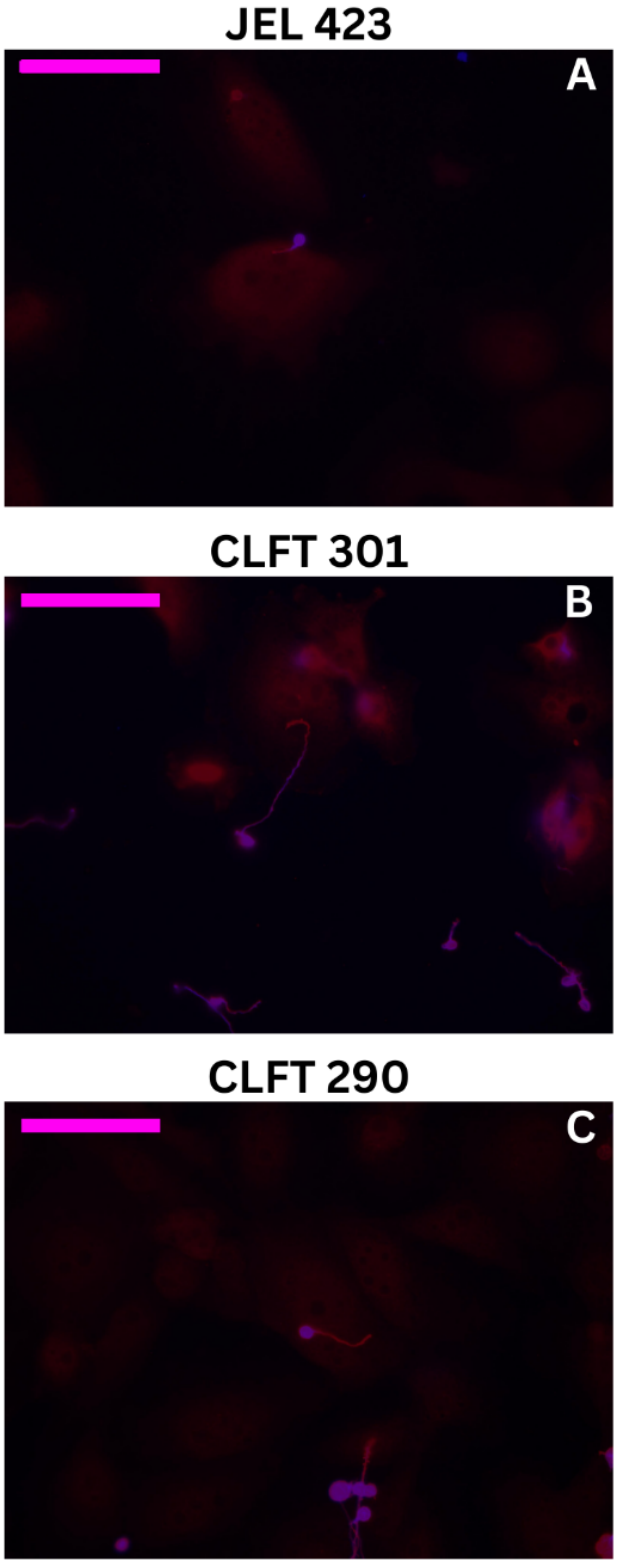

Figure 1. Formation and growth of the germ tube, and the germ tube penetration into the A6 cells for all isolates (A, B, C). Scale bar: 50 μm.

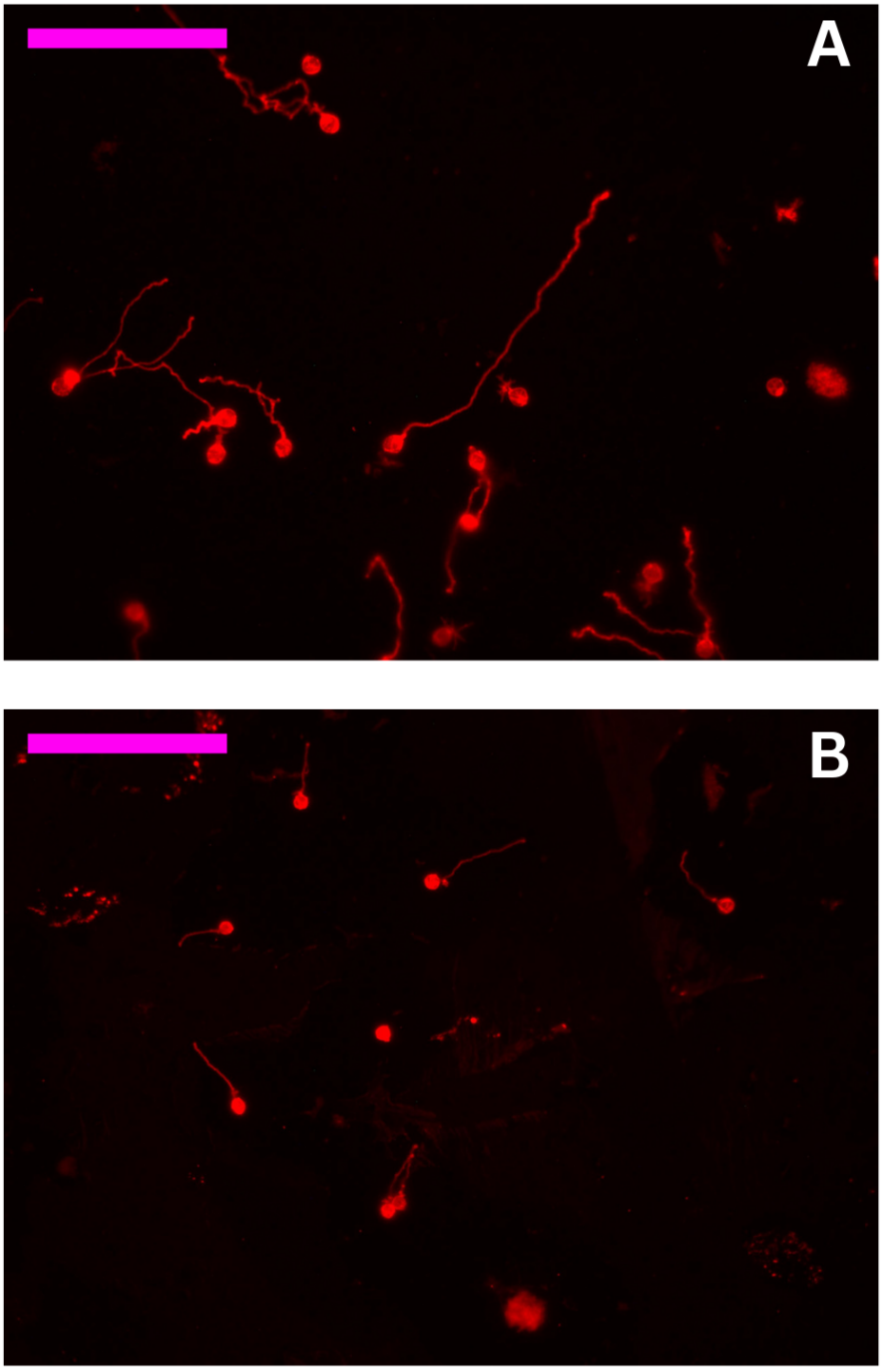

Figure 2. Long germ tubes of CLFT 301 (A) and JEL 423 (B) isolates in controls (coverslips without cells), 24 hours post-infection. Scale bar: 50 µm.

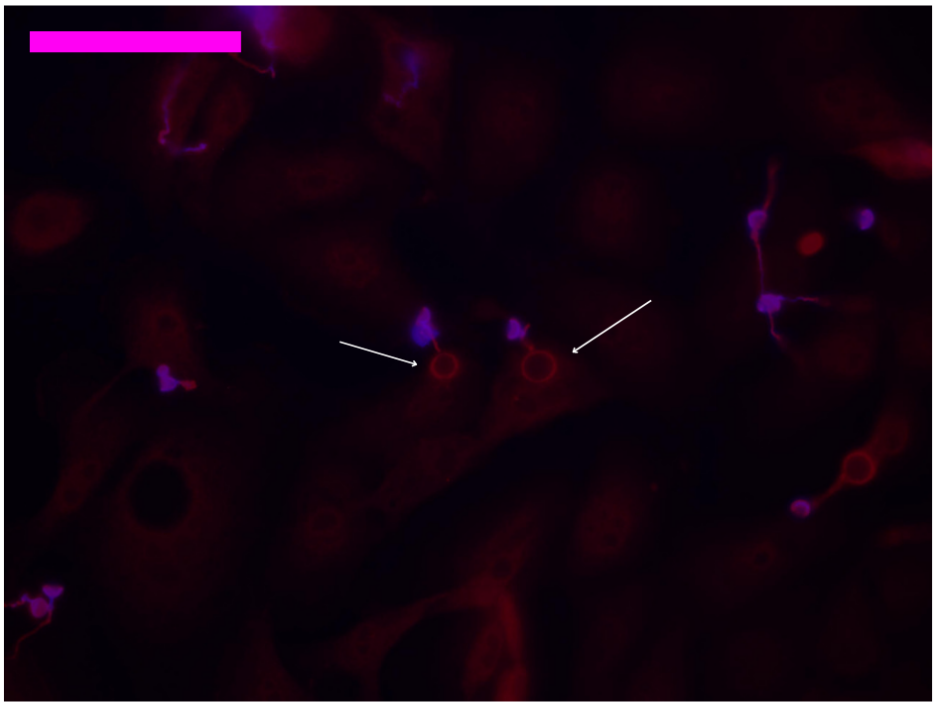

Figure 3. New intracellular *Bd* thalli formation of CLFT 301 isolate, 24 hours post-infection. Scale bar: 50 μm.

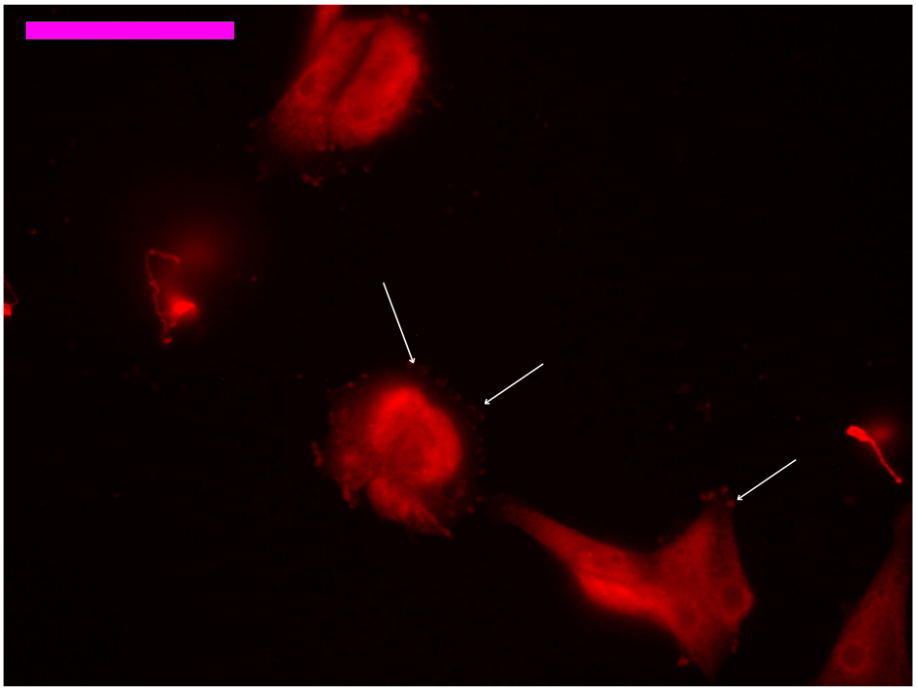

Figure 4. Contents released from the *Bd* isolate CLFT 301 into the A6 cells, 72 hours post-infection. Scale bar: 50 µm.

Supplementary material

Insights into *Batrachochytrium dendrobatidis* dynamics: *in vitro* models with Brazilian isolates

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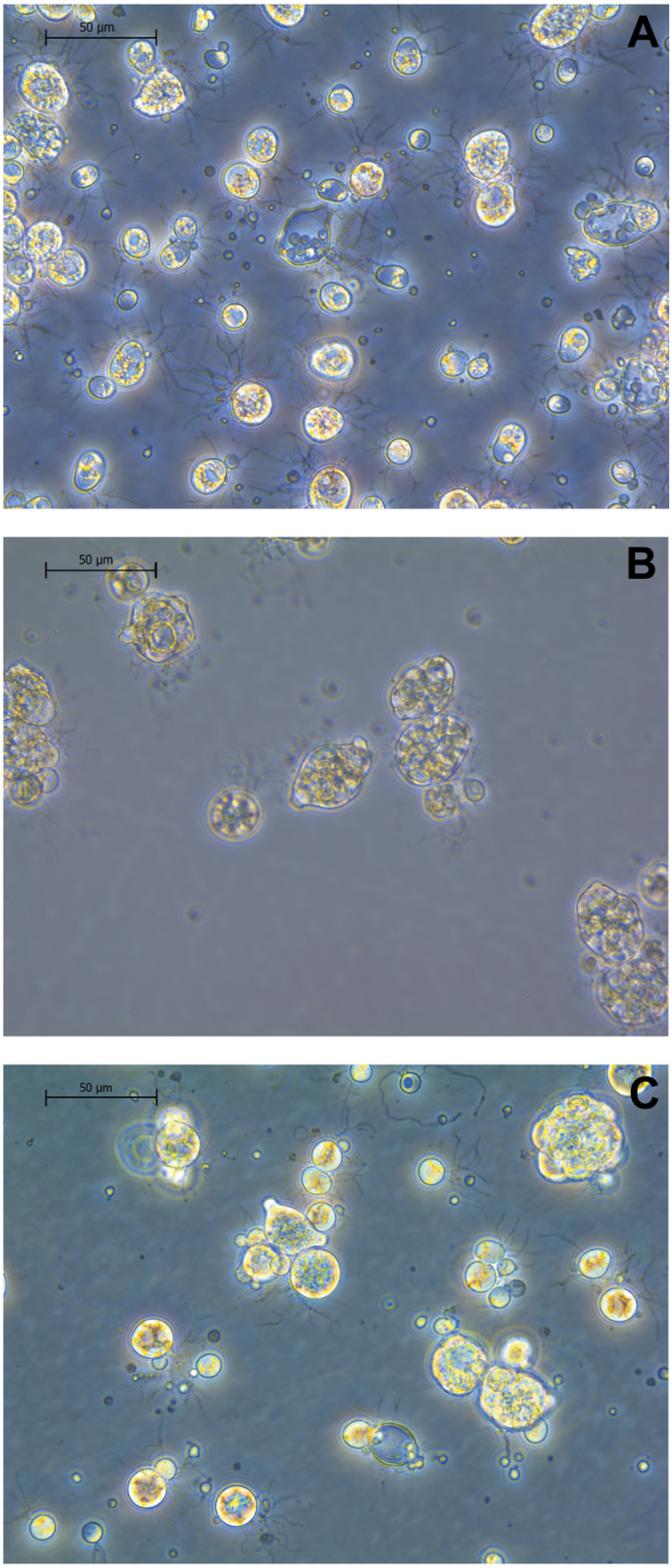

Figure S1. *Batrachochytrium dendrobatidis* full-grown cultures containing mature zoosporangia and zoospores. JEL 423 (A), CLFT 301 (B) and CLFT 290 (C) isolates. Scale bar = 50 µm.

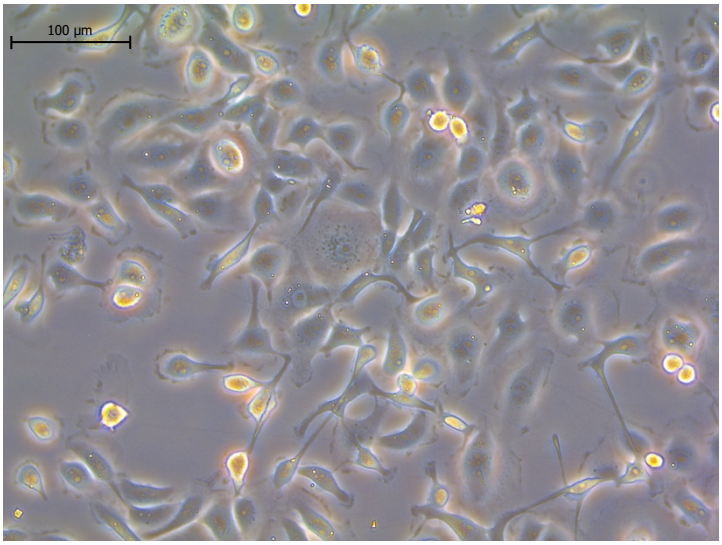

Figure S2. *Xenopus laevis* kidney epithelial cell line A6 (ATCC-CCL 102) in 74 % NCTC 109 medium. Scale bar = 100 µm.

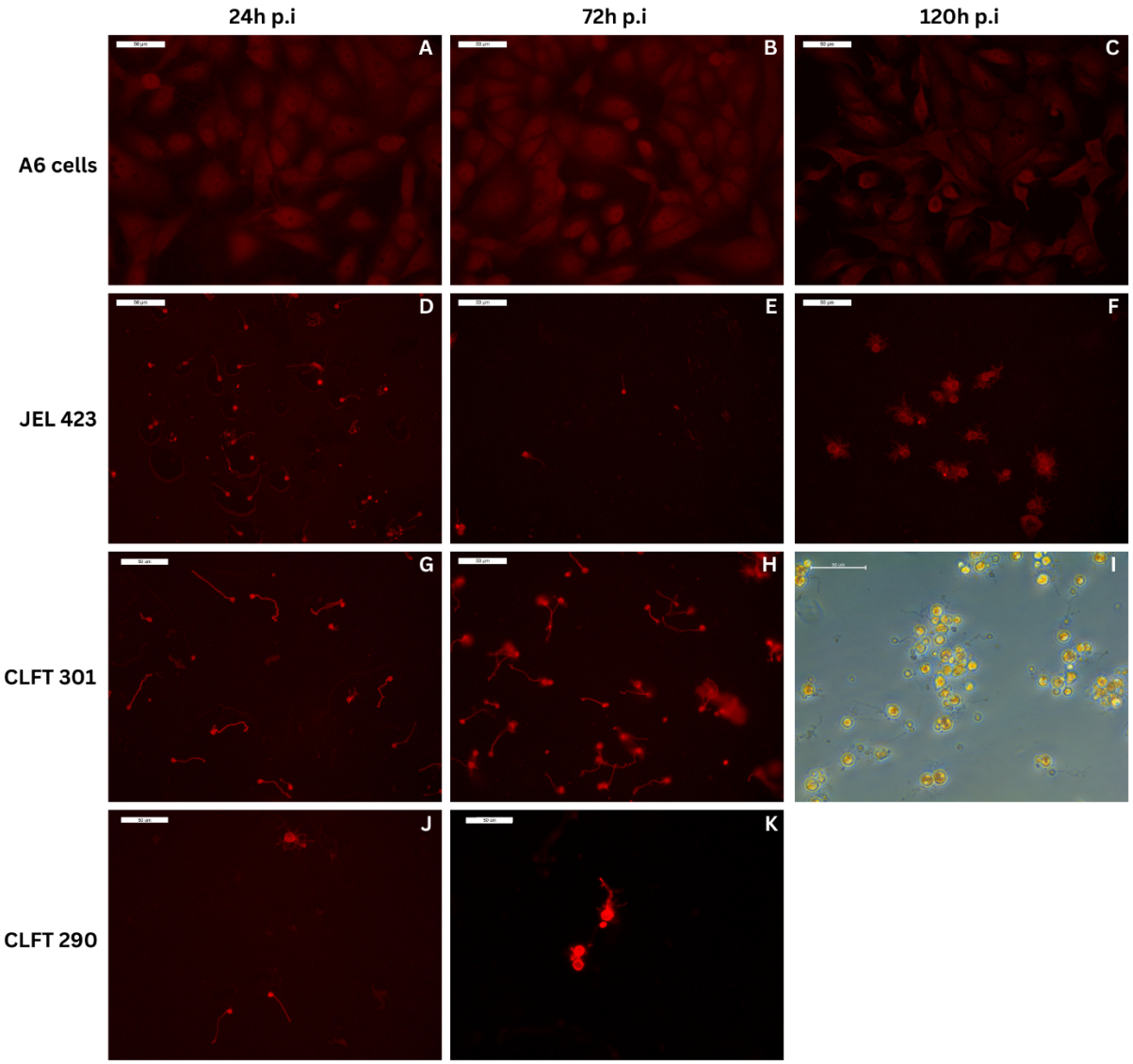

Figure S3. Negative controls of sham-infected A6 cells without the first antibody (A-C). JEL 423 (D-F) and CLFT 301 (G-I) isolates controls (coverslips without cells) in 24, 72 and 120 hours post infection. CLFT 290 isolate control in 24 and 72 hours post-infection (J-K). Scale bar = 50 μm.

CONCLUSÃO

As doenças infecciosas representam uma ameaça significativa aos anfíbios, grupo de vertebrados mais ameaçados do mundo (GAA2, Re:wild 2023). No Brasil, país com a maior diversidade de anfíbios (Segalla et al. 2021), o gênero *Melanophryniscus* se destaca como um grupo importante para os esforços de conservação (Anunciação et al. 2024). As espécies ameaçadas de extinção desse gênero apresentam microendemismo e/ou poucas populações conhecidas (Brasil 2022), e eventos estocásticos, como um surto de doenças, representam uma ameaça à existência desses animais.

A presente tese apresenta dados e informações sobre o fungo Bd em 10 populações de oito espécies do gênero *Melanophryniscus*, incluindo espécies ameaçadas de extinção. Elucidamos a importância da elevação na prevalência e carga de infecção por Bd, e demonstramos que, de modo geral, a prevalência e a carga de infecção por Bd foram baixas. Os hospedeiros infectados não apresentaram condição corporal reduzida, indicando que a infecção não causa grandes prejuízos a curto prazo para as espécies de *Melanophryniscus* estudadas. No entanto, encontramos que as populações de espécies ameaçadas de extinção têm maiores prevalências e cargas de infecção de Bd em relação às populações não ameaçadas. Desta forma, a presença deste patógeno em espécies ameaçadas deve ser monitorada ao longo do tempo, pois declínios causados por quitridiomicose podem ocorrer mesmo em populações historicamente infectadas (Carvalho et al. 2017). Uma única espécie, o ameaçado *M. biancae*, apresentou prevalência e cargas de infecção acima do esperado. Com a ampla amostragem realizada, foi possível entender também a distribuição espacial do Bd nesse gênero. Este trabalho representa o maior esforço amostral para diagnóstico do fungo Bd nos Sapinhos-de-barriga-vermelha do Brasil.

Além deste panorama geral sobre as taxas de Bd nas populações de *Melanophryniscus*, monitoramos o fungo Bd por mais de três anos na única população conhecida do criticamente ameaçado *M. admirabilis*. Encontramos que, em períodos mais secos e mais quentes, a prevalência de infecção por Bd é maior, sugerindo que as anomalias climáticas têm um papel crucial na dinâmica da transmissão de Bd nessa espécie. Esses resultados podem contribuir para o planejamento e elaboração de planos de manejo da espécie, oferecendo insights sobre os períodos em que os animais são mais suscetíveis às infecções.

Neste trabalho, registramos pela primeira vez, o genótipo híbrido do Bd fora da zona natural de sua ocorrência (Jenkinson et al. 2016, Carvalho et al. 2024). A presença deste genótipo em *M. biancae* (EN; IUCN 2023) é de especial preocupação, pois este genótipo pode

ser mais virulento e potencializar o risco de doenças nos hospedeiros (Greenspan et al. 2018). Recentemente, a espécie foi classificada como Criticamente Ameaçada de Extinção no estado do Paraná (Paraná 2024, Decreto nº 6.040). Adicionalmente, a presença de Rv nesta mesma população destaca a importância do planejamento de um esforço de conservação focado na espécie. Embora também tenhamos diagnosticado Rv em *M. admirabilis,* as taxas encontradas foram baixas. Os resultados sobre a presença de Rv encontrados neste trabalho, fornece pela primeira vez, informações de outro patógeno emergente em espécies em risco de extinção.

A detecção de Rv em espécies ameaçadas é de especial preocupação para conservação. Não existem trabalhos sobre como o Rv afeta anfíbios nativos do Brasil, e sobre as rotas de transmissão em ambientes naturais. Além disso, não há informações disponíveis sobre como a presença simultânea de ambos os agentes patogênicos pode prejudicar a existência de espécies nativas. Estudos futuros devem investigar questões sobre a sazonalidade de infecção, os padrões de transmissão e coocorrência com Bd a fim de evitar surtos ou interações negativas para os hospedeiros.

Neste trabalho, também foi possível compreender através de experimentos *in vitro,* que cepas de Bd isoladas da espécie invasora *Aquarana catesbeiana* podem apresentar características de alta virulência durante os processos iniciais de infecção celular. Identificamos que uma cepa isolada de rã-touro apresentou tubos germinativos mais longos, o que pode contribuir para um rápido processo de infecção na pele dos hospedeiros anfíbios. Esse resultado indica que cepas de Bd de anfíbios tolerantes à infecção podem representar um risco de doença significativo para espécies nativas. Considerando que a região sul do país contém inúmeras populações selvagens de rã-touro (Both et al. 2011), o mapeamento e entendimento da distribuição dessa espécie são ferramentas importantes para planejar ações de erradicação e evitar a dispersão de patógenos para espécies ameaçadas.

Baseado nas informações apresentadas nesta tese, sugerimos estudos futuros que investiguem a presença dos patógenos Bd e Rv nas outras espécies ameaçadas do gênero que não foram contempladas neste trabalho: *M. setiba*, *M. cambaraensis*, *M. klappenbachi* e *M. montevidensis.* Destacamos também a importância de ações de conservação direcionadas para *M. biancae*, como por exemplo, o desenvolvimento de um Plano Estratégico para Conservação de Anfíbios (PECAn). Este plano poderia incluir ações de monitoramento de patógenos, dado que este anuro está ameaçado de extinção (IUCN 2023, Paraná 2024, Decreto nº 6.040) e apresentou altas taxas de Bd, genótipo híbrido, e infecções por Rv.

O presente trabalho buscou contribuir de forma significativa para a conservação de anfíbios no Brasil através do fornecimento de informações sobre doenças em 10 populações de

Melanophryniscus. Alguns resultados obtidos aqui lançaram luz sobre algumas questões, e foram importantes para concepção de novos projetos focados em ações de conservação. Apesar dos diagnósticos positivos do Bd e Rv em várias populações do gênero *Melanophryniscus,* esta tese apresentou também ótimas notícias sobre a baixa prevalência do fungo no gênero. Este trabalho forneceu dados importantes para processos de elaboração de políticas públicas, como por exemplo, atualização da lista de espécies das espécies da fauna ameaçada de extinção no Estado do Paraná (Paraná 2024, Decreto nº 6.040). Todos os resultados obtidos e as experiencias adquiridas estão disponíveis para contribuir com a conservação dos Sapinhos-de-barriga-vermelha.

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Anexo I – Produção científica e outras atividades durante a vigência do curso de doutorado (08/2019 – 08/2024)

Produção científica

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5) Prado, J.S., Ernetti, J., **Pontes, M.R.** & Toledo, L.F. Chytrid in the clouds: an alternative passive transport of a lethal pathogen for amphibians. Hydrobiologia, v. 850, p. 2061-2073, **2023**.
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11) Queiroz, M.S., **Pontes, M.R.,** Chiquitelli, M., Campião, K.M. & Anjos L.A. Helminths of eight anuran species from a remnant riparian forest in the Cerrado biome, Brazil. Herpetology Notes, v. 13, p. 463-478, **2020**.
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1) Rebouças, R., Salla, R.F., **Retuci Pontes, M.** & Toledo, L.F. Anfíbios em ilhas brasileiras. Herpetologia para todos. Capítulo de livro. **2023**.

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Pontes, M.R, Abadie, M, Ribeiro, L.P, Augusto-Alves, Borges-Martins, M, Becker, C.G, Toledo, L.F. 2024. Climatic drivers of chytrid prevalence in the Critically Endangered Admirable Redbelly Toad. X World Congress of Herpetology (WCH10), 2nd Global Amphibian and Reptile Disease Conference (GARD2024), Kuching, Sarawak, Borneo, Malásia.

Pontes, M.R, Ruggeri, J, Rosa, G.M, Trafford, J, Teixeira, L, Bornshein, M.R, Toledo, L.F. 2023. Coinfecção de Ranavírus e fungo quitrídio em uma espécie de anuro ameaçada de extinção no Brasil: primeiro registro. X Congresso Brasileiro de Herpetologia, Porto Seguro, Bahia, Brasil.

Pontes, M.R, Abadie, M, Borges-Martins, M, Toledo, L.F. 2022. The pathogenic chytrid fungus in the threatened Admirable Redbelly Toad, *Melanophryniscus admirabilis*. First Global Amphibian and Reptile Disease Conference (online).

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Palestras ministradas

Pontes, M.R. 2024. Doenças emergentes em anfíbios brasileiros. Abertura da exposição *Ex libris*. Museu Biológico, Instituto Butantan, São Paulo, São Paulo, Brasil.

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Pontes, M.R. 2022. Doenças em anfíbios Brasileiros. II Congresso Brasileiro de Biodiversidade Virtual (online).

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Oficinas

Validação das avaliações das categorias de ameaças do táxon Amphibia. 2023. Parte do Projeto "Revisão, Atualização e Ampliação da Lista de Espécies da Fauna Silvestre Ameaçadas de Extinção no Estado do Paraná". Gerência de Biodiversidade - Diretoria do Patrimônio Natural, sendo executado pelo Mater Natura - Instituto de Estudos Ambientais, com apoio da WWF-Brasil Projeto Pró-Espécies. Resultado: Decreto nº 6.040. Art. 1º Reconhece as espécies da fauna ameaçada de extinção no Estado do Paraná. 05 de Junho de 2024 - Edição nº 11673.

Plano de Ação Nacional para a Conservação da Herpetofauna Ameaçada de Extinção do Sudeste (PANSE). 2023. Organização: Centro Nacional de Pesquisa e Conservação de Répteis e Anfíbios (RAN/ICMBio), Floresta Nacional de Ipanema, Iperó, São Paulo. Período: de 27 a 31 de março de 2023;

- Articuladora da Ação 4.3: "Executar pesquisas sobre a biologia e ecologia de *Melanophryniscus setiba* para subsidiar ações de conservação". (PANSE)

Webinars e workshops

IUCN Green Status of Species, online, 2024

Conservation Planning for Freshwater Ecosystems, online, 2024

Ranavirus Webinar & Virtual Workshop, online, 2023

I Workshop de Biogeografia da Unicamp, Campinas, SP, 2022

Conservation Needs Assessment, ouvinte, online, 2020

Contribuição à Sociedade Brasileira de Herpetologia (SBH) e à revista Herpetologia Brasileira (HB)

Membro editorial da revista Herpetologia Brasileira (HB), na seção "Notícias de Conservação" (agosto 2020 - atual).

Coordenação da Equipe de Divulgação da HB (junho 2022 – junho 2023).

Membro da Comissão de Redes Sociais da SBH (outubro de 2020 - junho 2023).

Membro da Comissão de Redes Sociais da HB (outubro de 2020 – junho 2023).

Revisor de periódico

Herpetological Journal

Scientific Reports

Marine and Freshwater Research

Global Ecology and Conservation

Participação em bancas de trabalho de conclusão de curso

Pontes, M.R, Rigacci, E.D.B, Faria, R.S. Aspectos da conservação da tartaruga-verde (*Chelonia mydas*) no município de Ubatuba, litoral norte de São Paulo. Graduação em Ciências Biológicas. Pontifícia Universidade Católica de Campinas. 2023.

Pontes, M.R**,** Augusto-Alves, G, Faria, R.S. Aspectos da análise de impactos ambientais na implementação de indústrias têxteis. Graduação em Ciências Biológicas - Pontifícia Universidade Católica de Campinas. 2023.

Associações

Sociedade Brasileira de Herpetologia (SBH)

Instituto Boitatá (Associação Instituto Boitatá de Etnobiologia e Conservação da Fauna; associada colaboradora)

Financiamentos e representações

Financiamento parcial do Global Amphibian & Reptile Disease Conference (GARD), para participação no GARD 2024, realizado em conjunto com o X World Congress of Herpetology (WCH10), de 4-10 de agosto de 2024, em Kuching, Sarawak, Malásia.

Financiamento integral do Resilience Institute Bridging Biological Training and Research (RIBBiTR), para participação no Amphibian Conservation Training Workshop, realizado de 26 de agosto a 01 de setembro de 2024, em Sierra Nevada, Califórnia, USA.

Financiamento parcial do SAVE THE FROGS! para uma visita técnica à University of Michigan, Ann Arbor, USA. 2023 e para participação no Global Amphibian & Reptile Disease Conference (GARD), realizado em conjunto com o X World Congress of Herpetology (WCH10), de 4-10 de agosto de 2024, em Kuching, Malásia.

Doação de equipamentos de campo por Idea Wild. 2020.

Representante discente (suplente) do programa de Pós-graduação em Ecologia, da Universidade Estadual de Campinas (UNICAMP). 2022.

Membro da Comissão de Finanças do Pós-graduação em Ecologia, da Universidade Estadual de Campinas (UNICAMP). 2019/2020.

Anexo II – Licença de coleta: Instituo Chico Mendes para a Conservação da Biodiversidade (SisBio # 72718-2)

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 72718-2	Data da Emissão: 12/02/2020 09:52:33	Data da Revalidação*: 22/11/2020
De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.		

Dados do titular

Nome: Mariana Retuci Pontes	CPF: 421.095.958-84
Título do Projeto: Fungos e vírus letais em espécies do gênero Melanophryniscus (Anura: Bufonidae)	
Nome da Instituição: UNIVERSIDADE ESTADUAL DE CAMPINAS	CNPJ: 46.068.425/0001-33

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Coleta de swabs de anfíbios	12/2019	12/2022
2	Coleta de girinos	12/2019	12/2022

Equipe

#	Nome	Função	CPF	Nacionalidade
1	GUILHERME AUGUSTO ALVES	Biólogo	401.739.638-03	Brasileira
2	JOICE RUGGERI GOMES	Bióloga	102.891.307-94	Brasileira
3	LUÍS FELIPE DE TOLEDO RAMOS PEREIRA	Pesquisador coordenador/biólogo	289.618.908-40	Brasileira
4	Márcio Borges Martins	Pesquisador/biólogo	646.691.060-15	Brasileira
5	Michelle Abadie de Vasconcellos	Bióloga	018.059.000-64	Brasileira
6	Luisa de Pontes Ribeiro	Bióloga	384.418.958-05	Brasileira
7	Tamilie Carvalho	Bióloga	367.720.108-09	Brasileira
8	Carlos Esequiel de Lima Toffolo	Auxiliar de campo	032.700.880-60	Brasileira
9	CAIO MARINHO MELLO	Biólogo	395.092.848-07	Brasileira
10	Valentina Zaffaroni Caorsi	Bióloga	018.516.050-61	Brasileira
11	NATÁLIA DALLAGNOL VARGAS	Bióloga	021.587.880-97	Brasileira
12	Patrick Colombo	Biólogo	927.385.590-34	Brasileira
13	VICTOR FÁVARO AUGUSTO	Biólogo	407.555.738-38	Brasileira
14	Daniel Christofer Medina López	Biólogo	241.963.528-04	Estrangeira
15	Felipe André Pavan	Biólogo	019.438.690-22	Brasileira
16	Camila Inés Zornosa Torres	Bióloga	236.898.378-39	Brasileira
17	Mário Júnior Nadaline Barbosa	Biólogo	083.359.399-42	Brasileira
18	Joelma Santos do Prado	Bióloga	448.035.668-10	Brasileira
19	Julia Renata Ernetti	Bióloga	093.349.799-76	Brasileira

Ministério do Meio Ambiente - MMA

Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio

Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 72718-2	Data da Emissão: 12/02/2020 09:52:33	Data da Revalidação*: 22/11/2020
De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.		

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Nome da Instituição: UNIVERSIDADE ESTADUAL DE CAMPINAS	CNPJ: 46.068.425/0001-33

Observações e ressalvas

1	A autorização não eximirá o pesquisador da necessidade de obter outras anuências, como: I) do proprietário, arrendatário, posseiro ou morador quando as atividades forem realizadas em área de domínio privado ou dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso; II) da comunidade indígena envolvida, ouvido o órgão indigenista oficial, quando as atividades de pesquisa forem executadas em terra indígena; III) do Conselho de Defesa Nacional, quando as atividades de pesquisa forem executadas em área indispensável à segurança nacional; IV) da autoridade marítima, quando as atividades de pesquisa forem executadas em águas jurisdicionais brasileiras; V) do Departamento Nacional da Produção Mineral, quando a pesquisa visar a exploração de depósitos fossilíferos ou a extração de espécimes fósseis; VI) do órgão gestor da unidade de conservação estadual, distrital ou municipal, dentre outras.
2	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infraestrutura da unidade.
3	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.
4	Este documento somente poderá ser utilizado para os fins previstos na Instrução Normativa ICMBio n° 03/2014 ou na Instrução Normativa ICMBio n° 10/2010, no que especifica esta Autorização, não podendo ser utilizado para fins comerciais, industriais ou esportivos. O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior.
5	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
6	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.
7	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
8	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico. Veja maiores informações em www.mma.gov.br/cgen.

Outras ressalvas

1	O pesquisador estrangeiro Daniel Christofer Medina López, que é membro da equipe de pesquisa, possui o vínculo junto ao Programa de Bolsas ou Auxílio à Pesquisa patrocinado pela FAPESP. Portanto, está dispensado de autorização do Ministério da Ciência, Tecnologia e Inovação.	COINF
2	OS QUANTITATIVOS AUTORIZADOS PARA COLETA SE RESTRINGEM A INDIVÍDUOS EM FASE LARVAL (GIRINOS). NÃO ESTÃO AUTORIZADAS COLETAS DE MELANOPHRYNISCUS ADULTOS.	RAN Goiânia-GO

Este documento foi expedido com base na Instrução Normativa nº 03/2014. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

Ministério do Meio Ambiente - MMA

Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio

Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 72718-2	Data da Emissão: 12/02/2020 09:52:33	Data da Revalidação*: 22/11/2020
De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.		

Dados do titular

Nome: Mariana Retuci Pontes	CPF: 421.095.958-84
Título do Projeto: Fungos e vírus letais em espécies do gênero Melanophryniscus (Anura: Bufonidae)	
Nome da Instituição: UNIVERSIDADE ESTADUAL DE CAMPINAS	CNPJ: 46.068.425/0001-33

Locais onde as atividades de campo serão executadas

#	Descrição do local	Município-UF	Bioma	Caverna?	Tipo
1	Todo Estado	Queluz-SP	Mata Atlântica	Não	Fora de UC Federal
2	Todo estado	Aiuruoca-MG	Mata Atlântica	Não	Fora de UC Federal
3	Todo estado	Tijucas do Sul-PR	Mata Atlântica	Não	Fora de UC Federal
4	Todo estado	Torres-RS	Mata Atlântica	Não	Fora de UC Federal
5	Serra do Papagaio	Alagoa-MG	Mata Atlântica	Não	Fora de UC Federal
6	Parque Estadual Paulo César Vinha	Guarapari-ES	Mata Atlântica	Não	Dentro de UC Estadual
7	Todo estado	Balneário Camboriú-SC	Mata Atlântica	Não	Fora de UC Federal
8	Ilha dos Marinheiros	Rio Grande-RS	Mata Atlântica	Não	Fora de UC Federal
9	Parque Estadual de Vila Velha	Ponta Grossa-PR	Mata Atlântica	Não	Dentro de UC Estadual
10	rio Forqueta (Perau de Janeiro)	Arvorezinha-RS	Mata Atlântica	Não	Fora de UC Federal
11	Parque Natural Municipal de Sert?o	Sertão-RS	Mata Atlântica	Não	Dentro de UC Municipal
12	Nova Teutônia - spectabilis	Seara-SC	Mata Atlântica	Não	Fora de UC Federal
13	Serra do Capivari	Campina Grande do Sul-PR	Mata Atlântica	Não	Fora de UC Federal
14	Serra do Quiriri	Garuva-SC	Mata Atlântica	Não	Fora de UC Federal
15	Passos Maia	Passos Maia-SC	Mata Atlântica	Não	Fora de UC Federal
16	Bom Jardim da Serra	Bom Jardim da Serra-SC	Mata Atlântica	Não	Fora de UC Federal
17	São Francisco de Paula	São Francisco de Paula-RS	Mata Atlântica	Não	Fora de UC Federal
18	Biturana	Bituruna-PR	Mata Atlântica	Não	Fora de UC Federal
19	Campos Novos	Campos Novos-SC	Mata Atlântica	Não	Fora de UC Federal
20	Morro dos Conventos	Araranguá-SC	Mata Atlântica	Não	Fora de UC Federal
21	Parque Estadual de Itapeva	Torres-RS	Mata Atlântica	Não	Dentro de UC Estadual
22	Gravataí	Gravataí-RS	Mata Atlântica	Não	Fora de UC Federal

Atividades

#	Atividade	Grupo de Atividade
1	Coleta/transporte de espécimes da fauna silvestre in situ	Fora de UC Federal
2	Coleta/transporte de amostras biológicas in situ	Fora de UC Federal
3	Captura de animais silvestres in situ	Fora de UC Federal
4	Coleta/transporte de material botânico, fúngico ou microbiológico	Fora de UC Federal

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Ministério do Meio Ambiente - MMA

Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio

Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

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Dados do titular

Nome: Mariana Retuci Pontes	CPF: 421.095.958-84
Título do Projeto: Fungos e vírus letais em espécies do gênero Melanophryniscus (Anura: Bufonidae)	
Nome da Instituição: UNIVERSIDADE ESTADUAL DE CAMPINAS	CNPJ: 46.068.425/0001-33

Atividades X Táxons

#	Atividade	Táxon	Qtde.
1	Captura de animais silvestres in situ	Melanophryniscus fulvoguttatus	-
2	Coleta/transporte de espécimes da fauna silvestre in situ	Melanophryniscus fulvoguttatus	30
3	Captura de animais silvestres in situ	Melanophryniscus setiba	-
4	Coleta/transporte de espécimes da fauna silvestre in situ	Melanophryniscus setiba	30
5	Captura de animais silvestres in situ	Melanophryniscus moreirae	-
6	Coleta/transporte de espécimes da fauna silvestre in situ	Melanophryniscus moreirae	30
7	Captura de animais silvestres in situ	Melanophryniscus milanoi	-
8	Coleta/transporte de espécimes da fauna silvestre in situ	Melanophryniscus milanoi	30
9	Captura de animais silvestres in situ	Melanophryniscus biancae	-
10	Coleta/transporte de espécimes da fauna silvestre in situ	Melanophryniscus biancae	30
11	Captura de animais silvestres in situ	Melanophryniscus alipioi	-
12	Coleta/transporte de espécimes da fauna silvestre in situ	Melanophryniscus alipioi	30
13	Captura de animais silvestres in situ	Melanophryniscus vilavelhensis	-
14	Coleta/transporte de espécimes da fauna silvestre in situ	Melanophryniscus vilavelhensis	30
15	Captura de animais silvestres in situ	Melanophryniscus admirabilis	-
16	Coleta/transporte de espécimes da fauna silvestre in situ	Melanophryniscus admirabilis	30
17	Captura de animais silvestres in situ	Melanophryniscus dorsalis	-
18	Coleta/transporte de espécimes da fauna silvestre in situ	Melanophryniscus dorsalis	30
19	Captura de animais silvestres in situ	Melanophryniscus devincenzii	-
20	Coleta/transporte de espécimes da fauna silvestre in situ	Melanophryniscus devincenzii	30
21	Captura de animais silvestres in situ	Melanophryniscus simplex	-
22	Coleta/transporte de espécimes da fauna silvestre in situ	Melanophryniscus simplex	30
23	Captura de animais silvestres in situ	Melanophryniscus spectabilis	-
24	Coleta/transporte de espécimes da fauna silvestre in situ	Melanophryniscus spectabilis	30
25	Captura de animais silvestres in situ	Melanophryniscus tumifrons	-
26	Coleta/transporte de espécimes da fauna silvestre in situ	Melanophryniscus tumifrons	30
27	Coleta/transporte de material botânico, fúngico ou microbiológico	Batrachochytrium dendrobatidis	-
28	Coleta/transporte de amostras biológicas in situ	Batrachochytrium dendrobatidis	-

Ministério do Meio Ambiente - MMA

Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio

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Nome: Mariana Retuci Pontes	CPF: 421.095.958-84
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Materiais e Métodos

#	Tipo de Método (Grupo taxonômico)	Materiais
1	Amostras biológicas (Anfíbios)	Outras amostras biológicas(Swabs e girinos)
2	Amostras biológicas (Fungos)	Outras amostras biológicas(Swabs de pele de anuros adultos)
3	Método de captura/coleta (Anfíbios)	Captura manual, Peneira

Destino do material biológico coletado

#	Nome local destino	Tipo destino
1	UNIVERSIDADE ESTADUAL DE CAMPINAS	Coleção

Ministério do Meio Ambiente - MMA

Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio

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Dados do titular

Nome: Mariana Retuci Pontes	CPF: 421.095.958-84
Título do Projeto: Fungos e vírus letais em espécies do gênero Melanophryniscus (Anura: Bufonidae)	
Nome da Instituição: UNIVERSIDADE ESTADUAL DE CAMPINAS	CNPJ: 46.068.425/0001-33

Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº03/2014, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	Qtde.	Tipo de Amostra	Qtde.	Data

* Identificar o espécime do nível taxonômico possível.

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Código de autenticação: 0727180220200212

Anexo III – Licença de coleta: Instituo Ambiental do Paraná (#51/19; atual Instituto Água e Terra)

IAP INSTITUTO AMBIENTAL DO PARANÁ

PARANÁ GOVERNO DO ESTADO

UNIDADES DE CONSERVAÇÃO DO PARANÁ

AUTORIZAÇÃO DE PESQUISA EM UNIDADE DE CONSERVAÇÃO DO PARANÁ

Número: 51/19	**Data de Emissão: 20/12/19**	**Protocolo: 16.231.487-4**

Dados do Pesquisador e da Pesquisa:

Nome: Mariana Retuci Pontes
RG: 40.999.713-4 CPF: 421.095.958-84
Título do projeto: Investigação da presença de fungos e vírus letais em espécies do gênero *Melanophryniscus* (Anura: Bufonidae).

Cronograma:

Unidade de Conservação:
Parque Estadual de Vila Velha
Parque Estadual Serra Graciosa

Equipe de trabalho:

Guilherme Augusto Alves	RG:36.011.933-5	CPF:401.739.638-03
Joice Ruggeri Gomes	RG:11.425.367-7	CPF:102.891.307-94
Caio Marinho Mello	RG:35.399.754-7	CPF:395.092.848-07
Luisa de Pontes Ribeiro	RG:46.822.713-1	CPF:384.418.958-05
Luis Felipe Toledo	RG:2.863.576-2	CPF:289.618.908-40

Observações:

1. Não é permitida a coleta de espécies ameaçadas ou em risco de extinção;
2. As gerências da(s) UC(s) devem ser comunicadas com antecedência sobre os trabalhos em campo a serem realizados na Unidade;
3. Esta autorização tem validade até **20/12/2020** podendo ser renovada no final do período.
4. Esta autorização **não da o direito do uso das imagens** oriundas desse trabalho.
5. O pesquisador titular fica inteiramente responsável por qualquer integrante da sua equipe de trabalho, sendo ele brasileiro ou estrangeiro.

Rafael Andreguetto
Diretor de Biodiversidade e Áreas Protegidas – DIBAP
Curitiba, 20 de dezembro de 2019.

Anexo IV – Parecer da Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas (CEUA/Unicamp #4983-1/2018)

UNICAMP

CEUA/UNICAMP

CERTIFICADO

Certificamos que a proposta intitulada **O fungo quitrídio e Ranavírus em espécies do gênero Melanophryniscus (Anura: Bufonidae)**, registrada com o nº **5581-1/2020**, sob a responsabilidade de **Prof. Dr. Luís Felipe de Toledo Ramos Pereira e Mariana Retuci Pontes**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal (CONCEA), tendo sido** aprovada pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP,** em reunião de **20/08/2020**.

Finalidade:	**( ) Ensino (X) Pesquisa Científica**
Vigência do projeto:	01/09/2020 a 30/04/2024
Vigência da autorização para manipulação animal:	20/08/2020 a 30/04/2024
Espécie / linhagem/ raça:	Anfíbio** / Melanoprhyniscus admirabilis
No. de animais:	60
Idade/Peso:	1.00 Anos / 3.00 Gramas
Sexo:	50 Machos 10 Fêmeas
Espécie / linhagem/ raça:	Anfíbio** / Melanoprhyniscus dorsalis
No. de animais:	60
Idade/Peso:	1.00 Anos / 2.00 Gramas
Sexo:	50 Machos 10 Fêmeas
Espécie / linhagem/ raça:	Anfíbio** / Melanoprhyniscus biancae
No. de animais:	60
Idade/Peso:	1.00 Anos / 1.00 Gramas
Sexo:	50 Machos 10 Fêmeas
Espécie / linhagem/ raça:	Anfíbio** / Melanoprhyniscus devincenzii
No. de animais:	60
Idade/Peso:	1.00 Anos / 2.00 Gramas
Sexo:	50 Machos 10 Fêmeas
Espécie / linhagem/ raça:	Anfíbio** / Melanoprhyniscus alipioi
No. de animais:	60
Idade/Peso:	1.00 Anos / 2.00 Gramas
Sexo:	50 Machos 10 Fêmeas
Espécie / linhagem/ raça:	Anfíbio** / Melanoprhyniscus vilavelhensis
No. de animais:	60
Idade/Peso:	1.00 Anos / 2.00 Gramas
Sexo:	50 Machos 10 Fêmeas
Espécie / linhagem/ raça:	Anfíbio** / Melanoprhyniscus tumifrons
No. de animais:	60
Idade/Peso:	1.00 Anos / 2.00 Gramas
Sexo:	50 Machos 10 Fêmeas
Espécie / linhagem/ raça:	Anfíbio** / Melanophryniscus simplex
No. de animais:	60
Idade/Peso:	1.00 Anos / 2.00 Gramas
Sexo:	50 Machos 10 Fêmeas
Espécie / linhagem/ raça:	Anfíbio** / Melanophryniscus spectabilis
No. de animais:	60
Idade/Peso:	1.00 Anos / 2.00 Gramas

Sexo:	50 Machos 10 Fêmeas
Espécie / linhagem/ raça:	Anfíbio** / Melanophryniscus moreirae
No. de animais:	60
Idade/Peso:	1.00 Anos / 2.00 Gramas
Sexo:	50 Machos 10 Fêmeas
Espécie / linhagem/ raça:	Anfíbio** / Melanophryniscus setiba
No. de animais:	60
Idade/Peso:	1.00 Anos / 2.00 Gramas
Sexo:	50 Machos 10 Fêmeas
Origem:	Natureza
Biotério onde serão mantidos os animais:	-Não se aplica-

A aprovação pela CEUA/UNICAMP não dispensa autorização a junto ao **IBAMA**,**SISBIO** ou **CIBio** e é **restrita** a protocolos desenvolvidos em biotérios e laboratórios da Universidade Estadual de Campinas.

Campinas, **29 de setembro de 2020**.

Prof. Dr. Wagner José Fávaro
Presidente

Rosangela dos Santos
Secretária Executiva

IMPORTANTE: Pedimos atenção ao prazo para envio do relatório final de atividades referente a este protocolo; até 30 dias após o encerramento de sua vigência, O formulário encontra-se disponível na página da CEUA/UNICAMP, área do pesquisador responsável. A não apresentação de relatório no prazo estabelecido impedirá que novos protocolos sejam submetidos.

Anexo V – Registro no Sistema Nacional de Gestão do Patrimônio Genético (SISGen #A3D44D1)

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Certidão

Cadastro nº A3D44D1

Declaramos, nos termos do art. 41 do Decreto nº 8.772/2016, que o cadastro de acesso ao patrimônio genético ou conhecimento tradicional associado, abaixo identificado e resumido, no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado foi submetido ao procedimento administrativo de verificação e não foi objeto de requerimentos admitidos de verificação de indícios de irregularidades ou, caso tenha sido, o requerimento de verificação não foi acatado pelo CGen.

Número do cadastro:	**A3D44D1**
Usuário:	**UNICAMP**
CPF/CNPJ:	**46.068.425/0001-33**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie

Batrachochytrium dendrobatidis
Melanophryniscus admirabilis
Melanophryniscus devincenzii
Melanophryniscus dorsalis
Melanophryniscus alipioi
Melanophryniscus biancae
Melanophryniscus vilavelhensis
Melanophryniscus tumifrons
Ranavirus

Título da Atividade:	**O fungo quitrídio e Ranavírus em espécies do gênero Melanophryniscus (Anura: Bufonidae)**

Equipe

MARIANA RETUCI PONTES	**UNICAMP**
LUÍS FELIPE DE TOLEDO RAMOS PEREIRA	**UNICAMP**
JOICE RUGGERI GOMES	**UNICAMP**
Michelle Abadie de Vasconcellos	**RAN ICMBio**
Márcio Borges Martins	**UFRGS**

Parceiras no Exterior

Institut National Polytechnique de Toulouse
Universiteit Gent
Zoological Society of London
Unidad Ejecutora Lillo (CONICET - FML)

Data do Cadastro: **13/01/2022 15:36:04**

Situação do Cadastro: **Concluído**

Conselho de Gestão do Patrimônio Genético

Situação cadastral conforme consulta ao SisGen em **14:35** de **17/06/2024**.

SISTEMA NACIONAL DE GESTÃO
DO PATRIMÔNIO GENÉTICO
E DO CONHECIMENTO TRADICIONAL
ASSOCIADO - **SISGEN**

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº A6737E5

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**A6737E5**
Usuário:	**UNICAMP**
CPF/CNPJ:	**46.068.425/0001-33**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie

Ranavirus
Batrachochytrium dendrobatidis
Melanophryniscus macrogranulosus
Melanophryniscus fulvoguttatus

Título da Atividade:	**O fungo quitrídio e Ranavírus em espécies do gênero Melanophryniscus (Anura: Bufonidae)**

Equipe

Mariana Retuci Pontes	**UNICAMP**
Pedro Carvalho Rocha	**Queensland University of Technology**
Diego José Santana Silva	**UFMS**
Luís Felipe de Toledo Ramos Pereira	**UNICAMP**
Guilherme Augusto Alves	**UNIFESP**
Márcio Borges Martins	**UFRGS**

Data do Cadastro:	**19/06/2024 11:12:54**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **11:13** de **19/06/2024**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN

Anexo VI – Declaração de Bioética e Biossegurança

COORDENADORIA DE PÓS-GRADUAÇÃO
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Universidade Estadual de Campinas
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DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Tese de Doutorado, intitulada "***THE CHYTRID FUNGUS AND RANAVIRUS IN SPECIES OF THE GENUS Melanophryniscus (ANURA: BUFONIDAE)***", desenvolvida no Programa de Pós-Graduação em Ecologia do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

Assinatura: _____
Nome do(a) aluno(a): Ma. Mariana Retuci Pontes

Assinatura: _____
Nome do(a) orientador(a): Dr. Luís Felipe de Toledo Ramos Pereira

Data: 17 de junho de 2024

Anexo VII – Declaração de Direitos Autorais

Declaração

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Campinas, 17 de junho de 2024

Assinatura: _____
Nome do(a) autor(a): **Ma. Mariana Retuci Pontes**
RG n.° 40.999.713-4

Assinatura: _____
Nome do(a) autor(a): **Dr. Luís Felipe de Toledo Ramos Pereira**
RG n.° 28.465.361-5