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CAMILA FERNANDA MOSER

# Impacto dos fatores antropogênicos e ambientais na dinâmica do microbioma e nas interações hospedeiro-patógeno em anfíbios

Belém, 2025

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Tese apresentada ao Programa de Pós-Graduação em Zoologia, do convênio da Universidade Federal do Pará e Museu Paraense Emílio Goeldi, como requisito parcial para obtenção do título de Doutor em Zoologia.
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**Orientador: Dr. Pedro Luiz Vieira Del Peloso**
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Prof. Dr. Pedro Luiz Vieira Del Peloso
Universidade Federal do Pará (Presidente)

Prof. Dr. Carlos Arturo Navas
Universidade de São Paulo

Prof. Dr. Luciano Alves dos Anjos
Universidade Estadual Paulista

Prof. Dr. Mariana Retuci Pontes
Universidade Estadual de Campinas

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# SUMÁRIO

# Impact of anthropogenic and environmental factors on microbiome dynamics and host-pathogen interactions in amphibians


## *ABSTRACT*

Amphibians are among the most threatened vertebrates, with 41% of species at risk of extinction due to habitat loss, climate change, invasive species, and emerging diseases. A key factor influencing their health is the skin microbiota, a community of symbiotic microorganisms that contribute to immunity and disease resistance. However, this microbiome is highly sensitive to environmental disturbances, which can alter its composition and reduce its protective functions. One of the major threats to amphibians is chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (*Bd*), a pathogen that disrupts skin integrity and weakens host defenses, leading to high mortality rates. This infection interacts with environmental stressors, including pollution and habitat degradation, increasing amphibian vulnerability. This thesis explores the composition and ecological drivers of amphibian skin microbiota, its interactions with *Bd*, and the effects of environmental disturbances. The results show that microbiome diversity varies across species, seasons, and environmental conditions. A case study on *Bd* dynamics across different species and environmental conditions demonstrated that infection prevalence and load were lower in warmer temperatures and in species with non-aquatic habits, suggesting that abiotic factors and host ecology significantly influence *Bd* susceptibility. Furthermore, seasonal variations in microbiota composition were observed, with microbial diversity generally decreasing in colder months. These seasonal shifts could be linked to changes in amphibian behavior and immune function, highlighting the need for long-term monitoring of microbiota-host-pathogen interactions. In conclusion, this thesis provides novel insights into the complex interactions between amphibian microbiota, environmental changes, and disease dynamics. Understanding how anthropogenic disturbances and seasonal variations shape microbiome diversity is essential for developing effective conservation strategies. Future research should focus on long-term monitoring of amphibian microbiomes, explore the role of larval-stage microbiota in pathogen resistance, and investigate microbiome-based interventions to support amphibian populations facing increasing environmental threats.



***Keywords:*** *Batrachochytrium dendrobatidis*; dysbiosis; microbiota; immune system; anurans; salamanders.

# Impacto dos fatores antropogênicos e ambientais na dinâmica do microbioma e nas interações hospedeiro-patógeno em anfíbios

## RESUMO

Os anfíbios estão entre os vertebrados mais ameaçados, com 41% das espécies em risco de extinção devido à perda de habitat, mudanças climáticas, espécies invasoras e doenças emergentes. Um fator crucial para sua saúde é a microbiota cutânea, uma comunidade de microrganismos simbióticos que contribuem para a imunidade e resistência a doenças. No entanto, esse microbioma é altamente sensível a distúrbios ambientais, que podem alterar sua composição e reduzir suas funções protetoras. Uma das principais ameaças aos anfíbios é a quitridiomicose, causada por *Batrachochytrium dendrobatidis* (*Bd*), um patógeno que compromete a integridade da pele e enfraquece as defesas do hospedeiro, levando a altas taxas de mortalidade. Essa infecção interage com estressores ambientais, como poluição e degradação do habitat, aumentando a vulnerabilidade dos anfíbios. Esta tese investiga a composição e os fatores ecológicos que influenciam a microbiota cutânea dos anfíbios, suas interações com *Bd* e os efeitos das perturbações ambientais. Os resultados mostram que a diversidade do microbioma varia entre espécies, estações do ano e condições ambientais. Um estudo de caso sobre a dinâmica do *Bd* em diferentes espécies e ambientes demonstrou que a prevalência e a carga da infecção foram menores em temperaturas mais altas e em espécies com hábitos não aquáticos, sugerindo que fatores abióticos e a ecologia dos hospedeiros influenciam significativamente a suscetibilidade ao *Bd*. Além disso, foram observadas variações sazonais na composição da microbiota, com uma redução na diversidade microbiana nos meses mais frios. Essas mudanças sazonais podem estar relacionadas a alterações no comportamento dos anfíbios e na função imunológica, ressaltando a importância do monitoramento de longo prazo das interações entre microbiota, hospedeiro e patógeno. Em conclusão, esta tese fornece novos insights sobre as complexas interações entre microbiota de anfíbios, mudanças ambientais e dinâmica de doenças. Compreender como as perturbações antrópicas e as variações sazonais moldam a diversidade do microbioma é essencial para o desenvolvimento de estratégias eficazes de conservação. Pesquisas futuras devem focar no monitoramento a longo prazo dos microbiomas dos anfíbios, explorar o papel da microbiota em estágios larvais na resistência a patógenos e investigar intervenções baseadas no microbioma para apoiar populações de anfíbios diante das crescentes ameaças ambientais.

**Palavras-chave:** *Batrachochytrium dendrobatidis*; disbiose; microbiota; sistema imune; anuros; salamandras.

# INTRODUÇÃO GERAL

A biodiversidade global enfrenta uma crise sem precedentes, com espécies sendo extintas a uma velocidade alarmante devido a uma combinação de fatores, como mudanças climáticas, perda de habitat, introdução de espécies invasoras e disseminação de patógenos emergentes (Dirzo et al. 2022, Munstermann et al. 2022, Luedtke et al. 2023). Entre os grupos de vertebrados, os anfíbios são os mais ameaçados, com quase 41% das espécies avaliadas pela União Internacional para a Conservação da Natureza (Luedtke et al. 2023, IUCN 2024) em risco de extinção. A fragilidade dos anfíbios pode ser atribuída a características biológicas específicas, como pele permeável, ciclos de vida dependentes de ambientes terrestres e aquáticos e interações complexas com comunidades microbianas simbióticas (Duellman & Trueb 1994, Wells 2007). Além disso, os anfíbios são indicadores cruciais de mudanças ambientais e desempenham papéis essenciais nos ecossistemas, como reguladores de populações de insetos e presas importantes para muitos predadores (Ceron et al. 2019). Sua saúde e sobrevivência estão, portanto, intrinsecamente ligadas à estabilidade dos ambientes onde vivem.

## Desafios para a sobrevivência dos anfíbios

Os anfíbios enfrentam diversas ameaças que comprometem sua sobrevivência em escala global, sendo a quitridiomicose uma das mais graves. Esta doença, causada pelo fungo *Batrachochytrium dendrobatidis* (*Bd*), infecta as células de queratina da pele dos anfíbios adultos (especialmente anuros) e aparato bucal de girinos, reduzindo suas defesas naturais e levando à mortalidade em massa (Woodhams et al. 2008, Lips 2016, Scheele et al. 2019). Esse fungo tem um crescimento ideal entre 15 e 25 °C em meios de cultura (Piotrowski et al. 2004, Stevenson et al. 2013), o que coincide com as temperaturas de montanhas tropicais e florestas onde os casos mais graves de quitridiomicose foram registrados (Piotrowski et al. 2004, Catenazzi et al. 2010, Stevenson et al. 2013, Hirschfeld et al. 2016, Muletz-Wolz et al. 2017). No entanto, sua presença também já foi confirmada em ambientes mais quentes, como na Caatinga brasileira (Benício et al. 2019). A infecção por *Bd* não apenas debilita a integridade da pele, mas também enfraquece o sistema imunológico, tornando os hospedeiros mais suscetíveis a outros patógenos (Voyles et al. 2009, Carver et al. 2010, Fites et al. 2013). Esta doença já foi definida como “a pior doença infecciosa já registrada entre vertebrados em termos de número de espécies impactadas e sua propensão a levá-las à extinção” (Fisher & Garner 2007). Exemplos dramáticos incluem espécies do gênero *Atelopus*, nativas da América Central, que sofreram declínios populacionais massivos e extinções locais (Lips et al. 2008).

No Brasil, a quitridiomicose também está associada a recentes declínios populacionais e extinções de espécies de anfíbios (Becker & Zamudio 2011, Lips 2016, Carvalho et al. 2017, Crawford et al. 2017).

Além das doenças infecciosas, alterações antropogênicas como desmatamento, urbanização e poluição, representam sérias ameaças à sobrevivência dos anfíbios (Ogutu et al. 2011, Torres et al. 2016, Neely et al. 2022). A dinâmica hospedeiro/patógeno é um exemplo de interação que, quando influenciada por alterações no habitat, pode aumentar a densidade de hospedeiros mais suscetíveis à doenças (Laliberté & Tylianakis 2010, Becker et al. 2016). Por exemplo, o desmatamento reduz a complexidade do habitat, rompendo interações ecológicas críticas, aumentando o estresse das espécies e intensificando os encontros entre hospedeiros e patógenos, o que afeta negativamente o sistema imunológico desses hospedeiros (Bradley & Altizer 2007, Johnson & Thieltges 2010, Cable et al. 2017). Embora estudos tenham mostrado que o risco de infecção por *Bd* pode ser menor em espécies generalistas que habitam áreas antropizadas (Becker & Zamudio 2011, Becker et al. 2012), os anfíbios ainda enfrentam impactos negativos mesmo em ambientes abertos e mais quentes, onde o patógeno é menos prevalente (Becker et al. 2010).

A exposição a pesticidas é outro fator alarmante que compromete a saúde dos anfíbios. Estudos demonstram que esses compostos químicos podem prejudicar a função imunológica dos hospedeiros e facilitar a dispersão de parasitas (Hayes et al. 2010). O enriquecimento de corpos d'água com nutrientes provenientes de práticas agrícolas pode alterar o equilíbrio ecológico, reduzindo populações de macroinvertebrados e modificando as comunidades microbianas ambientais, afetando sua saúde e resiliência (Loudon et al. 2014, Hasenbein et al. 2016). Apesar do aumento no número de estudos sobre os efeitos das mudanças ambientais na saúde dos anfíbios, ainda existem lacunas significativas no entendimento das interações entre os gradientes de antropização, o sistema imunológico dos anfíbios e as doenças infecciosas. Pesquisas que abordem essas questões em diferentes escalas de distúrbios ambientais são essenciais para compreender os impactos dessas ameaças e orientar estratégias eficazes de conservação.

## Microbioma

Os anfíbios, assim como outros animais, hospedam comunidades complexas de microrganismos simbióticos em diversas regiões do corpo, denominadas microbioma, que desempenham papéis essenciais na saúde do hospedeiro, como digestão, desenvolvimento, imunidade e resposta ao estresse (Robinson et al. 2010, Rebollar et al. 2020). A composição e diversidade do microbioma cutâneo dos anfíbios são influenciadas por uma série de fatores, tanto biológicos quanto

ambientais (Bird et al. 2019, Ruthsatz et al. 2020, Walke et al. 2021). A filogenia do hospedeiro, por exemplo, pode resultar em comunidades bacterianas distintas entre espécies (McKenzie et al. 2012), enquanto fatores como habitat, estágio de desenvolvimento, sexo, condição corporal e sazonalidade também moldam a configuração do microbioma (Rebollar et al. 2016, Hernández-Gómez et al. 2018, Douglas et al. 2021, Goodwin et al. 2022).

Em particular, a sazonalidade é um fator crucial para organismos ectotérmicos, como os anfíbios, cujas atividades fisiológicas e comportamentais dependem das variações de temperatura ambiente (Wells 2007, Douglas et al. 2021). Alterações na temperatura podem afetar a diversidade e composição das comunidades bacterianas, impactando a resistência a patógenos (Zhou et al. 2016, Muletz-Wolz et al. 2019). Essas interações complexas podem resultar em variações na diversidade do microbioma, tornando os anfíbios mais suscetíveis a infecções em determinados períodos do ano (Longo et al. 2015, Muletz-Wolz et al. 2017, Tong et al. 2020, Douglas et al. 2021, Walke et al. 2021), o que destaca a importância de compreender essas flutuações para entender os impactos da sazonalidade na saúde e resistência a doenças.

A composição do microbioma cutâneo dos anfíbios é especialmente relevante para sua saúde, pois atua na proteção contra patógenos (Robinson et al. 2010, Grice & Segre 2011). Membros bacterianos da pele têm demonstrado a capacidade de inibir o crescimento do fungo *Bd* por meio da produção de peptídeos antimicrobianos e metabólitos antifúngicos (Harris et al. 2009, Becker et al. 2010, Flechas et al. 2012, Woodhams et al. 2015). Pesquisadores classificam essas bactérias como *Bd*-inibidoras quando produzem altos níveis de metabólitos antifúngicos, ou *Bd*-facilitadoras quando apresentam baixa produção desses compostos (Wargo & Hogan 2006, Harris et al. 2009, Woodhams et al. 2015). O equilíbrio entre essas bactérias pode ser alterado por fatores ambientais, como a poluição. Em habitats contaminados, o microbioma cutâneo tende a ter menor riqueza e diversidade, o que favorece a colonização de bactérias facilitadoras de *Bd* e a homogeneização da comunidade bacteriana (Woodhams et al. 2015, Jani & Briggs 2018, Hughey et al. 2019, Preuss et al. 2020). Isso demonstra que, embora o microbioma cutâneo seja uma linha de defesa vital contra infecções, ele também pode ser vulnerável a alterações ambientais, comprometendo a saúde dos anfíbios (Lips 2016).

Apesar dos avanços na descrição da composição do microbioma e sua relação com a carga de patógenos, ainda são limitados os estudos sobre as funções específicas das bactérias para os hospedeiros. Alterações na diversidade microbiana podem levar à disbiose, caracterizada pela perda

de microrganismos benéficos e o aumento de patógenos, o que pode afetar a saúde do hospedeiro (Petersen & Round 2014, Zaneveld et al. 2017, Preuss et al. 2020). A identificação de disbiose pode ser desafiadora, pois sua definição varia entre hospedeiros e ecossistemas, e em alguns casos, a alta variabilidade do microbioma entre indivíduos pode indicar disbiose (Zaneveld et al. 2017). Esse fenômeno, descrito como o "Princípio de Anna Karenina", reflete a maior instabilidade nas comunidades microbianas de hospedeiros submetidos a altos níveis de estresse, resultando em maior dispersão nas métricas de diversidade do microbioma (Zaneveld et al. 2017). Esses achados ressaltam a necessidade de compreender a formação, manutenção e funções do microbioma, a fim de prever e mitigar os impactos de doenças, como a quitridiomicose. Estudar as interações entre microbiomas, doenças e mudanças ambientais é crucial para entender os desafios que os anfíbios enfrentam e para garantir a preservação desse grupo fundamental para os ecossistemas globais.

Dessa forma, a presente tese está dividida em quatro capítulos: o primeiro capítulo abrange uma revisão bibliográfica sobre os efeitos das atividades antrópicas no microbioma cutâneo e intestinal dos anuros; o segundo capítulo visa descrever e comparar a composição e diversidade do microbioma cutâneo de 10 espécies de anuros neotropicais; o capítulo três visa entender os efeitos da sazonalidade sobre o microbioma cutâneo dos anuros *Boana leptolineata* e *Scinax squalirostris*; e o quarto capítulo visa entender a influência do distúrbio do ambiente sobre a dinâmica de infecção por *Bd* em anfíbios de uma região temperada.

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# Capítulo 1

## The influence of anthropogenic activities on anuran amphibians' symbiotic communities and host-pathogen interactions

O capítulo I desta Tese foi elaborado e formatado conforme as normas da publicação científica *Environmental Research*, as quais se encontram em anexo (Anexo 1).

# The influence of anthropogenic activities on anuran amphibians' symbiotic communities and host-pathogen interactions

Camila F. Moser[a], Wesley J. Neely[b], Laura K. Schuck[c], Pedro Peloso[d,*] and C. Guilherme Becker[c]

[a] Programa de Pós-Graduação em Zoologia, Universidade Federal do Pará, Belém, BR.

[b] Department of Biology, Texas State University, San Marcos, Texas, USA.

[c] Department of Biology, The Pennsylvania State University, University Park, Pennsylvania, USA.

[d] Vertebrate Museum, Department of Biological Sciences, California State Polytechnic University, Arcata, California, USA.

* corresponding author: pedropeloso@gmail.com

**Abstract**

As one of the most threatened animal groups, understanding the impact of human activities on anuran amphibians is crucial for their conservation, especially in tropical forests where the amphibian killing fungus *Batrachochytrium dendrobatidis* (*Bd*) is more prevalent. This review analyzes 62 studies on how anthropogenic activities affect anuran skin and gut microbiomes. Most studies (45) focus on chemical exposure, while 17 examine disturbed environments. Generally, microbiome diversity decreases in disturbed environments, mainly due to chemical exposure, leading to impaired growth, development, and immune responses. However, some studies suggest an increase in microbial diversity, particularly from bacteria that degrade agrochemicals and inhibit pathogens. Heavy metals often raise the abundance of harmful Proteobacteria while reducing beneficial Fusobacteria. Fluctuating Firmicutes-to-Bacteroidetes ratios indicate gut microbiome instability, which may negatively impact host health. Despite this, some studies report microbiome stability even with chemical exposure. Further research is needed, particularly on the skin microbiome of tadpoles and the gut microbiome of adult anurans in tropical regions. These studies are essential for conservation strategies and for identifying microbiome shifts as early indicators of disease risk, including *Bd* infections. The findings highlight the need for policies addressing environmental disturbances that threaten anuran populations and their microbiomes.

**Keywords:** *Batrachochytrium dendrobatidis*, gut microbiome, microbiota, skin microbiome, tadpoles.

# 1. Introduction

Amphibians, like other animals, host diverse communities of symbiotic microorganisms, collectively referred to as *the microbiome*. This term encompasses bacteria, archaea, fungi, viruses, and their metabolic products, which inhabit specific environments within a given host and interact with both the host and the environment (Rebollar et al., 2020). Microbiomes play critical roles in a number of physiological processes, such as digestion, development, immunity, and stress responses (Rebollar et al., 2020; Robinson et al., 2010). High microbiome diversity is generally associated with ecological stability and resilience, aligning with the biodiversity-ecosystem functioning framework (Fassarella et al., 2021; Lozupone et al., 2012). This theory links biodiversity to functional diversity, and the diversity-stability hypothesis, which suggests that ecosystems with greater biodiversity are more stable (Loreau and Hector, 2001; McCann, 2000). Conversely, some theories propose that low microbiome diversity may increase susceptibility to diseases. Elton (1958) suggested that high parasite loads could reduce microbiome diversity, correlating it with higher risks of metabolic and immunological disorders (Bello et al., 2018). Similarly, the dilution effect hypothesis posits that greater microbiome diversity can mitigate pathogen transmission, thus reducing the chances of host pathology (Keesing and Ostfeld, 2021). Despite these theoretical frameworks, the relationship between microbiome diversity, pathogen defense, and host health remains incompletely understood (Greenspan et al., 2022).

Microbiome stability can be influenced by biotic and abiotic, with anthropogenic activities being particularly disruptive. Human-induced stressors can alter microbiome diversity and function, potentially diminishing mutualistic benefits between hosts and their microbiomes (Bernardo-Cravo et al., 2020; McCoy and Peralta, 2018; Rollins-Smith et al., 2011). Such disruptions may diminish the mutualistic benefits between hosts and their microbiomes (Figueiredo and Kramer, 2020). Therefore, this review focuses on how anthropogenic activities affect the skin and gut microbiomes of anuran amphibians, since both can directly influence host health, for example by defending against invading pathogens (Grice and Segre, 2011; Robinson et al., 2010).

Amphibians are especially relevant for microbiome studies due their global decline and the role of microbiomes in diseases resistance (Becker and Harris, 2010; Flechas et al., 2012; Harris et al., 2009; Woodhams et al., 2015, Greenspan et al., 2022). The skin of amphibians serves as the first line of defense against pathogens, producing antimicrobial peptides (Rollins-Smith et al., 2011) and hosting microbial communities that inhibit pathogens (Harris et al., 2006). Some members of the

amphibian skin bacterial community can inhibit the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), the causative agent of the disease chytridiomycosis (Becker and Harris, 2010; Flechas et al., 2012; Harris et al., 2009; Woodhams et al., 2015). Chytridiomycosis is a potentially deadly infectious disease considered a major driver of global amphibian population declines and extinctions (Scheele et al., 2019). Bacterial inhibition pathways of this pathogen can occur through production of antimicrobial peptides (AMPs) and antifungal metabolites (Becker and Harris, 2010; Flechas et al., 2012; Harris et al., 2009; Woodhams et al., 2015). Similarly, the gut microbiome is essential for digestion, vitamin synthesis, and metabolism of indigestible compounds, making its study crucial for amphibian conservation (Rooks and Garrett, 2016).

Environmental pollutants significantly impact microbial communities by increasing host susceptibility to diseases (McCoy and Peralta, 2018). Heavy metals, which are among one of the most harmful contaminants for anurans (Hacioglu and Tosunoglu, 2014), can alter both gut (Huang et al., 2022a; Wan et al., 2022; Ya et al., 2020; Yang et al., 2020) and skin microbiomes (Hernández-Gómez et al., 2020; Jiménez et al., 2020; Liu et al., 2022; Proença et al., 2021) in both adult and larval anurans. Agrochemicals also have a strong influence on the amphibian microbiota by reducing soil microbial diversity, thereby limiting microbial colonization of amphibian skin (Boccioni et al., 2021; Huang et al., 2021; Jiao et al., 2017; Li et al., 2022; Zhao et al., 2021). Additionally, agrochemicals induce host stress, further compromising microbiome stability and increasing disease susceptibility (Alverdy and Luo, 2017).

Other human activities, such as habitat loss and fragmentation, also destabilize anuran microbiomes, reducing microbial diversity in both skin (Bates et al., 2022; De Assis et al., 2021; Goff et al., 2020) and gut bacterial communities (Chang et al., 2016; Huang et al., 2018). Natural habitats tend to harbor higher microorganism diversity due to greater overall ecosystem diversity (Becker et al., 2017; De Assis et al., 2021; Jiménez et al., 2020). Alternatively, disturbed environments generally contain more diverse habitat types (e.g., open pasture, forest edge, etc.), which could result in higher diversity at these sites. However, previous research has shown that amphibian inhabiting highly disturbed environments have greater microbial community dispersion (i.e., microbiome in a state of dysbiosis), impairing host health (Jiménez et al., 2020; Neely et al., 2022).

Given the crucial role of microbiome composition and function in amphibian health, understanding which environmental factors influence the microbiome and identifying those with the greatest impact can improve models predicting the spread of infectious diseases, such as

chytridiomycosis (Jiménez and Sommer, 2017). This knowledge is essential for informing conservation policies and guiding future research efforts.

In this review, we compile and synthesize the available literature on the impacts of anthropogenic activities on the skin and gut microbiomes of anuran amphibians, encompassing studies published up to January 2024.

## 2. Land Use

Seventeen studies have examined the effects of land use on the anuran microbiome, where nine studies compared microbiomes in anthropogenic environments to those in natural environments (Bates et al., 2022; Chang et al., 2016; De Assis et al., 2021; Gabor et al., 2023; Huang et al., 2018; Jiménez et al., 2020; Krynak et al., 2016; Neely et al., 2022; Varela et al., 2018). Three studies focused on preserved versus disturbed forests (Becker et al., 2017; De Assis et al., 2020; Hughey et al., 2017; Neely et al., 2024), while another three assessed habitat quality factors such as landcover type, urban area percentage, canopy cover, and mesocosm conditions (Goff et al., 2020; Krynak et al., 2015; Preuss et al., 2020). Additional studies evaluated the effects of habitat disturbances, such as fire (Schuck et al., 2024) or compared microbiomes in different anthropogenic environments, such as agricultural versus urban areas (Carphio et al., 2021).

The effects of disturbed environments on anuran microbiome diversity generated mixed results. For instance, greater bacterial diversity in disturbed environments was observed in the gut microbiome of *Fejervarya limnocharis* (Chang et al., 2016; Huang et al., 2018) and *Babina adenopleura* (Huang et al., 2018), as well as in the skin microbiome of *Dendrobates auratus*, *Silverstoneia flotator*, *Allobates talamancae* (Varela et al., 2018), *Proceratophrys boiei* (De Assis et al., 2020) and *Dendropsophus minutus* (Preuss et al., 2020). Conversely, higher diversity in natural environments was found in the skin microbiome of *D. minutus* (Becker et al., 2017), *Pseudacris ornata* (Goff et al., 2020), *Scinax fuscovarius* (De Assis et al., 2021), and *Lithobates vibicarius* tadpoles and adults (Jiménez et al., 2020). Together, these findings suggest that landscape features influence the diversity of anuran skin bacterial communities, but the direction of effects are context dependent.

Among studies comparing the microbiome of anurans in forest/natural and agricultural/urban environments, *F. limnocharis* and *B. adenopleura* exhibited more habitat-specific and exclusive gut bacteria in agricultural areas (Chang et al., 2016; Huang et al., 2018). Most of the dominant bacterial

taxa found in anurans from forests were also common in *F. limnocharis* from agricultural areas (Chang et al., 2016). Furthermore, both species showed more specialized functional groups of bacteria in agricultural environments compared to forests, primarily associated with functions related to pesticide degradation and pathogenic diseases (Huang et al., 2018). The authors suggest that this reflects physiological, metabolic, and ecological responses to environmental disturbances (Huang et al., 2018). The greater number of specialized functional bacterial groups in agricultural areas supports the hypothesis of high "functional response diversity" in the gut microbiome as a compensatory mechanism for environmental disturbance (Elmqvist et al., 2003; Huang et al., 2018).

Interestingly, while dominant bacterial taxa in forest-dwelling anurans were also found in agricultural environments, the dominant taxa from agricultural habitats were absent in forest-dwelling individuals, suggesting that agricultural environments create unique ecological niches for these microbial groups (Chang et al., 2016). However, the gut microbiome diversity of adult *Pristimantis unistrigatus* showed no significant differences between agricultural and urban environments (Carphio et al., 2021), nor did *Bufo bufo* tadpoles between urban and natural habitats (Gabor et al., 2023). For instance, *B. bufo* tadpoles exhibited the highest abundance of the Sutterellaceae family in natural environments—a group known for its sensitivity to chemical compounds (Lin et al., 2022)—which may explain its reduced abundance in polluted urban environments (Gabor et al., 2023). Meanwhile, urban *B. bufo* tadpoles had a higher relative abundance of the genus *Desulfovibrio* (Gabor et al., 2023), also prevalent in the intestines of adult *Bufo raddei* from environments contaminated with heavy metals (Zhang et al., 2016). Additionally, *S. fuscovarius* tadpoles from livestock areas exhibited greater bacterial diversity compared to agricultural zones (e.g., soybean fields), suggesting that agricultural activities might induce the most significant alterations in their skin microbiota (De Assis et al., 2021).

Studies on the microbiome of anurans in continuous/preserved versus fragmented/disturbed forests report contrasting findings. For example, *P. boiei* had a more diverse microbiome in fragmented forests (De Assis et al., 2020), while *D. minutus* exhibited greater diversity in continuous forests (Becker et al., 2017). Additionally, there was no difference in *Eleutherodactylus coqui* alpha diversity between preserved and disturbed forests (Hughey et al., 2017). The findings of De Assis et al. (2020) contrast with expectations that preserved environments harbor greater microbial diversity (Becker et al., 2017; De Assis et al., 2021; Jiménez et al., 2020). It is hypothesized that the higher microbial diversity on the skin of *P. boiei* in fragmented forests could be linked to intrinsic host

characteristics, as soil bacterial communities did not differ between these environments (De Assis et al., 2020), which may explain the difference found between this species and *D. minutus*. Moreover, *P. boiei* in fragmented forests had a higher diversity of bacteria with antipathogenic properties in anurans, underscoring the influence of forest type on these communities (De Assis et al., 2020). In *D. minutus*, results indicated that greater habitat connectivity led to higher similarity in bacterial skin communities, with natural vegetation corridors facilitating microbial exchange between hosts and environments (Becker et al., 2017). More specifically, increased forest connectivity was linked to high bacterial skin similarity between host populations, where natural vegetation corridors maintain the movement of amphibian-associated bacteria through their hosts and/or environment (Becker et al., 2017). Although alpha diversity in *E. coqui* did not vary, beta diversity was influenced by landscape use, with certain taxa more abundant in intact forests than in disturbed ones, possibly reflecting environmental conditions or altered host physiology and immune function (Hughey et al., 2017).

In disturbed environments, microbial dispersion often increases. For instance, *Acris blanchardi* from natural habitats exhibited less variation and greater community stability compared to individuals from disturbed habitats (Krynak et al., 2016). The authors attribute these differences to variations in the availability of colonizing microorganisms rather than species-specific physiological differences (Krynak et al., 2016). Additionally, Varela et al. (2018) found that factors such as lower pH can stabilize microbiome composition, potentially enhancing host resistance to infections.

Intraspecific microbiome similarity within the same species generally indicates a stable microbiota and a more effective immune system (Jiménez et al., 2020; Neely et al., 2022; Zaneveld et al., 2017). Conversely, the high dispersion observed in the skin microbiomes of *Acris crepitans* and *L. vibicarius* is often a marker of negative health impacts under environmental stress (Jiménez et al., 2020; Neely et al., 2022). This suggests that frogs from less disturbed habitats exhibit greater resistance to infections than those from more disturbed areas. These findings align with the Anna Karenina principle, which posits that stressed microbiomes display greater variability, often leading to dysbiosis and heightened susceptibility to infections (Zaneveld et al., 2017). Accordingly, the authors propose that the microbiomes of both species in disturbed habitats are in a dysbiotic state (Jiménez et al., 2020; Neely et al., 2022), which likely increases their vulnerability to pathogenic infections threatening host health (Jiménez et al., 2020; Neely et al., 2022). Further supporting this, *S. squalirostris* exhibited a significant increase in microbiome dispersion and higher *Bd* loads

following experimental burning (Schuck et al., 2024). This effect is likely tied to abrupt environmental changes caused by the fire, prompting the species to migrate to new environments and thereby altering its microbiome (Schuck et al., 2024), highlighting that fire is a potential disturbance capable of driving microbiome variability and contributing to dysbiosis.

Several authors propose that environmental factors can influence the abundance of *Bd*-inhibiting and *Bd*-enhancing microbiota in anurans (Jiménez et al., 2020; Preuss et al., 2020; Varela et al., 2018). For example, soil pH has been identified as a key determinant of the skin microbiome in *D. auratus*. More acidic environments (lower pH values) were associated with a greater diversity of *Bd*-inhibiting bacteria in these frogs (Varela et al., 2018). This result possibly indicates that such conditions may favor anti-*Bd* bacterial communities (Varela et al., 2018). Furthermore, the influence of pH on anuran skin microbiomes is further supported by another study, which observed a significant microbial community shift in *Aquarana catesbeiana* tadpoles when the mean pH dropped from 7 to 6 (Krynak et al., 2015). Similarly, *L. vibicarius* individuals from agricultural and livestock areas exhibited a greater diversity of *Bd*-inhibiting bacteria in their skin microbiomes compared to those in natural environments (Jiménez et al., 2020). This increased richness and abundance of anti-*Bd* bacteria in disturbed environments may help frogs better resist fungal infections, thereby reducing the likelihood of disease (Varela et al., 2018; Jiménez et al., 2020).

However, contrasting results were observed in *D. minutus* populations inhabiting ponds with swine slurry, a more disturbed environment. These ponds hosted a higher proportion of *Bd*-facilitating bacteria in the anurans' skin microbiomes compared to ponds without slurry (Preuss et al., 2020). Swine slurry ponds exhibit characteristics of a highly eutrophic system, with poor water quality, an overabundance of dead organic matter, and a notable increase in coliform bacteria (Preuss et al., 2020). The authors suggest that this overabundance of coliform bacteria in the environmental microbial pool disrupted the recruitment of beneficial microbes to the anuran skin, favoring an increase in *Bd*-facilitating bacteria (Preuss et al., 2020).

Despite the contrasting findings among studies, it is evident that anthropogenic habitat changes can significantly influence the skin and gut microbiomes of anurans. These changes underscore the role of land-use intensification as a key driver of microbiome alterations in host organisms. In some cases, the increased microbiome diversity observed in anuran species from anthropogenically disturbed environments has been attributed to greater canopy openness, a characteristic of altered

habitats. This increased openness may contribute to heightened environmental variability, fostering a more diverse bacterial community in the surrounding environment (Goff et al., 2020).

# 3. Chemicals

## 3.1. Agrochemicals

### *3.1.1. Herbicides*

Exposure to herbicides can influence immune function in anurans, either depressing or stimulating it, which can increase or decrease resistance to diseases. Additionally, herbicides may alter the skin bacterial community, impacting protection against *Bd* or other pathogens (Krynak et al., 2017). Among the studies reviewed, seven specifically evaluated the effects of atrazine and glyphosate, two of the most widely used herbicides globally.

Atrazine poses significant environmental concerns due to its stability and resistance to degradation (Starr et al., 2017). With the rapid expansion of agriculture, atrazine usage has surged, heightening risks to both human and animal health, including adverse effects on anuran populations (Johnson et al., 2019). Atrazine contaminates aquatic ecosystems through precipitation, runoff, and leaching, resulting in surface water pollution and long-term persistence in soil and water—potentially lasting decades (Singh et al., 2018). Glyphosate is the most widely used herbicide worldwide (Benbrook, 2016) and is commonly applied for controlling weed plants in crops and emerging aquatic vegetation (Annett et al., 2014).

The impacts of atrazine on the gut microbiome were tested in both adults and tadpoles of *Osteopilus septentrionalis* (Knutie et al., 2018), adults and tadpoles of *P. nigromaculatus* (Huang et al., 2021; 2022a; Zhao et al. 2021; 2022). The effects of atrazine varied between developmental stages in *P. nigromaculatus*. In adults, atrazine exposure reduced gut bacterial diversity (Zhao et al., 2022, 2021), while in tadpoles, it increased diversity (Huang et al., 2022a, 2021). Furthermore, the short-term exposure to atrazine in *P. nigromaculatus* adults reduced the abundance of harmful bacteria (phylum Chlamydiae) but significantly increased beneficial bacteria (*Lactobacillus* and *Weissella)* (Zhao et al., 2021). However, prolonged exposure to 500 μg/L atrazine disrupted intestinal bacterial balance, exceeding tolerance thresholds in adults (Zhao et al. 2022).

In tadpoles, increased bacterial diversity was accompanied by a decline in beneficial groups such as Verrucomicrobia and Firmicutes, altering short-chain fatty acid (SCFA) content (Huang et

al., 2021). These differences may be attributed to variables such as exposure duration (short- versus long-term), developmental stage (tadpole versus adult), and diet (plant-based versus mixed feed) (Huang et al., 2022a). In contrast, no significant changes in gut bacterial diversity were observed in adults or tadpoles of *O. septentrionalis* following atrazine exposure (Knutie et al., 2018). The authors propose two hypotheses to explain why exposure to atrazine did not affect the microbiota of neither tadpoles or adults: either atrazine does not directly or indirectly impact gut bacterial communities, or the concentration used in their study was insufficient to provoke such changes (Knutie et al., 2018).

The effects of glyphosate on anuran microbiomes have been studied in the skin microbiome of adult and tadpole *A. blanchardi* (Krynak et al., 2017) and in the gut microbiome of *R. arenarum* tadpoles (Boccioni et al., 2021). In *A. blanchardi*, glyphosate exposure significantly altered the skin bacterial community in both adults and tadpoles (Krynak et al., 2017). However, the changes observed in tadpoles did not persist into the juvenile stage (Krynak et al., 2017). This finding led the authors to suggest that treating habitats with glyphosate earlier in the breeding season could have more detrimental effects on the species' disease resistance than treatments applied later in the season (Krynak et al., 2017). In *R. arenarum* tadpoles, exposure to glyphosate increased the bacterial diversity of the gut microbiome (Boccioni et al., 2021). Despite this increase, the authors classified the change as dysbiosis, highlighting the potential negative impacts of glyphosate on gut microbial balance (Boccioni et al., 2021).

#### *3.1.2. Other agrochemicals*

The effects of other agrochemicals on anuran microbiomes have been studied, including the fertilizers nitrate ($NO_3$) and nitrite ($NO_2$) on the gut microbiome of *B. gargarizans* tadpoles and adults (Yutian Liu et al., 2022 a; Xie et al., 2020; Zheng et al., 2020), the fungicide Chlorothalonil on the skin microbiome of *L. vibicarius* tadpoles (Jiménez et al., 2021), and the insecticide *cis*-bifenthrin on the gut microbiome of *Xenopus laevis* adults (Li et al., 2022).

Among the inorganic forms of nitrogen available for biological uptake are nitrate ($NO_3$) and nitrite ($NO_2$). $NO_3$ is the most stable and abundant form of nitrogen in aquatic environments, as nitrite and ammonia are readily converted into nitrate by bacteria and algae (Elisante and Muzuka, 2016). However, anthropogenic sources such as industrial effluents and agricultural fertilization can lead to contamination of surface and groundwater (Camargo and Alonso, 2006). $NO_2$, while generally present at low levels in aquatic systems, can become toxic under certain conditions, such as in lakes

near agricultural fields or aquaculture systems with high waste production (Madison and Wang, 2006).

Exposure to $NO_2$ reduced microbial richness but increased diversity in the gut microbiome of *B. gargarizans* tadpoles (Liu et al., 2022), a pattern similar to the effects of $NO_3$, which increased both richness and diversity in this species (Liu et al., 2022; Xie et al., 2020). These changes were associated with the expansion of pathogenic bacteria, suggesting that $NO_2$ exposure can increase disease susceptibility by disrupting the intestinal microbial community (Liu et al., 2022). Additionally, $NO_3$ exposure induced dysregulations in fatty acid and amino acid metabolism, with severe impacts observed under high exposure, potentially increasing the risk of metabolic disorders and diseases in tadpoles (Xie et al., 2020; Zheng et al., 2020).

*Cis*-bifenthrin (*cis*-BF) is one of the most widely used synthetic pyrethroid (SP) insecticides, valued for its insecticidal potency and faster degradation compared to organophosphate pesticides (Bertotto et al., 2018). However, SP contamination is pervasive, being detected in soil, water, river sediments, and indoor dust (Li et al., 2022). Exposure to cis-BF reduced bacterial diversity in the gut microbiome of *X. laevis* adults and significantly altered the structure and composition of gut microbial communities (Li et al., 2022). This disruption affected metabolites involved in lipid metabolic pathways, particularly bile acid metabolism (Li et al., 2022). The authors concluded that cis-BF exposure could lead to microbiome dysbiosis, increasing susceptibility to chronic diseases related to metabolic liver syndrome, thereby exposing unforeseen ecological risks associated with widely used pesticides (Li et al., 2022).

Fungicides can directly affect microbial communities by being toxic to fungi and bacteria, or indirectly by increasing nutrient availability for bacteria due to fungal death (Baćmaga et al., 2018). In soil, the fungicide chlorothalonil stimulates the growth of heterotrophic bacteria and actinobacteria while inhibiting fungal growth (Baćmaga et al., 2018). In the skin microbiome of *L. vibicarius* tadpoles, no changes in bacterial diversity or richness were observed after exposure to chlorothalonil (Jiménez et al., 2021). However, the composition of the microbiome was significantly altered, with suppression of bacterial strains believed to inhibit *Bd* (e.g. *Janthinobacterium* and *Acinetobacter* strains) at high concentrations (Jiménez et al., 2021). These shifts may disrupt the production of defensive bacterial metabolites essential for disease resistance, highlighting the potential indirect effects of chlorothalonil on anuran health (Jiménez et al., 2021).

### 3.2. Heavy metals

Heavy metals rank among the most harmful contaminants for anurans due to their direct interaction with the highly permeable skin of these animals, which facilitates rapid absorption from the environment (Hacioglu and Tosunoglu, 2014). The toxicity of heavy metals stems from their tendency to accumulate in organisms and their resistance to degradation (Aiman et al., 2016). Additionally, they are linked to a wide range of diseases through mechanisms like oxidative stress and long-term effects such as genotoxicity and carcinogenesis (Pratheeshkumar et al., 2014). Despite their known impacts, studies on the effects of heavy metals on the microbial communities of anurans remain limited (Zhang et al., 2016).

We found 21 studies that have investigated the impact of heavy metals on anuran microbiomes, seven focusing on adults and 14 on tadpoles (Table 2). Most studies reported that heavy metal exposure reduces microbiome diversity (Chai et al., 2022; Costa et al., 2016; Huang et al., 2022b; Liu et al., 2022b; Mu et al., 2018; Yao et al., 2019; Yang et al., 2020; Zhang et al., 2016) and bacterial richness (Shen et al., 2022; Ya et al., 2019, 2020; Zheng et al., 2021b). In contrast, three studies found an increase in bacterial diversity after exposure to heavy metals (Chai et al., 2023; Liu et al.. 2023; Lv et al., 2022).

#### *3.2.1. Lead*

Lead (Pb) was the most frequently studied heavy metal (Table 2). A widespread pollutant, Pb primarily enters aquatic ecosystems through industrial, urban, and mining wastewater (Xia et al., 2018). It is classified as one of the most toxic biological metals due to its frequency of exposure and inherent toxicity (Kim et al., 2014). Studies have demonstrated that Pb exposure adversely affects frogs, causing developmental delays, impairing growth and metamorphosis, and altering the gut microbiome of tadpoles (Chai et al., 2022; Zheng et al., 2021b; Huang et al., 2022b). For example, high concentrations of Pb in aquatic ecosystems can cause a wide range of adverse effects on growth, development and metamorphosis in anurans (Chai et al., 2023), in addition to modifying the gut microbiome of tadpoles (Huang et al., 2022b; Liu et al., 2022b).

Five studies tested the effects of Pb on the gut microbiome of *B. gargarizans* tadpoles (Chai et al., 2022, 2023; Liu et al., 2023; Zheng et al., 2021b; Zhu et al., 2023), generating contrasting results. Some studies reported reduced bacterial diversity (Zheng et al., 2021b; Chai et al., 2023), while others found increased diversity (Chai et al., 2023; Liu et al., 2023; Lv et al., 2022). For

instance, exposure to Pb was associated with a decrease in bacterial diversity in the gut microbiome of *B. gargarizans* tadpoles (Chai et al., 2022, 2023). However, an increase in microbial diversity was noted at a more advanced developmental stage, specifically at Gosner Stage 42 (Chai et al., 2023; Gosner, 1960). Additionally, Pb exposure led to reduced bacterial diversity in both the gut and skin microbiomes of *P. nigromaculatus* adults, regardless of whether the exposure was continuous or pulsed (Huang et al., 2022b; Liu et al., 2022b). Pulsed exposure was found to exacerbate Pb toxicity, making it more harmful to the host than short-term continuous exposure (Huang et al. 2022b; Liu et al., 2022b).

The increase in bacterial diversity reported in some studies is believed to result from the proliferation of potentially pathogenic bacteria and aerobic bacterial strains (Chai et al., 2023; Liu et al., 2023; Zhu et al., 2023). While increased diversity might seem beneficial, it is often associated with dysbiosis, loss of essential microbial functions, and higher susceptibility to infections and intestinal diseases, ultimately impairing host health (Chai et al., 2022; Zheng et al., 2021b; Huang et al., 2022b). Furthermore, one study linked gut microbiome changes induced by Pb exposure to alterations in bile acid profiles, potentially disrupting bile acid reabsorption in the gut (Liu et al., 2023). In contrast, increased bacterial diversity in the gut microbiome of *Rana omeimontis* tadpoles exposed to Pb was attributed to an attenuation of Pb toxicity due to concurrent exposure to sodium nitrate ($NaNO_3$) (Lv et al., 2022).

### *3.2.2. Cadmium*

Cadmium (Cd) is one of the most ecotoxic heavy metals (Genchi et al., 2020), and can enter the environment through pesticide application, fertilizer runoff, mining activities, sewage discharge, and irrigation. The high toxicity of Cd is primarily due to its teratogenic, mutagenic, and carcinogenic properties (Faroon et al., 2012). Moreover, Cd is highly persistent, difficult to degrade, and can accumulate in both organisms and the environment for many years (Ya et al., 2019). As a result, Cd poses significant threats to the survival of anurans, including embryos, tadpoles, and adults.

Seven studies have examined the effects of Cd on the anuran gut microbiome, including one study on adults of *P. nigromaculatus* (Wan et al., 2022), four on tadpoles of *B. gargarizans* (Ya et al. 2019; Zheng et al. 2020; Ya et al. 2020; Zheng et al. 2021b) and two on tadpoles of *Rana chensinensis* (Mu et al., 2018; Shen et al., 2022). Among those studies, only one study found an increase in bacterial diversity in the gut of *B. gargarizans* (Ya et al., 2020), a result that contradicted findings from their earlier study (Ya et al., 2019). However, despite this increase in diversity, the authors concluded that

the changes in microbial community structure induced by Cd exposure could be harmful to *B. gargarizans* tadpoles, as the gut microbiome of tadpoles at Gosner Stage 42 (Gosner, 1960) was significantly altered (Ya et al., 2020). In contrast, other studies found a reduction in bacterial diversity in *B. gargarizans* (Ya et al., 2019; Zheng et al., 2021b), as well as in two populations of *R. chensinensis* (Mu et al., 2018; Shen et al., 2022) and in *P. nigromaculatus* (Wan et al., 2022).

The alterations in taxonomic composition, community structure, and species abundance of the gut microbiota induced by Cd may lead to significant functional disruptions (Ya et al., 2019). In *R. chensinensis* tadpoles, changes in the gut microbiome may impair lipid storage and transport, as well as reduce anti-inflammatory capacity, potentially contributing to population declines (Shen et al., 2022). In *B. gargarizans* tadpoles, the microbiota composition became more varied with increasing Cd concentration, which could indicate dysbiosis (Ya et al., 2019). The authors suggested two factors contributing to the decline in bacterial diversity: first, histopathological damage in the intestines due to Cd exposure may cause severe deterioration of the intestinal microenvironment, leading to a loss of some commensal bacteria; second, Cd exposure may directly reduce or eliminate microbiota species that are less tolerant to the metal (Ya et al., 2019). Furthermore, short-term Cd exposure caused histopathological changes in the gut and increased mortality in *P. nigromaculatus* tadpoles, while also altering the gut microbiota community structure (Wan et al., 2022). Overall, exposure to cadmium tends to induce changes in microbial diversity, resulting in dysbiosis and negatively impacting energy balance and fitness (Mu et al., 2018; Ya et al., 2020; Zheng et al., 2021b). These studies characterize the effects of cadmium exposure as dysbiosis in the gut microbiome, which can ultimately harm host health.

#### *3.2.3. Copper*

Copper (Cu) is a heavy metal commonly found in the natural environment and serves as an essential nutrient for most organisms. However, it can become toxic when its concentration surpasses a critical threshold (Lee et al., 2019). Copper is widely utilized in various industries, including the production of batteries, fuels, pesticides, fertilizers, pigments, and paints. These anthropogenic activities are the primary sources of Cu pollution in aquatic ecosystems.

The effects of Cu on the gut microbiome of tadpoles have been investigated in five studies, four focusing on *B. gargarizans* (Chai et al., 2022; Zheng et al., 2020, 2021a; Liu et al., 2023) and one on *R. chensinensis* (Yang et al., 2020). All studies reported alterations in gut microbiome diversity following Cu exposure, with only one documenting an increase in diversity (Liu et al., 2023). Despite

the increase in the diversity of the gut microbiota after exposure to Cu, the authors suggest that the changes observed in the abundance of bacteria related to the biotransformation of bile acids in *B. gargarizans* may lead to inhibition of bile acid synthesis in the liver and reabsorption in the intestine (Liu et al., 2023). Moreover, Cu exposure was found to have a more significant impact on gut microbial populations than lead (Pb) exposure (Chai et al., 2022). In *B. gargarizans* tadpoles, changes in gut microbiome diversity and composition were linked to enhanced carbohydrate, lipid, and energy metabolic pathways, suggesting that heavy metal exposure induces dysbiosis and disrupts metabolic homeostasis in the host (Chai et al., 2022). Similarly, in *R. chensinensis* tadpoles, Cu exposure destabilized the gut microbiota and caused histological alterations, including epithelial cell degeneration, karyopyknosis, and disorganization of epithelial cell structures (Yang et al., 2020).

#### *3.2.4. Other heavy metals*

The effects of other heavy metals on the anuran microbiome, including arsenic, acid mine drainage (AMD), and chromium (Cr), have also been investigated. One study examined the effects of arsenic on the skin microbiome of a community of adult anurans, including *Boana faber*, *Bokermannohyla nanuzae*, *Ischnocnema izecksohni*, *Ololygon luizotavioi*, *Ololygon tripui*, *Rhinella crucifer*, and *Vitreorana uranoscopa* (Cordeiro et al., 2019). The effects of AMD were tested on the skin microbiome of *Pelophylax perezi* adults (Proença et al., 2021), while the effects of chromium were analyzed in the gut microbiome of *B. gargarizans* tadpoles (Yao et al., 2019; Zheng et al., 2020). Additionally, studies evaluated the cutaneous and gut microbiomes of *P. perezi* and *B. raddei* adults in regions contaminated by heavy metals in southern Portugal and China (Costa et al., 2016; Zhang et al., 2016).

Arsenic, a metalloid with wide geographic distribution, occurs in both soil and water, with contamination arising naturally or through anthropogenic activities (Wang and Zhao, 2009). In Brazil's Quadrilátero Ferrífero, a region of intensive mining activity in the state of Minas Gerais, arsenic is found in auriferous rocks rich in sulfides (De Figueiredo et al., 2007). One of the studies suggests that species with greater dependence on water, which are exposed for a longer time and to a higher level of contamination, presented bacteria with high resistance to arsenic, suggesting that this resistance may help to protect the anurans (Cordeiro et al., 2019). These results are consistent with the discussion raised in the study by Zhang et al. (2016), who proposed that microbiota may evolve to tolerate heavy metals, likely through detoxification mechanisms. Additionally, species more reliant

on aquatic environments exhibited greater biofilm production, which may act as a supplementary defense mechanism by reducing contaminant penetration through the skin (Cordeiro et al., 2019).

The skin microbiome of *P. perezi* adults from AMD-contaminated regions exhibited significantly reduced bacterial diversity compared to three reference areas (Proença et al., 2021). Frogs from metal-impacted sites also displayed fewer cultivable bacterial strains and lower overall diversity than those from uncontaminated sites (Costa et al., 2016). Similar to Proença et al. (2021), *B. raddei* individuals from polluted areas showed decreased microbial diversity in the gut microbiome (Zhang et al., 2016). Furthermore, Zhang et al. (2016) reported that heavy metal pollution caused a pronounced reduction in probiotic microorganisms and an increase in pathogenic bacteria abundance.

Chromium (Cr), a major inorganic heavy metal, is a significant environmental contaminant (Sforzini et al., 2017). It is primarily released into aquatic environments through effluents from industries such as textile manufacturing, leather tanning, metal finishing, chrome plating, and printing. Exposure to Cr caused a reduction in gut microbiome diversity in *B. gargarizans* tadpoles, with the disruption of microbiota homeostasis potentially leading to intestinal tissue damage (Yao et al., 2019).

### 3.3. Antibiotics

Antimicrobial compounds are widely used in agricultural livestock to treat and prevent bacterial infections. However, as a result of runoff events, these compounds are often dispersed throughout the environment, contaminating soil and aquatic systems (De Liguoro et al., 2007). The presence of antibiotics in aquatic environments has become a global concern due to the risk of bacteria acquiring antibiotic resistance (Kurenbach et al., 2018).

Exposure to antibiotics has been shown to reduce the gut microbiome diversity in tadpoles of *R. arenarum* (Boccioni et al., 2021) and *R. omeimontis* (Zhu et al., 2022). In *R. arenarum*, this state of dysbiosis was associated with a decrease in taxonomic diversity and an increase in the dominance of a single genus, *Aeromonas* spp. (Boccioni et al., 2021). For *R. omeimontis*, antibiotic exposure led to an enhanced ability of the intestinal microbiota to degrade xenobiotics, a reduction in resistance to other antibiotics, and a decreased capacity to synthesize certain antibiotics (Zhu et al., 2022). Furthermore, antibiotic pollution significantly reshaped the gut bacterial community of *R. omeimontis* tadpoles, increasing the relative abundance of harmful strains, which could negatively impact host health (Zhu et al., 2022). In contrast, antibiotic exposure did not significantly affect the bacterial

diversity of the skin microbiome in *Lithobates pipiens* tadpoles (Hernández-Gómez et al., 2020). However, changes in the composition of the microbial community were observed, suggesting that while diversity remained stable, the structure of the microbiome was altered (Hernández-Gómez et al., 2020)

## 4. Effects on microbiome composition

The composition of the microbiome varies greatly among different species and plays a critical role in the host's health and ecological fitness (Rollins-Smith et al., 2011). Despite similarities at the phylum level, each anuran species generally exhibits a unique microbial composition (Belden et al., 2015; McKenzie et al., 2012; Sabino-Pinto et al., 2017; Vences et al., 2015). These interspecific differences are associated with the physical and chemical properties of the host's skin, including the production of distinct antimicrobial peptides by different species (Woodhams et al., 2014). However, studies have shown that anurans sharing similar habitats tend to have more comparable microbiomes than species from different environments. This is likely due to their exposure to a shared pool of colonizing bacteria (Belden et al., 2015; Bletz et al., 2017; Kueneman et al., 2014; Muletz Wolz et al., 2018). Additionally, anthropogenic disturbances significantly influence the composition of both the gut and skin microbiomes of anurans (Becker et al., 2017; Goff et al., 2020; Krynak et al., 2016; Preuss et al., 2020). Understanding how environmental changes affect the anuran microbiome is therefore essential for their conservation.

### 4.1. Phyla

Among the studies describing the composition of the microbiome of anuran amphibians, the most relevant microbiome phyla were Proteobacteria, Fusobacteria, Bacteroidetes and Firmicutes. Proteobacteria is one of the most unpredictable phyla in the gut microbiome and is often associated with environmental disturbances. An increased relative abundance of Proteobacteria has been linked to microbiome dysbiosis, reflecting an unstable microbial community influenced by external factors (Shin et al., 2015). Several studies report that heavy metals, such as cadmium, copper, and lead, elevate Proteobacteria levels in tadpole microbiomes, despite some exceptions where reduced abundance was observed (Ya et al. 2019; Zheng et al. 2020, 2021a, 2021b; Chai et al. 2022; Lv et al. 2022; Liu et al. 2023). Similarly, agrochemicals like nitrate ($No_3$) (Xie et al., 2020; Zheng et al., 2020) and atrazine (Zhao et al., 2022) also promote increases in Proteobacteria in the microbiomes of *P. nigromaculatus* adults and *B. gargarizans* and *R. chensinensis* tadpoles (Liu et al., 2020; Wang et al.,

2019; Zhao et al., 2022). Elevated levels of this phylum pose risks to host health, including energy imbalances, metabolic diseases, immune dysfunction, and increased vulnerability to chytridiomycosis (*Bd*) infection (Jani and Briggs, 2014; Shin et al., 2015).

Fusobacteria contribute positively to host health by alleviating intestinal mucosa inflammation and producing butyric acids that regulate epithelial transport and strengthen the mucosal barrier (Sossai, 2012). Studies have consistently shown that exposure to heavy metals often leads to an increase in Fusobacteria abundance in the gut microbiome of both tadpoles and adult anurans (Mu et al., 2018; Yao et al., 2019; Yang et al., 2020; Zheng et al., 2020, 2021a, 2021b; Huang et al., 2022b; Wan et al., 2022). In contrast, a reduction in Fusobacteria abundance has been observed in anurans exposed to chemical agents such as diethylhexyl phthalate, polychlorinated biphenyls, and fluoride (Kohl et al., 2015; Shen et al., 2022; Wang et al., 2019), agrochemical (i.e. atrazine) (Huang et al. 2021, 2022a) and the heavy metal cadmium (Shen et al., 2022). Unlike Proteobacteria, there is a consensus regarding the positive impact of Fusobacteria on host health. Most studies associate a decrease in Fusobacteria abundance with structural disorders in the intestinal microbial community, leading to tissue damage and increased inflammation of the intestinal mucosa (Mu et al., 2018; Wang et al., 2019; Yang et al., 2020; Zheng et al., 2020, 2021a; Liu et al. 2022a; Wan et al., 2022; Huang et al. 2022a). Moreover, a higher relative abundance of Fusobacteria in tadpole intestines has been linked to lower *Bd* infection intensity, suggesting a protective role against chytridiomycosis (Knutie et al., 2017).

There is no consensus regarding the benefits or harms of the Bacteroidetes and Firmicutes phyla to their hosts. Bacteroidetes, for instance, are widely recognized as key contributors to overall health and the complex homeostasis maintained by the gut microbiome (Gibiino et al., 2018). However, bacteria within this phylum can also trigger inflammatory responses in the host, and increased lipopolysaccharide levels may impair the skin barrier and compromise intestinal barrier function (Yoshida et al., 2018). Furthermore, Bacteroidetes include numerous pathogenic bacteria linked to immune dysfunction, metabolic syndrome, and toxin production (Thomas et al., 2011). Most studies report an increase in Bacteroidetes abundance in the microbiome of anurans exposed to chemical compounds, particularly heavy metals (Liu et al., 2020, 2022b, 2023; Zheng et al., 2020; Gutierrez-Villagomez et al., 2021; Huang et al., 2021, 2022b). However, other studies have observed a decrease in this phylum's abundance following exposure to certain chemicals, including heavy metals (Chai et al., 2022), nitrite (Liu et al., 2022a; Xie et al., 2020) and the herbicide atrazine (Zhao

et al., 2022), as well as in anurans from agricultural environments (Chang et al., 2016). Some authors propose that an increase in Bacteroidetes abundance after exposure to chemical agents may benefit anurans by enhancing carbohydrate metabolism and energy production, potentially improving host physical fitness (Evariste et al., 2020). Others, however, interpret this increase as a sign of microbiome imbalance and heightened health risks (Ya et al., 2020). These differing perspectives highlight the need for further studies to clarify the role of Bacteroidetes in the microbiome and immune system of anurans. Conversely, a reduction in Bacteroidetes abundance in anurans following environmental disturbances has been associated with immune dysfunction, metabolic disruptions, increased inflammatory diseases, and reduced energy capture capacity (Chai et al., 2022; Gibiino et al., 2018; Liu et al., 2022b). These findings underline the complex and context-dependent role of Bacteroidetes in maintaining host health.

Firmicutes play a key role in nutrient absorption and metabolic control of energy storage (Turnbaugh et al., 2008). As major producers of butyrate, bacteria within this phylum are essential for maintaining gut health. Dysfunction in butyrate-producing bacteria is often associated with mucosal atrophy (Roediger, 1990) and inflammatory intestinal disease (Wang et al., 2014). Similar to Bacteroidetes, the studies reviewed here reveal contrasting effects of changes in Firmicutes abundance following environmental disturbances. For instance, exposure to heavy metals has been shown to decrease the abundance of Firmicutes in the skin microbiome of *P. nigromaculatus* adults (Liu et al., 2022b), and in the gut microbiome of *R. chensinensis* tadpoles (Mu et al., 2018; Shen et al., 2018) and *R. omeimontis* (Lv et al., 2022). Conversely, an increase in Firmicutes abundance was observed in the gut microbiome of *B. gargarizans* tadpoles exposed to similar pollutants (Zheng et al., 2021b; Liu et al., 2023). An increase in Firmicutes abundance can enhance energy storage and reabsorption but may also contribute to inflammatory intestinal diseases. On the other hand, a reduction in this phylum often indicates metabolic disturbances in the host. This suggests that exposure to different pollutants can disrupt butyrate production, increasing the risk of intestinal mucosa damage and inflammatory diseases (Shen et al., 2022). These findings highlight the complex role of Firmicutes in maintaining host health and the potential consequences of environmental disturbances on microbial homeostasis.

### 4.2. Genus

Among the evaluated studies, the genera *Aeromonas*, *Bacteroides*, *Flavobacterium*, and *Pseudomonas* stood out as particularly relevant, though their roles were discussed in contrasting ways

by the authors. For instance, a decrease in the abundance of *Aeromonas* was observed in the gut microbiome of *B. gargarizans* tadpoles following exposure to heavy metals (Ya et al., 2020; Yao et al., 2019; Zheng et al., 2021a) and in adults of *P. nigromaculatus* (Wan et al., 2022). Since *Aeromonas* is known to secrete immunomodulatory proteins essential for preventing intestinal inflammation (Rolig et al., 2018), this decline may indicate a compromised immune system in tadpoles (Ya et al., 2020). Conversely, some authors suggested that a reduction in *Aeromonas* abundance might serve as a protective mechanism, as excessive proliferation of this genus can turn it pathogenic (Zheng et al., 2021a). In contrast, other studies reported an increase in *Aeromonas* abundance following exposure to heavy metals in the gut microbiome of *R. chensinensis* tadpoles (Shen et al., 2022) and *B. gargarizans* tadpoles (Ya et al., 2019). Similarly, agrochemicals were found to elevate *Aeromonas* abundance in *R. arenarum* tadpoles (Boccioni et al., 2021) and *X. laevis* adults (Li et al., 2022). These contrasting results highlight the dual role of *Aeromonas* within the microbiome, which can either contribute to host health by supporting immune function or pose risks when its abundance becomes excessive, underlining the need for further research to clarify its ecological significance.

*Bacteroides* plays a complex and generally beneficial role in maintaining intestinal homeostasis by producing enzymes involved in protein metabolism and carbohydrate transport, which enhance digestion and nutrient absorption (He et al., 2018). Additionally, this genus can inhibit the adhesion of harmful microorganisms and help restore the balance of the intestinal microbial system when reintroduced into the flora (Guarner and Malagelada, 2003). However, contrasting effects of *Bacteroides* abundance have been observed in the gut microbiome of anurans exposed to environmental stressors. For instance, *Bacteroides* exhibited opposing responses in *B. gargarizans* and *P. nigromaculatus* exposed to the same chemicals. In *B. gargarizans* tadpoles exposed to heavy metals such as lead and copper, both an increase (Liu et al., 2023) and a decrease (Chai et al., 2022) in *Bacteroides* abundance were reported. Similarly, exposure to the agrochemical atrazine increased *Bacteroides* abundance in the gut microbiome of *P. nigromaculatus* tadpoles (Huang et al., 2021) but caused a decrease in adults of the same species (Zhao et al., 2022). In other studies, an increase in *Bacteroides* abundance was observed in response to lead and cadmium exposure in the gut microbiome of *Rana chensinensis* tadpoles (Mu et al., 2018) and *P. nigromaculatus* adults (Huang et al., 2022b), respectively. These results have been interpreted differently by various authors. A decrease in *Bacteroides* abundance following heavy metal exposure has been linked to compromised gut barrier and immune functions (Chai et al., 2022), while agrochemical exposure has been associated with disturbances in digestion, nutrient absorption, and immunity, potentially leading to

inflammation (Liu et al. 2022a; Zhao et al. 2022). On the other hand, an increase in *Bacteroides* abundance was sometimes viewed as an indicator of intestinal damage and weakened defense capacity, as observed in *P. nigromaculatus* adults exposed to lead (Huang et al., 2022b). Conversely, the increase in *Bacteroides* in *P. nigromaculatus* tadpoles exposed to atrazine was interpreted as an adaptive response to environmental stressors, consistent with suggestions from other studies (Huang et al., 2021; Mu et al., 2018).

*Flavobacterium* has been identified as an important component of host defense mechanisms in anurans (Zheng et al., 2020), although it has also been considered a potential pathogen for these animals (Yang et al., 2020). An increase in the abundance of *Flavobacterium* has been reported in the gut microbiome of *B. gargarizans* tadpoles exposed to heavy metals (Yang et al., 2020; Zheng et al. 2020, 2021b) and to nitrate (Zheng et al., 2020), as well as in the cutaneous microbiome of *Lithobates pipiens* tadpoles exposed to antibiotics (Hernández-Gómez et al., 2020). Notably, none of the reviewed studies reported a decrease in the abundance of this genus. The increased abundance of *Flavobacterium* in the gut microbiome of *B. gargarizans* tadpoles following exposure to heavy metals, such as copper, has been associated with physiological changes in the intestine, potentially elevating the risk of disease outbreaks and mortality in these animals (Yang et al., 2020; Zheng et al., 2021b). However, other studies have interpreted the rise in *Flavobacterium* abundance differently. For instance, its increase in *B. gargarizans* tadpoles exposed to various heavy metals, including copper, chromium, and cadmium, was viewed as a positive adaptive response. This suggests that the genus may play a role in enhancing resistance and adaptive tolerance to elevated chemical ion concentrations in the environment (Zheng et al., 2020).

*Pseudomonas* is known for its antibiotic properties, which can help protect anurans from infections, with some studies considering the presence of this genus beneficial to the hosts (Liu et al., 2022b; Coelho et al., 2022; Chai et al., 2023). However, other research characterizes *Pseudomonas* as a potential opportunistic pathogen (Bie et al., 2019; Chai et al., 2022; Kohl et al., 2015). In several studies, the abundance of *Pseudomonas* was reduced following exposure to heavy metals. For instance, it decreased in the cutaneous microbiome of *P. nigromaculatus* (Liu et al., 2022a) and in the gut microbiome of *B. gargarizans* tadpoles (Zhu et al., 2023). A reduction was also observed in *R. arenarum* adults and tadpoles after exposure to agrochemicals (Boccioni et al., 2021). In contrast, exposure to lead led to an increase in *Pseudomonas* abundance in the gut microbiome of *B. gargarizans* tadpoles (Chai et al., 2023), and both lead and copper were associated with increased

*Pseudomonas* in the microbiomes of other species, including *L. pipiens* (Kohl et al., 2015; Chai et al., 2022), and eucalypt ashes in *Rana iberica* (Coelho et al., 2022). While some studies view the presence of *Pseudomonas* as positive (Chai et al., 2023; Coelho et al., 2022; Liu et al., 2022a), the expansion of this genus following heavy metal exposure, such as lead and copper, has been linked to an increased risk of intestinal physiological disorders in *B. gargarizans* tadpoles (Chai et al., 2022). However, a later study suggested that the significant rise in *Pseudomonas* abundance in the intestines of *B. gargarizans* tadpoles after lead exposure may represent a self-protection mechanism, indicating a potential adaptive response (Chai et al., 2023).

## 5. Ratio Firmicutes x Bacteroidetes

Bacteroidetes and Firmicutes are common bacterial phyla found in anurans, predominantly in the digestive tract (Huang et al., 2021; Li et al., 2022; Liu et al., 2022a; Xie et al., 2020; Zheng et al., 2021b), but they are also present in the skin microbiome (Bie et al., 2019; Costa et al., 2016; Proença et al., 2021). Changes in the Firmicutes/Bacteroidetes (F/B) ratio are widely used as indicators of gut microbiome health (Zheng et al., 2021b), with a stable F/B ratio being crucial for maintaining intestinal microbiota balance (Guo et al., 2018). An elevated F/B ratio is typically associated with negative impacts on frog health, potentially leading to metabolic dysfunction, increased disease susceptibility, impaired nutrient absorption, and compromised intestinal permeability (Mariat et al., 2009). Conversely, a lower F/B ratio may reduce energy absorption (Turnbaugh et al., 2006), fat storage (Bäckhed et al., 2004), and lipid metabolism gene expression, while also decreasing levels of lipopolysaccharides and intestinal permeability (Lyu et al., 2017). Thus, a reduced F/B ratio could signal disruptions in lipid metabolic functions (Liu et al., 2020). In the studies reviewed, most (nine) reported a decreased F/B ratio following disturbances, five reported no variation, and three observed an increased F/B ratio in the gut microbiome of anurans after exposure to various stressors (Table 3).

The reduced F/B ratio in anuran gut microbiomes was most commonly linked to exposure to chemical agents, particularly heavy metals. This was observed in *B. gargarizans* tadpoles (Zheng et al., 2020, 2021a), *R. chensinensis* tadpoles (Liu et al., 2020; Mu et al., 2018), *P. nigromaculatus* tadpoles and adults (Huang et al., 2022b) and *B. raddei* adults (Zhang et al., 2016). Reduced F/B ratios were also noted in *B. gargarizans* and *P. nigromaculatus* tadpoles exposed to agrochemicals (Huang et al., 2021; Xie et al., 2020), and in *R. chensinensis* tadpoles (Liu et al., 2020) and *P. nigromaculatus* adults (Lin et al., 2022) exposed to other chemicals. These reductions were attributed to changes in the gut microbiota, likely resulting from the destruction of intestinal bacteria, damage

to the structural integrity of the intestine, and impaired host health (Huang et al., 2022 b; Liu et al., 2022b). However, exposure to copper did not affect the F/B ratio in *R. chensinensis* tadpoles in one study (Yang et al., 2020), suggesting that some tadpoles may be more sensitive to specific metals, such as cadmium (Mu et al., 2018) or octylphenol (Liu et al., 2020), than to copper. Additionally, exposure to agrochemicals and other chemical agents in *P. nigromaculatus* (Huang et al., 2021; Lin et al., 2022), *B. gargarizans* (Xie et al., 2020) and *R. chensinensis* (Liu et al., 2020) also reduced the F/B ratio, which was linked to dysbiosis in the gut microbiome, negatively affecting the frogs' ability to absorb and store energy (Huang et al., 2021; Lin et al., 2022; Liu et al., 2022a; Xie et al., 2020).

In contrast, an increase in the F/B ratio was observed in the gut microbiomes of *B. gargarizans* tadpoles following exposure to heavy metals (Chai et al., 2023; Zheng et al., 2021b), and in *F. limnocharis* adults from agricultural environments (Chang et al., 2016). In *B. gargarizans* tadpoles, this increase, particularly following exposure to cadmium and lead, was associated with a significant increase in body mass, which was linked to the loss of intestinal fitness and changes in digestive enzyme activity (Zheng et al., 2021b). This increase in the F/B ratio may reflect an improvement in the efficiency of calorie absorption, a phenomenon previously observed in humans (Ley et al., 2006). For *F. limnocharis*, the increase in the F/B ratio was associated with dietary changes in hosts from agricultural and forestry environments, which may have altered both the intestinal environment and its microbiota (Chang et al., 2016). However, no change in the F/B ratio was found in adults from another population of *F. limnocharis*, *Babina adenopleura* (Huang et al., 2018) and *B. bufo* tadpoles (Garbor et al., 2023), suggesting that diet may not differ significantly between environments for these species.

## 6. Concluding Remarks

Despite the recent surge in studies examining the impact of anthropogenic activities on the microbiome of anuran amphibians, our understanding remains limited, highlighting the urgent need for further research. As anurans are among the most threatened animal groups worldwide, it is crucial to assess the effects of human activities on their health and well-being. This area of research is vital not only for the conservation of amphibians but also for uncovering new avenues for scientific inquiry. Studies focused on the influence of anthropogenic environments are particularly needed, as only 17 studies have addressed this topic, compared to 45 studies examining the effects of chemicals on the anuran microbiome.

A microbiome with greater diversity is generally more beneficial to hosts, as it is associated with improved ecosystem functioning, greater stability, resilience (the diversity-stability hypothesis), and reduced pathogen transmission (the dilution effect) (Fassarella et al., 2021; Keesing and Ostfeld, 2021; Loreau and Hector, 2001; Lozupone et al., 2012; McCann, 2000). In contrast, reduced microbiome diversity is linked to increased metabolism and the development of immunological and cognitive disorders (Bello et al. 2018). Most studies have reported a reduction in microbiome diversity in disturbed environments or after exposure to chemical agents. Additionally, these studies highlight a range of harmful effects, such as impaired growth, development, and metamorphosis (Chai et al., 2023); disruption of lipid storage and transport, which directly impacts the host's energy balance and fitness (Mu et al., 2018; Shen et al., 2022; Zheng et al., 2021b); decreased anti-inflammatory capacity (Shen et al., 2022); reduction in probiotic microorganisms and an increase in pathogenic bacteria (Zhang et al., 2016); and histological changes, including degeneration and disruption of epithelial cells (Yang et al., 2019, 2020). These effects can enhance the likelihood of infections by opportunistic pathogens and the onset of diseases (Chai et al., 2022; Huang et al., 2022b; Shen et al., 2022; Zhang et al., 2016; Zheng et al., 2021b).

A very interesting topic that has been emerging is the assessment of the level of microbiome dispersion, considered an indicator of negative health in situations of environmental stress, where the greater the dispersion, the greater the chances of hosts being in dysbiosis (Zaneveld et al., 2017). Studies indicate that microbiome dispersion increases in disturbed environments and following heavy metal exposure, suggesting that anthropogenic activities contribute to dysbiosis in anurans (Jiménez et al., 2022; Neely et al., 2022; Krynak et al., 2016; Ya et al., 2019; Zaneveld et al., 2017). These

findings are valuable, and we strongly advocate for further research that evaluates microbiome dispersion in anurans.

Interestingly, some studies have observed increased microbial diversity in disturbed environments, attributing this to a higher abundance of bacteria that degrade agrochemicals and inhibit pathogen infections (Chang et al., 2016; Huang et al., 2018). These results may reflect physiological, metabolic, and ecological responses to environmental disturbances, supporting the hypothesis that high "functional response diversity" in the gut microbiome compensates for such perturbations (Elmqvist et al., 2003; Huang et al., 2018). Additionally, the pH of the environment plays a crucial role in shaping the skin microbiome of anurans, with more acidic conditions leading to changes in the skin microbiota of four anuran species (Krynak et al., 2015; Varela et al., 2018).

Regarding microbiome composition, there is general consensus that heavy metals increase the abundance of Proteobacteria, which is detrimental to host health due to its association with heightened *Bd* infections (Jani and Briggs, 2014). Fusobacteria, on the other hand, is considered beneficial for the amphibian microbiome, and its decreased abundance after exposure to heavy metals and agrochemicals may result in serious intestinal complications. However, there is no consensus regarding the effects of Bacteroidetes and Firmicutes. Studies have shown both increased and decreased abundances of these bacteria in response to heavy metals and other chemicals, leading to mixed interpretations. Similarly, the effects of anthropogenic factors on genera such as *Aeromonas*, *Bacteroides*, *Flavobacterium*, and *Pseudomonas*, which are predominant in the amphibian microbiome, remain inconsistent. Changes in the Firmicutes/Bacteroidetes (F/B) ratio have also been observed, with both increases and decreases after exposure to heavy metals and agrochemicals. Since a stable F/B ratio is essential for maintaining intestinal microbiota homeostasis, instability may have negative consequences for the host. However, some studies have reported no change in the ratio, even in disturbed environments or after chemical exposure. Thus, further research is needed to fully understand how chemical agents affect the anuran microbiome.

From this review, it is evident that anthropogenic activities generally have negative effects on the anuran microbiome, adversely affecting the health of these animals. However, most studies have been conducted in temperate regions, with few focusing on tropical forests, where *Bd* incidence is higher. We also emphasize the need for more studies on the impact of anthropogenic environments on the anuran microbiome, particularly the skin microbiome of tadpoles, which has been less studied than the gut microbiome or the effects of chemical exposure. Therefore, we strongly encourage more

research in tropical regions, with a focus on the skin microbiome of tadpoles and the gut microbiome of adult anurans.

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## Glossary

***Bd*-facilitating bacteria:** bacteria with properties capable of facilitate the proliferation of *Bd*.

***Bd*-inhibiting bacteria:** bacteria with properties capable of inhibiting the proliferation of *Bd*.

***Bd* intensity:** *Bd* load values.

**Dysbiosis:** changes in the composition of the host's microbiota relative to the community found in healthy hosts. It can also be seen as a reduction in beneficial or keystone species, a change in functional capacity, an increase in pathogenic species, or a change in diversity.

**Homeostasis:** the ability of an organism to remain constant so that its essential chemical functions and reactions are not influenced. Regulatory process that keeps the organism in constant balance.

**Host microbiome:** microbial communities that live somewhere on the host's body (e.g., skin microbiome or gut microbiome).

**Microbiome:** the communities of microorganisms including bacteria, yeast, fungi, protists, and archaea.

**Microbiota:** microorganisms that exist in a given environment, habitat, or host (inside or on the host).

**Microorganisms:** microscopic organisms that exist as single cells or clusters of cells, including bacteria, protozoa, archaea, microscopic fungi, and microscopic algae.

**Prevalence:** the number of individual frogs infected (e.g. five infected individuals out of a total of 10 individuals equals a prevalence of 50%).

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**Table 1:** Main results of the articles that evaluate the effect of anthropogenic environments or activities on the skin and gut microbiome of anuran amphibians.

| Skin microbiome of adult anurans | | | | |
|---|---|---|---|---|
| **Topic** | **Disturbances** | **Target species** | **Main results** | **Citation** |
| Anthropogenic and natural environments | High road and urban density environment and low road and urban density environment | *Duttaphrynus dhufarensis* | Alpha diversity was not affected. Beta diversity was driven by habitat disturbance. | Bates et al., 2022 |
| Preserved and disturbed forests | Fragmented and continuous forests | *Dendropsophus minutus* | Greater bacterial diversity in anurans from continuous forests. | Becker et al., 2017 |
| Preserved and disturbed forests | Fragmented and continuous forests | *Proceratophrys boiei* | Greater bacterial diversity in anurans from fragmented forests. | De Assis et al., 2020 |
| Preserved and disturbed forests | Disturbed and preserved forests | *Eleutherodactylus coqui* | Alpha diversity was not affected. Beta diversity was influenced by the type of environment, but at low intensity. | Hughey et al., 2017 |
| Anthropogenic and natural environments | Agriculture and livestock environments and natural environments | *Lithobates vibicarius* | Greater bacterial diversity in anurans from natural environments. Greater diversity of *Bd*-inhibitory bacteria in disturbed environments. | Jiménez et al., 2020 |
| Habitat quality | "Non-manipulated" mesocosmus and manipulated mesocosmus (higher water acidity and high and low levels of shade) | *Aquarana catesbeiana* | More acidic aquatic environments (lower pH) led to changes in the bacterial community on tadpole skin. | Krynak et al., 2015 |
| Anthropogenic and natural environments | Agricultural and urban environments and prairie and forest environments | *Eleutherodactylus coqui* | Anurans from natural environments had similar microbiomes, but different from those from disturbed environments. | Krynak et al., 2016 |

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|---|---|---|---|---|
| Anthropogenic and natural environments | agricultural, urban and altered environments and natural environment | *Acris crepitans* and *Aquarana catesbeiana* | High dispersion of the *Acris crepitans* microbiome from disturbed environments. | Neely et al., 2022 |
| Habitat quality | Ponds with swine slurry and without slurry | *Dendropsophus minutus* | Lower bacterial diversity and greater abundance of *Bd*-facilitating bacteria in anurans from ponds with swine slurry. | Preuss et al., 2020 |
| Habitat disturbance | Fire effect | *Boana leptolineata* and *Scinax squalirostris* | High dispersion of the *S. squalirostris* microbiome after fire. | Schuck et al., 2024 |
| Anthropogenic and natural environments | Urban environments and forests environments | *Dendrobates auratus, Silverstoneia flotator* and *Allobates talamancae* | Environments with more acidic soils (lower pH) led to greater alpha diversity in the skin of *D. auratus.* | Varela et al., 2018 |
| **Skin microbiome of tadpoles** | | | | |
| Anthropogenic and natural environments | Agricultural, livestock and natural environments | *Scinax fuscovarius* | Greater bacterial diversity in anurans from natural environments. | De Assis et al., 2021 |
| Habitat quality | Landcover type, percent of urban areas and canopy cover | *Pseudacris ornata* | Greater bacterial diversity and evenness in anurans from natural environments. | Goff et al., 2020 |
| Anthropogenic and natural environments | Agriculture and livestock environments and natural environments | *Lithobates vibicarius* | Greater bacterial diversity in anurans from natural environments. Greater diversity of Bd-inhibitory bacteria in disturbed environments. | Jiménez et al., 2020 |
| **Gut microbiome of adult anurans** | | | | |
| Anthropogenic environments | Agricultural and urban environments | *Pristimantis unistrigatus* | Bacterial richness was not affected. | Carphio et al., 2021 |
| Anthropogenic and natural environments | Agricultural and forest environments | *Fejervarya limnocharis* | Greater bacterial diversity in anurans from agricultural environments. | Chang et al., 2016 |

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| Anthropogenic and natural environments | Agricultural and forest environments | *Fejervarya limnocharis* and *Babina adenopleura* | Greater bacterial diversity and more specific bacteria in anurans from agricultural environments. | Huang et al., 2018 |
| **Gut microbiome of tadpoles** | | | | |
| Anthropogenic and natural environments | Agricultural, urban and natural environments | *Bufo bufo* | Bacterial diversity and richness were not affected. | Garbor et al., 2023 |

**Table 2:** Main results of articles that evaluate the effect of chemical substances on the skin and gut microbiome of anuran amphibians.

| **Skin microbiome of adult anurans** | | | | |
|---|---|---|---|---|
| **Category** | **Product tested** | **Target species** | **Main result** | **Citation** |
| Agrochemical (herbicide) | Glyphosate | *Acris blanchardi* | Microbiome was influenced by water quality and proportion of natural environments. | Krynak et al., 2017 |
| Heavy metal | - | *Pelophylax perezi* | Lower diversity and density of bacterial morphotypes in individuals from contaminated areas. | Costa et al., 2016 |
| Biocide | *Bacillus thuringiensis* | *Lithobates sphenocephalus* | Anurans previously exposed to the biocide had lower rates of *Bd* infection. | Weeks et al., 2020 |
| Heavy metal | Arsenic | *Boana faber*, *Bokermannohyla nanuzae*, *Ischnocnema izecksohni*, *Ololygon luizotavioi*, *Ololygon tripui*, *Rhinella crucifer* and *Vitreorana uranoscopa* | Species with greater dependence on water showed bacteria with high resistance to arsenic. | Cordeiro et al., 2019 |

| | | | | |
|---|---|---|---|---|
| Heavy metal | Acid mine drainage | *Pelophylax perezi* | Reduced bacterial diversity in anurans from contaminated area. | Proença et al., 2021 |
| Heavy metal | Lead (Pb) | *Pelophylax nigromaculatus* | Reduced bacterial diversity in treatment groups. | Liu et al., 2022b |
| By-product of coal combustion | Fly ash | *Pseudacris crucifer* | No effect detected on bacterial richness and diversity. | Hughey et al., 2016 |
| Chemical agent | Eucalypt ashes (AAEs) | *Rana iberica* | Increasing concentrations of AAEs led to an increase in the number of bacteria whose growth was negatively affected. | Coelho et al., 2022 |
| **Gut microbiome of adult anurans** | | | | |
| Agrochemical (herbicide) | Atrazine | *Osteopilus septentrionalis* | Exposure to atrazine did not affect bacterial diversity or community composition. | Knutie et al., 2018 |
| Chemical agent | Polychlorinated biphenis | *Lithobates pipiens* | Greater bacterial richness in the treatment groups. | Kohl et al., 2015 |
| Polyfluoroalkyl substances | Perfluorooctanoic acid, perfluorooctanesulfonic acid and chlorinated polyfluorinated ether sulfonate | *Pelophylax nigromaculatus* | Antibiotics increased the diversity and abundance of the gut microbiota. | Lin et al., 2022 |
| Agrochemical (herbicide) | Atrazine | *Pelophylax nigromaculatus* | Reduced bacterial diversity in the treatment group. | Zhao et al., 2021 |
| Heavy metal | - | *Strauchbufo raddei* | Reduced bacterial diversity in anurans from contaminated areas. | Zhang et al., 2016 |

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|---|---|---|---|---|
| Heavy metal | Cadmium (Cd) | *Pelophylax nigromaculatus* | Short-term Cd exposure changed the community structure of gut microbiota. | Wan et al., 2022 |
| Agrochemical (insecticide) | *Cis*-bifenthrin | *Xenopus laevis* | Reduced bacterial diversity in the treatment group. | Li et al., 2022 |
| Heavy metal | Lead (Pb) | *Pelophylax nigromaculatus* | Reduced bacterial diversity in the treatment group. | Huang et al., 2022a |
| Agrochemical (herbicide) | Atrazine | *Pelophylax nigromaculatus* | Reduced bacterial diversity in the treatment group. | Zhao et al., 2022 |
| **Skin microbiome of tadpoles** | | | | |
| Antibiotics | Sulfadimethoxine | *Lithobates pipiens* | Diversity and bacterial richness were not affected. | Hernández-Gómez et al., 2020 |
| Agrochemical (herbicide) | Glyphosate | *Acris blanchardi* | Microbiome was influenced by water quality and proportion of natural environments. | Krynak et al., 2017 |
| Agrochemical (fungicide) | Chlorothalonil | *Lithobates vibicarius* | Diversity and bacterial richness were not affected. | Jiménez et al., 2021 |
| Wastewaters | Chloride (Cl) | *Lithobates pipiens, L. sylvaticus* and *Pseudacris maculata* | Diversity and bacterial richness were not affected. | Tornabene et al., 2023 |
| Munitions compound | 2,4,6-trinitrotoluene (TNT) | *Rana pipiens* | Reduced alpha bacterial diversity in treatment groups. | Gust et al., 2021 |
| **Gut microbiome of tadpoles** | | | | |
| Heavy metal and chemical agent | Cadmium (Cd) and diethylhexyl phthalate | *Rana chensinensis* | Diversity and bacterial richness reduced in treatment groups. | Shen et al., 2022 |

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|---|---|---|---|---|
| Agrochemical (herbicide) | Atrazine | *Osteopilus septentrionalis* | Exposure to atrazine did not affect bacterial diversity or community composition. | Knutie et al., 2018 |
| Chemical agent | Polychlorinated biphenis | *Lithobates pipiens* | Greater bacterial richness in the treatment groups. | Kohl et al., 2015 |
| Agrochemical (herbicide) and antibiotic | Glyphosate and ciprofloxacin | *Rhinella arenarum* | Increased bacterial diversity and richness in groups treated with glyphosate. Decreased diversity in ciprofloxacin-treated groups. | Boccioni et al., 2021 |
| Heavy metal | Cadmium (Cd) | *Bufo gargarizans* | Diversity and bacterial richness reduced in treatment groups. | Ya et al., 2019 |
| Heavy metal | Cadmium (Cd) | *Bufo gargarizans* | Diversity and bacterial richness increased in the treatment group. | Ya et al., 2020 |
| Chemical agent | Octylphenol | *Rana chensinensis* | Reduced bacterial diversity in the treatment group. | Liu et al., 2020 |
| Heavy metal | Cadmium (Cd) | *Rana chensinensis* | Reduced bacterial diversity in treatment groups. | Mu et al., 2018 |
| Heavy metal | Copper (Cu) | *Rana chensinensis* | Reduced bacterial diversity and increased community uniformity in the treatment groups. | Yang et al., 2020 |
| Chemical agent | Boron nitride nanotubes (BNNT) | *Xenopus laevis* | Microbial richness was not affected. Evenness decreased in the treatment group. | Evariste et al., 2020 |
| Heavy metal | Chromium (Cr) | *Bufo gargarizans* | Reduced bacterial diversity in the treatment group. | Yao et al., 2019 |
| Heavy metal | Copper (Cu) | *Bufo gargarizans* | Change in bacterial richness, diversity and composition in treatment groups. | Zheng et al., 2021b |

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|---|---|---|---|---|
| Biocide | *Bacillus thuringiensis* var. *israelensis* | *Lithobates sylvaticus* and *Anaxyrus americanus* | Altered bacterial composition in the *A. americanus* treatment group. | Gutierrez-Villagomez et al., 2021 |
| Agrochemical (fertilizer) | Nitrite ($NO_2$) | *Bufo gargarizans* | Microbial richness was reduced in treatment groups, while diversity increased. | Liu et al., 2022a |
| Heavy metal | Lead (Pb) and Copper (Cu) | *Bufo gargarizans* | Reduced bacterial diversity in treatment groups. | Chai et al., 2022 |
| Heavy metal | Lead (Pb) | *Bufo gargarizans* | Bacterial diversity reduced in the Gs33 larval stage and increased in the Gs42 stage. | Chai et al., 2023 |
| Agroquímico (fertilizante) | Nitrate (NO3) | *Bufo gargarizans* | Diversity and bacterial richness increased in the treatment group. | Xie et al., 2020 |
| Heavy metal | Lead (Pb) and Copper (Cu) | *Bufo gargarizans* | Increased bacterial diversity in treatment groups. | Liu et al., 2023 |
| Agrochemical (herbicide) | Atrazine | *Pelophylax nigromaculatus* | Increased bacterial diversity in the treatment group. | Huang et al., 2021 |
| Heavy metal and chemical agent | Lead (Pb) and sodium nitrate (NaNO3) | *Rana omeimontis* | Increased alpha bacterial diversity in the treatment group. | Lv et al., 2022 |
| Heavy metal | Fluorine | *Bufo gargarizans* | Diversity and bacterial richness increased in treatment groups. | Wang et al., 2019 |
| Heavy metal | Cadmium (Cd) and lead (Pb) | *Bufo gargarizans* | Reduced microbial richness in treatment groups. | Zheng et al., 2021a |
| Agrochemical (herbicide) | Atrazine | *Pelophylax nigromaculatus* | Increased bacterial diversity in the treatment group. | Huang et al., 2022b |

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|---|---|---|---|---|
| Heavy metal | Lead (Pb) | *Bufo gargarizans* | High levels of Pb led to a low abundance of aerobic and probiotic bacteria, and a high abundance of pathogenic bacteria. | Zhu et al., 2023 |
| Heavy metal and chemical agent | Copper (Cu), chromium (Cr) cadmium (Cd) and nitrates | *Bufo gargarizans* | Microbiota distribution was positively correlated with increasing concentrations of chemical ions. | Zheng et al., 2020 |

**Table 3:** Main results found on the effects of chemical substances and anthropogenic activities on the Firmicutes/Bacteroidetes ratio (F/B).

| **Gut microbiome of tadpoles** | | | | |
|---|---|---|---|---|
| **Category** | **Product tested** | **Target species** | **Main result** | **Citation** |
| Heavy metal | Cadmium (Cd) and lead (Pb) | *Bufo gargarizans* | Increased F/B ratio in treatment groups. | Zheng et al., 2021b |
| Agrochemical (fertilizer) | Nitrite ($NO_2$) | *Bufo gargarizans* | Reduced F/B ratio in the treatment group. | Xie et al., 2020 |
| Agrochemical (herbicide) | Atrazine | *Pelophylax nigromaculatus* | Reduced F/B ratio in the treatment group. | Huang et al., 2021 |
| Heavy metal | Cadmium (Cd) | *Rana chensinensis* | Reduced F/B ratio in the treatment group. | Mu et al., 2018 |

| | | | | |
|---|---|---|---|---|
| Natural x anthropogenic environment | - | *Bufo bufo* | F/B ratio did not change. | Garbor et al., 2023 |
| Heavy metal | Copper (Cu) | *Rana chensinensis* | F/B ratio did not change. | Yang et al., 2020 |
| Chemical agent | Octilfenol (OP) | *Rana chensinensis* | Reduced F/B ratio in the treatment group. | Liu et al., 2020 |
| Chemical agent | Boron nitride nanotubes (BNNT) | *Xenopus laevis* | F/B ratio did not change. | Evariste et al., 2020 |
| Biocide | *Bacillus thuringiensis* var. *israelensis* | *Lithobates sylvaticus* and *Anaxyrus americanus* | F/B ratio did not change. | Gutierrez-Villagomez et al., 2021 |
| Heavy metal | Lead (Pb) | *Bufo gargarizans* | Increased F/B ratio in larval stage Gs42. | Chai et al., 2023 |
| Heavy metal | Copper (Cu) | *Bufo gargarizans* | Reduced F/B ratio in the treatment group. | Zheng et al., 2021a |
| Heavy metal and chemical agent | Copper (Cu), chromium (Cr) cadmium (Cd) and nitrate ($NO_3$) | *Bufo gargarizans* | F/B ratio increased in groups treated with Cd and $NO_3$ and reduced with Cu and Cr. | Zheng et al., 2020 |
| **Gut microbiome of adult anurans** | | | | |
| Heavy metal | Lead (Pb) | *Pelophylax nigromaculatus* | Reduced F/B ratio in treatment groups. | Huang et al., 2022b |
| Agricultural x natural environment | - | *Fejervarya limnocharis* | Increased F/b ratio in anurans from agricultural environments. | Chang et al., 2016 |

| | | | | |
|---|---|---|---|---|
| Heavy metal | - | *Strauchbufo raddei* | Reduced F/B ratio in anurans from contaminated area. | Zhang et al., 2016 |
| Chemical agent | Polifluoroalquil perfluorooctanoic acid, perfluorooctanesulfonic acid and chlorinated polyfluorinated ether sulfonate | *Pelophylax nigromaculatus* | Reduced F/B ratio in the treatment group. | Lin et al., 2022 |
| Agricultural x forest environment | - | *Fejervarya limnocharis* e *Babina adenopleura* | F/B ratio did not change. | Huang et al., 2018 |
| Heavy metal | - | *Strauchbufo raddei* | Reduced F/B ratio in the treatment group. | Zhang et al., 2016 |

# Capítulo 2

## Insights into the Skin Microbiome Diversity of Neotropical Amphibians

O capítulo II desta Tese foi elaborado e formatado conforme as normas da publicação científica *Biotropica*, as quais se encontram em anexo (Anexo 2).

# Insights into the Skin Microbiome Diversity of Neotropical Amphibians

Camila Fernanda Moser[1*], Laura Kauer Schuck[2], Carolina de Abreu Caberlon[3], Wesley J. Neely[4], Priscila Barth[3], C. Guilherme Becker[2,5] and Pedro Peloso[6]

[1]Universidade Federal do Pará, Programa de Pós-Graduação em Zoologia, R. Augusto Corrêa, Belém, PA 66075-110, BR.

[2]Department of Biology, The Pennsylvania State University, University Park, PA 16803, USA.

[3]Universidade do Vale do Rio dos Sinos, Programa de Pós-Graduação em Biologia, Av. Unisinos, São Leopoldo, RS 93022-750, BR.

[4]Department of Biology, Texas State University, San Marcos, TX 78666, USA.

[5]One Health Microbiome Center, Center for Infectious Disease Dynamics, Ecology Institute, Huch Institutes of the Life Sciences, The Pennsylvania State University, University Park, PA 16803, USA.

[6]Department of Biological Sciences, California State Polytechnic University, Arcata, CA 95521, USA.

[*]corresponding author: camila-moser@hotmail.com

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**Abstract**

Describing the skin microbiome of amphibians is essential for understanding the complex microbial interactions that occur on their skin and their critical role in health and immunity. The composition and function of a species' basal microbiome are key to detecting changes that may arise due to environmental disturbances, such as climate change, habitat loss, or the introduction of new pathogens. This study provides an initial description of the skin microbiome of 10 anuran species from the Atlantic Forest, including two endemic species, *Physalaemus carrizorum* and *Leptodactylus plaumanni*. Our findings revealed that the primary taxa comprising the skin microbiome of these species belongs to the family Comamonadaceae, the genus *Pseudomonas*, and the species *Stenotrophomonas rhizophila*. These taxa, known for their antifungal and antioxidant properties, are widespread among various hosts and play a vital role in infection protection. Additionally, we observed significant interspecies differences in microbiome composition, emphasizing that, despite similarities at the phylum level, each species harbors a distinct microbiome shaped by factors such as ecology and life history. Our findings underscore the diversity of microbial communities and their potential role in protecting amphibians against pathogens. These results lay a foundation for future research aimed at understanding how microbiomes can be leveraged to improve amphibian health in both natural and captive settings, contributing to conservation strategies for species threatened by environmental disturbances.

**Keywords:** Antifungal metabolites, microbiota, OTUs, immune system.

AMPHIBIANS, LIKE OTHER ANIMALS, HARBOR COMPLEX COMMUNITIES OF SYMBIOTIC MICROORGANISMS IN DIFFERENT PARTS OF THE BODY—I.E., MICROBIOMES (Rebollar, Martínez-Ugalde, & Orta, 2020). The amphibian skin microbiome is essential in several aspects of the health of these animals and is considered a vital part of the amphibian immune system (Rebollar et al., 2020; Robinson, Bohannan, & Young, 2010). The microbiota present on the skin of amphibian hosts works in conjunction with the innate immune system to protect these animals from colonization, growth and infection by pathogens (Kau, Ahern, Griffin, Goodman, & Gordon, 2011; Thaiss, Zmora, Levy, & Elinav, 2016). For example, several species of bacteria have beneficial effects on the host, which can compete against invading pathogens and produce antioxidant and antifungal compounds that can inhibit infection by pathogens such as the fungus *Batrachochytrium dendrobatidis* (*Bd*) (Barnes & Lewis, 2021; Kueneman et al., 2016; Piovia-Scott et al., 2017), which is linked to amphibian population declines and/or extinction globally (Kueneman et al., 2019; Longo & Zamudio, 2017; Rebollar et al., 2020). Therefore, it is essential that we know the composition of bacteria in the amphibian skin microbiome so that we can also understand their interactions with the host.

The main taxa that inhabit the skin of these hosts belong to the phyla Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes and Proteobacteria (Belden et al., 2015; Longo, Savage, Hewson, & Zamudio, 2015; McKenzie, Bowers, Fierer, Knight, & Lauber, 2012; Walke et al., 2017). Regardless of similarity at the phylum level, in general, each amphibian species tends to have exclusive composition of microorganisms (Belden et al., 2015; McKenzie et al., 2012; Sabino-Pinto et al., 2017; Vences et al., 2015). These differences seem to be related to the physical and chemical properties of the host skin, where different amphibian species can produce distinct sets of antimicrobial peptides (Woodhams et al., 2014). On the other hand, amphibian species have differences in their ecology, inhabiting different types of environments and with different behaviors (Wells, 2007), which can also influence the formation of the microbiota, since hosts are exposed to different microbial reservoirs (Loudon et al., 2014). For example, recent studies observed that amphibian species with similar ecologies had a more similar microbiome when compared to species with different ecologies (Bletz, Perl, & Vences,

2017; Muletz Wolz, Yarwood, Campbell Grant, Fleischer, & Lips, 2018). In the same sense, other studies found that species with very different behaviors (e.g., aquatic, terrestrial or arboreal) differed in the composition and structure of the skin microbiome (Belden et al., 2015; Kueneman et al., 2014). Thus, it is plausible to consider that species with similar ecologies also have a similar microbiota.

Despite the importance of the skin microbiome for amphibians, there are still significant gaps in our understanding of the composition and diversity of the microbiome of Neotropical amphibians, especially in regions such as the extreme south of the Atlantic Forest (Ruthsatz et al., 2020). Knowing which microorganisms make up the basal microbiome of species is essential, as this information provides data needed to identify variations associated with environmental changes or disturbances, such as population decline (Jiménez & Sommer, 2017). In addition, this knowledge can support conservation strategies, such as the use of microbiological supplementation in captive breeding programs, where the controlled environment can harm the microbial diversity of individuals, or even in interventions to reintroduce endangered species into natural environments (Becker & Harris, 2010; Bletz et al., 2013; Kueneman et al., 2014, 2016).

Our study contributes to closing these gaps by providing a detailed description of the basal microbiome of anuran species from the southern Atlantic Forest, a region with high biodiversity but limited microbiome data. These findings not only advance our understanding of amphibian-microbiome interactions but also highlight potential pathways for integrating microbiome research into conservation strategies, particularly for endemic species threatened by environmental change. Therefore, our objectives are (1) to describe the composition of the skin core microbiome of 10 frog species from the southern Atlantic Forest and (2) compare the similarity of the core microbiome between these species.

## METHODOLOGY

STUDY SITE. — Sampling was carried out in September, October, November and December 2021, and January and February 2022 in a region in the south of the Atlantic Forest, in the municipality of São Francisco de Paula, Rio Grande do Sul, Brazil (29°27'–29°35'S, 50°08'–50°15'W). Specifically, we carried

out the study at the Center for Research and Conservation of Nature Pró-Mata (CPCN Pró-Mata), a preserved area with approximately 4,500 ha. This region is characterized by a mosaic of natural grasslands and subtemperate forests. The vegetation phytophysiognomy known as Mixed Ombrophilous Forest predominates in the sampled habitat, which is dominated by *Araucaria angustifolia* (Bertol.) Kuntze (i.e., Araucaria Forest). The region's climate is temperate, with an average annual temperature of 14.4°C and average annual rainfall of 2162 mm evenly distributed over the year (Maluf, 2000).

DATA COLLECTION. — We collected 28 skin samples from 10 frogs species (Table 1), where: three individuals of *Aplastodiscus perviridis* (Canebrake Treefrog), one *Boana faber* (Blacksmith Treefrog), one *Dendropsophus microps* (Nova Friburgo Treefrog), six *Dendropsophus minutus* (Lesser Treefrog), three *Leptodactylus luctator* (Wrestler Frog), three *Leptodactylus plaumanni* (Gray-striped Frog), one *Ololygon aromothyella* (Treefrog), two *Ololygon rizibilis* (Santo Andre Snouted Treefrog), two *Physalaemus carrizorum* (Frog) and six *Scinax granulatus* (Treefrog). We collected the skin mucus using rayon swabs (Medical Wire) following standard methods (Boyle, Boyle, Olsen, Morgan, & Hyatt, 2004). Swabs were stored at -20ºC in the field, then transported to the lab on ice and stored at -80ºC until extraction.

MOLECULAR METHODS AND DATA ANALYSIS. — DNA from all amphibian skin swabs was isolated using IBI extraction kits, following standard protocol. We followed the Earth Microbiome Project 16S Illumina Amplicon protocol to perform metabarcoding of bacterial communities from skin swabs (Caporaso et al., 2012; Kozich, Westcott, Baxter, Highlander, & Schloss, 2013), targeting the V4 region of the bacterial 16S rRNA gene using a dual-index approach with primers with barcode 515F and 806R. Extracted DNA was amplified by PCR in duplicate plates using the following recipe per sample: 12.2 µL UltraPure water, 4 µL Phire 5X reaction buffer (Thermo Scientific), 0.4 µL 2.5 mM dNTPs (Invitrogen), 0.4 µL of Phire Hot Start II DNA Polymerase (Thermo Scientific), 0.5 µL of each of the 10 µM barcoded forward and reverse primers (Integrated DNA Technologies), and 2 µL of DNA sample. We ran duplicate PCR plates

in SimpliAmp thermal cyclers (Thermo Scientific) according to the following protocol: 98°C for 3 min, 38 cycles of 98°C for 5 s, 50°C for 5 s, and 72°C for 15 s, then 72°C for 3 min before holding at 12°C. To monitor any potential contamination of the PCR reagents, we included a negative control (water without template DNA) in each plate. We combined duplicate plaques and amplicons visualized on a 1% agarose gel to confirm DNA amplification, which revealed highly variable amplification between samples. We repeated all samples with weak amplification with doubled DNA concentration (4 µL instead of 2 µL) and halved DNA concentration (1:2 dilution) to compensate for low DNA concentrations and PCR inhibition. After this process, we quantified the DNA concentration for each of the samples using the Qubit 2.0 fluorometer with a high-sensitivity dsDNA assay kit (Invitrogen) and pooled equimolar amounts of each sample (~10 nM) into a single amplicon library. We purified the library using a QIAquick gel extraction kit (Qiagen) and then measured the concentration of the amplicon library using the Qubit 2.0 fluorometer with a dsDNA Broad Range Assay Kit (Invitrogen). The concentrations of the purified library were 45.7 nM (11.1 ng/µL). The 16S library was sequenced using Illumina MiSeq at the Huck Institutes of Life Sciences Genomics Core Facility at Pennsylvania State University, State College, PA, USA. All bacterial sequences have been deposited in the NCBI Sequence Read Archive (BioProject PRJNA999620).

The limited number of individuals sampled for most species in this study prevented us from defining a core microbiome, which, in some studies, is characterized as the set of Operational Taxonomic Units (OTUs) present in at least 90% of the individuals of a host species (Hernandez-Agreda, Gates, & Ainsworth, 2017; Jani et al., 2021). Therefore, to describe the microbiome and compare composition and abundance across species, we focused on OTUs that represented at least 2% of the total microbiome abundance. OTUs with less than 2% abundance were grouped under the category "Other." To create taxonomic bar plots, we used the *ggplot* package in R software (R Core Team, 2013).

## RESULTS

The microbiome of the evaluated anuran community presented nine main OTUs, which had an abundance greater than 2%: Comamonadaceae (14.6%), *Stenotrophomonas rhizophila* (9.6%),

Caulobacteraceae (8.6%), *Pseudomonas* (7.9%), *Devosia* (7.2%), *Janthinobacterium* (5.3%), *Variovorax* (4.7%), Burkholderiaceae (3.9%) and *Bradyrhizobium* (3.5%) (Figure 1). In total, these 9 main taxa represented 65.3% of the total abundance of the community microbiome, while the remaining taxa (N = 1311) represented an abundance of 34.7%.

The Comamonadaceae family was the most abundant taxon in six of the 10 species evaluated: *Dendropsophus microps* (21.7%), *Leptodactylus luctator* (18.4%), *Aplastodiscus perviridis* (17.1%), *Dendropsophus minutus* (16.4%), *Scinax granulatus* (16.2%) and *Leptodactylus plaumanni* (13%) (Figure 2C, 2E, 2A, 2D, 2J and 2F, respectively). For the other species, this taxon was the second most abundant in the microbiome of *Ololygon rizibilis* (12.9%), the third in *Physalaemus carrizorum* (8.1%) and the fourth most abundant in *Boana faber* (4.8%) (Figure 2H, 2I and 2B, respectively). Regarding *Ololygon aromothyella*, this taxon was included in the "Other" group, since its abundance was less than 2%.

The genus *Pseudomonas* was the taxon with the highest abundance in relation to the others, but only in the species *O. aromothyella* (31.5%) and *O. rizibilis* (29.5%) (Figure 2G and 2H). Meanwhile, the other species presented an abundance of less than 8.2% of this genus in their microbiome, which was not present among the most abundant taxa in the *B. faber* microbiome.

The species *Stenotrophomonas rhizophila* was abundant in the microbiome of most species, with emphasis on *O. aromothyella* (26.5%), where this taxon was the second most abundant (Figure 2G), as well as for *A. perviridis* (10.5%) and *L. luctator* (8.8%) (Figure 2A and 2E). This taxon was the third most abundant in the microbiome of *D. microps* (12.5%), *O. rizibilis* (11.2%), *D. minutus* (10.5%) and *L. plaumanni* (8.2%) (Figure 2C, 2H, 2D and 2F, respectively), and the fourth most abundant in *S. granulatus* (7.8%) (Figure 2J). Like the genus *Pseudomonas*, the species *S. rhizophila* was not present among the most abundant taxa in the microbiome of *B. faber*.

The Caulobacteraceae family was the most abundant taxon in the microbiome of *B. faber* (7.1%) (Figure 2B). However, this taxon presented higher abundance values in the microbiome of the species *D. minutus* (15.9%) and *S. granulatus* (10.6%), being the second most abundant taxon in these anurans (Figure 2D and 2J), and was the fourth most abundant in *O. rizibilis* (8.9%) and L. *plaumanni* (7.4%)

(Figure 2H and 2F). Regarding the other species, this taxon represented less than 4% of the total abundance of the microbiome and was considered as "Other" in *L. luctator*.

The genus *Devosia* was the third most abundant taxon in the microbiome of *A. perviridis* (10.2%) and *S. granulatus* (9.4%) (Figure 2A and 2J), the fourth most abundant in *D. microps* (11.7%) and *D. minutus* (8%) (Figure 2C and 2D), the fifth most abundant in *L. luctator* (6%) (Figure 2E) and the sixth most abundant in *L. plaumanni* (Figure 2F). The hosts *B. faber*, *O. riziblis* and *P. carrizorum* had an abundance of less than 3.8% of this genus in their microbiome (Figure 2B, 2H and 2I), while this taxon was included as "Other" in *O. aromothyella*.

The Burkholderiaceae family was the second most abundant taxon in the microbiome of *D. microps* (14%) and *L. plaumanni* (9.4%) (Figure 2C and 2F), the third most abundant in *B. faber* (5.8%) (Figure 2B) and the fifth most abundant in *S. granulatus* (7.2%) (Figure 2J). Meanwhile, this taxon had low abundance values in the microbiome of *A. perviridis*, *D. minutus*, *L. luctator* and *O. aromothyella* (Figure 2A, 2D, 2E and 2G), and was included as "Other" in *O. rizibilis* and *P. carrizorum*.

The genera *Janthinobacterium* and *Variovorax* had similar abundances in the microbiome of most species, where they were considered as "Other" only in *B. faber*, in addition to *O. aromothyella* in relation to *Variovorax* (Figure 2). The genus *Sanguibacter* was the third most abundant in the microbiome of *O. aromothyella* (15.4%) (Figure 2G), *Pedobacter* was the most abundant taxon in *P. carrizorum* (15.3%) (Figure 2I), and the order Burkholderiales was the second most abundant taxon in *B. faber* (Figure 2B), while all three of these taxa were considered as "Other" in the other species.

## DISCUSSION

Our results provide a foundational description of the basal skin microbiome of 10 species of amphibians from the Atlantic Forest, two of which are endemic to this biome, *Physalaemus carrizorum* and *Leptodactylus plaumanni*. Understanding the basal microbiome of a species is essential for detecting changes driven by environmental disturbances, such as climate change, habitat loss, and the introduction of pathogens (Belden & Harris, 2007; Colston & Jackson, 2016; Jiménez & Sommer, 2017; Zaneveld et

al., 2016). The importance of this baseline has been extensively discussed in vertebrate literature, including in humans, where health and disease are closely linked to microbiome stability (Bäckhed et al., 2012; Turnbaugh et al., 2007; Zhao, 2013). For example, in medicine, data on healthy gut microbiomes have been used to restore microbial communities in individuals suffering from dysbiosis, highlighting microbiome-based interventions as a means of restoring health after disease or imbalance (Strati et al., 2017; Suez et al., 2014; Watanabe, Fukiya, & Yokota, 2017). In this context, our data serves as a valuable baseline for monitoring and mitigating the effects of global change scenarios, helping to preserve the functional integrity of microbiomes that are essential for amphibian health and survival. Thus, in addition to advancing the understanding of microbial ecology, these findings hold relevance for conservation strategies aimed at amphibians.

***Amphibian community core microbiome diversity***

Our results show that the skin microbiome of this anuran community is predominantly composed of nine OTUs, each with abundances greater than 2%, cumulatively accounting for approximately 70% of the total microbiome abundance. These taxa include: Comamonadaceae, *Stenotrophomonas rhizophila*, Caulobacteraceae, *Pseudomonas*, *Devosia*, *Janthinobacterium*, *Variovorax*, Burkholderiaceae, and *Bradyrhizobium*. Notably, most of these taxa, excluding Caulobacteraceae and the genera *Devosia*, *Sanguibacter*, and *Variovorax*, are associated with the production of antioxidant and antifungal compounds that inhibit pathogens (Barnes, Carter, & Lewis, 2020; Barnes & Lewis, 2021; Bletz et al., 2017; Walke et al., 2017; Woodhams et al., 2015; Wuerthner, Hua, & Hernández-Gómez, 2022).

Among these taxa, three—Comamonadaceae, *Pseudomonas*, and *S. rhizophila*—showed the highest relative abundances, each surpassing 15% and collectively representing approximately 50% of the total microbiome. These taxa are commonly found on the skin of various amphibian species (Abarca et al., 2018; Bates et al., 2018; Chen et al., 2022; Kouete, Bletz, LaBumbard, Woodhams, & Blackburn, 2023; Medina et al., 2019). Despite their antifungal properties (Barnes et al., 2020; Campos, Lucid, Ehlers, & Walke, 2024; Muletz-Wolz et al., 2017; Woodhams et al., 2015; Wuerthner et al., 2022), the abundance of Comamonadaceae has been linked to increased *Bd* infection intensity (Campos et al., 2024; Goodwin,

Hutchinson, & Gompert, 2022). Conversely, *Pseudomonas* demonstrates antimicrobial activity against *Aeromonas salmonicida* (Magalhães, Garda, Marques, & Brandão, 2016), and *S. rhizophila* exhibits traits beneficial to amphibians, such as heavy-metal resistance, rapid growth, and the ability to thrive in low-nutrient environments (Ryan et al., 2009).

Although Caulobacteraceae are not known for antifungal metabolite production, they play essential roles in nutrient cycling in aquatic ecosystems by breaking down complex organic compounds (Poindexter, 1981; Schmidt, Cordovez, De Boer, Raaijmakers, & Garbeva, 2015), indirectly benefiting amphibians. Their abundance has also been linked to immune processes in fish (Fuess et al., 2021). Given their prominence in this community and the limited understanding of their role in amphibians, further studies are warranted to explore their potential contributions to amphibian health.

Burkholderiaceae, commonly found as symbionts on amphibian skin (Medina, Greenspan, Carvalho, Becker, & Toledo, 2021; Ramsey, Mercurio, Holland, Harris, & Minbiole, 2015), occupy diverse ecological niches and thrive in varied environments (Depoorter et al., 2016; Willems, De Ley, Gillis, & Kersters, 1991). Unlike Comamonadaceae, higher abundances of Burkholderiaceae have been linked to reduced fungal presence on amphibian skin (Kueneman et al., 2016). However, other studies suggest that their increased abundance in *Bd*-infected amphibians may reflect opportunistic colonization, as these bacteria proliferate when other taxa decline (Campos et al., 2024; Ellison, Knapp, Sparagon, Swei, & Vredenburg, 2019; Jani & Briggs, 2014).

***Comparison of the skin microbiome between species***

Although environmental factors influence the skin microbiome of amphibians (Walke et al., 2021), their microbiota is generally species-specific. Different amphibian species harbor distinct bacterial communities, even closely related species or those living in similar environments (McKenzie et al., 2012; Medina et al., 2019; Walke et al., 2014). For instance, while *Dendropsophus microps* and *Dendropsophus minutus* are congeneric species inhabiting similar environments (Kwet, Lingnau, & Di Bernardo, 2010), notable differences in their skin microbiome composition were observed. *Dendropsophus minutus* exhibited high abundances of Caulobacteraceae and *Bradyrhizobium*, which were underrepresented in *D.*

*microps*. Conversely, *D. microps* had Burkholderiaceae as its second most abundant taxon, a group with low representation in *D. minutus*. Similarly, within the genus *Ololygon*, *O. rizibilis* showed high abundances of Comamonadaceae and Caulobacteraceae, which were not abundant in *O. aromothyella*. In turn, *O. aromothyella* presented *Sanguibacter* as its third most abundant taxon. These findings underscore the species-specific specialization of amphibian skin microbiomes, shaped by environmental pressures and the complex interactions between amphibians and their microbial communities.

The identification of species-specific bacterial taxa opens new avenues for conservation strategies, such as harnessing the microbiome to enhance resistance to pathogens and improve the health of at-risk species. Our study recorded 12 taxa exclusive to specific hosts. Notable examples include *Pedobacter*, the most abundant taxon in *P. carrizorum*; *Sanguibacter*, the third most abundant in *O. aromothyella*; and Burkholderiales, the second most abundant taxon in *B. faber*. The high abundance of exclusive taxa like *Pedobacter* and *Sanguibacter* reinforces the species-specificity of microbiomes (Jiménez & Sommer, 2017). This exclusivity may relate to the chemical and physical properties of amphibian skin, as some species produce unique antimicrobial peptides that either inhibit or promote bacterial colonization (Jiménez, Alvarado, Sandoval, & Sommer, 2020; Woodhams et al., 2014). For example, peptides produced by *P. carrizorum* and *O. aromothyella* may create favorable conditions for the colonization of symbiotic bacteria, such as *Pedobacter* and *Sanguibacter* (Demori et al., 2019; Flechas et al., 2019; Greenspan et al., 2022).

Environmental factors and natural history may also explain some microbiome differences between species. For instance, the genus *Brevundimonas*, found abundantly only in *B. faber*, may be associated with biofilms on tree bark—a habitat commonly used by this tree frog (Dreyling, Schmitt, & Dal Grande, 2022). Similarly, the exclusivity of *Pedobacter* in *P. carrizorum* could be tied to the marshes and mudflats it inhabits, environments where this genus is commonly found (Margesin & Shivaji, 2015). Additionally, both *P. carrizorum* and *O. rizibilis* produce foam nests for egg-laying. These nests, built with proteins exhibiting antimicrobial properties, might influence microbiome composition by deterring bacterial colonization (Fleming, Mackenzie, Cooper, & Kennedy, 2009).

Differences in microbial abundance across species may also reflect the microenvironments they occupy. Amphibian species living in similar environments are likely to access similar bacterial pools (Bastos, Haddad, & Pombal Jr, 2010; Kwet et al., 2010). For example, while *Pseudomonas* was present in the core microbiomes of all species, it was notably more abundant in both *Ololygon* species. This pattern may result from peptides produced by these frogs that favor colonization by *Pseudomonas*. A similar trend was observed in two *Leptodactylus* species, which shared similar microbiomes except for a *Caulobacteraceae* taxon abundant only in *L. plaumanni*.

Comparing the skin microbiome of individual species to the broader amphibian community revealed striking differences, particularly between *B. faber* and *O. aromothyella*. Despite both species being hylids with arboreal habits outside the breeding season (Kwet et al., 2010), *B. faber* had nine dominant taxa (>2% abundance) accounting for only 38% of its total microbiome—the lowest percentage among the species studied. In contrast, *O. aromothyella* had seven dominant taxa representing 88% of its microbiome, the highest percentage among the species. This indicates that *O. aromothyella* has a microbiome with lower evenness, while *B. faber* shows higher evenness. Typically, greater microbiome evenness is associated with functional diversity, stability, and resilience (Greenspan et al., 2022; Jiménez et al., 2020). However, in *O. aromothyella*, low evenness is likely driven by the dominance of *Pseudomonas* and *S. rhizophila*, which produce antimicrobial and antifungal compounds that enhance immunity and may inhibit pathogens like *Bd* (Barnes et al., 2020; Campos et al., 2024; Muletz-Wolz et al., 2017; Woodhams et al., 2015; Wuerthner et al., 2022). Despite its lower evenness, the dominance of these beneficial bacteria likely provides *O. aromothyella* with a robust defense against infection.

## CONCLUSIONS

Our study provides unprecedented insights into the skin microbiome composition of an amphibian community from the extreme southern portion of the Atlantic Forest, highlighting species-specific differences that underscore the complexity and uniqueness of amphibian-microbiome interactions. This

foundational knowledge is essential for future research aiming to evaluate the impacts of environmental changes—such as habitat destruction, climate change, and pathogen introduction—on amphibian health.

We identified key bacterial taxa consistently abundant across species, many of which are known for their antifungal properties and may provide protection against pathogens like *Bd*. Approximately 50% of the skin microbiome abundance in the community is composed of bacterial taxa that produce antifungal compounds, suggesting a potential benefit to amphibian health. However, the species-specific microbiomes also harbored unique taxa, likely influenced by the host's natural history, microenvironments, and production of antimicrobial peptides, reinforcing the critical role of host biology in shaping microbial communities.

The identification of species-exclusive taxa presents new opportunities for conservation strategies. These microbial signatures, particularly those linked to pathogen resistance, could play a pivotal role in enhancing amphibian resilience to environmental stressors and disease. Our findings enrich the growing body of knowledge on amphibian microbial ecology and provide valuable insights for the development of microbiome-based conservation efforts, aiming to safeguard the functional integrity and survival of these species in dynamic and changing ecosystems.

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**Author Contributions**

All authors contributed to the study conception and design. Material preparation and data collection were performed by Laura K. Schuck, Carolina de A. Caberlon, Priscila C. Barth and Camila F. Moser. Analysis was performed by Camila F. Moser, Laura K. Schuck and Wesley J. Neely. The first draft of the manuscript was written by Camila F. Moser and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**Conflict of Interest**

The authors declare that they have no competing financial or non-financial interests directly or indirectly related to the work submitted for publication.

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**Table 1:** *Values of diversity metrics for each host species, where N = number of individuals sampled, $\bar{x}$ = mean values, ± = standard deviation.*

| **Host species** | **N** | **OTUs richness** $\bar{x}$ **(±)** | **Shannon** $\bar{x}$ **(±)** | **Faith's PD** $\bar{x}$ **(±)** | **Evenness** $\bar{x}$ **(±)** |
|---|---|---|---|---|---|
| *A. perviridis* | 3 | 356 (167.3) | 5.6 (1.4) | 26.9 (10) | 0.7 (0.12) |
| *B. faber* | 1 | 383 | 7.3 | 32.8 | 0.8 |
| *D. microps* | 1 | 231 | 4.5 | 17 | 0.6 |
| *D. minutus* | 6 | 98.7 (61.6) | 3.8 (1.1) | 10.8 (5.7) | 0.6 (0.09) |
| *L. luctator* | 3 | 343.3 (177.3) | 6 (1.5) | 24.9 (11.9) | 0.7 (0.11) |
| *L. plaumanni* | 3 | 247.3 (176.9) | 5.6 (1.6) | 20.5 (8.2) | 0.7 (0.1) |
| *O. aromothyella* | 1 | 97 | 3.2 | 12.5 | 0.5 |
| *O. rizibilis* | 2 | 100.5 (7.5) | 3.8 (0.1) | 11.5 (0.4) | 0.6 (0.03) |
| *P. carrizorum* | 2 | 416 (197) | 6.4 (1.5) | 33.5 (11.9) | 0.7 (0.12) |
| *S. granulatus* | 6 | 138 (57.6) | 4.6 (0.7) | 14.4 (4) | 0.6 (0.05) |

**FIGURES**

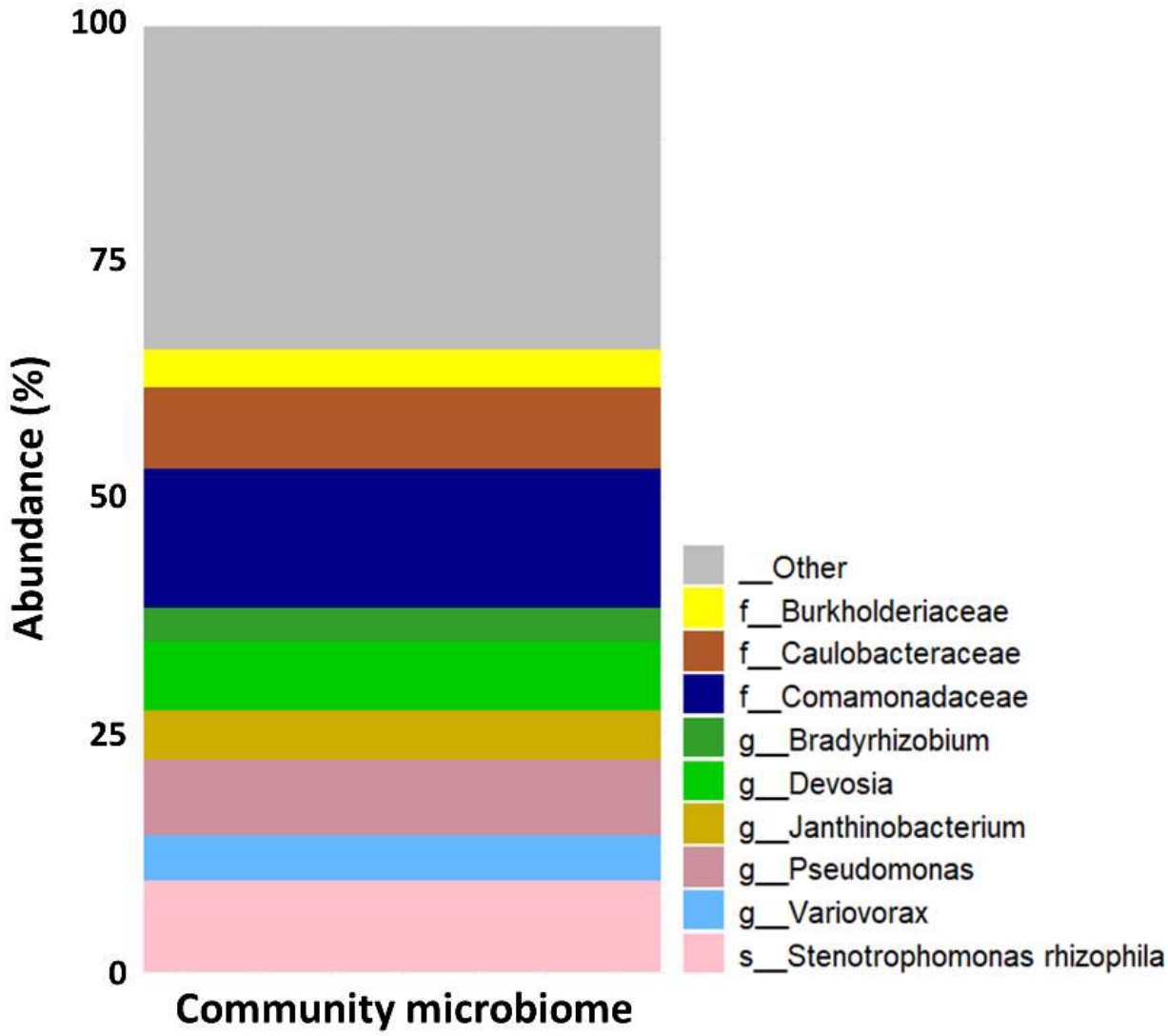


**FIGURE 1.** Composition of the skin microbiome of the anuran community evaluated, where we considered only taxa with abundance greater than 2%, while taxa with lower abundance were grouped as "__Other". "f__" = family, "g__" = genus and "s__" = species.

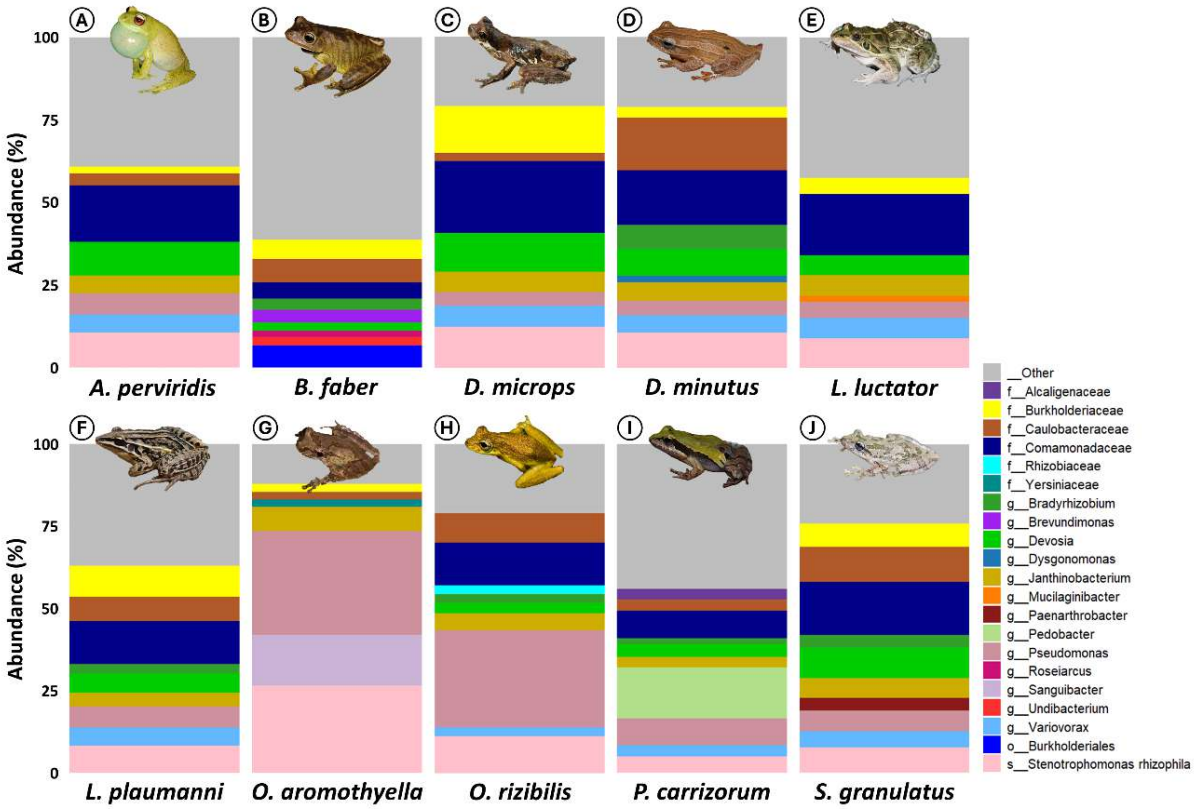


**FIGURE 2.** Skin microbiome of the species *A. perviridis* (N = 3), *B. faber* (N = 1), *D. microps* (N = 1), *D. minutus* (N = 6), *L. luctator* (N = 3), *L. plaumanni* (N = 3), *O. aromothyella* (N = 1), *O. rizibilis* (N = 2), *P. carrizorum* (N = 2) and *S. granulatus* (N = 6), where "f__" = family, "g__" = genus, and "o__" = order. Copyright on species images: *A. perviridis* by Germano Woehl Junior, *B. faber* by Marco Vicariotto, *D. microps* by Renato Martins, *D. minutus*, *L. luctator*, *P. carrizorum* and *S. granulatus* by Daniel Loebmann, *L. plaumanni* by Fernando Farias, *O. aromothyella* by Alyssa Freitas, *O. rizibilis* by Daniel Perrella.

# Capítulo 3

## Seasonal dynamics of skin microbiomes in two Neotropical tree frogs: Insights into host-microbe interactions

O capítulo III desta Tese foi elaborado e formatado conforme as normas da publicação científica *Environmental Research Communications*, as quais se encontram em anexo (Anexo 3).

# Seasonal dynamics of skin microbiomes in two Neotropical tree frogs: Insights into host-microbe interactions

Camila F. Moser[1], Laura K. Schuck[2], Carolina de A. Caberlon[3], Wesley J. Neely[4]*, Guilherme Becker[2,5] and Pedro Peloso[6]

[1] Instituto de Ciências Biológicas, Universidade Federal do Pará, Belém, Pará, BR

[2] Department of Biology, The Pennsylvania State University, University Park, Pennsylvania, USA.

[3] Laboratório de Ecologia de Vertebrados Terrestres, Universidade do Vale do Rio dos Sinos, São Leopoldo, Rio Grande do Sul, BR.

[4] Department of Biology, Texas State University, San Marcos, USA.

[5] One Health Microbiome Center, Center for Infectious Disease Dynamics, Ecology Institute, Huch Institutes of the Life Sciences, The Pennsylvania State University, University Park, Pennsylvania, USA.

[6] Department of Biological Sciences, California State Polytechnic University, Arcata, California, USA.

E-mail: wesleyjneely@gmail.com

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**Abstract**

Seasonality is one of the many factors that shape amphibian skin microbiomes, driven by temperature's dual impact on microorganisms and their hosts. This study explored seasonal microbiome dynamics in two Neotropical tree frogs, *Boana leptolineata* and *Scinax squalirostris*, across winter, spring, and summer. We tested if seasonality (1) affects alpha-diversity metrics in the species' skin microbiome, (2) affects the dispersion of their microbiomes, and (3) compare the microbiome composition across seasons. *Scinax squalirostris* displayed higher microbial diversity and evenness during winter, suggesting cold-adaptive strategies that bolster pathogen resistance, while *B. leptolineata* exhibited greater diversity in spring, possibly due to milder conditions favorable to bacterial growth. Although microbiome dispersion showed no significant variation across seasons, differences emerged between species in spring. Boana leptolineata exhibited lower dispersion compared to *S. squalirostris,* indicating potentially distinct behavioral and microhabitat factors. Seasonal shifts revealed unique taxa that may be adapted to fluctuating conditions, highlighting their importance for host resilience. These findings illustrate how temperature and environmental changes shape amphibian skin microbiomes, influencing stability and species adaptability, with broader implications for ecological resilience and pathogen defense.

Keywords: *Boana leptolineata*, dysbiosis, microbiome dispersion, *Scinax squalirostris*, skin-associated bacteria

## 1. Introduction

The skin of amphibians, like other animals, harbors a diverse community of symbiotic microorganisms that can form a species-specific skin microbiome (Rebollar et al. 2020). This microbiome plays a pivotal role in amphibian health, contributing to critical functions such as respiration, development, immunity and stress response (Rebollar et al., 2020; Robinson et al., 2010). Among its most remarkable features is the production of antifungal metabolites that protect the host from invading pathogens, including *Batrachochytrium dendrobatidis* (*Bd*), the deadly fungus responsible for chytridiomycosis (Grice and Segre, 2011; Robinson et al., 2010). This disease has driven massive population declines and even extinctions of amphibians worldwide (Lips, 2016; Scheele et al., 2019; Woodhams et al., 2015).

In general, amphibian skin microbiota tends to be host-specific, with distinctive bacterial assemblages observed even among coexisting species, as the skin microenvironment selects particular taxa (McKenzie et al., 2012). However, bacterial communities on amphibian skin are influenced by a complex interplay of factors, including host phylogeny and environmental conditions (Bird et al., 2019; Ruthsatz et al., 2020; Walke et al., 2021). Although these aspects have been widely studied, their relative contributions to microbiota development remain partly unresolved (Bletz et al., 2017). Among elements that affect the composition and diversity metrics of skin microbiome are as habitat type (Rebollar et al., 2016), developmental stage (Goodwin et al., 2022), sex (Douglas et al., 2021), body condition (Hernández-Gómez et al., 2018), and seasonal changes (Douglas et al., 2021). Among these, seasonality emerges as a particularly significant factor in shaping the microbiome of ectothermic organisms like amphibians, whose physiological and behavioral activities are closely tied to environmental temperatures (Wells, 2007). Shifts in temperature can substantially alter their skin microbiota composition and diversity by affecting environmental bacterial communities (Muletz-Wolz et al., 2019; Zhou et al., 2016). Although previous studies have documented seasonal variations in amphibian skin microbiome composition (Douglas et al., 2021; Longo et al., 2015; Walke et al., 2021), the impact of these fluctuations on amphibian health and disease resistance is not fully understood. Seasonal variations in microbiome diversity can affect pathogen defense, as observed in several studies (Douglas et al., 2021; Longo et al., 2015; Muletz-Wolz et al., 2017; Tong et al., 2020; Walke et al., 2021), underscoring the need to understand periods of greater susceptibility to infections.

Obtaining data that generates insights into how seasonality influences the amphibian skin microbiome can reveal conditions under which amphibians are more prone to pathogen infections (Raffel et al., 2006). Nonetheless, the extent of seasonality's impact on microbiome diversity remains uncertain (Douglas et al., 2021). In this study, we used two species of Neotropical tree frogs, *Boana leptolineata* and *Scinax squalirostris* (Hylidae), to further understand how seasonality influences the

dynamics of the anuran skin microbiome. Our goals are to (1) test whether there is an influence of seasonality on the alpha-diversity metrics of the skin microbiome of both species; (2) test whether seasonality has an influence on the beta-diversity dispersion of the species' microbiome; (3) compare the composition of the skin microbiome between the seasons for each species. We hypothesize that alpha diversity metrics will decrease during colder seasons due to limited resource availability (e.g., bacterial reservoirs) and reduced. Additionally, we predict increased microbiome dispersion during these colder periods, as reduced mobility and hiding behaviors may limit bacterial colonization, resulting in greater variability among individuals. By shedding light on the interplay between seasonality and microbiome dynamics, this research aims to contribute to a deeper understanding of how fluctuations in environmental temperature influence host-microorganism interactions.

## 2. Methodology

### *2.1. Study site*

Our samplings were carried out in the months of September, October, November and December 2021, and January and February 2022. The samplings carried out during October, November and December 2021 were considered as spring, the samplings in January and February 2022 as summer, and sampling carried out in September 2021 as winter. Sampling took place in a region in the south of the Atlantic Forest, in the municipality of São Francisco de Paula, Rio Grande do Sul, Brazil (29°27'–29°35'S, 50°08'–50°15 'W). Specifically, we carried out the study at the *Centro de Pesquisa e Conservação da Natureza Pró-Mata*, a preserved area with approximately 4,500 ha. This region is characterized by a mosaic of natural grasslands and subtemperate forests. Despite the presence of forests in the region, the samplings were concentrated in field environments, with tall shrub vegetation and close to aquatic environments, such as lagoons, marshes and mudflats. The region's climate is classified as super-humid temperate, with an average annual temperature of 14.4°C, where the temperature during the summer reaches approximately 35ºC, with extreme winters, where the temperature often drops below 0ºC, while spring and autumn have mild temperatures (between 15ºC and 20°C), and average annual rainfall of 2162 mm evenly distributed over the year (Maluf, 2000).

### *2.2. Data collection*

We collected skin mucus samples from amphibians using rayon swabs (Medical Wire) following standard methods (Boyle et al., 2004). After sampling, swabs were stored at -20ºC in the field, then transported to the laboratory on ice and stored at -80ºC until extraction. We collected a total of 97 skin mucus samples of the species in the spring and winter of 2021 and in the summer of 2022, as follows: 51 samples during the summer (23 of *Boana leptolineata* and 28 of *Scinax squalirostris*), 28

samples in the spring (11 of *B. leptolineata* and of 17 *S. squalirostris*), and 18 samples in the winter (two of *B. leptolineata* and 16 of *S. squalirostris*).

*2.3. Molecular methods*

DNA from all swabs was extracted using IBI extraction kits, following standard protocol. We followed the Earth Microbiome Project 16S Illumina Amplicon protocol to perform metabarcoding of bacterial communities from skin swabs (Caporaso et al., 2012; Kozich et al., 2013), targeting the V4 region of the bacterial 16S rRNA gene using a dual-index approach with primers with barcode 515F and 806R. Extracted DNA was amplified by PCR in duplicate plates using the following recipe per sample: 12.2 µL UltraPure water, 4 µL Phire 5X reaction buffer (Thermo Scientific), 0.4 µL 2.5 mM dNTPs (Invitrogen ), 0.4 µL of Phire Hot Start II DNA Polymerase (Thermo Scientific), 0.5 µL of each of the 10 µM barcoded forward and reverse primers (Integrated DNA Technologies), and 2 µL of DNA sample. We ran duplicate PCR plates in SimpliAmp thermal cyclers (Thermo Scientific) according to the following protocol: 98°C for 3 min, 38 cycles of 98°C for 5 s, 50°C for 5 s, and 72°C for 15 s, then 72°C for 3 min before holding at 12°C. To monitor any potential contamination of the PCR reagents, we included a negative control (water without template DNA) in each plate. We combined duplicate plaques and amplicons visualized on a 1% agarose gel to confirm DNA amplification, which revealed highly variable amplification between samples. We repeated all samples with weak amplification with doubled DNA concentration (4 µL instead of 2 µL) and halved DNA concentration (1:2 dilution) to compensate for low DNA concentrations and PCR inhibition. After this process, we quantified the DNA concentration for each of the samples using the Qubit 2.0 fluorometer with a high-sensitivity dsDNA assay kit (Invitrogen) and pooled equimolar amounts of each sample (~10 nM) into a single amplicon library. We purified the library using a QIAquick gel extraction kit (Qiagen) and then measured the concentration of the amplicon library using the Qubit 2.0 fluorometer with a dsDNA Broad Range Assay Kit (Invitrogen). The concentrations of the purified library were 45.7 nM (11.1 ng/µL). The 16S library was sequenced using Illumina MiSeq at the Huck Institutes of Life Sciences Genomics Core Facility at Pennsylvania State University, State College, PA, USA. All bacterial sequences have been deposited in the NCBI Sequence Read Archive (BioProject PRJNA999620).

*2.4. Data analysis*

To evaluate the influence of seasonality (i.e., winter, spring and summer) on the alpha-diversity metrics of the skin microbiome of the anuran community: Operational Taxonomic Units (OTUs) richness, Shannon diversity, Faith's phylogenetic diversity and Evenness), we ran ANOVA variance analyzes for normal variables (OTUs richness), followed by the Tukey HSD post hoc test, and Kruskal-Wallis test for non-normal variables (Shannon diversity, Faith's phylogenetic diversity and

Evenness), followed by Dunn's post hoc test. For these analyses, due to the low number of *B. leptolineata* individuals sampled during winter (N = 2), we excluded these samples and evaluated microbiome diversity metrics only during spring and summer for this species. We considered *p* values lower than 0.05 as significant.

We calculated microbiome dispersion based on Bray–Curtis dissimilarity matrices grouped by host species and by seasons using the betadisper function (Table S1). Higher values indicate samples that are further from the average community composition. We used the first two axes of a PCoA based on the Bray-Curtis dissimilarity matrix to construct scatterplots for each species in each season (Table S2). To test whether there is a difference in microbiome dispersion between seasons for each species and between species, we used the ANOVA test, followed by the Tukey HSD post hoc test for the species *S. squalirostris* (Table S1). To test whether microbiome dispersion between species is different in spring and summer, we also used the ANOVA test.

To compare the microbiome composition and abundance of each species between seasons, we only considered OTUs from the core microbiome. In this study, we considered as part of the core microbiome only those OTUs that had a 90% or more frequency of occurrence in the microbiome of each species and each season. Due to the low number of *B. leptolineata* individuals sampled during winter (N = 2), in this case we considered as core microbiome the OTUs with percentage abundance greater than 1%. To create the taxonomic bar plots, we used the ggplot package. All analyses and images were generated in the R environment, version 4.3.3 (R Core Team, 2023).

## 3. Results

### *3.1. Influence of seasonality on diversity metrics*

The ANOVA and Kruskal-Wallis tests indicated that seasonality had influence on all alpha diversity metrics of the *B. leptolineata* skin microbiome, where both the richness of OTUs (spring: $\overline{x}$ = 270.9, ± 150.1; summer: $\overline{x}$ = 151.5 ± 76) (F = 5.665, df = 12.39, *p* = 0.03), the Shannon diversity ($\overline{x}$ = 5.5 ± 0.95; $\overline{x}$ = 4.5 ± 0.76, respectively) (H = 7.522, *p* = 0.006), phylogenetic diversity ($\overline{x}$ = 24.6, ± 8.6; $\overline{x}$ = 14.7, ± 6.1) (H = 10.38, *p* = 0.001), and evenness ($\overline{x}$ = 0.69 ± 0.07; $\overline{x}$ = 0.64 ± 0.06) (H = 4.174, *p* = 0.04) had higher values during spring than during summer (Figure 1). However, for *S. squalirostris*, our data suggests the influence of seasonality only on Shannon diversity (H = 6.962, *p* = 0.03) and in the evenness (H = 6.65, *p* = 0.04) (Figure 2B and 2D). The Shannon diversity of the skin microbiome of S. squlirostris was higher during winter ($\overline{x}$ = 4.7, ± 1.1) than during summer ($\overline{x}$ = 4.2 ± 0.9) (*p* = 0.008), while evenness was also higher during winter ($\overline{x}$ = 0.65 ± 0.09) than during summer ($\overline{x}$ = 0.61, ± 0.07) (p = 0.02) and spring ($\overline{x}$ = 0.59 ± = 0.07) (*p* = 0.02) (Figure 2B and 2D),

but there was no difference in OTUs richness (F = 0.681, df = 33.59, $p$ = 0.5) or phylogenetic diversity (H = 2.248, $p$ = 0.3) (Figure 2A and 2C).

*3.2. Influence of seasonality on microbiome dispersion*

Our results indicated that the dispersion of the *B. leptolineata* skin microbiome during spring and summer was similar (β = 0.29 and 0.30, respectively), and the ANOVA test indicated that there is no difference in the dispersion of the microbiome between seasons in this species ($p$ = 0.9) (Figure 3A). Likewise, there was no difference in the dispersion of the *S. squalirostris* microbiome during spring (β = 0.40), summer (β = 0.31) and winter (β = 0.41) ($p$ = 0.08), result that was also confirmed by the Tukey test (Figure 3B). However, when we compared the dispersion of the two species in each season, we observed that *S. squalirostris* had higher microbiome dispersion than *B. leptolineata* during spring ($p$ = 0.04) (Figure 4A), but there was no difference in microbiome dispersion between the two species during the summer ($p$ = 0.8) (Figure 4B).

*3.3. Community core microbiome*

We identified that the core microbiome of *B. leptolineata* and *S. squalirostris* was composed of, respectively, 29 and 14 OTUs, which represented about 75% and 64% of the relative abundance of the skin microbiome of these species (Figure 5A and 5B). In general, the composition of the core microbiome between species was similar, where both *B. leptolineata* and *S. squalirostris* had similar abundances of the seven main OTUs: Comamonadaceae (17% and 14%, respectively), *Pseudomonas* (7.4% and 11.1%), *Stenotrophomonas rhizophila* (9.8% and 10.4%), *Devosia* (9.4% and 7%), *Janthinobacterium* (5.8% and 6.1%), Caulobacteraceae (5.4% and 5%) and *Variovorax* (5.4% and 4.3%). However, *B. leptolineata* presented five unique OTUs: Yersiniaceae (1.1%), *Staphylococcus* (1%), Microbacteriaceae (0.9), *Acinetobacter* (0.3%) and *Roseomonas* (0.2%), which represented around 3.5% of the total abundance of the microbiome of this species (Figure 5A), while *S. squalirostris* did not present exclusive taxa in its core microbiome.

*3.4. Influence of seasonality on the core microbiome*

For *B. leptolineata*, the microbiome during spring had a higher richness of OTUs (N = 22) than during summer (N = 18) and winter (N = 12) (Figure 6A, B and C). Although richness was lower during winter, the core microbiome accounted for approximately 65% of the total microbiome abundance, similar to the values observed for spring (73%) and summer (70%). Notably, *B. leptolineata* exhibited three unique taxa in spring (Beijerinckiaceae, *Brevundimonas* and *Streptococcus*), while there were no unique taxa during summer and winter. The taxon Comamonadaceae was abundant across all three seasons for *B. leptolineata*, but its abundance was higher during summer (17%) compared to winter (12%), while the genus *Pseudomonas* and the family

Caulobacteraceae were more abundant during winter (12% and 12.1%, respectively) compared to summer (7.5% and 5.4%). The genera *Janthinobacterium* and *Variovorax* were more abundant during the spring (5% and 5%, respectively) and summer (6% and 5.4%) than during the winter (2.8% and 2.2%).

For *S. squalirostris*, the core microbiome exhibited similar richness during winter (N = 16) and spring (N = 15), representing a total abundance of about 58% and 52%, respectively, while richness was lower during summer (N = 12) (Figure 6D, E and F). Despite this lower richness, the 12 taxa present in the summer core microbiome accounted for 70% of the total abundance, a higher proportion than observed in other seasons with greater richness. The composition of the primary taxa in the skin microbiome of this species was consistent, including Caulobacteraceae, Comamonadaceae, *Devosia*, *Janthinobacterium*, *Pseudomonas* and *Stenotrophomonas rhizophila*. However, the taxa Comamonadaceae, *Pseudomonas*, *S. rhizophila*, and *Janthinobacterium* were more abundant during summer (16.8%, 14.1%, 14.8%, and 8.2%, respectively) compared to spring (10.7%, 7.1%, 6.4%, and 4.1%). We also recorded four unique taxa in the core microbiome of *S. squalirostris* during winter (*Brevundimonas*, *Massilia*, *Methylobacterium*, and *Staphylococcus*), three during spring (*Variovorax*, Yersiniaceae, and Microbacteriaceae), and only one during summer (*Herbaspirillum*). Among these exclusive taxa, all had an abundance of less than 1%, except for *Variovorax* (4%).

When comparing the core microbiome between the two species, we found that *B. leptolineata* had higher OTU richness than *S. squalirostris* during both spring (N = 22 and 15, respectively) and summer (N = 18 and 12). Conversely, *S. squalirostris* had higher richness during winter compared to *B. leptolineata* (N = 16 and 12). Despite these differences, the core microbiomes of both species during spring and winter shared similar composition and abundance of the five main taxa: Caulobacteraceae, Comamonadaceae, *Devosia*, *Pseudomonas*, and *S. rhizophila*. Notably, the Comamonadaceae family was the most abundant taxon in both species across all evaluated seasons. However, during the summer, some notable differences emerged: while *S. squalirostris* exhibited higher abundance of *S. rhizophila* and *Pseudomonas* (15% and 14.1%, respectively) than *B. leptolineata* (4.6% and 7.5%), the genus *Variovorax* had an abundance of 5.4% in the microbiome of *B. leptolineata* but was not detected in *S. squalirostris* during this season.

## 4. Discussion

Our findings reveal a significant influence of seasonality on the skin microbiome of the evaluated species, with unexpected patterns. Contrary to our initial expectations, *Scinax squalirostris* exhibited higher bacterial diversity and evenness during the colder winter months, potentially reflecting adaptations that enhance survival and pathogen resistance in harsher conditions. For *Boana leptolineata*, although winter data on alpha diversity metrics were unavailable, we found that OTUs

richness, Shannon diversity, Faith's phylogenetic diversity, and evenness peaked during spring—a season characterized by milder temperatures, ideal for bacterial growth. While seasonality did not impact microbiome dispersion within each species, an interspecific comparison revealed that *B. leptolineata* exhibited significantly lower dispersion than *S. squalirostris* during spring, hinting at differences in behavior or habitat use. These results emphasize the nuanced and species-specific ways in which environmental factors shape amphibian skin microbiomes.

### *4.1.* ***Influence of seasonality on skin microbiome diversity***

The diversity of the amphibian skin microbiome plays a crucial role in the stability and resilience of species against various challenges, such as stress, environmental changes, pathogen invasions, and seasonal temperature fluctuations (Bernardo-Cravo et al., 2020; Hernández-Gómez and Hua, 2023). Seasonal changes can significantly alter microbial community dynamics on amphibian skin, affecting microbial growth or favoring the colonization of certain bacterial species (Longo et al., 2015). Furthermore, some studies suggest that seasonality exerts a more pronounced influence on the skin microbiome than pathogen infections (Le Sage et al., 2021; Longo et al., 2015).

Amphibians typically experience reduced immunity during winter due to low temperatures that inhibit bacterial growth, potentially leading to a less diverse and more unstable microbiome (Ribas et al., 2009). However, our results showed a contrasting pattern: the evenness and Shannon diversity of the skin microbiome of *S. squalirostris* were higher during winter, indicating a more diverse and evenly distributed bacterial community. This increased microbial diversity may enhance the host's resilience to infections and environmental stressors, providing a robust microbial "safety net" that supports immune function (Kueneman et al., 2019; Rebollar et al., 2016). Such findings could suggest that *S. squalirostris* may have developed adaptive strategies to cope with colder temperatures, offering protection against pathogens, such as the *Batrachochytrium dendrobatidis* (*Bd*) fungus, which thrives in cooler climates (Savage and Zamudio, 2011). These findings diverge from studies in temperate regions where microbial diversity declines during winter (Le Sage et al., 2021; Tong et al., 2020). This discrepancy could reflect the subtropical context of our study area, where milder winters promote more stable microbial dynamics. However, differences in precipitation patterns and host phylogenies may also contribute, suggesting the need for broader comparisons across subtropical ecosystems.

In contrast, *B. leptolineata* showed higher alpha diversity metrics in spring compared to summer. Although we lack winter data for this species, the milder spring temperatures compared to summer (Maluf, 2000) could imply that cooler temperatures may also influence its microbiome. The greater skin microbiome diversity observed during spring in this species may reflect adaptation to favorable environmental conditions for bacterial proliferation, resulting in a broader bacterial community

(Erwin et al., 2015; Romanowicz and Kling, 2022). Seasonal changes in behavior and the use of different microenvironments could lead to alterations in bacterial communities, as exposure to various pools of colonizing bacteria changes with habitat shifts (Estrada et al., 2019). Thus, the lack of significant variation in OTUs richness and phylogenetic diversity in *S. squalirostris* may be related to behavioral or habitat preferences, regardless of the season (Estrada et al., 2019), which suggests ecological and behavioral differences from *B. leptolineata*.

Our findings diverge from studies in temperate regions, where bacterial diversity decreases in winter, as observed in *Rana dybowskii* in China (Tong et al., 2020) and *Lithobates sphenocephalus* in the U.S. (Le Sage et al., 2021). A potential explanation for this discrepancy lies in the contrasting climates: while temperate regions experience extreme seasonal shifts, our study took place in a subtropical region with milder winters (Maluf 2000). For example, Longo et al. (2015) found that microbial diversity remained stable across seasons in Arizona's subtropical climate, characterized by hot summers and mild winters, suggesting that areas with less drastic winter temperature changes may experience more stable bacterial colonization patterns. Additionally, amphibians from the Atlantic Forest tend to exhibit higher OTUs richness in colder temperatures compared to warmer ones (Ruthsatz et al., 2020), potentially hosting bacterial communities that enhance immune responses and pathogen resistance (Maniero and Carey, 1997). Furthermore, precipitation differences could also contribute to these variations. For instance, winter rainfall in our study area (1.5 mm) was significantly lower than in regions studied by Tong et al. (2020) and Le Sage et al. (2021), where winter rainfall reached 5.06 mm and 3.37 mm, respectively, higher values than our study area (1.5 mm) (Varejão Silva, 2001). Beyond climatic variation, it is important to emphasize that host phylogeny may also play a critical role in these differences (Belden et al., 2015; McKenzie et al., 2012; Sabino-Pinto et al., 2017; Vences et al., 2015). These findings provide new insights into the interplay between climate, host traits, and microbiome dynamics, emphasizing the complex factors driving skin microbiome diversity in amphibians.

### *4.2.* ***Influence of seasonality on microbiome dispersion***

Microbiome dispersion reflects the stochasticity of a population's microbiome and serves as a potential indicator of species health (Jiménez et al., 2020). High dispersion values can signal dysbiosis, a state in which changes in the microbiota composition deviate from the community structure observed in healthy hosts (Zaneveld et al., 2017). This imbalance can lead to a reduction in beneficial bacteria on amphibian skin and an increase in pathogenic taxa (Jiménez et al., 2020; Neely et al., 2022; Preuss et al., 2020; Zaneveld et al., 2017). This interpretation aligns with the Anna Karenina Principle (Zaneveld et al., 2017), which suggests that microbiomes under stress exhibit higher variability compared to the microbiomes of healthy individuals.

While we did not find significant seasonal effects on microbiome dispersion within either species, our data revealed that *B. leptolineata* had lower dispersion during spring (β = 0.30) compared to *S. squalirostris* (β = 0.40). This difference was unexpected, given that both species share similar natural histories, inhabit overlapping environments, and have comparable reproductive periods (Kwet et al., 2010). A plausible explanation is that *S. squalirostris* interacts with a broader range of bacterial reservoirs due to behavioral differences, such as increased movement or the use of more diverse microhabitats, which could contribute to higher microbiome variability. In contrast, the more stable dispersion observed in *B. leptolineata* may reflect consistent habitat use or physiological traits that buffer against microbial variability.

Interestingly, Schuck et al. (2024) reported higher *Bd* infection rates in *S. squalirostris* during winter, coinciding with increased microbiome dispersion. Conversely, *B. leptolineata* exhibited few signs of Bd infection during the same period (Schuck et al., 2024). This raises the possibility that Bd presence could influence microbiome dynamics in *S. squalirostris*, potentially driving shifts in the abundance and composition of bacterial taxa, either by favoring protective symbionts or enabling opportunistic pathogens (Campos et al., 2024; Goodwin et al., 2022). While these findings do not fully align with the Anna Karenina Principle (Zaneveld et al., 2017), they highlight that interspecific traits and behaviors, rather than seasonal changes, are likely the primary drivers of microbiome dispersion differences.

However, results of other studies further emphasize the role of environmental stressors in amplifying microbiome variability. For instance, *Lithobates yavapaiensis* inhabiting Arizona's extreme summer heat and mild winters showed increased dispersion, potentially in response to thermal stress (Longo et al., 2015). Similarly, *Ambystoma altamirani* in temperate regions showed greater microbiome dispersion in winter compared to spring during pre-metamorphic stages (Martínez-Ugalde et al., 2022). These findings highlight how dispersion dynamics vary across climatic regions, life stages, and host species. In subtropical ecosystems, such as those inhabited by *B. leptolineata* and *S. squalirostris*, stable environmental conditions with milder seasonal fluctuations may lead to less pronounced variability in microbiomes, contributing to more consistent community structures.

The results of these studies underscore the complexity of amphibian microbiome responses to seasonal and ecological changes, emphasizing the importance of comparing microbiome variability across species and climatic regions. Further research is essential to clarify how these differences influence the stability and functionality of amphibian microbiomes throughout the year. As less stable bacterial communities have been shown to exhibit reduced functional redundancy, this instability may negatively impact host health and resilience (Biggs et al., 2020).

### *4.3.* ***Influence of seasonality on the skin microbiome composition***

Comparing the diversity and abundance of microorganisms on anuran skin across different seasons provides valuable insights into the dynamic interactions between bacteria and their amphibian hosts (Longo and Zamudio, 2017; Muletz-Wolz et al., 2019). This study demonstrated that seasonal variations significantly influenced the skin microbiome composition of both *B. leptolineata* and *S. squalirostris*, highlighting how fluctuations in temperature and environmental conditions drive changes in the richness, abundance, and structure of the core microbiome.

In *B. leptolineata*, spring showed the highest richness of OTUs in the core microbiome, with 10 additional taxa compared to winter. This pattern suggests that transitional conditions in spring promote the establishment and persistence of a more diverse microbial community (Estrada et al., 2019; Longo and Zamudio, 2017). Despite the lower richness observed in winter, the core microbiome remained stable across seasons in terms of abundance, indicating the presence of functionally important taxa that confer adaptive advantages under varying environmental conditions (Bell-Dereske et al., 2023). Conversely, *S. squalirostris* exhibited the highest richness during winter (16 OTUs), followed by spring (15 OTUs), and the lowest during summer (12 OTUs). Notably, the summer core taxa comprised 70% of total abundance, suggesting a microbiome dominated by fewer, potentially more specialized taxa adapted to the harsher environmental conditions of this season (Romanowicz and Kling, 2022).

Seasonal variation in unique taxa was observed for both species. For instance, *S. squalirostris* harbored *Brevundimonas*, *Massilia*, *Methylobacterium*, and *Staphylococcus* during winter, whereas *Variovorax*, Yersiniaceae, and Microbacteriaceae appeared exclusively in spring. In contrast, *B. leptolineata* exhibited three unique taxa in spring: Beijerinckiaceae, *Brevundimonas*, and *Streptococcus*. The presence of these unique taxa likely reflects their physiological tolerance to temperature fluctuations (Erwin et al., 2015) and the skin microbiome's adaptive capacity to respond to seasonal environmental changes (Douglas et al., 2021). Additionally, the presence of exclusive taxa—such as *Acinetobacter* and *Streptococcus* in *B. leptolineata* and *Massilia* and *Methylobacterium* in *S. squalirostris*—may be influenced by antimicrobial peptides uniquely synthesized by each species (Demori et al., 2019; Flechas et al., 2019; Greenspan et al., 2022). These peptides act selectively, promoting or inhibiting the colonization of certain bacterial taxa, and may play a crucial role in shaping the observed microbial profiles (Jiménez et al., 2020; Woodhams et al., 2014).

The consistent presence of key taxa, such as Caulobacteraceae, Comamonadaceae, *Devosia*, *Janthinobacterium*, *Pseudomonas*, and *Stenotrophomonas rhizophila* across all seasons and both species underscores the existence of a conserved functional microbiome. These core taxa likely play critical roles in maintaining host health and microbiome stability (Tong et al., 2023; Woodhams et

al., 2015), as most of them produce antifungal compounds (Barnes et al., 2020; Campos et al., 2024; Muletz-Wolz et al., 2017; Woodhams et al., 2015; Wuerthner et al., 2022). The similarity in core microbiome composition and abundance between the species suggests overlapping functional roles and shared microbial strategies for resilience (Belden et al., 2015).

Despite these shared patterns, notable interspecific differences emerged, particularly in summer. *Scinax squalirostris* exhibited higher abundances of *S. rhizophila* and *Pseudomonas*, whereas *B. leptolineata* showed a prominent presence of *Variovorax*, which was absent in *S. squalirostris*. Such differences reflect species-specific microbiome dynamics and suggest that environmental conditions and host-specific factors interact to shape the microbiome structure (McKenzie et al., 2012; Medina et al., 2019; Walke et al., 2021, 2014).

## 4. Conclusion

Our findings emphasize the adaptive nature of the amphibian skin microbiome in response to seasonal changes. The observed seasonal shifts in diversity and community composition reflect adaptive responses to fluctuating environmental conditions, with each species maintaining a microbiome that may confer resilience against pathogens and environmental stressors. However, the persistence, and sometimes dominant presence, of certain taxa (e.g., Comamonadaceae, *Pseudomonas* and *Stenotrophomonas rhizophila*) suggests that these taxa may confer critical protective functions, particularly in seasons that favor pathogenic challenges. Despite the lack of significant changes in microbiome dispersion, the lower variability observed in *B. leptolineata* during spring compared to *S. squalirostris* suggests that ecological and behavioral differences between those species may influence microbiome dynamics. These findings contribute to our understanding of how seasonality impacts microbial communities and host resilience in amphibians, emphasizing the need for further studies on the effects of climate on microbial ecology and host-pathogen interactions.

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**Figures**

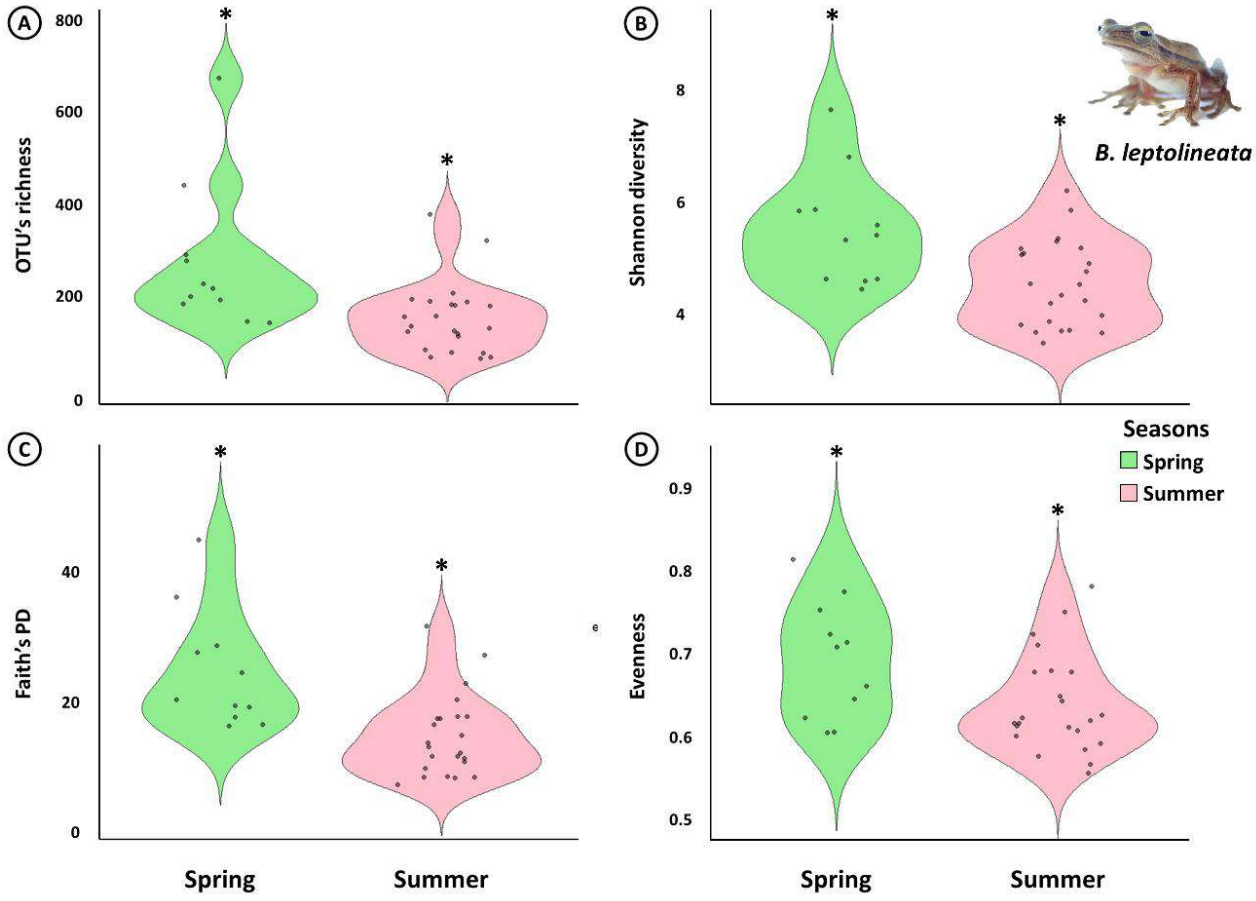


**Figure 1.** Values of (A) OTUs richness, (B) Shannon diversity, (C) Faith's phylogenetic diversity and (D) Evenness between spring (green) and summer (pink) for *Boana leptolineata*. Asterisks indicate significant differences between groups ($p < 0.05$).

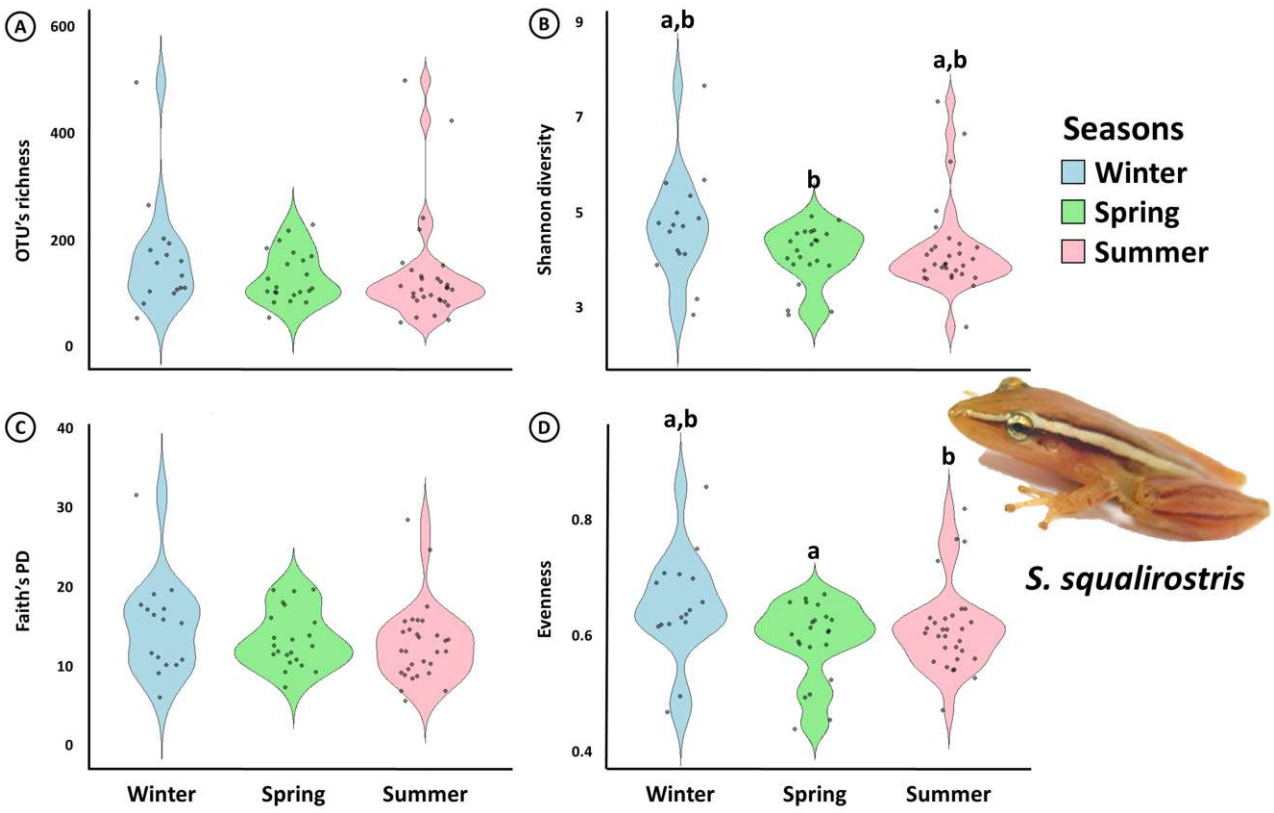


**Figure 2.** Values of (A) OTUs richness, (B) Shannon diversity, (C) Faith's phylogenetic diversity and (D) Evenness between winter (blue), spring (green) and summer (pink) for *Scinax squalirostris*. Lowercase letters indicate significant differences between groups ($p < 0.05$).

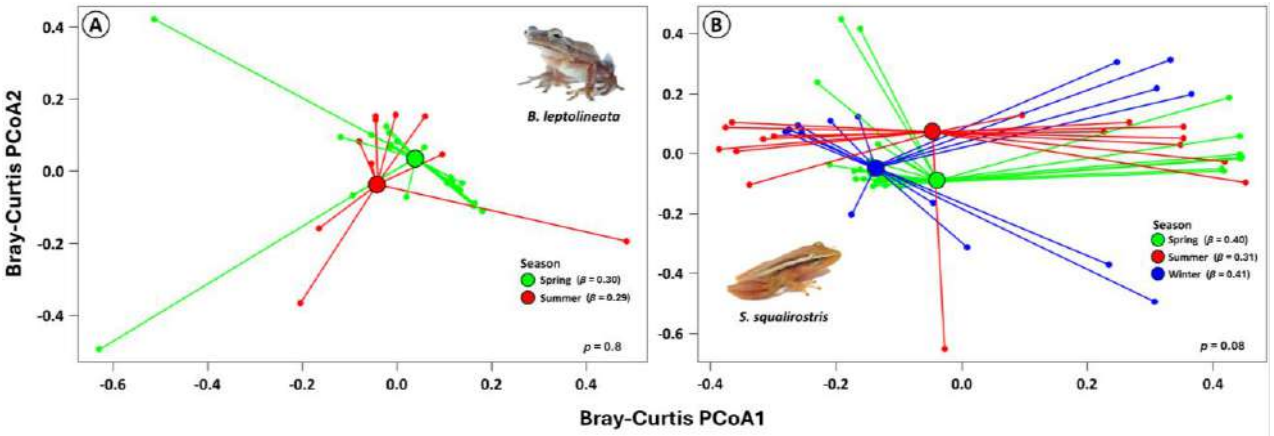


**Figure 3.** Influence of seasonality on microbiome dispersion ($\beta$), based on the Bray-Curtis dissimilarity matrix, in (A) *Boana leptolineata* and (B) *Scinax squalirostris* during spring (green), summer (red) and winter (blue), where the larger points represent the centroid value for each station, and smaller points represent the distance of each individual in relation to the centroid.

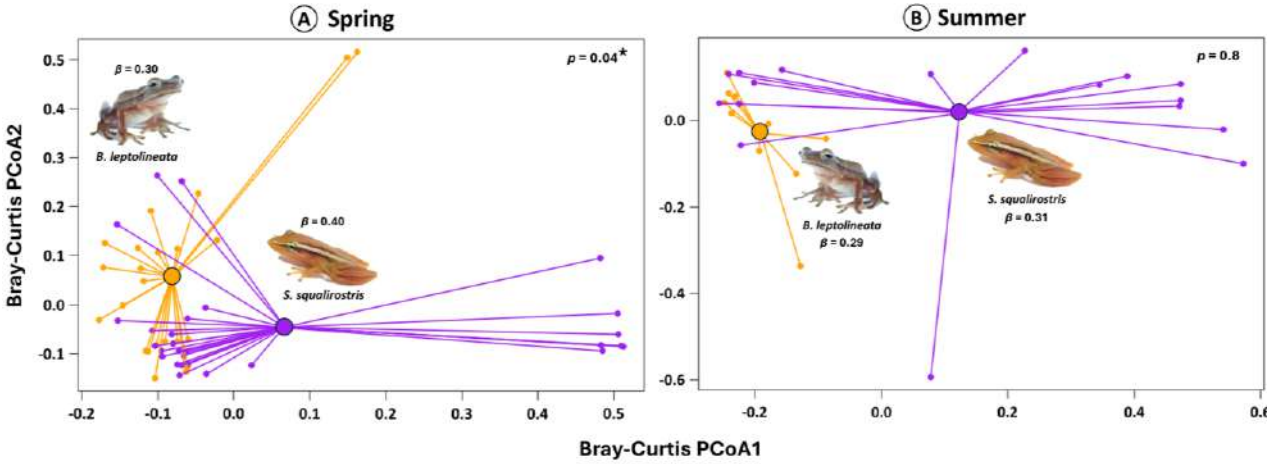


**Figure 4:** Microbiome dispersion ($\beta$) during (A) spring and (B) summer of *Boana leptolineata* (orange) and *Scinax squalirostris* (purple), based on the Bray-Curtis dissimilarity matrix, where the larger points represent the centroid value for each station, and smaller points represent the distance of each individual sample in relation to the centroid. Asterisks indicate significant differences between groups ($p < 0.05$).

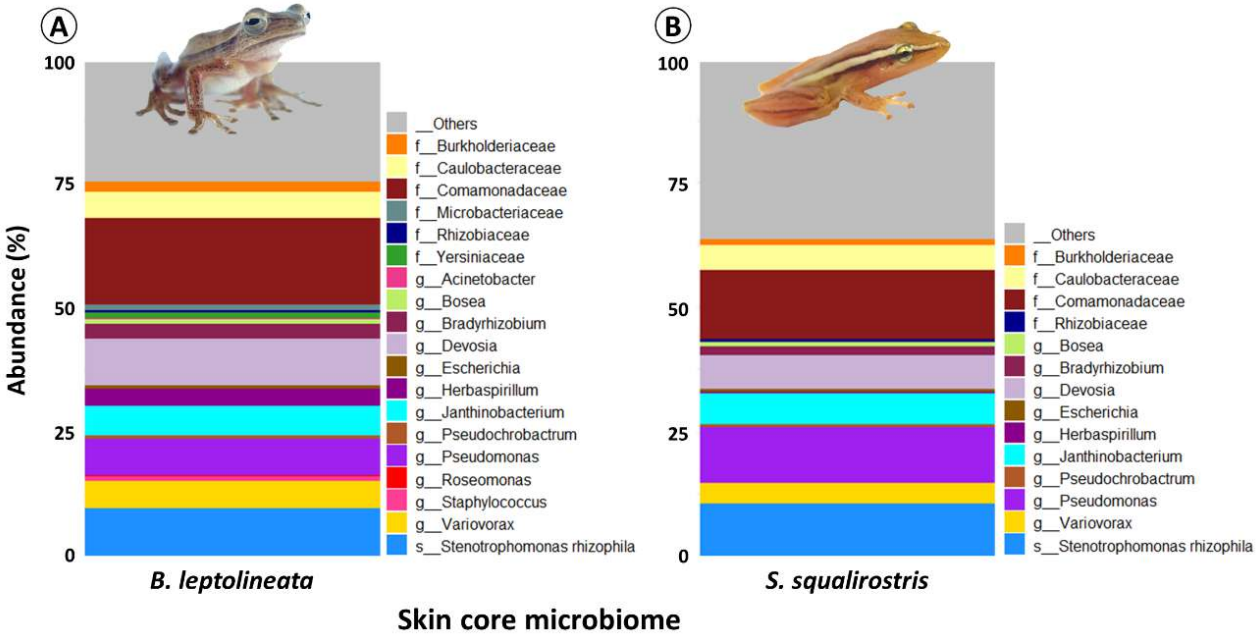


**Figure 5.** Skin core microbiome of (A) *Boana leptolineata* and (B) *Scinax squalirostris*.

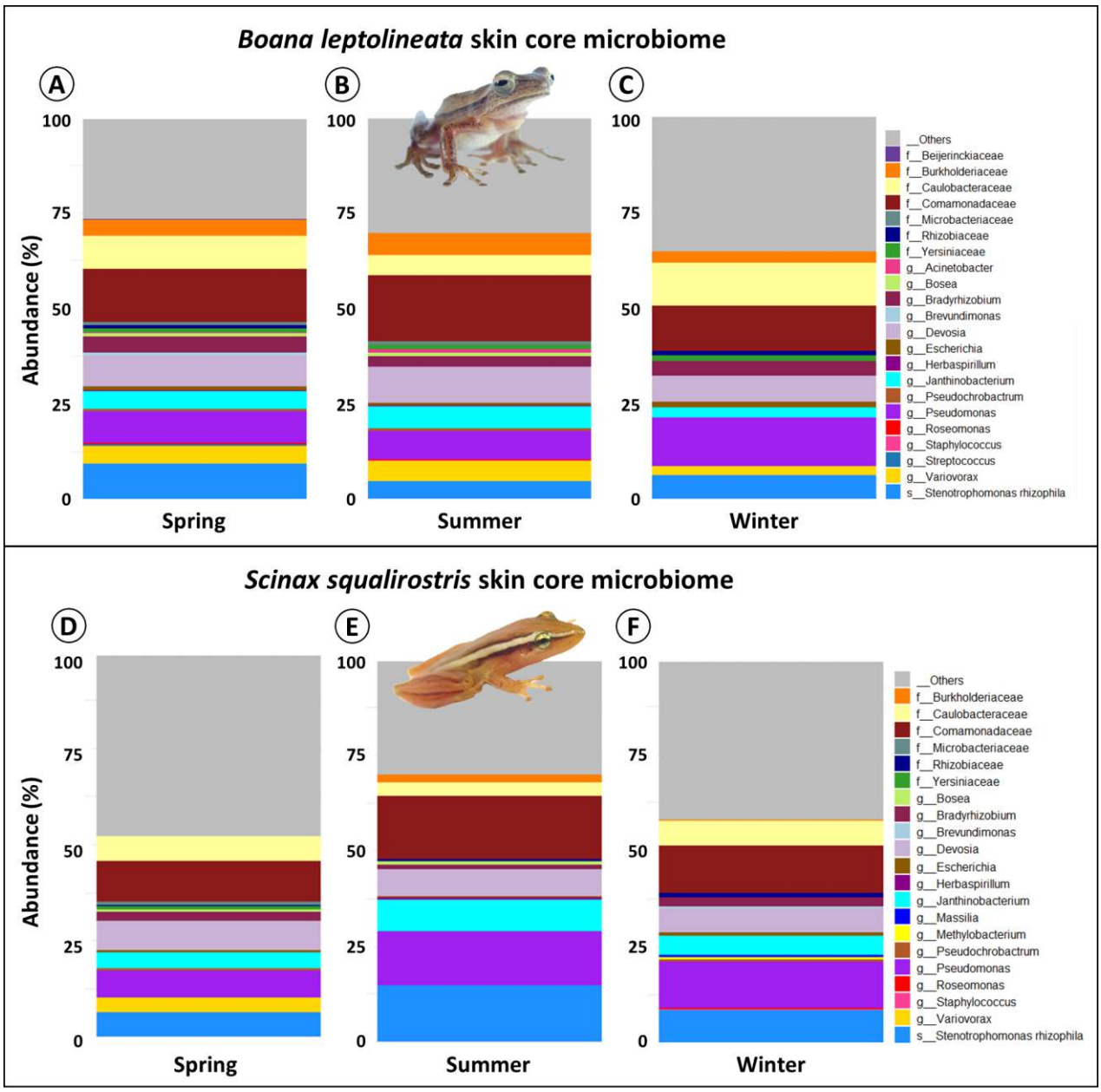


**Figure 6.** Skin core microbiome of *Boana leptolineata* (A, B and C) and *Scinax squalirostris* (D, E and F) during (A, D) spring, (B, E) summer and (C, F) winter.

# Capítulo 4

## Infection dynamics of

## *Batrachochytrium dendrobatidis*

## in temperate amphibian communities

O capítulo IV desta Tese foi elaborado e formatado conforme as normas da publicação científica *Diseases of Aquatic Organisms*, as quais se encontram em anexo (Anexo 4).

# Infection dynamics of *Batrachochytrium dendrobatidis* in temperate amphibian communities

Camila F. Moser[1], Wesley J. Neely[2*], Leigha M. Stahl[2], Laura K. Schuck[3], Henrique Schipanski[4], C. Guilherme Becker[3], Pedro Peloso[5]

[1] Department of Zoology, Federal University of Pará (UFPA), Belém, Pará, BR.

[2] Department of Biology, The University of Alabama, Tuscaloosa, Alabama, USA.

[3] Department of Biology, The Pennsylvania State University, University Park, Pennsylvania, USA.

[4] Department of Zoology, Federal University of Paraná (UFPR), Curitiba, Paraná, BR.

[5] Vertebrate Museum, Department of Biological Sciences, California State Polytechnic University, Arcata, California, USA.

* corresponding author: wesleyjneely@gmail.com

**Running page head:** Infection of *Bd* in temperate amphibians

**Abstract**

*Batrachochytrium dendrobatidis* (*Bd*) is a pathogenic fungus linked to population declines and extinctions of amphibians globally. Anthropogenic habitat disruptions can directly and indirectly influence host-pathogen interactions by altering wetland microclimates, physicochemical profiles, and amphibian communities. To assess the combined effects of host and environmental factors on *Bd* infection dynamics, we sampled 581 amphibians from 12 species in Tuscaloosa County, Alabama, USA. We tested the influence of host traits (body size and life history) and environmental factors (habitat disturbance and surface water temperature) on *Bd* prevalence and infection loads. *Bd* was detected in 127 individuals (22% prevalence) across six frogs and two salamander species. Aquatic amphibians showed higher *Bd* prevalence and loads than terrestrial and arboreal species, with no significant difference between the latter two. Higher water temperatures were associated with lower *Bd* prevalence and loads, while body size and habitat disturbance had no significant effect. These results emphasize the influence of host traits and environmental factors on *Bd* dynamics, expanding our understanding of amphibian host-pathogen.

**Keywords:** Anura, Caudata, Chytridiomycosis, Habitat disturbance, Salamander, Pathogens

## 1. INTRODUCTION

Understanding host-pathogen dynamics is crucial for advancing disease ecology and conservation efforts, as these interactions are influenced by a complex combination of environmental factors and host-specific characteristics (Fisher et al. 2012, Voyles et al. 2018). In amphibians, the most threatened group of vertebrates in the world (IUCN 2024), understanding host-specific responses to pathogens is crucial for the conservation of these animals due to the recent rise in diseases, like chytridiomycosis, for example. (Scheele et al. 2019). The disease is caused by *Batrachochytrium dendrobatidis* (*Bd*), an aquatic fungus, and can cause damage to the keratinocytes in amphibian skin, reducing physiological function and potentially leading to mortality (Woodhams et al. 2008, Lips 2016). This pathogen can act synergistically with other stressors such as temperature and habitat loss to drive population declines and extinctions (Cohen et al. 2017, Scheele et al. 2019, Neely et al. 2022, Becker et al. 2023). Despite this, amphibians have several mechanisms that can contribute to the inhibition of *Bd* infections, such as antimicrobial interactions with host-associated microbes peptides produced in the skin of these animals (Rollins-Smith & Conlon 2005, Richmond et al. 2009, Harris et al. 2009, Lam et al. 2010, Savage & Zamudio 2011). However, these defense mechanisms can entail high metabolic costs for amphibians, which can lead to negative consequences on the fitness of individuals, affecting their ability to survive and reproduce (Rohr et al. 2010, Gervasi et al. 2013).

Host-pathogen dynamics are strongly influenced by environmental temperature, as changes in water temperature impact both hosts and pathogens (Kärvemo et al. 2018, Rohr & Cohen 2020). *Batrachochytrium dendrobatidis* (Bd) thrives at lower temperatures, with optimal growth around 21°C (Stevenson et al. 2013), whereas higher temperatures can reduce its proliferation (Piotrowski et al. 2004, Longo et al. 2009, Bustamante et al. 2010). Amphibians from cooler or moderately warm environments (~20°C) are thus more susceptible to infection (Piotrowski et al. 2004, Berger et al. 2004, Kriger & Hero 2007, Becker & Harris 2010, Raffel et al. 2010, Robak et al. 2023). Anthropogenic disturbances, such as deforestation, can elevate environmental temperatures beyond *Bd*'s thermal optimum, potentially reducing pathogen persistence (Piotrowski et al. 2004, Longo et al. 2009, Bustamante et al. 2010). However, habitat alteration can also increase host stress (Wikelski & Cooke 2006, Navas & Otani 2007) and facilitate pathogen transmission by modifying host behavior and immune responses (Bradley & Altizer 2007, Johnson & Thieltges 2010, Cable et al. 2017). These contrasting effects may explain the variability in *Bd* prevalence across disturbed

and natural habitats (Adams et al. 2010, Murray et al. 2011, Saenz et al. 2015, Preuss et al. 2020, Kriger & Hero 2007, Raffel et al. 2010, Becker & Zamudio 2011, Becker et al. 2012, 2015).

The probability of *Bd* infection can also be influenced by host life history (Searle et al. 2011). Since *Bd* is an aquatic-borne pathogen, with zoospores generally found in aquatic environments prior to infection, amphibians with more aquatic habits may have a higher risk of infection than terrestrial or arboreal species (Lips et al. 2003, Kriger & Hero 2007, Toledo et al. 2023). Consequently, aquatic species are suspected to suffer disproportionately greater negative impacts of *Bd*-related population decline than the non-aquatic species assessed (Lips et al. 2003, Bielby et al. 2008, Greenberg et al. 2017; Toledo et al. 2023). However, other authors indicate that aquatic amphibians may have acquired a certain resistance to the fungus due to constant contact with this pathogen, which can reduce the chances of contracting chytridiomycosis compared to species that have less contact with *Bd* (Chestnut et al. 2014, Becker et al. 2015). The host's body size is also a factor that can influence the prevalence of pathogens (Searle et al., 2011), once larger animals are predicted to have higher pathogen loads as they provide greater space for microorganism colonization than smaller animals (Kuris et al., 1980; Searle et al., 2011; Lenker et al., 2014).

While chytridiomycosis can lead to mortality in many species, more information on the impact of *Bd* on populations in North America is needed. In the United States, the state of Alabama is a known hotspot of amphibian biodiversity, with 73 native species of amphibians (Jenkins et al. 2015, Mirarchi & Shelton-Nix 2017, IUCN 2024). However, there are few studies that evaluate the infection of *Bd* and its impacts on amphibians in Alabama (e.g., Byrne et al. 2008, Bakkegard & Pessier 2010, Chiari et al. 2017, Neely et al. 2022). Alabama's climate is classified as humid-subtropical, with high average annual rainfall (1,500 mm) and has 69% forest cover which provides cool microclimates even during the hot summers (Hartsell 2017). Therefore, this region offers optimal environmental conditions for *Bd* proliferation, and we can expect high prevalence in native amphibian communities.

The goals of our study are to (1) estimate *Bd* prevalence and *Bd* loads in amphibian communities across a gradient of habitat loss in Tuscaloosa county, Alabama, (2) test for links between the level of habitat disturbance and average *Bd* prevalence and *Bd* loads in the assessed amphibian communities, (3) test whether there is an influence of water temperature on the *Bd* prevalence and *Bd* loads, (4) test whether there is a difference between *Bd* prevalence and *Bd* loads in species with different life histories (aquatic, terrestrial and arboreal), and (5) test whether amphibian body size has an influence on *Bd* prevalence and loads. From our literature review, we expect that (1) *Bd* loads and prevalence will be lower in amphibians from more disturbed habitats,

(2) higher water temperatures will be linked with lower *Bd* loads and prevalence, since the incidence of *Bd* is generally higher in cool, moist microclimates, (3) species with more aquatic habits will have a higher *Bd* prevalence (due to greater contact with water), but lower *Bd* loads (due to acquired resistance through constant contact with the fungus), than species with more terrestrial and arboreal habits, and (4) amphibians with larger body size will have higher *Bd* prevalence and loads (due to the greater space available for colonization of microorganisms).

## 2. MATERIALS & METHODS

### 2.1. Study site and study design

Sampling took place from April to May 2019 during peak anuran breeding season in 30 ponds in Tuscaloosa County, Alabama, U.S.A. (Figure 1, Table S1). This region has a humid subtropical climate, with hot summers and cool winters (Chen 2018). The average annual temperature ranges from approximately 18°C to 19°C, exceeding 30°C in summer, while winter minimums average around 5°C (Climate-Data 2025). Annual regional rainfall varies between 1,500 mm to 1,600 mm (Carter & Seaquist 1984).

We conducted nocturnal surveys by searching along pond edges, capturing amphibians using either dip nets or by hand. Sampling was random with regards to sex and size. Field equipment (e.g. boots, waders, and nets) were sterilized with 0.10% bleach between sample sites to prevent spread of microorganisms. Dip nets were rinsed and new plastic bags were placed over the searcher's hand between individuals to avoid cross contamination. We temporarily held frogs in sterile plastic bags until processing. Each individual was weighed, measured (snout-vent length), and swabbed, then released at the location of capture. Prior to swabbing, we rinsed amphibians with sterile distilled water to remove debris. We swabbed individuals using a sterile swab (Medical Wire) 5 times on the underside of each foot and 5 times on each side of the venter for a total of 30 passes (Hyatt et al. 2007). We dry-stored swabs in sterile tubes on ice while in the field and moved them to a -20 ºC freezer upon return to the lab, where they remained until DNA extraction. We also assessed surface water temperature at 3 locations in each pond using a YSI Pro Plus multiparameter meter (Yellow Springs Inc., Yellow Springs, OH). All field work was conducted under appropriate state permits (Alabama Scientific Collecting Permit #2019069449668680) and institutional animal care and use approval (IACUC #18-11-1721).

We calculated the percentage of habitat disturbance (i.e. the proportion of non-natural habitat) within a 500 m radius of each sampled pond using Arc-GIS (version 10.5.1). Land cover was classified as either natural (e.g. aquatic vegetation, forest, and woodland, introduced and semi natural vegetation, nonvascular and sparse vascular rock vegetation, or shrubland and grassland) or

disturbed (e.g. agricultural vegetation, developed and other human use, or recently disturbed or modified), based on the GAP Land Cover Data (Homer et al. 2015). These classifications were used to generate a continuous variable representing the percentage of disturbed habitat. For statistical analysis, this continuous disturbance percentage was used to classify each site into one of two categories: natural (< 45% disturbance) or disturbed (> 45% disturbance) (Table S1) (Becker et al. 2017). This binary classification was included in the models as the predictor "Environmental type".

### 2.2. Molecular methods

We extracted DNA from skin swabs using the Qiagen DNeasy Blood & Tissue kit following a modification of the standard protocol. After addition of proteinase K, we incubated at 56ºC for 12 hours instead of 4 hours to increase DNA yield (Caligiuri et al. 2019). We included negative controls of sterile water to monitor potential contamination of extraction reagents.

For qPCR analysis of *Bd* from skin swab DNA, we diluted DNA 1:10 to reduce reaction inhibition and quantified *Bd* loads using Taqman qPCR assays on *Bd*-specific ITS and 5.8S genes (Boyle et al. 2004) and used gBlock synthetic *Bd* standards (Integrated DNA Technologies) diluted from $10^6$ to $10^2$ gene copies (g.c.). We ran plates in duplicate, with mismatching samples (positive on only one plate) run in triplicate. Only samples that were positive on 2 plates were recorded as positive. *Bd* loads were averaged across the duplicate plates and log10-transformed to correct for non-normal residual distributions.

### 2.3. Statistical Analyses

To investigate the influence of environmental type (disturbed or natural), body size, average water temperature, and host life history (aquatic, arboreal, and terrestrial habits) on *Bd* prevalence, we applied a generalized linear mixed model (GLMM) with a binomial distribution and a logit link function, using the *glmer* function from the *lme4* package. To examine the factors affecting *Bd* loads, we employed a zero-inflated generalized linear mixed model (GLMM-ZI) with a negative binomial distribution (family *nbinom2*) to account for overdispersion in the data. The model was fitted using the *glmmTMB* function.

We tested eight models for both response variables (*Bd* prevalence and loads), considering the sampling area (Site) as a random factor: (1) *Bd* ~ body size + Host habit + Environmental type + Water temperature + (1|Site), (2) *Bd* ~ Environmental type + Water temperature + body size + (1| Site), (3) *Bd* ~ Environmental type + Water temperature + (1| Site), (4) *Bd* ~ Host habit + (1| Site), (5) *Bd* ~ body size + Host habit + (1| Site), (6) *Bd* ~ Host habit + Environmental type + (1| Site), (7) *Bd* ~ body size + Host habit + Environmental type + (1| Site), and (8) *Bd* ~ body size + (1| Site) (Tables S2 and S3). The collinearity of the model was checked using the *Variance Inflation Factor*

(VIF) in the *vif* function of the car package (Fox et al. 2012) with a cutoff threshold value of 3 (Zuur et al. 2009). To determine the best-fitting final model, we used the Akaike Information Criterion (AIC) (Akaike 1974). Additionally, we assessed the residual diagnostics using the *DHARMa* package to ensure model validity (Hartig 2022). This package estimates the residuals from a set of simulated data using the model that best fits the data and compares the estimates with the observed residuals. Significant deviations in relation to the distribution of simulated residuals indicate that the model is not valid (Hartig 2022). From this, the model chosen for both response variables was the model with formula (1) (Table S4 and S5).

To test the significance of fixed effects in the final models, we used Type II Wald chi-square tests (function *Anova* from the *car* package), appropriate for generalized linear mixed models with non-Gaussian distributions. In addition, to explore pairwise differences between host habit, we conducted post hoc comparisons using the *emmeans* package, applying Tukey's correction for multiple testing. Furthermore, we assessed the correlation between average water temperature and (1) the degree of environmental disturbance and (2) *Bd* loads in each of the three host habitats (i.e., aquatic, arboreal, and terrestrial) using Pearson's linear correlation. All analyses were run in the R environment, version 3.2.2 (R Core Team 2013). We considered results significant when *p* values were lower than 0.05.

Regarding the host's ecological habits, we classified *Acris crepitans*, *Aquarana catesbeiana*, *Aquarana clamitans*, *Eurycea cirrigera* and *Notophthalmus viridescens* species as mostly aquatic (Blem et al. 1978, Willson & Dorcas 2003, Roe & Grayson 2008, Pitt et al. 2017, Sepulveda 2018), *Anaxyrus* sp., *Eurycea guttolineata*, *Gastrophryne carolinensis* and *Lithobates sphenocephalus* as mostly terrestrial (Dodd Jr 1995, Freeman & Bruce 2001, Liang 2013), and *Dryophytes chrysoscelis*, *Dryophytes cinereus* and *Dryophytes gratiosus* as mostly arboreal (Oldham & Gerhardt 1975, Pittman et al. 2008). We categorized species according to their known ecological habits as described in the literature and their typical microhabitats, which were confirmed by the specific habitats where individuals were sampled.

## 3. RESULTS

We sampled the skin of 581 amphibian specimens: five individuals were of the species *Notophthalmus viridescens* (Eastern Newt) (Caudata, Salamandridae), one *Eurycea cirrigera* (Southern Two-lined Salamander), one *Eurycea guttolineata* (Three-lined Salamander) (Caudata, Plethodontidae), two *Gastrophryne carolinensis* (Eastern Narrowmouth Toad) (Anura, Microhylidae), 59 *Anaxyrus* sp. (North American Toads) (Anura, Bufonidae), 20 *Dryophytes*

*chrysoscelis* (Cope's Gray Treefrog), 108 *Dryophytes cinereus* (Green Treefrog), 20 *Dryophytes gratiosus* (Barking Treefrog), 156 *Acris crepitans* (Northern Cricket Frog) (Anura, Hylidae), 72 *Aquarana clamitans* (Green Frog), 17 *Lithobates sphenocephalus* (Southern Leopard Frog) and 120 *Aquarana catesbeiana* (American Bullfrog) (Anura, Ranidae). These 12 species represent distributed in two orders (Anura and Caudata), six families (Bufonidae, Hylidae, Microhylidae, Plethodontidae, Ranidae and Salamandridae) (Table 1).

We detected *Bd* in 127 of 581 (22%) individuals sampled. Of the 12 species sampled, six species of frogs (51 *Ac. crepitans*, two *Anaxyrus* sp., one *D. cinereus*, 53 *Aq. catesbeiana*, 10 *Aq. clamitans* and six *L. sphenocephalus*) and two salamander species (one *E. cirrigera* and three *N. viridescens*) were infected with *Bd* (Table 1). We did not detect *Bd* in three frogs (*D. chrysoscelis*, *D. gratiosus*, and *G. carolinensis*) and one salamander (*E. guttolineata*).

Among the species with more than one individual sampled, the salamander *N. viridescens* presented the highest prevalence of *Bd* (60%, samples = 5, *Bd* + = 3), followed by the frogs *Aq. catesbeiana* (43.3%, samples = 120, *Bd* + = 53), *L. sphenocephalus* (35.3%, samples = 17, *Bd* + = 6), *Ac. crepitans* (33%, samples = 156, *Bd* + = 51), and *Ac. clamitans* (14%, samples = 72, *Bd*+ = 10) (Table 1).

Both our models chosen for *Bd* prevalence and *Bd* loads had the same variables: body size, host habit (i.e. arboreal and terrestrial compared to aquatic habit), environmental type (i.e. disturbed or natural), and average water temperature, with sites as a random factor. Regarding *Bd* prevalence model, the analysis indicated that amphibians with arboreal (Slope = -3.81, Std. Error = 1.02, $z$ = -3.72, $p < 0.001$) and terrestrial habits (Slope = -1.29, Std. Error = 0.41, $z$ = -3.14, $p = 0.002$) have lower *Bd* prevalence when compared with aquatic amphibians (Table S4, Figure 2A). We also found a lower prevalence at higher water temperatures (Slope = -0.46, Std. Error = 0.12, $z$ = -1.20, $p = 0.008$) (Table S4, Figure 2A and 3), while we did not detect significant influences of body size and habitat type on prevalence ($p > 0.05$) (Table S4, Figure 2A). The results of the ANOVA test confirmed what was observed in the model, where only the variables habit ($Chi^2 = 22.50$, Df = 2, $p < 0.0001$) and average water temperature ($Chi^2 = 7.01$, Df = 1, $p = 0.008$) were significant (Table S6). Furthermore, the post-hoc test revealed that aquatic amphibians had a higher *Bd* prevalence compared to arboreal amphibians (Slope = 3.81, Std. Error = 1.02, z = 3.72, $p < 0.001$) and terrestrial amphibians (Slope = 1.3, Std. Error = 1.08, z = 3.14, $p = 0.005$) (Table S7). However, no significant difference was found between terrestrial and arboreal amphibians ($p = 0.052$) (Table S7).

As in the *Bd* prevalence model, the *Bd* loads model with the lowest AIC value contained the independent variables host habit, habitat disturbance, and average water temperature, considering

the species as a random factor (Table S2 and S5). The chosen model indicated lower *Bd* loads were found at higher water temperatures (Slope = -0.17, Std. Error = 0.07, $z$ = -2.61, $p$ = 0.009) (Table S5, Figure 2B). The variables body size, host habit and environmental type had no influence on *Bd* loads ($p > 0.05$) (Table S5, Figure 2B). The ANOVA test confirmed the significance of the habit and temperature variables on the *Bd* loads (Table S8). However, the post-hoc test showed that there is a difference between aquatic and arboreal habits (Slope = 3.94, Std. Error = 0.73, $z$ = 5.40, $p <$ 0.001) and between arboreal and terrestrial habits (Slope = 3.62, Std. Error = 0.79, $z$ = -4.60, $p <$ 0.001), but not between amphibians with aquatic and terrestrial habits ($p$ = 0.61).

Pearson's linear correlation revealed that water temperature was higher in less disturbed environments ($R^2$ = -0.11, $p$ = 0.006). Additionally, aquatic ($R^2$ = -0.16, $p$ = 0.003) and terrestrial amphibians ($R^2$ = -0.44, $p < 0.001$) exhibited higher *Bd* loads in ponds with lower temperatures, whereas no correlation was found between mean water temperature and *Bd* loads in arboreal amphibians ($p > 0.05$).

## 4. DISCUSSION

Our findings indicate that *Bd* prevalence and *Bd* loads are lower in amphibians exposed to higher surface water temperatures, and *Bd* loads are lower in arboreal and terrestrial amphibians compared to aquatic species. These results partially corroborate our initial hypothesis and provide insights into the complex interactions between environmental variables, amphibian traits, and *Bd* infection dynamics.

Environmental temperature is a major driver of host-pathogen interactions and is especially important in mediating interactions between amphibians and *Bd* because both are physiologically sensitive to shifts in temperature (Turner et al. 2021). Higher environmental temperatures may benefit amphibian hosts, since *Bd* is typically more pathogenic at lower temperatures, usually between 15 °C and 25 °C (Kriger & Hero 2007, Stevenson et al. 2013). Our results support this pattern, as we observed lower *Bd* prevalence and loads at higher water temperatures. Similar trends have been documented in other studies, where elevated water temperatures improved amphibian immune function and reduced *Bd* fitness (Sapsford et al. 2013, Bradley et al. 2019, Neely et al. 2020, Turner et al. 2021). However, it is important to note that our sampling was conducted during a relatively warm period of the year, which may not have been favorable for *Bd* growth to begin with.

The influence of water temperature on *Bd* prevalence and loads may also be related to habitat alterations. Deforested or urbanized habitats often experience higher levels of chemical

pollution, increased water turbidity, and altered thermal regimes, which can create conditions that either enhance or inhibit *Bd* persistence (Raffel et al. 2010, Becker et al. 2012, 2023). Interestingly, our data showed that water temperatures were higher in less disturbed environments. One possible explanation for this pattern is that in more preserved areas, dense vegetation around water bodies may contribute to greater heat retention. In contrast, disturbed environments, with reduced vegetation cover, are more exposed to wind, which enhances evaporative cooling and results in lower water temperatures (Manteghi et al. 2015).

Amphibians rely on the environment to regulate their body temperature and immune functions, so fluctuations in temperature can significantly impact their ability to resist infections (Raffel et al. 2013, Voyles et al. 2017). While elevated temperatures may reduce *Bd* fitness, they can also disrupt amphibians' physiological functions, including immune responses (Sapsford et al. 2013). This relationship highlights the potential influence of climate and habitat modifications on shaping *Bd* infection dynamics, particularly in environments experiencing anthropogenic disturbances that alter thermal conditions.

The higher *Bd* loads in aquatic hosts did not corroborate our initial hypothesis that was based on the premise that frequent contact with the fungus would increase resistance to infection (Chestnut et al. 2014; Becker et al. 2015). However, constant contact with *Bd* can lead aquatic amphibians to develop an immune response effective enough to control the infection without eliminating it (Savage & Zamudio 2011; Gervasi et al. 2013; McKnight et al. 2019). For example, the two species with the highest number of infected individuals, *Acris crepitans* and *Aquarana catesbeiana*, are regarded by several researchers as *Bd*-tolerant species, capable of maintaining high zoospore loads without significant negative effects on health or mortality (Steiner & Lehtinen 2008, Duncan Pullen et al. 2010, Gahl et al. 2012, Brannelly et al. 2012, 2018, Hanlon et al. 2015, Miaud et al. 2016). Studies show that some amphibian populations have co-evolved with *Bd* and developed tolerance mechanisms, allowing them to carry the fungus without manifesting deleterious effects (Woodhams et al. 2007, McKnight et al. 2019). Examples of tolerance mechanisms include the production of antimicrobial peptides in the skin (Rollins-Smith 2017) and interactions with beneficial bacteria that may decrease the impact of *Bd* (Bletz et al. 2013). Thus, our results suggest that even aquatic species considered tolerant to *Bd*, such as *Ac. crepitans* and *Aq. catesbeiana* (Hanlon et al. 2015, Miaud et al. 2016, Brannelly et al. 2018), may be able to sustain stable populations despite high *Bd* loads, making them potential vectors and reservoirs of *Bd* (Rizkalla 2009, Gahl et al. 2012, Gervasi et al. 2013). Moreover, the fact that aquatic species had a higher *Bd* loads highlight that aquatic species are indeed more prone to get infected by *Bd*—which can be potentially devastating to non-resistant aquatic taxa.

We observed that, while aquatic species have higher *Bd* prevalence and loads than terrestrial and arboreal species, arboreal hosts have significantly lower *Bd* prevalence and loads than species of other habits. These results are probably related to the aquatic habitat of *Bd*, where arboreal amphibians have less contact with the pathogen than aquatic amphibians, a pattern supported by past research (Lips et al. 2003, Kriger & Hero 2007, Gervasi et al. 2013, Toledo et al. 2023). In addition, the lower number of *Bd*-positive arboreal amphibians may be related to increased exposure to ultraviolet radiation, which may contribute to *Bd* inhibition (Bletz et al. 2017).

In contrast to our initial expectations, we did not find a significant effect of habitat type or body size on *Bd* prevalence and loads. Previous studies have reported conflicting results regarding habitat disturbance and *Bd* infection. Some authors suggest that amphibians in natural environments host higher *Bd* loads due to stable environmental conditions that support *Bd* persistence (Kriger & Hero 2007, Raffel et al. 2010, Becker & Zamudio 2011, Becker et al. 2012, 2015). However, other studies have found the opposite pattern, with lower *Bd* loads in disturbed environments, possibly due to higher water turbidity, increased temperatures, and chemical pollutants that negatively impact *Bd* (Adams et al. 2010, Murray et al. 2011, Saenz et al. 2015, Preuss et al. 2020).

Regarding body size, although it has been shown to influence pathogen prevalence and loads, with larger animals generally hosting more pathogens due to greater space for microorganism colonization (Searle et al. 2011, Lenker et al. 2014) and longer exposure times (Kuris et al. 1980), we did not find any effect of host size on *Bd* prevalence or loads in our study. This is consistent with Lenker et al. (2014), who also observed no clear relationship between body size and *Bd* infection in amphibians. A possible explanation for this result is that larger amphibians may be healthier and better able to resist infections (Carey et al. 1999), while those in poorer body condition might have reduced immune responses (Searle et al. 2011, Wilcoxen et al. 2010). These results highlight the importance of local environmental conditions and host-pathogen dynamics in determining infection outcomes.

## 5. CONCLUDING REMARKS

Overall, our study reinforces the critical role of water temperature and amphibian habitat in shaping *Bd* infection patterns. The lower *Bd* prevalence and loads at higher water temperatures and in non-aquatic species suggest that both abiotic and biotic factors contribute to the observed variation in *Bd* dynamics. Our findings have important implications for understanding the potential impacts of climate change and habitat alterations on *Bd*-host interactions, especially considering that these changes could lead to shifts in pathogen distribution and host susceptibility. Further research is needed to explore the long-term consequences of these patterns, focusing on the

influence of fluctuating environmental conditions and the evolving strategies amphibians may develop to cope with *Bd*. Additionally, our study emphasizes the need for more comprehensive monitoring of *Bd* dynamics across different ecosystems and amphibian species to inform conservation strategies and mitigate the risk of amphibian population declines caused by this deadly pathogen.

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**Table 1:** Relationship between the host species sampled and the presence of *Bd*, where: N = Number of specimens sampled, *Bd* + = Number of infected specimens, Prev = Prevalence of *Bd*, *Bd* loads (Log 10) = Log normalized *Bd* load, % = Percentage values, ± = Standard deviation.

| Group | Host | Life history | N | *Bd* + | Prev (%) | *Bd* loads (gene copies) | *Bd* loads (Log 10) |
|---|---|---|---|---|---|---|---|
| Anura | *Acris crepitans* | Aquatic | 156 | 51 | 33 | 2534 ± 13804 | 2.8 ± 1 |
| Anura | *Anaxyrus* sp. | Terrestrial | 59 | 2 | 3.4 | 84.8 ± 628.7 | 2.9 ± 0.8 |
| Anura | *Aquarana catesbaeiana* | Aquatic | 120 | 53 | 43.3 | 2354 ± 22785 | 2.3 ± 0.8 |
| Anura | *Aquarana clamitans* | Aquatic | 72 | 10 | 13.9 | 40 ± 222.8 | 2.1 ± 0.5 |
| Anura | *Dryophytes chrysoscelis* | Arboreal | 20 | 0 | 0 | 0 ± 0 | 0 |
| Anura | *Dryophytes cinereus* | Arboreal | 108 | 1 | 0.9 | 2354.1 | 1.96 |
| Anura | *Dryophytes gratiosus* | Arboreal | 20 | 0 | 0 | 0 ± 0 | 0 |
| Anura | *Gastrophyrne carolinensis* | Terrestrial | 2 | 0 | 0 | 0 ± 0 | 0 |
| Anura | *Lithobates sphenocephalus* | Terrestrial | 17 | 6 | 35.3 | 5099 ± 17735 | 3.0 ± 1.2 |
| Caudata | *Eurycea cirrigera* | Aquatic | 1 | 1 | 100 | 7.4 ± 0 | 0.92 |
| Caudata | *Eurycea guttolineata* | Terrestrial | 1 | 0 | 0 | 0 ± 0 | 0 |
| Caudata | *Notophthalmus viridescens* | Aquatic | 5 | 3 | 60 | 1272 ± 2188 | 2.7 ± 0.9 |

**Table 2:** Relationship between the host species sampled and the presence of *Bd* in natural and disturbed environments, where: N = Number of individuals, *Bd* + = *Bd*-positive individuals, Prev = Prevalence, % = Values in percentage, ± = Standard deviation, $\bar{x}$ = average of the values.

| Host | Natural environment | | | | Disturbed environment | | | |
|---|---|---|---|---|---|---|---|---|
| | N | *Bd* + | Prev (%) | *Bd* load (Log 10) | N | *Bd* + | Prev (%) | *Bd* load (Log 10) |
| *Ac. crepitans* | 65 | 27 | 41.5 | 1.3 ± 1.6 | 91 | 24 | 26.4 | 0.7 ± 1.3 |
| *Anaxyrus* sp. | 39 | 1 | 2.6 | 2.12 | 20 | 1 | 5 | 3.69 |
| *Aq. catesbaeiana* | 74 | 41 | 55.4 | 1.3 ± 1.3 | 46 | 12 | 26.1 | 0.5 ± 0.9 |
| *Aq. clamitans* | 62 | 9 | 14.5 | 0.3 ± 0.7 | 10 | 1 | 10 | 0.2 ± 0.6 |
| *D. chrysoscelis* | 6 | 0 | 0 | 0 | 14 | 0 | 0 | 0 |
| *D. cinereus* | 47 | 0 | 0 | 0 | 61 | 1 | 1.6 | 1.96 |
| *D. gratiosus* | 1 | 0 | 0 | 0 | 19 | 0 | 0 | 0 |
| *E. cirrigera* | 0 | 0 | - | - | 1 | 1 | 100 | 0.9 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *E. guttolineata* | 0 | 0 | - | - | 1 | 0 | 0 | 0 |
| *G. carolinensis* | 2 | 0 | 0 | - | 0 | 0 | - | - |
| *L. sphenocephalus* | 9 | 2 | 22.2 | 0.5 ± 1.05 | 8 | 4 | 50 | 1.7 ± 1.9 |
| *N. viridescens* | 5 | 3 | 60 | 1.6 ± 1.5 | 0 | 0 | - | - |
| **Total ($\bar{x}$)** | **302** | **(7.4)** | **(20.1)** | **(0.5)** | **303** | **(4.5)** | **(22)** | **(0.4)** |
| **±** | **-** | **13.6** | **23.4** | **0.6** | **-** | **7.3** | **30.3** | **0.5** |

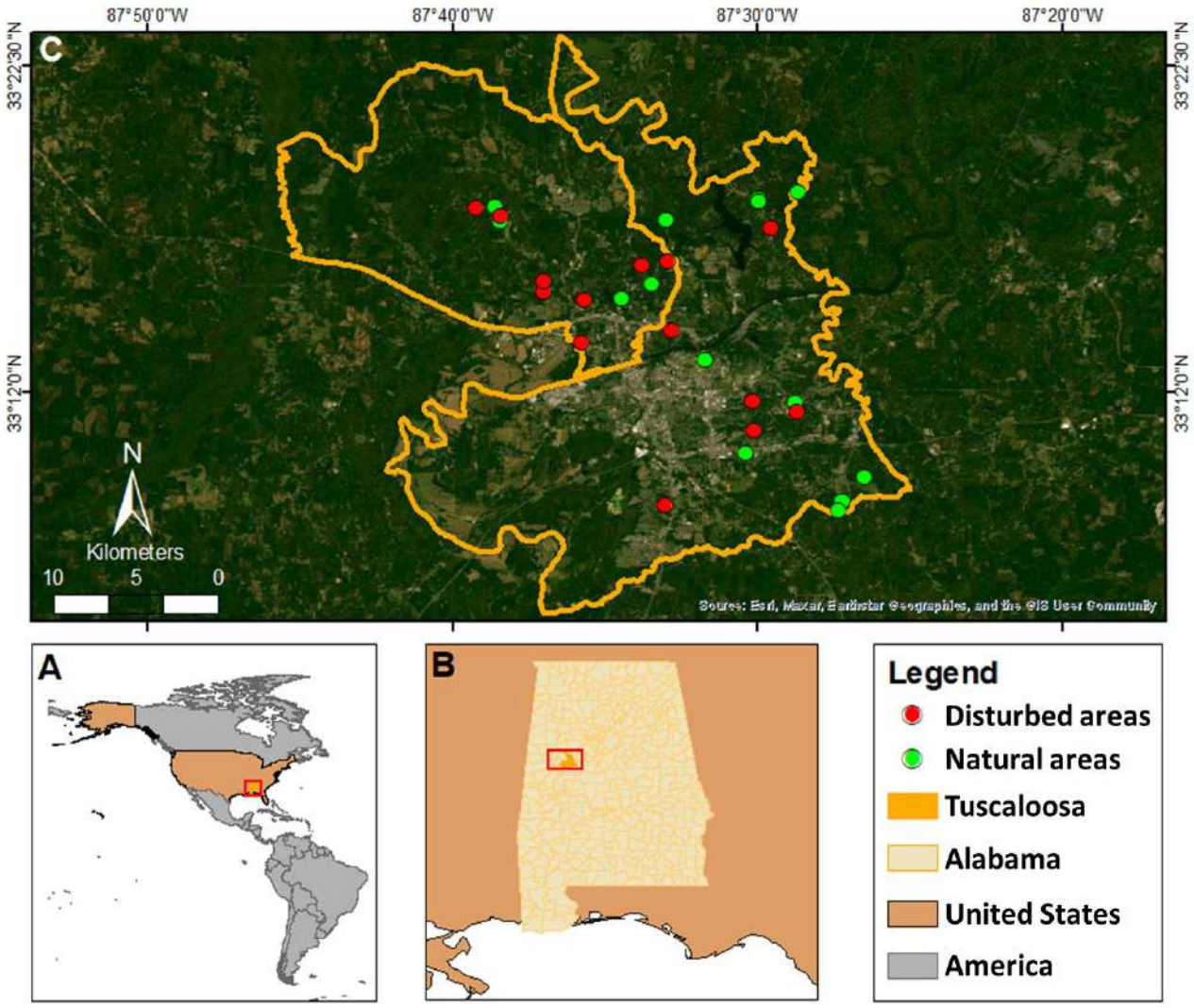


**Figure 1:** Location of sampled areas, where (A) is a map of the American continent highlighting the United States (light brown), (B) highlights the state of Alabama (light orange) and Tuscaloosa County (red line), and (C) highlights the boundaries of Tuscaloosa County (orange), with the points of the sampled ponds, where the red dots indicate areas with a high level of habitat disturbance, and green dots indicate areas with a low level of habitat disturbance. Map created by Gabriela Morais Olmedo.

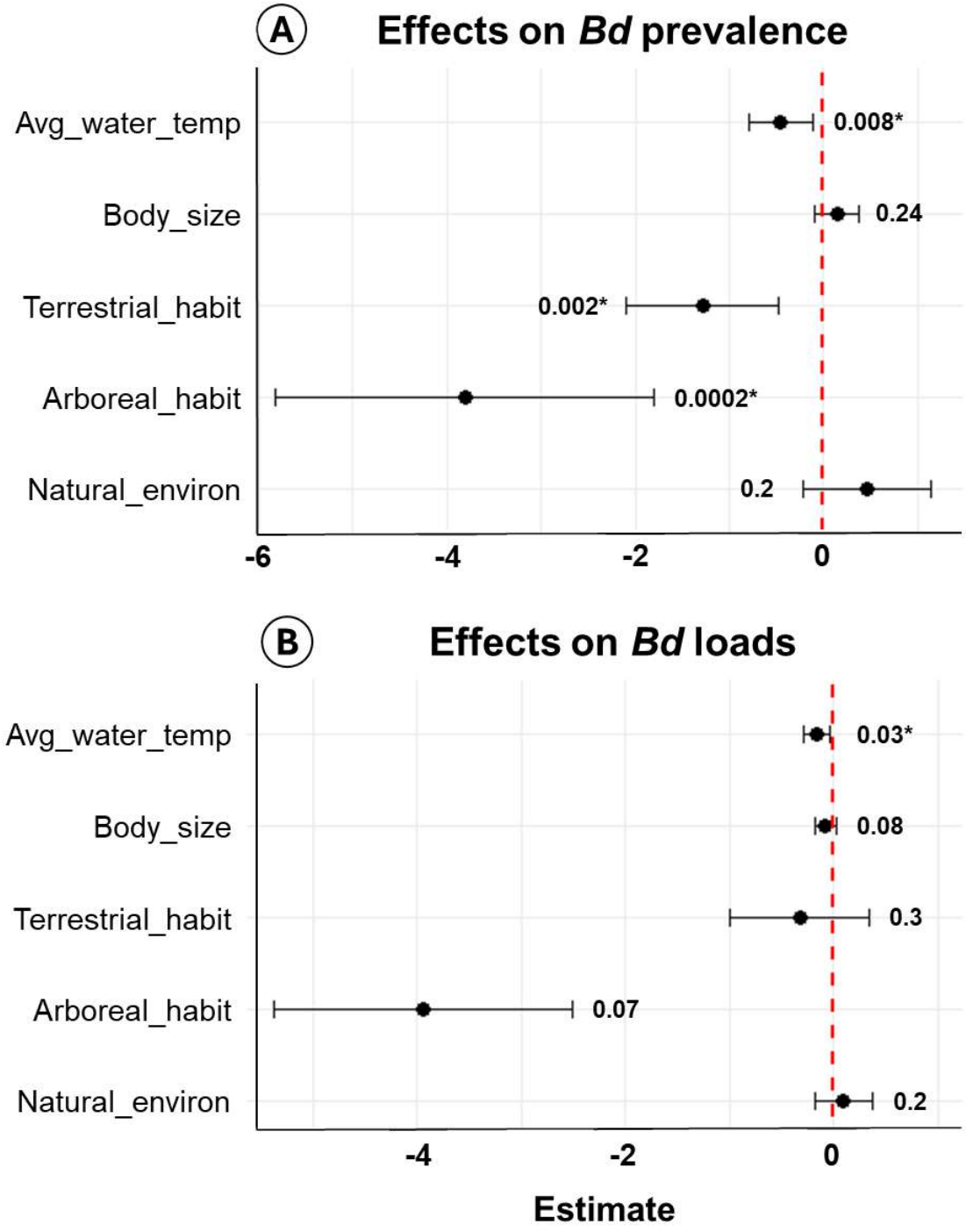


**Figure 2:** Environmental and ecological coefficients estimated by the best fitting GLMM on the effect on *Bd* prevalence (A) and *Bd* loads (Log 10) (B) in the evaluated amphibian community. The confidence interval is represented by the black bar. The *p* value is located to the right of each bar. The red dashed line indicates no effect (positive or negative). Asterisks (*) represent significant *p*-values.

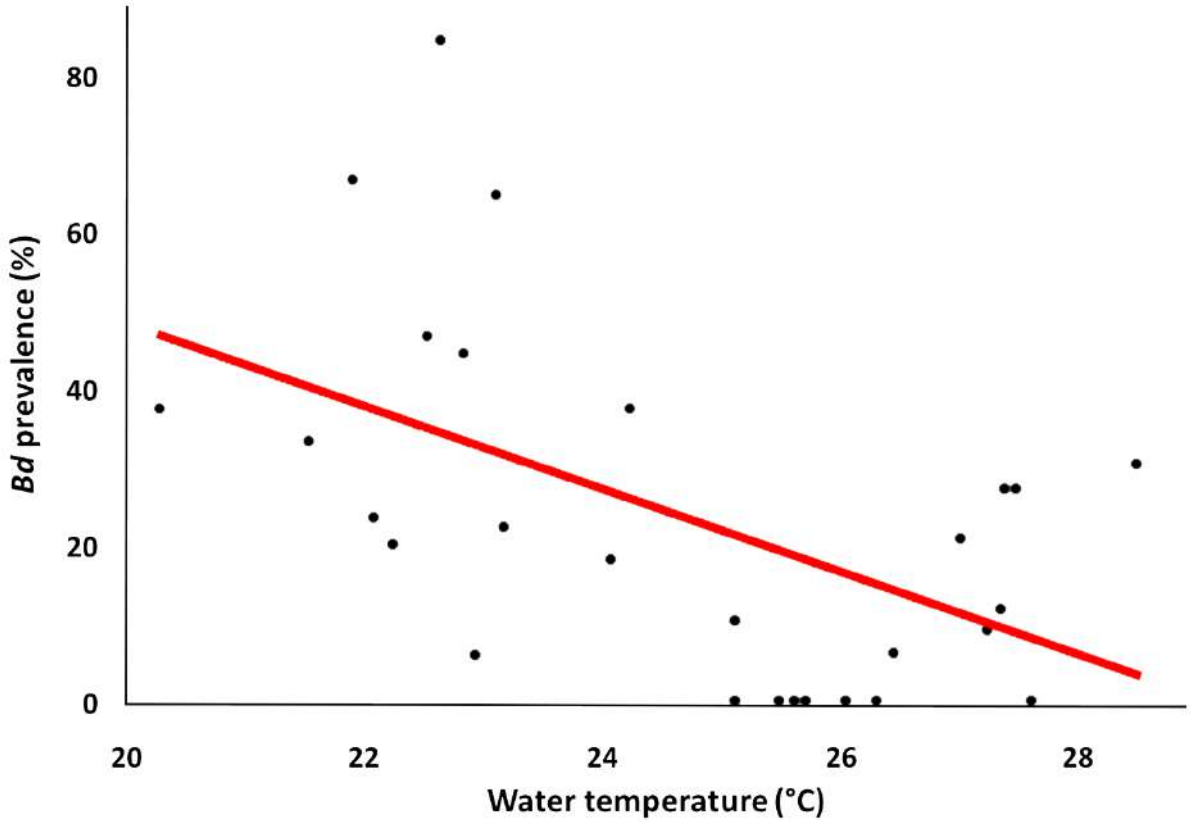


**Figure 3:** Correlation between *Bd* prevalence (%) and average water temperature (°C), where each point represents the *Bd* prevalence of each sampled site.

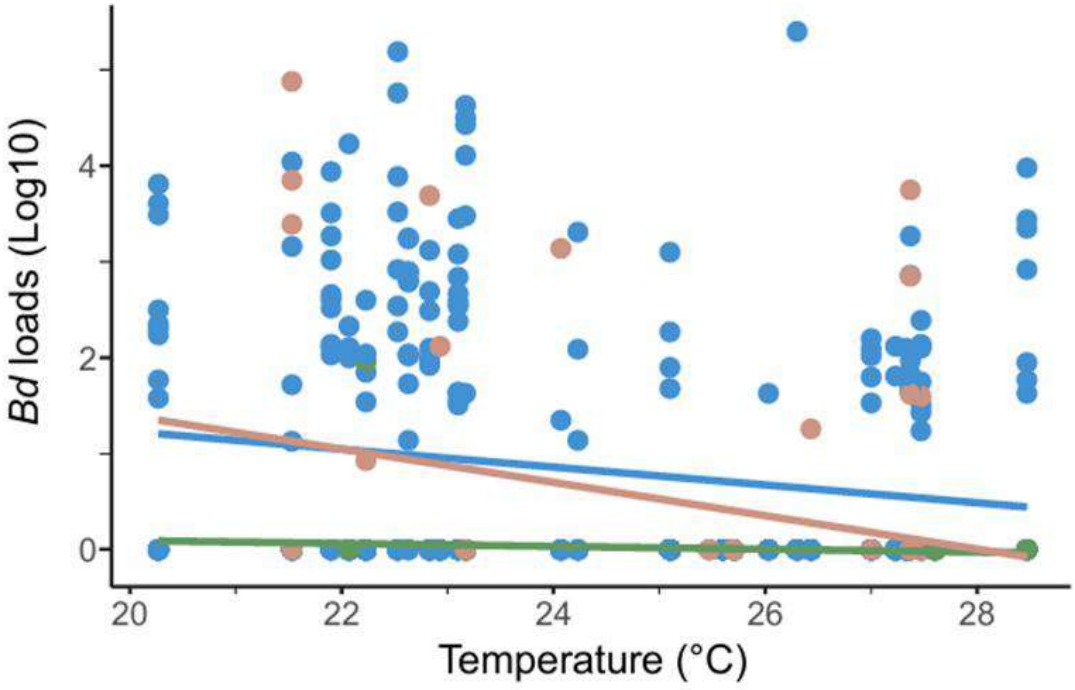

**Figure 4:** *Bd* loads in species with aquatic (blue), arboreal (green) and terrestrial (brown) habits and the influence of water temperature.

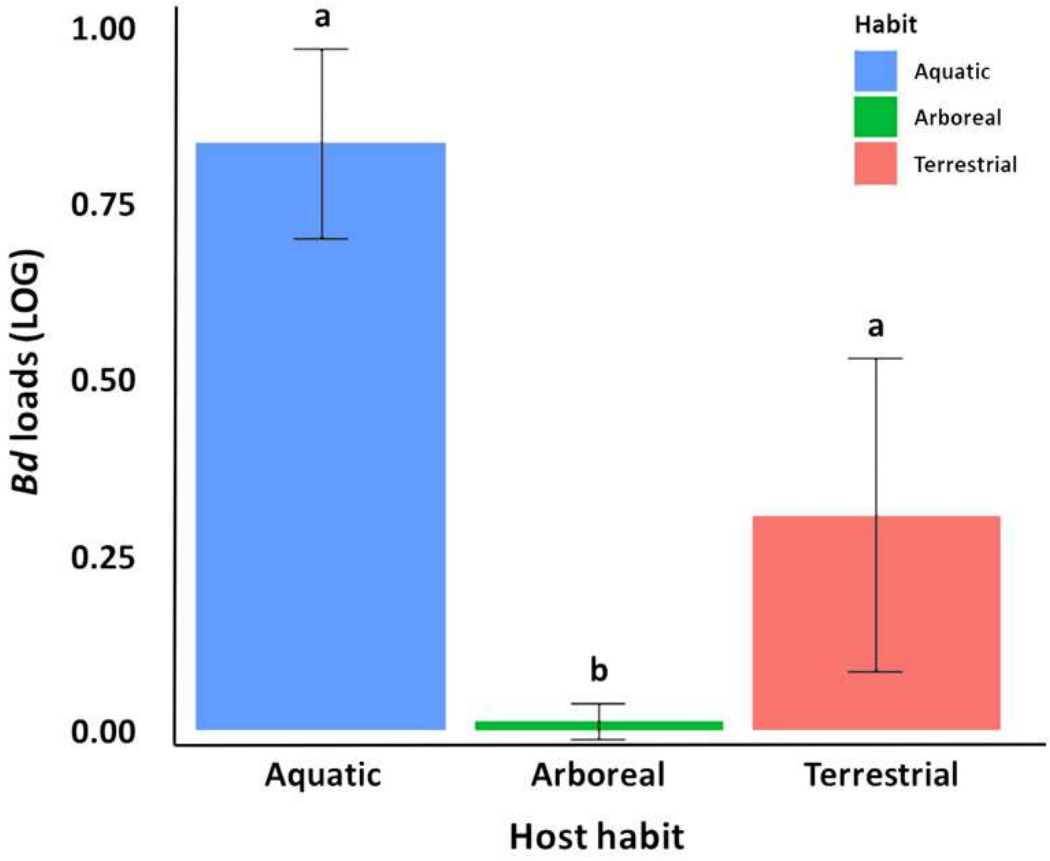


**Figure 5:** *Bd* loads (LOG) in species with aquatic (blue), arboreal (green) and terrestrial (brown) habits.  and the influence of water temperature. Different lowercase letters indicate significant difference between groups ($p < 0.05$).

## Supplementary material

**Table S1:** Geographic coordinates of each site sampled in this study, where: AWT = Average Water Temperature, *Bd* + = number of *Bd*-positive individuals, Bd - = number of *Bd*-negative individuals, Prev = *Bd* prevalence, % = percentage values.

| Code | Latitude | Longitude | AWT (°C) | Disturbed (%) | Natural (%) | *Bd* + | *Bd* - | Prev (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | 33,19499 | -87,50294 | 27.6 | 47.85 | 52.15 | 0 | 17 | 0 |
| 2 | 33,2879 | -87,49301 | 27 | 60.11 | 39.89 | 1 | 11 | 8.33 |
| 3 | 33,19468 | -87,4792 | 26 | 21.36 | 78.64 | 1 | 6 | 14.29 |
| 4 | 33,29932 | -87,64387 | 21.9 | 42.92 | 57.08 | 10 | 5 | 66.67 |
| 5 | 33,1893 | -87,47858 | 24.2 | 67.63 | 32.37 | 3 | 5 | 37.50 |
| 6 | 33,29471 | -87,64098 | 22.5 | 70.98 | 29.02 | 7 | 8 | 46.67 |
| 7 | 33,22635 | -87,59643 | 22.8 | 59.73 | 40.27 | 8 | 10 | 44.44 |
| 8 | 33,29132 | -87,64048 | 25.5 | 41.34 | 58.66 | 0 | 3 | 0 |
| 9 | 33,15444 | -87,44148 | 28.5 | 42.52 | 57.48 | 7 | 8 | 46.67 |
| 10 | 33,14181 | -87,45345 | 23.2 | 41.11 | 58.89 | 6 | 21 | 22.22 |
| 11 | 33,29879 | -87,65406 | 22.2 | 50.80 | 49.20 | 6 | 24 | 20 |
| 12 | 33,21735 | -87,52857 | 25.1 | 30.90 | 69.10 | 1 | 31 | 3.13 |
| 13 | 33,23289 | -87,54722 | 27.3 | 86.35 | 13.65 | 2 | 15 | 11.76 |
| 14 | 33,17963 | -87,50209 | 26.4 | 64.62 | 35.38 | 1 | 15 | 6.25 |
| 15 | 33,27034 | -87,54972 | 21.5 | 47.61 | 52.39 | 7 | 14 | 33.33 |
| 16 | 33,24999 | -87,57451 | 24.1 | 32.64 | 67.36 | 2 | 9 | 18.18 |
| 17 | 33,25402 | -87,61708 | 27.2 | 52.19 | 47.81 | 2 | 20 | 9.09 |
| 18 | 33,13665 | -87,45613 | 22.1 | 42.06 | 57.94 | 4 | 13 | 23.53 |
| 19 | 33,25918 | -87,61743 | - | 67.01 | 32.99 | 0 | 17 | 0 |
| 20 | 33,29209 | -87,5504 | 27.4 | 13.92 | 86.08 | 10 | 27 | 27.03 |
| 21 | 33,24927 | -87,59496 | 25.7 | 74.94 | 25.06 | 0 | 56 | 0 |
| 22 | 33,16711 | -87,50623 | 22.9 | 24.57 | 75.43 | 1 | 16 | 5.88 |
| 23 | 33,27034 | -87,54972 | 26.3 | 37.39 | 62.61 | 1 | 18 | 5.26 |
| 24 | 33,13943 | -87,55115 | 25.6 | 73.58 | 26.42 | 0 | 13 | 0 |
| 25 | 33,26777 | -87,56335 | 25.1 | 50.63 | 49.37 | 3 | 26 | 10.34 |
| 26 | 33,25811 | -87,55817 | 20.3 | 25.20 | 74.80 | 9 | 15 | 37.50 |
| 27 | 33,30361 | -87,49925 | 27.5 | 38.81 | 61.19 | 9 | 24 | 27.27 |
| 28 | 33,13943 | -87,55115 | 22.6 | 32.13 | 67.87 | 11 | 2 | 84.62 |
| 29 | 33,30225 | -87,49933 | 23.1 | 42.05 | 57.95 | 11 | 6 | 64.71 |
| 30 | 33,30752 | -87,47818 | - | 7.35 | 92.65 | 6 | 1 | 85.71 |

**Table S2:** Models generated from generalized linear mixed models (GLMM) analysis for the dependent variable *Bd* prevalence, where SVL = snout-vent length (host body size), Environmental type = disturbed or natural, Avg_Temp = average water temperature, Site = sampling area, AIC = Akaike Information Criterion, BIC = Bayesian Information Criterion, KS = Kolmogorov-Smirnov test, Dispersion = dispersion of simulated residuals based on Bray-Curtis similarity. Candidate model selected through AIC (best fit model) represented with an asterisk (*) next to the AIC value.

| Models | Formula | AIC | BIC | Deviance | Residual | KS | Dispersion |
|---|---|---|---|---|---|---|---|
| **1** | Prevalence ~ SVL + Host habit + Environmental type + Avg_Temp + (1\|Site) | 500* | 530.5 | 486 | 571 | $p = 0.55$ | $p = 0.804$ |
| **2** | Prevalence ~ Environmental type + Avg_Temp + SVL + (1\| Site) | 539.8 | 561.6 | 529.8 | 573 | $p = 0.12$ | $p = 0.505$ |
| **3** | Prevalence ~ Environmental type + Avg_Temp + (1\| Site) | 546.6 | 564 | 538.6 | 577 | $p = 0.08$ | $p = 0.824$ |
| **4** | Prevalence ~ Host habit + (1\| Site) | 506.8 | 524.3 | 498.8 | 577 | $p = 0.13$ | $p = 0.96$ |
| **5** | Prevalence ~ SVL + Host habit + (1\| Site) | 504.1 | 525.9 | 494.1 | 573 | $p = 0.65$ | $p = 0.956$ |
| **6** | Prevalence ~ Host habit + Environmental type + (1\| Site) | 507.4 | 529.2 | 497.4 | 576 | $p = 0.08$ | $p = 0.856$ |
| **7** | Prevalence ~ SVL + Host habit + Environmental type + (1\| Site) | 504.6 | 530.7 | 492.6 | 572 | $p = 0.80$ | $p = 0.92$ |
| **8** | Prevalence ~ SVL + (1\| Site) | 545.6 | 558.6 | 539.6 | 575 | $p = 0.096$ | $p = 0.668$ |

**Table S3:** Models generated from generalized linear mixed models (GLMM) analysis for the dependent variable *Bd* loads (LOG), where SVL (LOG) = snout-vent length (host body size), Environmental type = disturbed or natural, Avg_Temp = average water temperature, Site = sampling area, AIC = Akaike Information Criterion, BIC = Bayesian Information Criterion, KS = Kolmogorov-Smirnov test, Dispersion = dispersion of simulated residuals based on Bray-Curtis similarity. Candidate model selected through AIC (best fit model) represented with an asterisk (*) next to the AIC value.

| Models | Formula | AIC | BIC | Deviance | Residual | KS | Dispersion |
|---|---|---|---|---|---|---|---|
| 1 | Bd_loads ~ SVL + Host habit + Environmental type + Avg_Temp + (1\|Site) | 891.9* | 939.9 | 869.9 | 567 | $p = 0.14$ | $p = 0.18$ |
| 2 | Bd_loads ~ Environmental type + Avg_Temp + SVL + (1\| Site) | 1033.6 | 1059.7 | 1021.6 | 572 | $p = 0.69$ | $p = 0.07$ |
| 3 | Bd_loads ~ Environmental type + Avg_Temp + (1\| Site) | 1040.6 | 1062.4 | 1030.6 | 576 | $p = 0.68$ | $p = 0.08$ |
| 4 | Bd_loads ~ Host habit + (1\| Site) | 944.1 | 979.3 | 928.1 | 597 | $p = 0.38$ | $p = 0.17$ |
| 5 | Bd_loads ~ SVL + Host habit + (1\| Site) | 937.1 | 976.7 | 919.1 | 593 | $p = 0.83$ | $p = 0.12$ |
| 6 | Bd_loads ~ Host habit + Environmental type + (1\| Site) | 945.2 | 984.9 | 927.2 | 596 | $p = 0.34$ | $p = 0.17$ |
| 7 | Bd_loads ~ SVL + Host habit + Environmental type + (1\| Site) | 937.9 | 981.9 | 917.9 | 592 | $p = 0.73$ | $p = 0.14$ |
| 8 | Bd_loads ~ SVL + (1\| Site) | 1090.4 | 1108.0 | 1082.4 | 598 | $p = 0.00$ | $p = 0.948$ |

**Table S4:** Results of generalized linear mixed models (GLMM) analyzes for the dependent variable *Bd* prevalence, where SVL = snout-vent length (host body size), Environmental type = disturbed or natural, Avg_Temp = average water temperature, Site = sampling area, AIC = Akaike Information Criterion, BIC = Bayesian Information Criterion, df = degrees of freedom. Asterisks (*) indicate significant values ($p < 0.05$).

| **Formula =** Prevalence ~ SVL + Host habit + Environmental type + Avg_Temp + (1\|Site) | | | | |
|---|---|---|---|---|
| **AIC** = 500.0, **BIC** = 530.5, **logLik** = -243.0, **deviance** = 486.0, **df** = 571 | | | | |
| | **Slope** | **Std. Error** | **z value** | **Pr(>\|z\|)** |
| **(Intercept)** | -1.2010 | 0.2775 | -4.329 | < 0.0001* |
| **SVL** | 0.1420 | 0.1198 | 1.185 | 0.008* |
| **Terrestrial habit** | -1.2874 | 0.4102 | -3.138 | 0.0017* |
| **Arboreal habit** | -3.8102 | 1.0244 | -3.719 | 0.0002* |
| **Habitat natural** | 0.4600 | 0.3492 | 1.317 | 0.1878 |
| **Avg_Temp** | -0.4576 | 0.1728 | -2.648 | 0.0081 |

**Table S5:** Results of generalized linear mixed models (GLMM) analyzes for the dependent variable *Bd* loads (LOG), where SVL = snout-vent length (host body size), Environmental type = disturbed or natural, Avg_Temp = average water temperature, Site = sampling area, AIC = Akaike Information Criterion, BIC = Bayesian Information Criterion, df = degrees of freedom. Asterisks (*) indicate significant values ($p < 0.05$).

| **Formula =** Bd_LOG ~ Host habit + Disturbed.or.Natural + Avg_Temp + (1\|Site) | | | | |
|---|---|---|---|---|
| **AIC** = 500.0, **BIC** = 530.5, **logLik** = -243.0, **deviance** = 486.0, **df** = 571 | | | | |
| | **Slope** | **Std. Error** | **z value** | **Pr(>\|z\|)** |
| **(Intercept)** | 2.217670 | 0.637754 | 3.477 | 0.0005* |
| **SVL** | -0.003093 | 0.001797 | -1.722 | 0.0851 |
| **Terrestrial habit** | 0.255593 | 0.245201 | 1.042 | 0.297 |
| **Arboreal habit** | -0.400278 | 0.966625 | -0.414 | 0.679 |
| **Habitat natural** | 0.184978 | 0.145346 | 1.273 | 0.203 |
| **Avg_Temp** | -0.058017 | 0.027320 | -2.124 | 0.034* |

**Table S6:** Results of ANOVA test for the dependent variable *Bd* prevalence, where SVL = snout-vent length (host body size), Environmental type = disturbed or natural, Avg_Temp = average water temperature, df = degrees of freedom. Asterisks (*) indicate significant values ($p < 0.05$).

| **Variables** | **Chisq** | **df** | **Pr(>Chisq)** |
|---|---|---|---|
| **SVL** | 1.4050 | 1 | 0.235884 |

| | | | |
|---|---|---|---|
| **Host habit** | 22.5051 | 2 | 1.297e-05* |
| **Avg_Temp** | 7.0108 | 1 | 0.008102* |
| **Environmental type** | 1.7351 | 1 | 0.187761 |

**Table S7:** Results of the post-hoc test for the dependent variable *Bd* prevalence between host habits. SE = standard error, z ratio = test statistic (Estimate / SE). Asterisks (*) indicate significant values ($p < 0.05$).

| Host habit | Estimate | SE | z ratio | p value |
|---|---|---|---|---|
| **Aquatic - Arboreal** | 3.81 | 1.02 | 3.719 | 0.0006* |
| **Aquatic - Terrestrial** | 1.29 | 0.41 | 3.138 | 0.0048* |
| **Arboreal - Terrestrial** | -2.52 | 1.08 | -2.329 | 0.0518 |

**Table S8:** Results of ANOVA test for the dependent variable *Bd* loads, where SVL = snout-vent length (host body size), Environmental type = disturbed or natural, Avg_Temp = average water temperature, df = degrees of freedom. Asterisks (*) indicate significant values ($p < 0.05$).

| Variables | Chisq | df | Pr(>Chisq) |
|---|---|---|---|
| **SVL** | 2.9756 | 1 | 0.08453 |
| **Host habit** | 1.3052 | 2 | 0.52068 |
| **Avg_Temp** | 4.5081 | 1 | 0.03373* |
| **Environmental type** | 1.6314 | 1 | 0.20151 |

**Table S9:** Results of the post-hoc test for the dependent variable *Bd* loads between host habits. SE = standard error, z ratio = test statistic (Estimate / SE). Asterisks (*) indicate significant values ($p < 0.05$).

| Host habit | Estimate | SE | z ratio | p value |
|---|---|---|---|---|
| **Aquatic - Arboreal** | 3.94 | 0.732 | 5.377 | <.0001* |
| **Aquatic - Terrestrial** | 0.32 | 0.340 | 0.940 | 0.6148 |
| **Arboreal - Terrestrial** | -3.62 | 0.792 | -4.566 | <.0001* |

## CONCLUSÕES GERAIS

Ao longo dos quatro capítulos desta tese, foram avaliados diversos aspectos relacionados à microbiota cutânea em anfíbios. A análise da microbiota cutânea dos anfíbios revela a complexidade e a diversidade das comunidades microbianas associadas a essas espécies, com importantes implicações para a saúde e resistência a patógenos. A composição microbiana é moldada por diversos fatores, incluindo a ecologia, a história de vida e as características comportamentais das espécies, além de influências ambientais e sazonais. A presença de bactérias com propriedades antifúngicas, como membros da família Comamonadaceae, do gênero *Pseudomonas* e da espécie *Stenotrophomonas rhizophila*, pode oferecer proteção contra patógenos, como o fungo *Batrachochytrium dendrobatidis* (*Bd*). No entanto, as interações entre os microrganismos e seus hospedeiros são altamente específicas, e diferenças na diversidade microbiológica podem ser observadas entre espécies e estações do ano, refletindo adaptações aos desafios ambientais sazonais.

Além disso, através de um estudo de caso e a comparativas com a literatura atual, foi demonstrado que fatores abióticos e bióticos, como temperatura da água e hábitos ecológicos dos anfíbios, também influenciam a dinâmica da infecção por *Bd*. A menor prevalência e carga de *Bd* em temperaturas mais altas e em espécies não aquáticas sugere que variações ambientais podem afetar a distribuição do patógeno e a suscetibilidade dos hospedeiros. Essas descobertas têm implicações importantes para compreender os impactos potenciais das mudanças climáticas e das alterações de habitat nas interações entre *Bd* e seus hospedeiros.

O impacto das atividades antropogênicas sobre as comunidades microbianas dos anfíbios também foi analisado, destacando-se a diminuição da diversidade microbiana em ambientes perturbados, especialmente devido à exposição a agentes químicos. A exposição a metais pesados e agroquímicos tem sido associada a um aumento da abundância de bactérias potencialmente prejudiciais, como as do filo Proteobacteria. Esses distúrbios podem afetar negativamente a saúde dos anuros, comprometendo seu sistema imunológico, crescimento e desenvolvimento, além de aumentar a suscetibilidade a doenças.

A necessidade de monitoramento contínuo da presença e da dinâmica do *Bd* em diferentes ecossistemas e espécies de anfíbios torna-se evidente para informar estratégias de conservação e mitigar o risco de declínio populacional causado por esse patógeno. Pesquisas futuras devem focar nas consequências de longo prazo dessas variações ambientais, investigando como os anfíbios podem desenvolver estratégias para lidar com a infecção. Além disso, estudos sobre a microbiota cutânea de girinos e o microbioma intestinal de anuros adultos são essenciais para um entendimento mais

abrangente da interação hospedeiro-patógeno e para o desenvolvimento de abordagens de conservação baseadas em microbiomas.

## ANEXOS

**Anexo 1** - Normas da *Environmental Research*, na qual foi encaminhado para publicação o capítulo I dessa Tese.

### WRITING AND FORMATTING

#### File format

We ask you to provide editable source files for your entire submission (including figures, tables and text graphics). Some guidelines:

- Save files in an editable format, using the extension .doc/.docx for Word files and .tex for LaTeX files. A PDF is not an acceptable source file.
- Lay out text in a single-column format.
- Remove any strikethrough and underlined text from your manuscript, unless it has scientific significance related to your article.
- Use spell-check and grammar-check functions to avoid errors.

#### Title page

You are required to include the following details in the title page information:

- Article title. Article titles should be concise and informative. Please avoid abbreviations and formulae, where possible, unless they are established and widely understood, e.g., DNA).
- Author names. Provide the given name(s) and family name(s) of each author. The order of authors should match the order in the submission system. Carefully check that all names are accurately spelled. If needed, you can add your name between parentheses in your own script after the English transliteration.
- Affiliations. Add affiliation addresses, referring to where the work was carried out, below the author names. Indicate affiliations using a lower-case superscript letter immediately after the author's name and in front of the corresponding address. Ensure that you provide the full postal address of each affiliation, including the country name and, if available, the email address of each author.
- Corresponding author. Clearly indicate who will handle correspondence for your article at all stages of the refereeing and publication process and also post-publication. This responsibility includes answering any future queries about your results, data, methodology and materials. It

is important that the email address and contact details of your corresponding author are kept up to date during the submission and publication process.

- Present/permanent address. If an author has moved since the work described in your article was carried out, or the author was visiting during that time, a "present address" (or "permanent address") can be indicated by a footnote to the author's name. The address where the author carried out the work must be retained as their main affiliation address. Use superscript Arabic numerals for such footnotes.

**Abstract**

You are required to provide a concise and factual abstract which does not exceed 250 words. The abstract should briefly state the purpose of your research, principal results and major conclusions. Some guidelines:

- Abstracts must be able to stand alone as abstracts are often presented separately from the article.
- Avoid references. If any are essential to include, ensure that you cite the author(s) and year(s).
- Avoid non-standard or uncommon abbreviations. If any are essential to include, ensure they are defined within your abstract at first mention.

**Keywords**

You are required to provide 1 to 7 keywords for indexing purposes. Keywords should be written in English. Please try to avoid keywords consisting of multiple words (using "and" or "of").

We recommend that you only use abbreviations in keywords if they are firmly established in the field.

**Tables**

Tables must be submitted as editable text, not as images. Some guidelines:

- Place tables next to the relevant text or on a separate page(s) at the end of your article.
- Cite all tables in the manuscript text.
- Number tables consecutively according to their appearance in the text.
- Please provide captions along with the tables.
- Place any table notes below the table body.
- Avoid vertical rules and shading within table cells.

We recommend that you use tables sparingly, ensuring that any data presented in tables is not duplicating results described elsewhere in the article.

**Figures, images and artwork**

Figures, images, artwork, diagrams and other graphical media must be supplied as separate files along with the manuscript. We recommend that you read our detailed artwork and media instructions. Some excerpts:

When submitting artwork:

- Cite all images in the manuscript text.
- Number images according to the sequence they appear within your article.
- Submit each image as a separate file using a logical naming convention for your files (for example, Figure_1, Figure_2 etc).
- Please provide captions for all figures, images, and artwork.
- Text graphics may be embedded in the text at the appropriate position. If you are working with LaTeX, text graphics may also be embedded in the file.

**Figure captions**

All images must have a caption. A caption should consist of a brief title (not displayed on the figure itself) and a description of the image. We advise you to keep the amount of text in any image to a minimum, though any symbols and abbreviations used should be explained.

Provide captions in a separate file.

**Supplementary material**

We encourage the use of supplementary materials such as applications, images and sound clips to enhance research. Some guidelines:

- Cite all supplementary files in the manuscript text.
- Submit supplementary materials at the same time as your article. Be aware that all supplementary materials provided will appear online in the exact same file type as received. These files will not be formatted or typeset by the production team.
- Include a concise, descriptive caption for each supplementary file describing its content.
- Provide updated files if at any stage of the publication process you wish to make changes to submitted supplementary materials.

- Do not make annotations or corrections to a previous version of a supplementary file.
- Switch off the option to track changes in Microsoft Office files. If tracked changes are left on, they will appear in your published version.

## ARTICLE STRUCTURE

### Article sections

- Divide your article into clearly defined and numbered sections. Number subsections 1.1 (then 1.1.1, 1.1.2, ...), then 1.2, etc.
- Use the numbering format when cross-referencing within your article. Do not just refer to "the text."
- You may give subsections a brief heading. Headings should appear on a separate line.
- Do not include the article abstract within section numbering.

### Glossary

Please provide definitions of field-specific terms used in your article, in a separate list.

### Acknowledgements

Include any individuals who provided you with help during your research, such as help with language, writing or proof reading, in the acknowledgements section. Acknowledgements should be placed in a separate section which appears directly before the reference list. Do not include acknowledgements on your title page, as a footnote to your title, or anywhere else in your article other than in the separate acknowledgements section.

## REFERENCES

### References within text

Any references cited within your article should also be present in your reference list and vice versa. Some guidelines:

- References cited in your abstract must be given in full.
- We recommend that you do not include unpublished results and personal communications in your reference list, though you may mention them in the text of your article.
- Any unpublished results and personal communications included in your reference list must follow the standard reference style of the journal. In substitution of the publication date add "unpublished results" or "personal communication."

- References cited as "in press" imply that the item has been accepted for publication.

**Reference format**

This journal does not set strict requirements on reference formatting at submission. Some guidelines:

- References can be in any style or format as long as the style is consistent.
- Author names, journal or book titles, chapter or article titles, year of publication, volume numbers, article numbers or pagination must be included, where applicable.
- Use of DOIs is recommended.

Our journal reference style will be applied to your article after acceptance, at proof stage. If required, at this stage we will ask you to correct or supply any missing reference data.

**Reference style**

All citations in the text should refer to:

- Single author: the author's name (without initials, unless there is ambiguity) and the year of publication.
- Two authors: both authors' names and the year of publication.
- Three or more authors: first author's name followed by 'et al.' and the year of publication.

Citations can be made directly (or parenthetically). Groups of references can be listed either first alphabetically, then chronologically, or vice versa. Examples: "as demonstrated (Allan, 2020a, 2020b; Allan and Jones, 2019)" or "as demonstrated (Jones, 2019; Allan, 2020). Kramer et al. (2023) have recently shown".

The list of references should be arranged alphabetically and then chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

**Anexo 2** - Normas da *Biotropica*, na qual foi encaminhado para publicação o capítulo II dessa Tese.

**Manuscript Format**

Use 8.5" x 11" page size (letter size) with a 1" margin on all sides. Align left and do not justify the right margin. Number all pages starting with the title page and include continuous line numbers.

Double space throughout the manuscript, including tables, figures and title legends, abstract, and literature cited

Use Times New Roman 12-point font throughout except in figures, for which Arial is preferred.

Use the abbreviations provided in Section D (below) throughout the text.

**Assembling your Manuscript:**

**Assemble manuscripts in this order:**

1. Title page
2. Abstract (s)
3. Keywords
4. Text
5. Tables
6. Figure legends
7. Figures
8. Acknowledgments
9. Disclosure Statements
10. References
11. Supplementary Information (to be supplied as separate files)

**1. TITLE PAGE**

**Running Heads:** The authors' family name should be included as left and right running heads. It is set in small caps. The format is as follows:

LRH and RRH: YAZ and PEIGH

(may not exceed 50 characters, two or more authors use YAZ et al.)

**Title:** No more than 12 words (usually), flush left, near the middle of the page. Use Bold Type.

Where species names are given in the title, it should be clear to general readers what type(s) of organism(s) are being referred to, either by using Family appellation or common name:

'Invasion of African savanna woodlands by the Jellyfish tree *Medusagyne oppositifolia*', OR 'Invasion of African savanna woodlands by *Medusagyne oppositifolia* (Medusagynaceae)'

Titles that include a geographic locality should make sure that this is clear to the general reader:

'Effect of habitat fragmentation on pollination networks on Flores, Indonesia', NOT

'Effect of habitat fragmentation and pollination networks on Flores'.

**Authors:** Below title, include the author(s) full name(s), affiliation(s), and unabbreviated complete address(es). Use superscript number(s) following author(s) name(s) to indicate present address(s) if different than above. In multi-authored papers, additional footnote superscripts may be used to indicate the corresponding author and e-mail address. Although geographical place names should use the English spelling in the text (e.g., Zurich, Florence, Brazil), authors may use their preferred spelling when listing their affiliation (e.g., Zürich, Firenze, Brasil).

**Submission and Acceptance Dates:** At the bottom of the title page every article must include: Received: _____; Revised:____(optional); Accepted:_____. (*Biotropica* will fill in the dates.)

**2. ABSTRACT PAGE**

Abstracts have maximum of 250 words for papers and reviews and 50 words for Insights. There is no abstract for Commentary papers.

The Abstract should include brief statements about the intent or purpose, materials and methods, results, and significance of findings. Abstract can be given as multiple paragraphs (with subheadings such as Aim, Methods, Results, and Conclusion) or as a single paragraph. Do not use abbreviations in the abstract.

Publication must be in English, but a second abstract in other languages (such as Spanish, French, Portuguese, Hindi, Arabic, Chinese etc.) may be published as online Supporting Information.

**3. KEYWORDS**

Provide up to eight keywords after the abstract, separated by a comma (,). Keywords should be in English (with the exception of taxonomic information) and listed alphabetically.

Include the location of the study as a key word if it is not already mentioned in the title (see example below). Key words should *not* repeat words used in the title. Avoid words that are too

broad or too specific. (e.g., keywords: Melastomataceae, *Miconia argentea*, Panama, seed dispersal, tropical wet forest).

**4. TEXT**

**Headings**

Main headings are 1. INTRODUCTION, 2. METHODS, 3. RESULTS, and 4. DISCUSSION in bold, capital letters, numbered, and flush left.

Indent all but the first paragraph of each section.

Leave one blank between main heading and text.

Second level headings should be in Initial caps, bold, numbered, and flush left. (e.g., 2. Inventory technique.)

First three headings are numbered and fourth and fifth order headings are unnumbered.

Insights submissions do not use any subject headings.

When using previously published data in analyses please cite both the data archive(s) and the original manuscript(s) for which they were collected in the text: “We used previously archived data (Bruna et al., 2011a,b) in our simulations.”, where a is the data archive and b is the publication. Be sure both citations are included in the literature cited.

Do not use footnotes in the main text.

Refer to figures as ‘Figure 1’, and tables as ‘Table 1’. Reference to online Supporting Information is referred to as ‘Figure S1’ or ‘Table S1’.

**Units, Abbreviations, and style**

**Abbreviations:** year(s), month(s), week(s), day(s), hr, min, s, km, cm, mm, ha, kg, g, L, g/m2

**Units:** Use solidus style for simple units (e.g., m/s) and follow negative indices style for compound units (e.g., nmol · $hr^{-1}$ · $mg^{-1}$)

Write out other abbreviations the first time they are used in the text and abbreviate thereafter: "El Niño Southern Oscillation (ENSO) . . ."

**Numbers:** Write out one to 9 unless a measurement or in combination with other numbers: four trees, 6 mm, 35 sites, 7 year, 10 × 5 m, 7 m, ± *SE*, 5 bees and 12 wasps).

Use a comma as a separator in numbers with four or more digits: 1,000 vs. 10,000

**Decimals:** 0.13 (leading zero and points, never commas)

**Temperature:** 21°C (no space after the degree symbol)

Use dashes to indicate a set location of a given size (*e.g.,* 1-ha plot).

Spell out ‘percent’ when used at the beginning of a sentence and use symbols when used in number combinations (e.g., “there was a 5% increase…”, “plants were grown at high light levels (20%)…”, 95% CI.)

**Statistical abbreviations:**

- Use italics for *P, N, t, F, R2, r, G, U, N,* $\chi^2$ (italics, superscripts non-italics)
- Use italic for: *df, SD, SE, SEM*
- Use roman for CI, two-way ANOVA, ns

**Dates:** 10 December 1997

**Times:** 0930 h, 2130 h

**Latitude and Longitude:** 10°34′21″ N, 14°26′12″ W

**Above sea level:** a.s.l.

**5. TABLES**

While Biotropica does have word limits that differ by manuscript category, there are not have strict limits on the number of tables and/or figures. However, printed manuscripts rarely exceed 32 pages in length, and we encourage authors to submit only necessary tables and figures. Additional information, figures, and tables should appear in the Supporting Information."

Each table must start on a separate page

Number tables with Arabic numerals followed by a period. Capitalize ‘Table’ (e.g., Table 1, Table 2, etc.).

Indicate footnotes by lowercase superscript letters

Do not use vertical lines in tables.

**6. FIGURE LEGENDS (Continue page numbering)**

Type figure legends in paragraph form, starting with ‘Figure’ and number.

Do not include symbols (lines, dots, triangles, etc.) in figure legends; either label them in the figure or refer to them by name in the legend.

Label multiple plots/images within one figure as a, b, c etc., and please ensure the panels of each plot include these labels and are referred to in the legend (e.g., Figure 1 Fitness

of *Medusagyne oppositifolia* as indicated by (a) seed set and (b) seed viability', making sure to include the labels in the relevant plot.)

**7. FIGURES**

Please make sure figures are accessible by following our Figures Guidelines.

Please consult Wiley Author Services' figures and illustrations guide (PDF) for more detailed information about submitting electronic artwork. Authors are encouraged to utilize online Supporting Information for tables and figures that do not have central importance to the manuscript.

All figures and photographs are referred to as 'Figures' in the text.

**8. ACKNOWLEDGEMENTS**

Authors are encouraged to acknowledge funding, general supervision of the research group, or general support, in addition to any writing assistance, technical editing, language editing, and proofreading provided outside of the typical production process. Where applicable, please identify the relevant authorities granting permission for the research and provide research, collecting, and export permit numbers in this section

**Anexo 3 -** Normas da *Environmental Research Communications*, na qual foi encaminhado para publicação o capítulo III dessa Tese.

**Article format and templates**

You can format your paper in the way that you choose! It is not necessary to try to produce pages that look like published journal pages, as the detailed design (typesetting) work will be undertaken by IOP as part of the production process.

If you would prefer to work from a template, we do provide this for both LaTeX and Word.

LaTeX template
Word template

When submitting a new article, we only require you to upload a single PDF file (and any relevant supplementary data). Check the peer review model for the journal you are submitting to. If the peer review model is single-anonymous then your PDF will need to contain the names and institutes of authors at the start of the text. Figures and tables also need to be included within the text. If double-anonymous then you will need to anonymise your manuscript.

We do ask that you consider the readability for reviewers when formatting your manuscript. For example, please use a reasonable font size (at least 12 point) and line spacing. There is no need for you to include line numbers in your manuscript as these will automatically be added on submission. Figures and tables should be embedded at the appropriate point within the text, rather than placed at the end of the manuscript. Papers must be written in English.

When writing your article, please only use Roman characters and do not include Chinese, Japanese or Korean characters in the body of the manuscript, including the reference list. Chinese, Japanese or Korean characters are only permitted in the author list.

**Article structure**

You should consider the best way to structure your article before you begin writing. If you wish to use a LaTeX template to format your manuscript (this is optional, you are not obliged to do so) then the files are available in zipped format and Unix tar gzipped format here. Your article should follow the Introduction, Methods, Results and Discussion system, and usually consist of the following sections:

**Title**

The title should be concise, informative and meaningful to the whole readership of the journal. It should include key terms, to help make it more discoverable when people search online. Please

avoid the use of long systemic names and non-standard or obscure abbreviations, acronyms or symbols.

**Authors**

Check the peer review model for the journal you are submitting to when preparing the PDF version of your manuscript. If double-anonymous then you will need to anonymise your manuscript.
If single-anonymous then you need to list all authors' full names and institutions. Authors in all IOP journals have the option to include names in Chinese, Japanese or Korean characters in addition to the English name. The names will be displayed in parentheses after the English name. During the submission process, we recommend you supply ORCID identifiers for all authors to avoid ambiguity. If an author's current address is different from the address where the work was carried out, this should be explained in a footnote or acknowledgement. We encourage authors to make specific attributions of contribution and responsibility in the acknowledgements of the article, otherwise all co-authors will be taken to share full responsibility for all of the paper. Authors may wish to use a taxonomy such as CRediT to describe the contributions of each author. More guidance on authorship, including the responsibilities of the corresponding author, can be found here.

**Keywords**

When you submit an article, you will be asked to supply some keywords relevant to your work. If your article is accepted for publication, we will display these keywords on the published article, and they will be used to index your article, helping to make it more discoverable. When choosing keywords, think about the kinds of terms you would use when searching online for related articles.

**Abstract**

Your abstract should give readers a brief summary of your article. It should concisely describe the contents of your article, and include key terms (especially in the first two sentences, to increase search engine discoverability). It should be informative, accessible and not only indicate the general aims and scope of the article, but also state the methodology used, main results obtained and conclusions drawn. The abstract should be complete in itself; it should not contain undefined acronyms/abbreviations and no table numbers, figure numbers, references or equations should be referred to. Articles relying on clinical trials should quote the trial registration number at the end of the abstract. The abstract should be suitable for direct inclusion in abstracting services and should not normally be more than 300 words. If you submit an article with an abstract longer than 300 words, we may rescind the manuscript and ask you to re-write it. Some journals ask for abstracts to follow a particular structure. Check the instructions for specific journals to see if you need to submit a structured abstract.

**Introduction**

This should be concise and describe the nature of the problem under investigation and its background. It should also set your work in the context of previous research, citing relevant references. Introductions should expand on highly specialised terms and abbreviations used in the article to make it accessible for readers.

**Method**

This section should provide sufficient details of the experiment, simulation, statistical test or analysis carried out to generate the results such that the method can be repeated by another researcher and the results reproduced.

**Results**

The results section should detail the main findings and outcomes of your study. You should use tables only to improve conciseness or where the information cannot be given satisfactorily in other ways such as histograms or graphs. Colour should not be used in tables, if you need to denote different things in a table then you can use bold or italics etc. providing no coloured text or shading is included. Tables should be numbered serially and referred to in the text by number (table 1, etc.). Each table should have an explanatory caption which should be as concise as possible.

**Discussion**

This should discuss the significance of the results and compare them with previous work using relevant references.

**Conclusion**

This section should be used to highlight the novelty and significance of the work, and any plans for future relevant work.

Acknowledgements

Check the peer review model for the journal you are submitting to when preparing the PDF version of your manuscript. If double-anonymous then do not include any author names or institution information in the Acknowledgements section of your manuscript. Author names and Funding information should be removed and can be re-added later in the peer review process. For single-anonymous please include an acknowledgements section before the References section in your PDF manuscript.

During the submission process all authors and co-authors are required to disclose any potential conflict(s) of interest when submitting an article (e.g. employment, consulting fees, research

contracts, stock ownership, patent licences, honoraria, advisory affiliations, etc). This information should be included in an acknowledgements section at the end of the manuscript (before the references section). All sources of financial support for the project must also be disclosed in the acknowledgements section. The name of the funding agency and the grant number should be given, for example: *This work was partially funded by the National Institutes of Health (NIH) through a National Cancer Institute grant R21CA141833.* When completing the online submission form, we also ask you to select funders and provide grant numbers in order to help you meet your funder requirements. We encourage authors to use the acknowledgements section of the article to make specific attributions of author contribution and responsibility, otherwise all co-authors will be taken to share full responsibility for all of the paper.

**References**

This section should be used to list all relevant work. More information on referencing. However, check the peer review model for the journal you are submitting to. If double-anonymous then when referring to thesis/unpublished work, please avoid identifying information. You should include non-identifiable information e.g. journal name, year etc...

If you need more information or guidance about any of the above then please contact the journal to which you are submitting.

**Figures**

Carefully chosen and well-prepared figures, such as diagrams and photographs, can greatly enhance your article. You are encouraged to prepare figures that are clear, easy to read and of the best possible quality and resolution.

To make your figures accessible to as many readers as possible, try to avoid using colour as the only means of conveying information. For example, in charts and graphs use different line styles and symbols. Where colours are used try to ensure that:

- there is good contrast between adjacent colours;
- colours are distinguishable if the figure is converted to greyscale;
- different line styles, fill styles, symbols or labels are used in addition to different colours.

We accept that it is not always possible to follow these guidelines, for example with figures that use colour gradient scales to convey information, or for photographic images. As with all figures, it is important to use the figure caption to describe the information conveyed by the figure. See below for further details.

Figures are converted and sized to the journal template as part of the production process for accepted articles, but they are not normally edited further. It is your responsibility to ensure that the figures you supply are legible and technically correct.
Characters should appear as they would be set in the main body of the article. Aim for text sizes of 8 to 12 pt at the final figure size: typically 8.5cm for a small/single-column figure and 15cm for a large/double-column figure. Micrographs should include a scale bar of appropriate size, e.g. 1 μm. Figures should be numbered in the order in which they are referred to in the text, using sequential numerals (e.g. figure 1, figure 2, etc.).

If there is more than one part to a figure (e.g. figure 1(a), figure 1(b), etc.), the parts should be identified by a lower-case letter in parentheses close to or within the area of the figure.

**Anexo 4** - Normas da *Diseases of Aquatic Organisms*, na qual foi encaminhado para publicação o capítulo IV dessa Tese.

**1. Manuscript length and structure**

The target lengths for the different manuscript types (Research Article, Note, Review, Comment, Reply Comment and Opinion Piece) are listed in 'Author guidelines'.

**Manuscripts should be structured as follows:**

- Title page (title, author list, author affiliations, corresponding author email, running page head, abstract, key words)
- Main text (see below)
- Acknowledgements
- Literature cited
- Tables (with legends as separate, regular text above each respective table)
- Figures (with legends as separate, regular text below each respective figure)
- Appendices (length: up to 2 printed pages; longer materials should be submitted as online supplementary materials in a separate file)

**Main text:**

- Research Articles and Notes should follow the IMRAD format (Introduction, Materials & Methods, Results, Discussion and an optional Conclusions section). Note that separate Results and Discussion sections are strongly preferred, but exceptions are possible if the manuscript's content warrants a combined Results & Discussion section.
- Reviews, Comments, Reply Comments and Opinion Pieces may deviate from the IMRAD format as necessary.

**2. Title page**

**Title:** The title should be concise and informative, i.e. summarizing either the subject or the most important findings of the study rather than merely the hypothesis addressed. It should have around 100 characters (ca. 15 words), and 150 characters at most (including spaces). Avoid 'A', 'An', 'The', 'On', etc. at the beginning. Avoid questions in the title.

Provide a running page head with 3 to 6 words; e.g. 'Detection of shrimp WSSV'.

**Authors and addresses:**

If a manuscript has several authors from different institutions:

- use superscript numerals for identification;
- provide the address of each author's institution, identifying any affiliations as 'present address' if applicable. Include zip or postal code but not street address or PO box number;
- use an asterisk (*) to refer to a footnote that identifies the single corresponding author and provide a contact e-mail address.

**Abstract:** Limit length to 250 words. Provide concise information on your work: background/reason for your work, basic approach, its principal results and its broader significance. Ensure strong opening and closing statements for maximum impact. Avoid literature cites, series of data or detailed statistical results, or meaningless clauses such as 'the results are discussed'.

**Key words:** Supply 3 to 8 key words, listed in order of importance.

**3. Text**

Please use continuous line numbering throughout your manuscript, 12 point font, double spacing and numbered sections. Manuscripts that do not use correct English grammar, spelling and punctuation will be returned to authors without review; if you are not a native English speaker, you should have the text edited by someone who is, before submitting your manuscript. You may also wish to consult a 'How to' book such as Day & Gastel (2011; How to write and publish a scientific paper, 7th edn. Greenwood Press, Santa Barbara, CA).

**Section headings:** Main sections (IMRAD) should be numbered '1. INTRODUCTION', '2. MATERIALS & METHODS', etc. Subsections should be numbered as e.g. '2.1. Study site', '2.2. Sample collection', etc. Avoid going beyond third-level subsections (e.g. 3.1.1.).

**Verbosity:** Please eliminate verbiage; example:

**Verbose –** 'The speed was chosen because past studies by Miller (1995) and Smith (1998) have shown this to be slightly greater than the maximum sustained swimming speed.'

**Not verbose –** 'The speed is slightly greater than the maximum sustained swimming speed (Miller 1995, Smith 1998).'

**Verbose –** 'It has been shown that boat noise affects whale behaviour (Smith 1994).' (and similar phrases such as 'it has been reported/found that', 'it is possible/suspected that', 'results show that')

**Not verbose –** 'Boat noise influences whale behaviour (Smith 1994).'

**Genus and species names** must be in italics; write the genus name in full at first mention in each section (Abstract, Introduction, Materials & Methods, Results, Discussion) and abbreviate whenever mentioned again in the same section. When referring to a species, do not use the genus

name alone, unless you have previously defined it that way; be precise when using 'sp.' (singular) and 'spp.' (plural).

**At first mention in a section –** 'The filter feeding of blue mussels *Mytilus edulis* was examined'.

**After first mention in a section –** 'Filter feeding rates of *M. edulis* increased with increasing temperature.'

**Abbreviations:** Define abbreviations and acronyms in the Abstract and at first mention in the main text, and thereafter use only the abbreviation / acronym.

**Equations and units:** Use standard SI units. Relations or concentrations (e.g. mg per l) must be given as 'mg $l^{-1}$' (not mg/l). Variables are usually italicised (except for Greek letters). Italicisation should be consistent in normal, superscript, and subscripted text. Example of proper spacing: 'p = 0.047, $r^2$ = 0.879' (not 'p=0.047, $r^2$=0.879'); but: 'we studied organisms of size <0.5 µm'

**Figures and tables:** Figures, tables, and their legends should be self-explanatory; e.g. any abbreviations and acronyms used in figures or tables must be defined there. Tables need to be editable (not embedded as an image). Legends should succinctly describe table/figure content, but not summarize methods or results. Legends must not be embedded in the tables or figures but be presented as regular text above tables or below figures. For table footnotes, use superscripted lower case letters; asterisks can be used to indicate statistical significance (must be defined in the legend). Please consult 'Figures' for details on figure preparation.

**Statistics:** In the Materials & Methods, clearly state which statistical analyses you performed and the programme(s) (name and version number as well as reference where applicable) used; report how any relevant assumptions (e.g. for parametric statistics) were tested, the outcome and the solution (e.g. data transformation or an alternate test); state the significance value (alpha) you used. In the Results, clarify which analysis a result is from and report all relevant values (e.g. for most tests, the test-statistic, df value(s) and p-value); ideally, report exact p-values (not levels) to 3 decimal places (exception: $p < 0.001$ or $< 0.0001$ for very small values).

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