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LUISA DE PONTES RIBEIRO

IMPLICAÇÕES DO COMÉRCIO NACIONAL E INTERNACIONAL DE RÃ-TOURO NA DISPERSÃO E TOLERÂNCIA ADQUIRIDA AO FUNGO QUITRÍDIO E MEDIDAS PARA CONSERVAÇÃO DE ANUROS

IMPLICATIONS OF NATIONAL AND INTERNATIONAL BULLFROG TRADE IN SPREAD AND TOLERANCE ACQUIRED TO CHYTRID FUNGUS AND CONSERVATION MEASURES OF ANURANS

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Orientador: PROF. DR. LUÍS FELIPE DE TOLEDO RAMOS PEREIRA

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"A beleza da biodiversidade está na diferença, na variedade de formas de vida que coexistem e se complementam."
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RESUMO

A crise de extinção global, impulsionada pela atividade humana, representa uma ameaça significativa à biodiversidade. Os anfíbios, como a classe de vertebrados mais ameaçada, têm 40 % das suas espécies sob risco de extinção. Fatores como a perda de habitat, doenças infecciosas, espécies invasoras e a ranicultura emergem como ameaças críticas para esses animais. Apesar do crescimento global da ranicultura, a falha regulamentação e conhecimento sobre sua influência nos ecossistemas levanta preocupações sobre os impactos nas espécies nativas. A espécie *Aquarana catesbeiana*, conhecida como rã-touro, destaca-se como uma das principais espécies utilizada na ranicultura, mas também é reconhecida como uma das piores espécies invasoras do mundo. Esta espécie desempenha um papel central na disseminação do fungo *Batrachochytrium dendrobatidis* (Bd), responsável pela quitridiomicose, doença que contribuiu para o declínio e extinção de diversas espécies de anfíbios. A linhagem Bd-GPL (*Global Panzootic Lineage*) está amplamente distribuída e tem sido associada a declínios e extinções, enquanto outras linhagens geograficamente mais restritas careçam de informações. Nesta tese, exploramos os impactos do comércio de rã-touro na disseminação do fungo Bd, propondo medidas para conservar as espécies nativas. Nossos resultados evidenciam o Brasil como um grande produtor de rã-touro, com informações compiladas de 151 ranários brasileiros que geraram 400 toneladas de rã-touro em 2019, movimentando US$ 1,9 milhão. Além disso, identificamos a necessidade de melhorias nos mercados consumidores e nos processos produtivos para garantir a sustentabilidade econômica e ambiental dessa atividade. Propomos um novo método eficiente para identificar as linhagens do Bd, o qual empregamos para genotipar o fungo em ranários brasileiros. Nossos resultados revelaram a presença das linhagens Bd-GPL, Bd-BRAZIL, além de híbridos do fungo nos ranários do país. Ressaltamos a importância da genotipagem na compreensão da distribuição global dessas linhagens. Com base em dados históricos da presença do fungo quitrídio e nas rotas comerciais de rã-touro, apontamos a provável origem da linhagem Bd-BRAZIL no Brasil e sua disseminação através do comércio de rã-touro. Ao analisar o impacto da produção em massa de rã-touro na infecção por Bd, identificamos baixa resistência à infecção nas rãs-touro de ranários, indicando seu potencial como superdisseminadoras de Bd. Este estudo destaca os impactos ambientais da ranicultura e a importância de práticas sustentáveis para reduzir riscos de doenças e promover a conservação de anfíbios. Por fim, apresentamos estratégias regulatórias para a ranicultura brasileira, com foco na conservação dos anfíbios nativos. Almejamos que os resultados apresentados nesta tese possam contribuir de forma significativa para o avanço científico e político da conservação de anfíbios, especialmente no contexto da produção animal e da ecologia de doenças.

Palavras-chave: *Batrachochytrium dendrobatidis*, *Aquarana catesbeiana*, dinâmica de infecção por Bd, linhagens de Bd, métodos de genotipagem, quitridiomicose, regulamentação da ranicultura, sustentabilidade na produção animal.

ABSTRACT

The global extinction crisis, driven by human activity, poses a significant threat to biodiversity. Amphibians, as the most threatened class of vertebrates, have 40 % of their species at risk of extinction. Factors such as habitat loss, infectious diseases, invasive species, and frog farming emerge as critical threats to these animals. Despite the global growth of frog farming, inadequate regulation and understanding of its influence on ecosystems raise concerns about impacts on native species. The species *Aquarana catesbeiana*, commonly known as bullfrog, stands out as a primary species used in frog farming but is also recognized as one of the worst invasive species globally. This species plays a central role in spreading the fungus *Batrachochytrium dendrobatidis* (Bd), responsible for chytridiomycosis, a disease that has contributed to the decline and extinction of numerous amphibian species. The Bd-GPL (Global Panzootic Lineage) is widely distributed and has been associated with declines and extinctions, while other geographically restricted lineages lack information. In this thesis, we explore the impacts of bullfrog production on the spread of Bd, proposing measures to amphibian conservation. Our results highlight Brazil as a major bullfrog producer, compiling data from 151 Brazilian bullfrog farms that generated 400 tons of bullfrogs in 2019, totalling 1.9 million USD. Additionally, we identify the need for improvements in consumer markets and production processes to ensure economic and environmental sustainability of this activity. We propose a new efficient method to identify Bd lineages, which we used to genotype the fungus in Brazilian bullfrog farms. Our findings revealed the presence of Bd-GPL, Bd-BRAZIL, and hybrids in the country's bullfrog farms. We emphasize the importance of genotyping in understanding the global distribution of these lineages. Based on historical data on the presence of the chytrid fungus and bullfrog trade routes, we pinpoint the likely origin of the Bd-BRAZIL lineage in Brazil and its spread through bullfrog trade. Analysing the impact of mass bullfrog production on Bd infection, we identify low resistance to infection in farmed bullfrogs, indicating their potential as supershedders of Bd. This study underscores the environmental impacts of bullfrog farming and the importance of sustainable practices to reduce disease risks and promote amphibian conservation. Finally, we present regulatory strategies for Brazilian bullfrog farming, focusing on the conservation of native amphibians. We aim for the results presented in this thesis to significantly contribute to the scientific and political advancement of amphibian conservation, particularly within the context of animal production and disease ecology.

Keywords: *Batrachochytrium dendrobatidis*, *Aquarana catesbeiana*, Bd infection dynamics, Bd lineages, genotyping methods, chytridiomycosis, bullfrog farming regulation, sustainability in animal production.

RESUMEN

La crisis de extinción global, impulsada por la actividad humana, representa una amenaza significativa para la biodiversidad. Los anfibios, como la clase de vertebrados más amenazada, tienen el 40 % de sus especies en peligro de extinción. Factores como la pérdida de hábitat, enfermedades infecciosas, especies invasoras y la ranicultura emergen como amenazas críticas para estos animales. A pesar del crecimiento global de la ranicultura, la falta de regulación y conocimiento sobre su influencia en los ecosistemas plantea preocupaciones sobre los impactos en las especies nativas. La especie *Aquarana catesbeiana*, conocida como rana toro, destaca como una de las principales especies utilizadas en la ranicultura, pero también es reconocida como una de las peores especies invasoras del mundo. Esta especie desempeña un papel central en la propagación del hongo *Batrachochytrium dendrobatidis* (Bd), responsable de la quitridiomicosis, una enfermedad que ha contribuido al declive y extinción de diversas especies de anfibios. El linaje Bd-GPL (*Global Panzootic Lineage*) está ampliamente distribuida y ha sido asociada a declives y extinciones, mientras que otros linajes geográficamente más restringidos carecen de información. En esta tesis, exploramos los impactos del comercio de ranas toro en la propagación del hongo Bd, proponiendo medidas para conservar las especies nativas. Nuestros resultados evidencian a Brasil como un gran productor de ranas toro, con información recopilada de 151 criaderos brasileños que generaron 400 toneladas de ranas toro en 2019, moviendo US$ 1,9 millones. Además, identificamos la necesidad de mejoras en los mercados consumidores y en los procesos productivos para garantizar la sostenibilidad económica y ambiental de esta actividad. Proponemos un nuevo método eficiente para identificar los linajes de Bd, el cual empleamos para genotipar el hongo en los criaderos brasileños. Nuestros resultados revelaron la presencia de los linajes Bd-GPL, Bd-BRAZIL, además de híbridos del hongo en los criaderos del país. Destacamos la importancia de la genotipificación para comprender la distribución global de estos linajes. Basándonos en datos históricos de la presencia del hongo quitrídio y en las rutas comerciales de las ranas toro, señalamos el probable origen del linaje Bd-BRAZIL en Brasil y su propagación a través del comercio de ranas toro. Al analizar el impacto de la producción masiva de ranas toro en la infección por Bd, identificamos una baja resistencia a la infección en las ranas toro de los criaderos, indicando su potencial como superdiseminadoras de Bd. Este estudio destaca los impactos ambientales de la ranicultura y la importancia de prácticas sostenibles para reducir los riesgos de enfermedades y promover la conservación de anfibios. Finalmente, presentamos estrategias regulatorias para la ranicultura brasileña, centradas en la conservación de los anfibios nativos. Esperamos que los resultados presentados en esta tesis puedan contribuir de manera significativa al avance científico y político en la conservación de anfibios, especialmente en el contexto de la producción animal y la ecología de enfermedades.

Palabras clave: *Batrachochytrium dendrobatidis*, *Aquarana catesbeiana*, dinámica de infección por Bd, linajes de Bd, métodos de genotipificación, quitridiomicosis, regulación de la ranicultura, sostenibilidad en la producción animal.

SUMÁRIO

INTRODUÇÃO GERAL

A atual sexta extinção em massa, acelerada pelo homem, representa uma ameaça às espécies e aos serviços ecossistêmicos, evidenciando a urgência global na conservação da biodiversidade (Ceballos et al. 2020). Segundo a mais recente avaliação global de anfíbios, revelou-se que os anfíbios são a classe de vertebrados mais ameaçada, com 40,7 % das espécies em risco (Luedtke et al. 2023). Entre 1980 e 2004, a perda de habitat e doenças corresponderam a 91 % das perdas registradas (Luedtke et al. 2023). Além disso, fatores como espécies exóticas invasoras e aquicultura, ou seja, a produção de organismos aquáticos, também estão associados a altos níveis de ameaça para os anfíbios (Falaschi et al. 2020; Greenville et al. 2021).

Os anfíbios têm sido usados como alimento globalmente por anos (Teixeira et al. 2001; Warkentin et al. 2009; FAO 2024), tornando sua produção e comércio capazes de atender à demanda local, nacional e até mesmo internacional (Altherr et al. 2011). A ranicultura, como é conhecida a produção especializado em rãs está inserida no âmbito da aquicultura, que se refere à criação e cultivo de organismos aquáticos. Diversos países e regiões, como Taiwan, Península da Coreia, Malásia, Tailândia, Brasil, Uruguai, México e Equador, criam anfíbios para comercialização, prática conhecida como ranicultura, que está em crescimento significativo em todo o mundo (Altherr et al. 2011; FAO 2024). Apesar dos dados apontarem um aumento na produção, as estatísticas de mercado para anfíbios são escassas e pouco confiáveis (FAO 2024), principalmente devido à falta de regulamentação ao longo do processo de produção e comercialização, seja para venda local ou exportação.

A falta de protocolos de biossegurança na infraestrutura e na comercialização de anfíbios aumenta a preocupação sobre os possíveis impactos negativos nas espécies nativas. O comércio global se tornou um dos principais contribuintes para a crise global dos anfíbios (Kats & Ferrer 2003; Fisher & Garner 2007; Laufer et al. 2008; Schloegel et al. 2009; Carpenter et al. 2014; GISD 2018; O'Hanlon et al. 2018). A espécie *Aquarana catesbeiana*, conhecida popularmente como rã-touro, é nativa no leste da América do Norte, do México ao Canadá (Frost 2024), e foi introduzida em muitos países, sendo uma das principais espécies utilizada na ranicultura (Schlaepfer et al. 2005; FAO 2018).

No decorrer da produção, as rãs frequentemente escapam dos ranários e invadem os ambientes naturais (Garner et al. 2006; Fisher & Garner 2007; Both et al. 2011), tornando-as uma das principais espécies invasoras de anfíbios globalmente (Kraus 2015; Falaschi et al. 2020). No Brasil, a rã-touro foi inicialmente introduzida no estado do Rio de Janeiro, espa-

lhando-se posteriormente para outras regiões, e atualmente está presente em 14 estados, abrangendo quase todos os biomas brasileiros, exceto o Pantanal (Both et al. 2011; Forti et al. 2017; Melo-Dias et al. 2023).

As rãs-touro impactam negativamente as populações de anfíbios nativos de diversas maneiras, incluindo interferências na comunicação (Forti et al. 2017; Medeiros et al. 2017), predação (Toledo et al. 2007; Leivas et al. 2013) e competição (Kiesecker et al. 2001; Boone et al. 2004). Além disso, essa espécie desempenha um papel crucial na dinâmica do fungo quitrídio, *Batrachochytrium dendrobatidis* (doravante Bd) (Longcore et al. 1999), responsável pela quitridiomicose, principal doença infecciosa emergente que levou ao declínio de mais de 500 espécies, com pelo menos 90 delas já extintas em todo o mundo (Scheele et al. 2019; Fisher & Garner 2020). Estudos indicam que as rãs-touro possuem mecanismos eficientes de defesa à infecção pelo quitrídio, atuando assim como reservatório e vetor do patógeno para outras espécies (Daszak et al. 2004; Hanselmann et al. 2004; Garner et al. 2006; Eskew et al. 2015).

O fungo Bd é um patógeno cuja fase infectante é aquática, dependendo da presença de água ou do contato direto entre indivíduos para sua transmissão (Berger et al. 2005). A manifestação da quitridiomicose caracteriza-se pela infecção e proliferação do fungo na epiderme do anfíbio adulto hospedeiro, ocasionando hiperqueratose e morte celular (Pessier et al. 1999; Voyles et al. 2011; Brannelly et al. 2017). Isso compromete funções osmorregulatórias (Voyles et al. 2007), eletrolíticas e cardíacas (Carver et al. 2010; Salla et al. 2015), potencialmente levando à morte os animais (Voyles et al. 2007, 2009).

A quitridiomicose também pode agir silenciosamente, impactando de forma subletal parâmetros críticos para o equilíbrio hídrico, como a resistência da pele e a perda de água por evaporação (Bovo et al. 2016). Tais mudanças fisiológicas subletais podem resultar na diminuição da aptidão física e na alteração da abundância populacional (Longo & Burrowes 2010; Bovo et al. 2016). Além disso, o Bd pode produzir toxinas (Brutyn et al. 2012; Fites et al. 2013) que interferem na proliferação e atividade dos linfócitos, reduzindo as respostas imunes do hospedeiro (Fites et al. 2014; Young et al. 2014).

Compreender os mecanismos de defesa, como resistência e tolerância, contra patógenos é crucial para o manejo das populações. No entanto, o detalhamento das respostas das espécies de anfíbios à infecção por Bd ainda representa um desafio. Hospedeiros resistentes são capazes de reduzir ativamente a carga e a replicação do patógeno, enquanto os hospedeiros tolerantes conseguem limitar os danos causados por ele (Råberg et al. 2009; Grogan et al. 2023). Espécies dos gêneros *Atelopus* e *Dendrobates* são notavelmente suscetíveis à quitridiomicose em seus ambientes naturais (Nichols et al. 2001; Lips et al. 2008; Cheng et al. 2011). Da mesma

forma, espécies da superfamília Brachycephaloidea, conhecidas por seu desenvolvimento direto, frequentemente mostram susceptibilidade a infecções por Bd (Mesquita et al. 2017; Moura-Campos et al. 2021).

Por outro lado, certas espécies, como a rã-touro, têm demonstrado tolerância ou até mesmo resistência às infecções por Bd, conforme evidenciado em estudos prévios (Daszak et al. 2004; Hanselmann et al. 2004; Schloegel et al. 2010; Eskew et al. 2015). Entretanto, apesar das evidências que sustentam a resistência e a tolerância ao Bd em rãs-touro, os mecanismos subjacentes à capacidade delas de resistir a essas infecções, particularmente no que se refere às respostas imunes, ainda permanecem incertos. Diferentes hospedeiros apresentam respostas imunológicas e fisiológicas variadas, influenciadas por seu hábito de vida, exposição ao patógeno ao longo da vida e condições ambientais (Gervasi et al. 2013; Savage et al. 2015; Mesquita et al. 2017). Além disso, o fungo Bd apresenta distintas linhagens que podem exibir diferenças na virulência, podendo acentuar ou reduzir a gravidade da doença (Greenspan et al. 2018; Ribeiro et al. 2019; Carvalho et al. 2023).

A principal linhagem de Bd, associada a declínios e extinções em massa, é a Bd-GPL (*Global Panzootic Lineage*), amplamente distribuída pelo mundo (Scheele et al. 2019). No entanto, existem outras quatro linhagens principais de Bd (O'Hanlon et al. 2018; Byrne et al. 2019). Entre elas, está o Bd-CAPE, encontrado na África do Sul, na África Ocidental e, localmente, na Europa (Farrer et al. 2011), além de ter sido identificado recentemente na América Central (Byrne et al. 2019). O Bd-Asia-1 foi identificado na Península Coreana, enquanto o Bd-BRAZIL está presente no Brasil, nos Estados Unidos da América, na Península da Coreia (Rodriguez et al. 2014; O'Hanlon et al. 2018) e, possivelmente, no Japão (Goka et al. 2021). Por fim, o Bd-Asia-3 foi encontrado no Sudeste Asiático (Byrne et al. 2019).

Com base em dados genômicos, identificou-se que a Bd-GPL surgiu no continente asiático por volta do meio do século XX, espalhando-se globalmente através do comércio de anfíbios (O'Hanlon et al. 2018). Apesar disso, ainda há uma lacuna significativa na investigação da origem e disseminação das linhagens endêmicas ou geograficamente restritas de Bd. No entanto, recentes avanços nos métodos de genotipagem do Bd e na compreensão de sua complexa diversidade genética, aliados ao aumento dos estudos sobre registros de comercialização de anfíbios, têm direcionado novas investigações nesse campo (Farrer et al. 2011; Schloegel et al. 2012; Bataille et al. 2013; Rosenblum et al. 2013; Byrne et al. 2019; Monzon et al. 2020). Por exemplo, a linhagem Bd-BRAZIL, anteriormente considerada endêmica do Brasil, foi recentemente identificada em outros países, possivelmente devido ao comércio internacional de rãs-touro (Schloegel et al. 2012).

Em ranários brasileiros, já foram identificadas duas linhagens do fungo: Bd-GPL e Bd-BRAZIL (Ribeiro et al. 2019). Estudos também apontaram para uma alta prevalência e carga de infecção por Bd em rãs-touro mantidas em ranários (Schloegel et al. 2009, 2010; Ribeiro et al. 2019; Santos et al. 2020). A disseminação do Bd e suas linhagens para novos locais é preocupante, uma vez que populações previamente não expostas ao fungo têm maior risco de enfrentar uma epizootia em comparação com aquelas que coexistem com o patógeno há mais tempo (Vredenburg et al. 2010; Talley et al. 2015; Yap et al. 2018). Apesar de existirem evidências da importância do comércio de rã-touro na disseminação do Bd, informações detalhadas sobre os criadouros dessa espécie, relacionadas à produção e comércio, bem como a caracterização do Bd, são cruciais para compreender a dinâmica de infecção e dispersão do fungo Bd pelas rãs-touro.

Assim, esta tese foi organizada em cinco capítulos com o propósito de aprofundar nossa compreensão sobre as implicações do comércio de rã-touro na disseminação do fungo Bd, visando propor e implementar medidas para a conservação de anfíbios. O primeiro capítulo, através de uma extensiva revisão, objetivou compilar e detalhar os dados atuais sobre a produção e o comércio (nacional e internacional) de rã-touro no país. Destacamos aspectos-chave que requerem melhorias para expandir e dar visibilidade à ranicultura, considerando tanto aspecto econômico quanto ambiental relacionados à criação de rãs no Brasil. No capítulo II, focamos na identificação de linhagens do fungo Bd em ranários brasileiros, utilizando diversas ferramentas moleculares, como qPCR e dPCR. Nosso intuito é ampliar o conhecimento sobre a distribuição das linhagens de Bd nos ranários e propor um novo método de genotipagem do fungo. O capítulo III reúne registros históricos da presença do fungo quitrídio e do comércio global de rã-touro no intuito de investigar a origem e disseminação da linhagem Bd-BRAZIL. No capítulo IV, através de uma abordagem experimental, investigamos os impactos da produção em massa de rã-touro nos mecanismos de defesa contra a quitridiomicose e a disseminação do fungo quitrídio. Por fim, o capítulo V oferece um resumo histórico das principais estratégias e ações para regulamentar a ranicultura brasileira, nas quais estivemos ativamente envolvidos.

CHAPTER I

AN OVERVIEW OF THE BRAZILIAN FROG FARMING

Uma revisão sobre a ranicultura no Brasil

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An overview of the Brazilian frog farming

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Abstract

World population growth demands an accelerated increase in food production especially that of animal origin. One of the main challenges worldwide is to couple such a production with environmental health. The production of aquatic animals, i.e., aquaculture, tends to be less harmful to the environment due to the reduction of crop yield requirements and the decrease in land areas for cultivation and grazing. Furthermore, it is associated with a wide range of cultivated species and production systems, which is related to the resilience of the global food system. The bullfrog, *Aquarana catesbeiana* (Shaw, 1802), production, known as frog farming, could represent an economically competitive and environmentally conscious production opportunity. Brazil a pioneer and one of the world's largest bullfrog producers. However, there is no data compilation about its current and past production. Here, we present current data on bullfrog production, national and international trade, and relevant environmental issues related to frog farming in this country. We compiled information about Brazilian bullfrog farms from February 2019 to January 2020, and obtained information on production and trade directly from the producers. We described the frog farming workflow and classified the farms by the processes they accomplish. We mapped 151 bullfrog farms distributed mostly in southeastern Brazil. In 2019 we estimated a production of about 400 tons (gross weight), which generated roughly 200 net tons of bullfrog meat, moving about 1.9 million USD. In addition, we mapped the local, interstate, and international bullfrog production commercial routes. Brazilian frog farming has great expansion potential; nevertheless, improvements are needed, both in the consumer market, as well as in the production and overall frog farming workflow. We highlight the main aspects that need improvement to expand and generate visibility for frog farming in Brazil, from an economic and environmental feasible perspective.

Keywords: Bullfrog farm, commercial routes, animal protein alternative, national economy, production.

1. Introduction

Human population growth and the increase of individual income in many countries are intensifying the global consumption of animal source food (Godfray et al., 2018; Steinfeld et al., 2006; Vranken et al., 2014). Feeding a growing population and reducing the pressures of food production on the environment is a major challenge (Froehlich et al., 2018b; Herrero et al., 2015; Macdiarmid et al., 2016). The assessment of impacts caused by animal production is generally centered on land use (Froehlich et al., 2018b). Livestock, for instance, uses about 80 % of the planet's agricultural land, the vast majority of which are represented by extensive ruminant systems (Herrero et al., 2015). There are major differences in impacts, which can vary according to the species produced, the type of product, and the applied production method (Hilborn et al., 2018). A more efficient and diversified food system, whether due to the number of species produced, places of production and/or feeding strategies is linked to resilience in the global food system (Troell et al., 2014).

The adoption of a diversified diet, consisting of small daily portions of different types of meat and products of animal origin (Willett and Skerrett, 2005) implies in a reduction of the required areas of arable land and pasture (Stehfest et al., 2009). The land area required for food production will increase in all future scenarios, but millions of hectares could be spared by an increase in human diets based on a higher proportion of aquatic animal protein (Froehlich et al., 2018b). Human diets in which aquatic organisms are dominant when compared to livestock could reduce annual crop yield requirements by 34 million tons and decrease land areas for cultivation and grazing, saving ca. 750 million hectares (Froehlich et al., 2018b). Furthermore, while terrestrial food production is based on a limited number of species, aquaculture uses a wide range of cultured species and different production systems (Subasinghe, 2005). More than 330 species are farmed, including a variety of taxa, such as crustaceans, mollusks, fish, amphibians, and alligators (FAO, 2019; Metian et al., 2020).

Currently, aquaculture represents the fastest growing food production sector in the world, increasing its global production almost fivefold from 1990 to 2015, and it is expected to double by the middle of this century (Froehlich et al., 2018a; Ottinger et al., 2018; Tacon, 2020). In 2012 the cultivation of aquatic species exceeded the production of beef, which has always remained the main protein source in the world (Larsen and Roney, 2013). In 2017 aquaculture production was about 80 million tons, with over 66.7 % represented by fish, followed by mollusks (21.7 %), crustaceans (10.5 %) and others, such as frogs, turtles, sea cucumbers, and sea urchins (1.1 %) (FAO, 2019). Most of this production comes from developing countries (Subasinghe, 2005), especially in South America, which are recognized for their large

production and high economic impact on local and global aquaculture (Valladão et al., 2018). In addition to extensive fish production, South American countries also produce different aquatic organisms, including shrimp, shellfish, and frogs (Valladão et al., 2018).

Aquaculture systems require large amounts of good quality water sources to sustain the long-term growth and health of the produced species (Qing et al. 2021). Besides, environmental impacts are associated with aquaculture such as the degradation and contamination of water systems due to the increase in nutrient concentration in the aqueous medium, which relates to the accumulation of excretion and uneaten feed residues (Qing et al. 2021, Viegas et al. al. 2021). Organic and inorganic nutrients figure among the main contaminants, such as ammonia, nitrite, and phosphorus, as well as a diversity of microorganisms, including pathogens (Diaz et al. 2011, Rosa et al. 2020).

Frog production, frog farming, or ranaculture, has been recorded since the 20th century (Altherr et al., 2011; FAO, 2021), although frog consumption dates back at least to the 16th century (Neveu, 2004). Frog meat, especially frog legs, has white color, soft texture, and mild flavor (Herbst, 1995), being considered a delicacy in Europe (Tyler et al., 2007). In recent years, it has attracted the attention of consumers who are increasingly concerned with health, due to its nutritional characteristics, such as low lipids percentage and high quality of proteins and amino acids (Oliveira et al., 2017; Paixão and Bressan, 2009). In the 1980's, Asian countries had high exports, which had been accompanied a world consumption of 6,500 tons of frog legs per year (Beebee, 1996). Frog production requires special facilities for different life stages (tadpole *vs.* post-metamorphic) and several specifications for breeding practices, such as temperature and water quality control, nutrition, and adequate sanitation (Cribb et al., 2013; Lutz and Avery, 1999). Prominent technological advances have maximized frog farming expansion, increasing the production under controlled conditions (Olvera-Novoa et al., 2007). Currently, the main frog producers are based in Asia (Taiwan and China) and Latin America (especially Brazil and Mexico) (FAO, 2019; Mello et al., 2016), while Europe and the USA represent the largest consumers and importers in the world (Altherr et al., 2011; FAO, 2019; Neveu, 2004).

Some frog species, including the European green frog [*Pelophylax ridibundus* (Pallas, 1771)], the East Asian bullfrog [*Hoplobatrachus rugulosus* (Wiegmann, 1834)], and other *Rana* spp. are used as a meat source and produced. However, the North American bullfrog (*Aquarana catesbeiana*; hereafter bullfrog), is the most farmed amphibian globally (FAO, 2021) (**Fig. 1A**). Due to its ease of handling, rapid growth, prolificacy, large size, and fleshy legs, it is favored by producers (Cribb et al., 2013; Lutz and Avery, 1999). As a result, it has been

introduced in more than 40 countries for frog farming (FAO, 2021; Frost, 2021; García et al., 2020).

Frog production has either increased or maintained stable over the years. We observed an increase of over 100 % in world production in 2018 when compared to 2010. From 2010 to 2018 the world average production was approximately 3,200 tons of bullfrog per year, led by Taiwan, but with great contributions from Malaysia, Singapore, Brazil, Ecuador, and Mexico (FAO, 2021) (**Figs. 1B and 1C**). Even though bullfrog production is still not as representative as other aquatic species, its worldwide production is growing and has contributed to the global economy with an average of 11 million USD per year movement from 2010 to 2018, reaching its peak in 2013, with 14.5 million USD (FAO, 2021) (**Fig. 1B**). The bullfrog is ecologically relevant as well, as the increase in its production and consumption could reduce beef's and add resilience to the food system (Froehlich et al., 2018b; Troell et al., 2014). On the other hand it represents one of the worst invasive species in the world, a condition directly related to the development of frog farming and its (in)consequent introduction in several countries (Ficetola et al., 2007; Govindarajulu et al., 2006; Kraus, 2015; Lowe et al., 2000; Lutz and Avery, 1999).

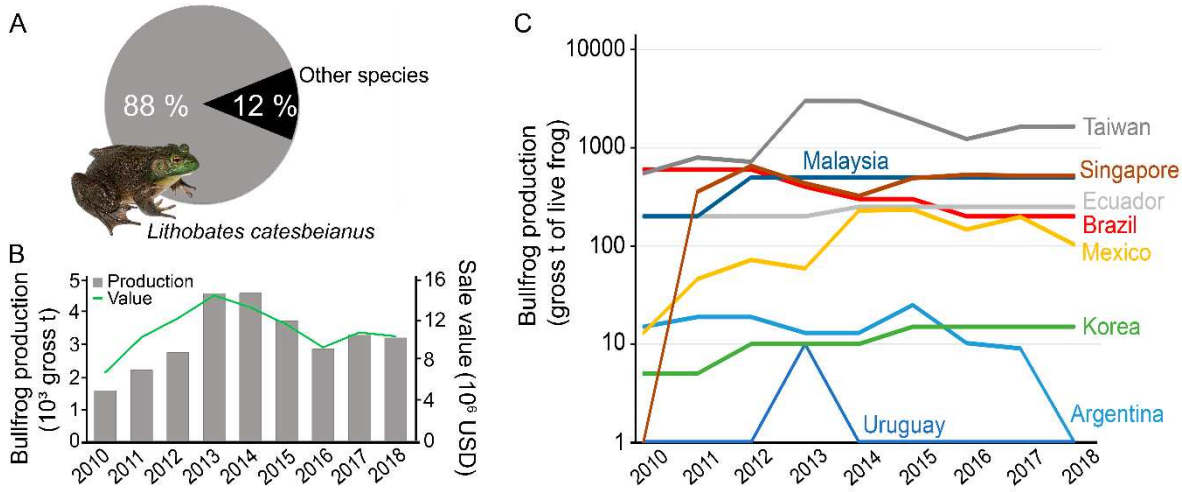

Fig 1. Worldwide frog production. Proportion of frog species produced from 2010 to 2018 in the world (**A**); total bullfrog production (gross weight) and sale value in the world (**B**); total bullfrog production (gross weight) in Asian and American countries (**C**).

Bullfrog is well adapted to Brazilian climatic conditions, where numerous feral populations were reported (Both et al., 2011). Also, a model predicted high likelihood rates of occurrence and success in the establishment of bullfrog in Brazilian regions, especially in the

south and southeast (Giovanelli et al., 2008). Invasive species play an important role in the global amphibian crisis and can directly or indirectly affect native amphibian fitness, population size and dynamics, and community structure (Carpenter et al., 2014; Falaschi et al., 2020; Fisher and Garner, 2007; Manenti et al., 2020). Bullfrogs can prey on native anurans (Boelter et al., 2012; Leivas et al., 2013; Silva et al., 2011; Toledo et al., 2007), interfere on native amphibian acoustic communication (Both and Grant, 2012; Forti et al., 2017; Medeiros et al., 2017), and act as pathogens carriers (Brunner et al., 2019; O'Hanlon et al., 2018). Therefore, frog farms must avoid bullfrog escapes into the surrounding environments.

Brazil was one of the first countries to import live bullfrogs (FAO, 2005; Schloegel et al., 2010b), and since then it represents one of the largest contributors and suppliers of technology for frog farming (Pahor-Filho et al., 2019). Brazilian bullfrog farms have promising infrastructure, environmental conditions, and a potential market in several regions of the country (Feix et al., 2006; Rodrigues et al., 2010; Sousa and Maltarolo, 2019). However, the slow process of environmental licensing and producer registration and the difficulty in products transportation (Cribb et al., 2013) lead to the instability in the sector, scarcity of production and trade data, or even the difficulty to access them. As a result, there is great difficulty in carrying out studies and research for innovations, improvements in the activity, and in the development of action plans aimed at the conservation of native species. Hence, we here present an updated data on the number and distribution of bullfrog farms in Brazil, as well as economic and environmental aspects of the Brazilian frog farming, with perspectives for the sustainable development of this activity.

2. Material and methods

We used a database provided by FAOSTAT, accessed in June 2020, to estimate the proportion of frog species produced in the world, the volume of global bullfrog production, and the sale value between 2000 and 2018. Regarding Brazilian frog farming, we carried out an extensive search to compile information about the number and distribution of bullfrog farms from February 2019 to January 2020. We searched for bullfrog farms available on the internet (Google, YouTube, and diverse social media), including advertising, posts, or videos to obtain farm distribution data. We used the following keywords (in Brazilian Portuguese): aquaculture, bullfrog, bullfrog farm, producer associations, frog culture, frog farming, frog meat, frog production, frog slaughter, and frog trade. We also included combinations of such keywords associated with Brazilian regions, states or municipalities to refine the search. We used these words to search for journal articles in the databases of Web of Science and Google Scholar. In

addition, we extracted quantitative information by directly contacting governmental agencies in charge of producers' registration, such as the Ministério da Agricultura, Pecuária e Abastecimento (MAPA) – national – and Órgãos Executores de Sanidade Agropecuária (OESA) of each state. We also collected data from previously known bullfrog producers, researchers, and professionals. We created all maps in ArcGIS®.

In addition to the farm locations, we surveyed production, commercial, and biological aspects relevant to understand the current scenario of Brazilian frog farming. We contacted producers to obtain qualitative information, especially about production volumes and commercial routes. We used the interrogative method and developed a semi-structured questionnaire to interview bullfrog producers (Oliveira, 2008). The questionnaire contained 28 questions to address topics such as farm location and structure, start and end period (if inactive) of activity, property licensing, first frog individuals' acquisition, production steps, handling techniques, capitation and disposal of water, sanitary problems, consumer market, and marketed products. We were unable to obtain the complete information for all farms. Thus, we show the number of farms that we obtained full information in each topic below.

Finally, we converted bullfrog sale values in Brazil from reais (BRL) to American dollars (USD). We corrected past values in reais according to inflation and converted them using a single exchange rate, i.e., 1 BRL = 0.2481 USD. This quotation refers to 31 December 2019. We used the Banco Central do Brasil website for all conversions.

3. Production processes and bullfrog trade

Some native large-sized frog species have been suggested for commercial farming, as the pepper frog [*Leptodactylus labyrinthicus* (Spix 1824)], smoky jungle frog [*Leptodactylus pentadactylus* (Laurenti, 1768)], and the butter frog [*Leptodactylus latrans* (Steffen 1815)] (Cribb et al., 2013; Ferreira et al., 2002; Lima et al., 1987; Lima and Agostinho, 1992; Weigert et al., 1998). However, currently Brazilian frog farming consists of the production in captivity of a single species, the North-American bullfrog, *Aquarana catesbeiana* (Brasil, 1998). In general, the bullfrog farm chain is made up of the suppliers of inputs and equipment, the bullfrog farm, the slaughter and processing industry, and the traders of the final products (Seixas Filho et al., 2017). The farm aims to produce bullfrogs in captivity and selling live individuals with the desired slaughter weight to the slaughter and processing industries (Cribb et al., 2013; Seixas Filho et al., 2017). However, we observed a multitude of implementation options in the Brazilian frog farming; ranging from farms that are dedicated to only one phase of the production, to those that incorporated all stages of the production chain. We obtained

information on the production processes of 76 farms that are currently operating in Brazil, and divided the production (phases 1, 2, and 3) and trade (live frog purchase, live frog sales, and processed frog sales) processes into distinct phases (**Table 1, Fig. 2**).

Producers must purchase the frogs as a first step to start a bullfrog farm (trade - live frog purchase). Most of the times, bullfrogs were acquired by buying individuals from other existing farms. In Brazil, there is no genetically improved bullfrog lineage for production (Marcantonio et al., 2002). Therefore, a common recommendation made to producers is to acquire males and females from different farms to minimize inbreeding. While some producers buy bullfrogs from farms, others have captured bullfrogs from feral populations. Even though bullfrogs are considered an exotic species, many wild populations are already established in the country (Both et al., 2011). After the acquisition of the bullfrogs, production phase 1 begins, which covers from the animal reproduction to metamorphosis. The reproduction is generally divided into two sectors: maintenance, where males and females are kept apart until they reach sexual maturity, which is diagnosed by the development of secondary sexual characteristics (Figueiredo et al., 2001); and mating, where couples are selected to mate after maintenance (Cribb et al., 2013). Breeding individuals must be up to four years old, due to reduced fertility after that (Seixas Filho et al., 2017). In the mating sector, two or three females are usually kept with each male, containing an area with aquatic habitat for spawning (Cribb et al., 2013). In all reproduction sectors, abiotic parameters, such as temperature and photoperiod (essential for triggering reproduction) must be controlled (Figueiredo et al., 2001; Fontanello et al., 1984). Breeding usually takes place between the months of September and February (Fontanello et al., 1984), but in Brazilian regions with less climatic variations, such as the north, northeast, and midwest, individuals reproduce all year (Cribb et al., 2013). Artificial reproduction techniques for bullfrogs have been developed (Agostinho et al., 2011, 2000), even though we have not found any farms that apply such methods in our survey.

Table 1. Production workflow in bullfrog farms, their respective processes (trade and production) and stages of processes, mandatory activities, and the marketed products.

Type of production	Type of processes	Stages of processes	Mandatory activity	Products marketed
Complete	Commerce phase 1	Live frog purchase	Yes	Tadpole, metamorphic or adult
	Production phase 1	Reproduction, eggs development, tadpoles raising, metamorphosis	Yes	-
	Production phase 2	Initial growth, final growth	Yes	-
	Production phase 3	Slaughtering	No	-
	Commerce phase 3	Processed frog sales	No	Meat or co-products
	Commerce phase 2	Live frog sales	No	Tadpole, metamorphic or adult
First-stage	Commerce phase 1	Live frog purchase	Yes	Tadpole, metamorphic or adult
	Production phase 1	Reproduction, eggs development, tadpoles raising, metamorphosis	Yes	-
	Commerce phase 2	Live frog sales	Yes	Tadpole, metamorphic or adult
Growth	Commerce phase 1	Live frog purchase	Yes	Metamorphic or adult
	Production phase 2	Initial growth, final growth	Yes	-
	Production phase 3	Slaughtering	No	-
	Commerce phase 3	Processed frog sales	No	Meat or co-products
	Commerce phase 2	Live frog sales	No	Adult
Slaughtering	Commerce phase 1	Live frog purchase	Yes	Adult
	Production phase 3	Slaughtering	Yes	-
	Commerce phase 3	Processed frog sales	Yes	Meat or co-products

Female bullfrogs produce an average of 10,000 viable oocytes, which, after fertilization (i.e., eggs) will be collected from the mating area and incubated for a period of 48 to 72 h (Cribb et al., 2013). After the eggs hatch, tadpole raising begins and ends with the end of the metamorphosis. The producers usually divide tadpole development cycle into four phases: G1, roughly equivalent to Gosner's (1960) stages 17 to 23; G2, (= Gosner's stages 24 to 37); G3, (= Gosner's stages 38 to 40); and G4 (= Gosner's stages 41 to 46). Tadpoles generally take from three to four months to complete their development under ideal captivity conditions (Cribb et al., 2013). During these phases, it is essential to monitor and control water quality, as well as to renew tank water daily (Castro and Pinto, 2000). In phases G1 and G4 tadpoles also consume the energetic reserves from the vitellogenic sac and from the re-absorption of the tail, respectively (Gosner, 1960). Phases G2 and G3 represent the predominant period of development and growth, in which tadpoles are exclusively exotrophic, thus dependent on feed supply, which is generally fish feed, as there is no table of nutritional requirements for bullfrogs available, one of the obstacles to the activity (Seixas Filho et al., 2008). However, several studies on zootechnical performance have been carried out with tadpoles, evaluating feed digestibility, ideal protein levels, and daily supply needs (Barbosa et al., 2005; Lima et al., 2003a; Secco et al., 2005). The ideal water temperature for tadpole development during raising phase is about 23 ºC (Cribb et al., 2013). Increase in temperature within the limits of thermal tolerance, accelerates the metabolic rates of individuals, completing metamorphosis faster (Hoffmann et al., 1988). On the contrary, the decrease in water temperature can extend the period of the tadpole phase and can be used as a management strategy, ensuring a tadpole stock during the coldest months of the year, when reproduction decreases (Álvarez and Nicieza, 2002; Seixas Filho et al., 2017).

After the complete absorption of the tail (end of the metamorphosis), production phase 2 begins, which is the individual's growth, from metamorphic to large adults. The first stage of this process, called initial growth, comprises the growth of individuals from eight to 50 g. After reaching 50 g, the second stage begins (final growth), which will last until the individual reaches the desired slaughter weight (Ferreira et al., 2002). Unlike tadpoles, which are omnivorous, post-metamorphic frogs are exclusively carnivorous and require a high protein diet, which comes from commercial fish feed (Casali et al., 2005a). At the initial growth phase, individuals are highly susceptible to protein content in the diet. Approximately 40 % of protein is recommended for better growth rates, efficient use of feed, and reduced vulnerability to diseases (Olvera-Novoa et al., 2007). Feed used in the final growth phase can have a higher

percentage of raw protein, which guarantees greater weight gain with less daily consumption when compared to the initial growth (Lima et al., 2003b).

Some commercial rations have been reported to present good nutritional performance (Fenerick Junior and Stéfani, 2005), although they are not accompanied by information on their efficiencies (Casali et al., 2005a). A diet with such commercial rations can represent low efficiency of protein deposition and high efficiency in fat deposition (Pereira et al., 2015), in addition to histopathological changes and mortality (Hipolito et al., 2004; Seixas Filho et al., 2009, 2008). Feeding represents one of the main costs of a bullfrog farming, which will only be economically competitive using feed with more efficient rates of feed conversion (Moreira et al., 2013). Therefore, it is essential to devise a specific bullfrog diet, by investigating the necessary requirements of proteins, amino acids, minerals and vitamins, and the digestibility of feed. This will reduce the production costs and improve the nutritional quality of the meat (Olvera-Novoa et al., 2007; Pereira et al., 2015).

The control of temperature, animal density, and regularly screening of individuals to form homogeneous batches encourage the simultaneous consumption of feed and reduce losses due to cannibalism (Braga and Lima, 2001; Casali et al., 2005b; Fontanello et al., 1993), a problem commonly reported by producers. Production phase 2, under ideal conditions, should last from three to four months. Thus, the period from egg laying to slaughtering should be due in six to eight months (Cribb et al., 2013). Besides that, genetic improvement research and development can assist in reducing the completion time of the production cycle, in addition to optimizing feed conversion (Marcantonio et al., 2002).

The ideal bullfrog weight for slaughtering according to the Brazilian standards is between 250 and 300 g (Seixas Filho et al., 2017), but it can vary according to the requirements of the slaughter industry, reaching up to 380 g. Production phase 3 (slaughtering) begins with the selection of individuals at the desired weight. In Brazil, the slaughtering and processing of bullfrog meat must be carried out in establishments under an official health inspection regime, respecting quality and hygienic-sanitary standards defined by the Regulamento e Inspeção Industrial e Sanitária de Produtos de Origem Animal (RIISPOA) of the MAPA (Brasil, 2017). The main methods of individuals stunning prior to slaughtering are: i) thermal, immersion in cold water or salt solution bath, and ii) electrical, stunning with electric shocks (Ramos et al., 2004). After stunning, individuals are hung up upside down in hooks, where bleeding occurs, followed by skin removal and evisceration, separation of the head and viscera, packaging of the final product, and freezing (Cribb et al., 2013; Seixas Filho et al., 2017). We recorded reports from many producers about difficulties, bureaucracy, and delay in the certification process. We

only registered three establishments in the Serviço de Inspeção Federal (SIF). However, many Brazilian producers carry out the entire process, including the slaughtering (production phase 3), and even direct product marketing, which usually takes place locally (**Table 1, Fig. 2**).

We divided the sales into two types: i) live bullfrog's sales and ii) meat and/or co-products sales (**Table 1, Fig. 2**). In live sales, tadpoles or metamorphic may be sold to other farms who will complete the metamorphosis and growth process, respectively. Adult frogs with weight for slaughter are sold to frog slaughter industries. Generally, individuals can be sold at any stage of development to producers who are starting their farms or need supplementary individuals. Meat and/or co-products sales happen either between business to consumer (B2C) or between business-to-business (B2B), generally in larger scales.

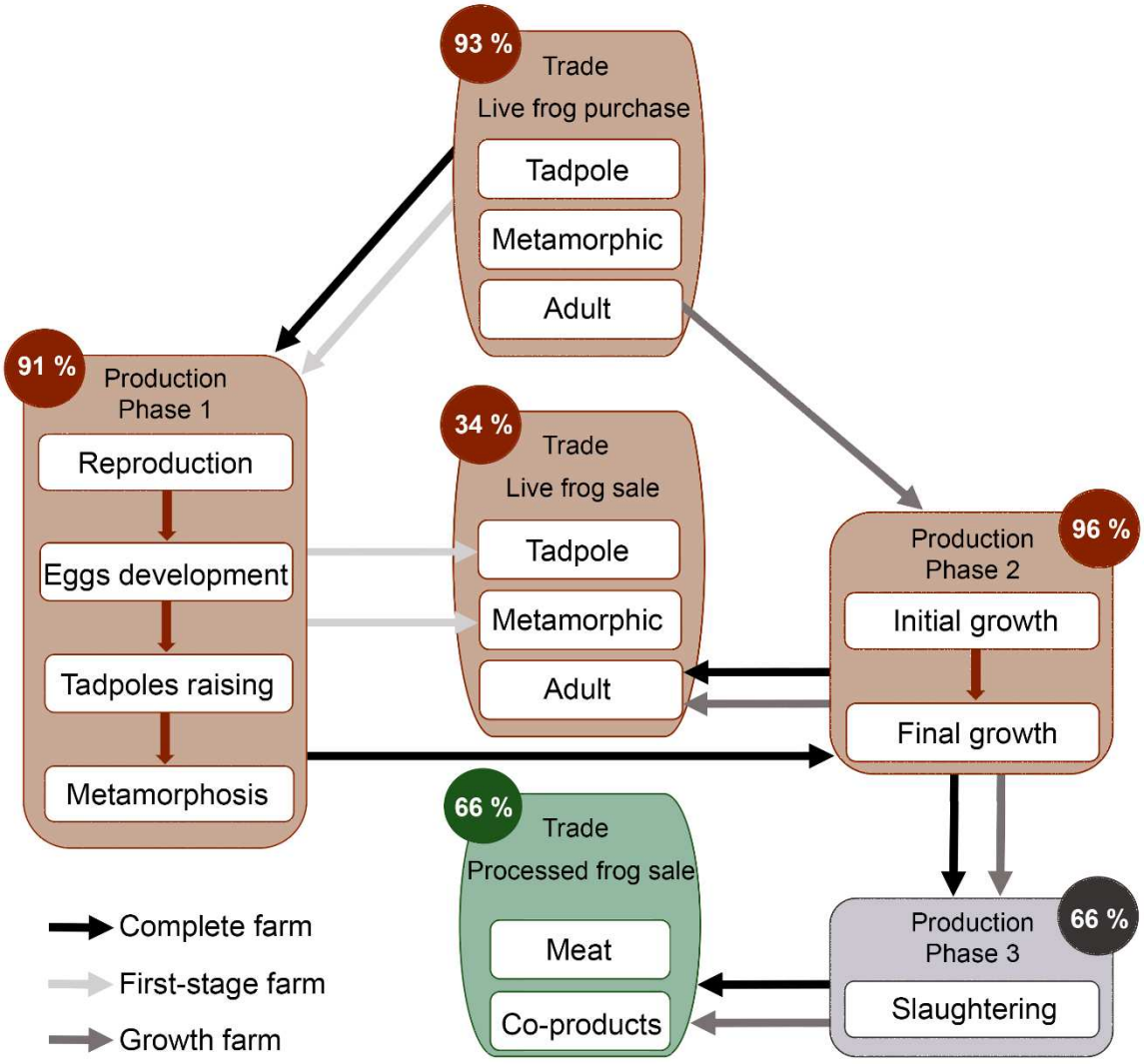

Fig 2. Production and trade processes in bullfrog farms and the representation of the number of farms (highlighted percentages) carrying out each type of process. Arrows represent the paths taken by each type of production workflow: black arrows represent complete production, with

the possibility of slaughtering; light grey arrows represent first-stage production; and dark grey arrows represent growth, with or without slaughtering in the same farm.

4. Types of bullfrog production workflow

We classified the farms regarding their production phases and products marketed: i) *complete production farms*, those that present all three phases, from reproduction to marketing live frogs, meat and/or co-products; ii) *first-stage production farms*, which deals only with the reproduction, eggs development, and tadpoles raising phases, selling live tadpoles and/or metamorphic to other farms; iii) *growth production farms*, where producers acquire tadpoles and/or metamorphic from other farms and perform only the frog growth phase, selling live individuals to slaughterhouses or selling frog meat and/or co-products locally; iv) *slaughtering factories*, where there are no frog development or maintenance, restricted only to the acquisition of individuals with slaughter weight from other farms, locally slaughtering and commercializing meat or co-products; and v) *scientific production* farms, where all stages of production can occur, however these frogs will serve to scientific purposes rather than commercial, as for research and teaching in universities.

We obtained information from 76 farms that are currently operating in Brazil. Most of them (81.6 %) are complete farms, with or without their own slaughtering (**Table 1, Fig. 2, Supplementary Table S1**). The fact that most producers perform the entire production chain can be one of the major obstacles to the activity. The different stages and processes of production require very different facilities, management, and nutrition techniques (Cribb et al., 2013; Seixas Filho et al., 2017). For example, tadpoles raising requires large water supplies, with strict quality parameters, but can be done in smaller spaces (Cribb et al., 2013; Seixas Filho et al., 2017). On the other hand, the growing sector does not require such conditions, but can represent about 70 % of the farm constructed area (Ostrensky et al., 2008). An integration model, as used in other types of animal production, such as swine and poultry would be an alternative. It involves less investment and risks, generating specialization of production and gain in scale, which could boost frog farming (Belusso and Hespanhol, 2010; Moreira, 2011). In addition, the improvement in the activity results in greater investments in input industries, genetic improvement, and breeding technology (Moreira, 2011).

There are currently few producers specialized in particular production processes. We found greater representation in the growth production, followed by first-stage production, and slaughtering (**Table 1, Fig. 2, Supplementary Table S1**). We also classified and quantified bullfrog breeding in terms of production for scientific purposes (**Supplementary Table S1**).

Research institutions and universities have been involved in improving frog farming since the late 1970s (Cribb et al., 2013). They kept on playing an important role in Brazilian frog farming, especially regarding research on nutrition (Mansano et al., 2020), health (Antonucci et al., 2014; Oliveira et al., 2020), reproduction (Agostinho et al., 2011), animal welfare (Alves et al., 2020), slaughter (Ramos et al., 2004), and processing (Nascimento et al., 2019; Seixas Filho et al., 2020).

Although we classified bullfrog farms in these five categories, they represent the main activities of the farms, and not necessarily the only ones. The current bullfrog farming does not follow standards, and, depending on the production volume, producers can slaughter the bullfrogs in their own farms, or sell a surplus to slaughterhouses when production is high. We observed a lot of flexibility, especially in trade, in which the sale of live individuals is recurrent, even if it is not the main activity of a particular farm.

5. Distribution of bullfrog farms in Brazil

The pathways of introduction of bullfrogs in Brazil are not yet a consensus in the literature (e.g., Jorgewich-Cohen et al., 2020). Some authors mention that the first introduction of the bullfrog came from the United States of America (Sousa and Maltarolo, 2019), while others suggest that the origin is Canada (Cribb et al., 2013). Indeed, the first 300 bullfrog couples were brought from North America to Brazil in 1935 by a Canadian technician (Ferreira et al., 2002). Shortly after, the first commercial farm was implemented in Brazil, in the state of Rio de Janeiro, followed by São Paulo, in 1939 (Silva et al., 2013). From the 1940s onwards, the Divisão de Caça e Pesca, at the time belonging to the Departamento de Produção Animal of the Ministério da Agricultura (now MAPA), extensively encouraged the practice of bullfrog farming through the free supply of bullfrog tadpoles to anyone interested in starting a production (Fontanello and Ferreira, 2007).

Three different dates in the literature report secondary imports: 1955 (imported from the United States; FAO, 1988), 1970's (Schloegel et al., 2010b; Mazzoni, pers. Com.), and 1980's (Kraus, 2009; Jorgewich-Cohen et al., 2020). However, we only have confirmation (from the importer; L.D. Vizotto) for a second import in the 1970s. In addition, there is an indication that this batch contained 20 individuals and came from Michigan, USA (Schloegel et al., 2010b; Jorgewich-Cohen et al., 2020). In fact, the Brazilian bullfrog market expanded in the early 1980s, with the increase in technology and facilities improvement (Cribb et al., 2013). The activity peaked at the end of the 1980 decade, with about 2,000 farms operating in the country (Lima and Agostinho, 1989), while in the next decade (1990's) there are records of only 170

active farms (Rodrigues et al., 2010). Over the years, frog farming has faced several obstacles, such as lack of investment, lack of government incentives, difficulties in management, and high production costs, which reflected in the large fluctuation in the number of producers (Lima et al., 1999; Moreira et al., 2013; Rodrigues et al., 2010).

There is general high interest and financial dazzle when it comes to starting a bullfrog production in Brazil, but the proper zootechnical, logistical, and environmental instruction is usually lacking. Adequate planning for the practice of frog farming is essential and must be carried out carefully. For example, the farm geographic location, as well as the abiotic conditions, the availability and quality of water, local legislation, market research, management practices, sanitary conditions, commercialization, and financial aspects, altogether are rarely considered before starting (Rodrigues et al., 2010). Frog farming projects without such complex planning have resulted in a considerable number of producers that left the activity in the first years of the business (Cribb et al., 2013; Feix et al., 2006). We obtained information about the installation date of 69 bullfrog farms. Approximately 61 % of these have started their activities recently (in the 2010's), of which ca. 95 % remain active today (**Supplementary Fig. S1**). Therefore, the market is full of recent producers. In addition to the financial loss of an unsuccessful business, the end of farm activities can lead to environmental irresponsibility, mainly due to the release of bullfrogs into the wild, maintaining a severe biological invasion that impact negatively native amphibian populations (Cunha and Delariva, 2009; Falaschi et al., 2020; Garner et al., 2006).

We mapped 151 bullfrog farms in Brazil during the sampling period and classified these farms into three categories: i) active, when they were in operation, i.e., producing and commercializing, or in the installation process of the farm, but without commercialization yet (60.9 % of the farms); ii) inactive, when we have evidence that the farm existed, but it is no longer in operation (13.2 % of the farms); iii) no information, when we have evidence of the farm existence, but could not access the information of its operation, if any (25.8 % of the farms).

The mapped bullfrog farms are distributed in the five Brazilian regions, with greatest concentration in the southern (S) and southeastern (SE) (136 farms). The northern (N), northeastern (NE), and midwestern (MW) regions had for only 11 farms. Besides, we were not able to locate four farms. These are the ones for which there is evidence of existence, but information on its location is missing (**Fig. 3, Supplementary Table S2**). In 2017, 53 farms were recorded in the SE, 17 in the S, and 7 for the N, NE, and MW regions (IBGE, 2021). The SE region has always been significant in Brazilian frog farming (Ostrensky et al., 2008). In

2010, the region presented 145 farms, most of them in the states of São Paulo and Rio de Janeiro. Even though the state of Espírito Santo presented only four farms, it has the best climatic conditions for bullfrog production and great potential for expanding frog farming (Rodrigues et al., 2010). Nowadays, there are, at least, twice the number of active farms in the state of Espírito Santo (**Fig. 3, Supplementary Table S2**).

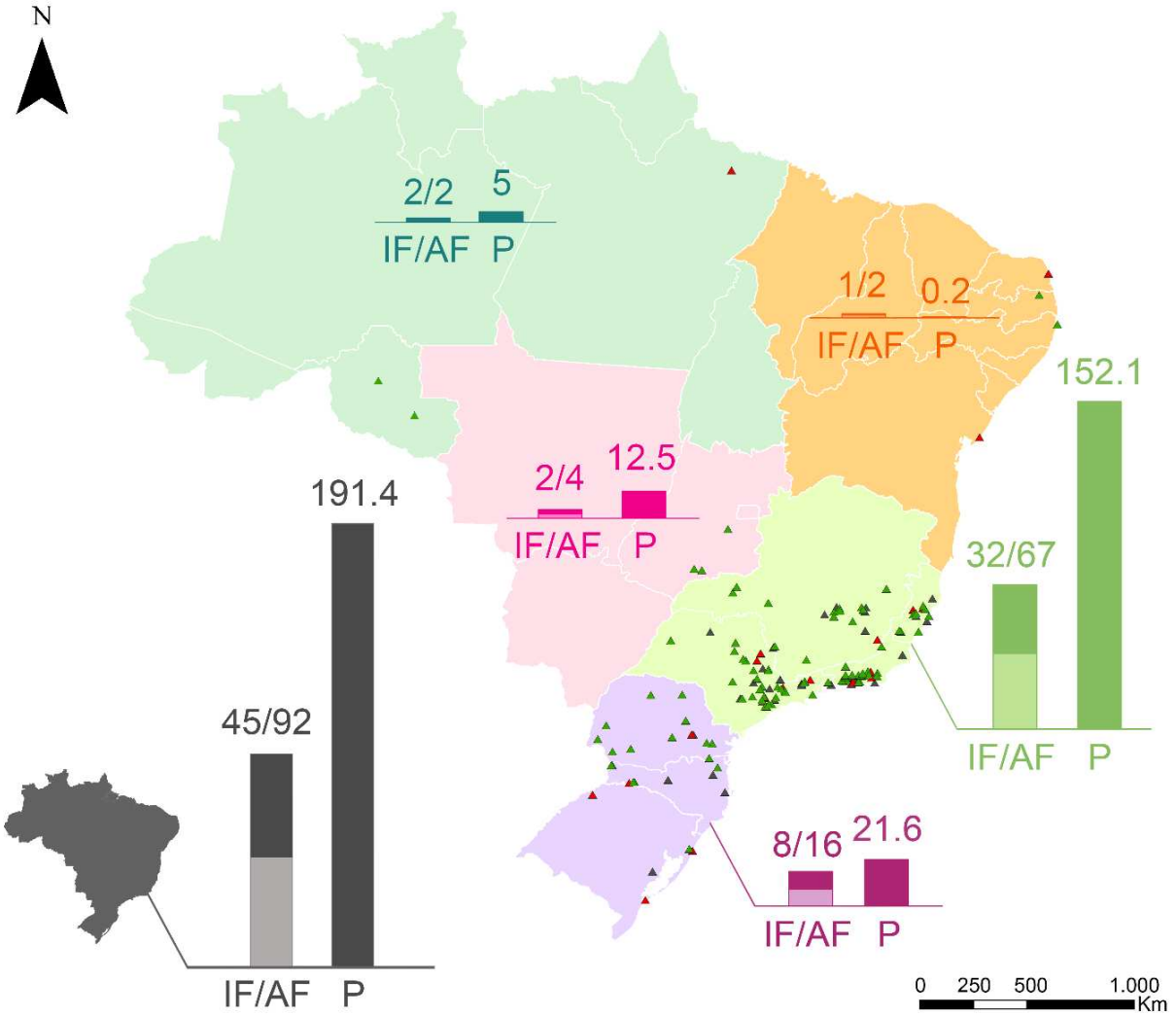

Fig 3. Distribution, status, and production of Brazilian bullfrog farms. Green triangles represent active farms, red triangles are inactive farms, and grey triangles are farms that we could not obtain information. The inset graphs represent the number of farms with known production information (IF), the total number of active farms (AF), and the production expressed in net tons of bullfrog meat per year (P).

Not surprisingly, the consumption of bullfrog meat is also higher in the SE region, especially in the states of São Paulo and Rio de Janeiro (Cribb et al., 2009). In Brazil, the

demand for bullfrog meat is mostly restricted to middle to upper-class restaurants, denoting that the economic potential of commercializing bullfrog meat is centered in large cities (Gavião, 2016; Moreira, 2011). The gourmet character of bullfrog meat, coupled with high production costs, raise the meat price and limit consumption practices (Feix et al., 2006; Ferreira et al., 2002). Nonetheless, Brazil demonstrates the potential for expansion of the activity, since consumer market's demand is three times greater than the offer (Cribb et al., 2009). If that expansion happens, it is reasonable to expect a cultural change in Brazilians, accepting more this sort of meat, which could in turn lower the prices of the production, and consequently the meat and its co-products.

6. Quantitative and economic aspects of the bullfrog production

Production is estimated based on the number of individuals that reach slaughter weight, the weight of these individuals (gross weight), or the weight of bullfrog meat produced (net weight). Due to these divergences, we standardized and calculated all production volume data based on live bullfrogs and bullfrog meat and expressed this data in (gross and net, respectively) tons per year. For this, we established that the slaughtered bullfrogs would weight 275 g each, which is an ideal average slaughter weight for Brazilian conditions (Seixas Filho et al., 2017). The carcass yield, that is, how much a live individual generates meat and co-products, averages 49 % in the main production systems in Brazil (Nascimento et al., 2019). In addition, we calculated the volume of the produced meat and multiplied it by the product price in each Brazilian region. We averaged the sale price of bullfrog meat based on the information we have gotten from producers. For the SE, S, and MW regions, the sale price of bullfrog meat was 9.9 USD / kg, in the NE 11.2 USD / kg, and in the N 13.6 USD / kg (**Table 2**).

The first FAO record of bullfrog production in Brazil dates back to 1986, when 10 tons of live weight was produced in Brazil, generating 50,000 USD (FAO, 2021). According to the FAO data, until 2012 Brazil represented the second largest bullfrog producer in the world, producing 670 gross tons and raising about 3.3 million USD in 2000 (FAO, 2021; Ostrensky et al., 2008). Brazilian production decreased to 200 tons of live weight per year from 2016 to 2018, resulting in 613, 671, and 586,000 USD income, respectively (FAO, 2021). Nevertheless, data recorded by FAO in recent years are rough estimates, as Brazil does not reported official production data to this organization since 2014 (FAO, 2020). In 2017 the Instituto Brasileiro de Geografia e Estatística (IBGE) recorded the selling of 129.3 net tons of bullfrog meat, representing 1.3 million USD (IBGE, 2021). In 2004 the SE region represented 69 % of national production (Ostrensky et al., 2008) and 52 % in 2017 (IBGE, 2021).

Based on our sampling, in 2019 we recorded an annual production of 390.6 gross tons of live weight, which generated 191.4 net tons of bullfrog meat (**Table 2, Fig. 3**). The selling of this amount of meat would have generated about 1.9 million USD in 2019 (**Table 2**). However, we registered production data for approximately half of the active farms in the sampled period. If we estimate an average production value for the farms that we did not obtain information, Brazil likely produced 798.6 tons of live weight and 391.3 tons of bullfrog meat in 2019. The SE region also led the production in 2019, with 79 % of the active farms, followed by the S, MW, N, and NE, which was the region with the smallest proportion of farms (0.6 %) (**Table 2, Fig. 3**). The SE had the largest representation, with 1.5 million USD, followed by the S with about 214,000 USD, MW with 124,000 USD, N with 68,000 USD, and the NE with 2,000 USD (**Table 2)**. In addition, these farms are certainly moving the local commerce with the purchase and sale of live individuals. We were unable to estimate the values of this type of sales due to high variability and low sample sizes in our datasets. However, live bullfrog trade is quite expressive. Prices range from 23.6 to 248.1 USD per 1,000 tadpoles, 79.4 to 310.1 USD per 1,000 juveniles, 12.4 USD each adult or about 5 USD / kg of the adult in slaughter weight, as breeder frogs can cost from 9.9 to 26.1 USD.

Table 2. Bullfrog production (alive or meat) expressed in net tons per year in bullfrog farms, as well the sale price of bullfrog meat and amount generated from their sale. The total number of active farms and the number of farms with known production information for each Brazilian region are also presented.

Regions	Active farms	Farms with known production information	Bullfrog production (tons/year)	Bullfrog meat production (tons/year)	Sale price of bullfrog meat (USD/kg)	Amount generated from the sale of bullfrog meat (USD/year)
North	2	2	10.2	5	13.6	68,000
Northeast	2	1	0.4	0.2	11.2	2,000
Midwest	4	2	25.5	12.5	9.9	124,000
Southeast	67	32	310.5	152.1	9.9	1,500,000
South	16	8	44.1	21.6	9.9	214,000
Unknown	1	0	0	0	-	-
Total	92	45	390.6	191.4	-	1,900,000

Brazilian frog farming is still predominantly composed by small producers, often amateurs, who generally practice frog farming as a secondary activity, with the help of family labor (Corrêa et al., 2008; Cribb et al., 2013; Sousa and Maltarolo, 2019). An alternative for leveraging their production is cooperativism or associativism. This model brings together producers who work in solidarity in carrying out all stages of the production chain, from the joint purchase of inputs to the products sale (Almeida et al., 2017; Moreira, 2011). Few examples of associations took place in Brazil, such as the Cooperativa Regional de Piscicultores e Ranicultores do Vale do Macacu e Adjacências (COOPERCRAMMA) from Rio de Janeiro, the Cooperativa de Ranicultores do Vale do Paraíba from São Paulo, and the Associação Pernambucana de Criadores de Rãs (APECRÃ) from Pernambuco (Ferreira et al., 2002; Moura, 2003). However, cooperativism faced difficulties, mainly due to governance issues, resulting in the end of these associations (Moreira, 2011). Currently, there is no active association, as Brazil beholds a novel scenario of individuality. Producers are moving away from collective objectives, a pattern already evidenced for a specific region in Rio de Janeiro (Borin and Lima, 2013).

A key economic aspect in aquaculture systems is the care and maintenance of water quality, not only the water used for production, but also its effluents (Qing et al., 2021). The discharge of wastewater, which contains high concentrations of organic and inorganic compounds and microorganisms (pathogenic or not), presents serious environmental impacts (Mercante et al., 2014; Viegas et al., 2021). Alternatives for wastewater treatment in aquaculture have been recently investigated, such as the use of microalgae and humic acid to remove organic compounds and pollutants, respectively (for details, see Chianese et al., 2020; Qing et al., 2021; Salvestrini et al., 2020; Viegas et al., 2021). Besides, treated wastewater can be of economic interest, as they can be used as biostimulants in seed germination (Viegas et al., 2021). Another economically advantageous approach for efficiently using and disposing wastewater is recirculating aquaculture systems (RAS), since the availability of quality water is decreasing and the costs are increasing (Ben-Asher and Lahav, 2016; Qing et al., 2021).

The accumulation of metabolites in production systems also represents a crucial challenge to overcome. It is important to know the maximum levels of metabolite accumulation, such as carbon dioxide (CO2), not to compromise physiology, performance, or welfare of the farmed species. Particularly in the case of the bullfrog, the respiratory signaling of locus coeruleus neurons in the brainstem, which senses alterations in CO2/pH and influences ventilatory adjustments, is affected in conditions of hypercapnic acidosis (Santin and Hartzler, 2013). Alternatives in aquaponics, i.e., the combination of aquaculture and horticulture within

a single recirculating aquaponic system (SRAPS), provide sustainable approaches, in which plants can fix the CO2 released by the farmed species (Kloas et al., 2015).

In summary, wastewater treatment is essential to minimize the impact caused by frog farming on receiving water bodies, promoting the sustainability of this activity in Brazil (Freitas Borges and Tavares, 2017). The main environmental impact in the aquaculture industry has its origin in both technical and allocative inefficiency, which are significant in explaining the level and variation in farm costs (Asche et al., 2009). In this sense, the development of frog farming in this country should be accompanied by technology and innovation.

7. Commercial routes and marketed products

We recorded 190 commercial routes and classified them as follows: i) intrastate, when the product is commercialized within the state of origin (n = 113); ii) interstate, when the product is commercialized between different states (n = 72), and iii) international, when the product is commercialized between Brazil and other countries (n = 5) (**Fig. 4, Supplementary Table S3**). In addition, we categorized the products marketed for each route as live bullfrogs, meat or co-products, and both. The commercial routes represent occasional purchases and sales, which occurred throughout the history of Brazilian frog farming. For example, trades from one state to another can occur periodically, however, these routes are dynamic and the data we express here do not necessarily represent current trading.

The bullfrog arrived in Brazil by international routes to start the national production or introduce genetic diversity in the first farms. The routes of trade from Brazil to other countries represent the supplying of bullfrogs for human consumption. For many years, Brazil supplied live bullfrogs to the United States, but Brazilian producers ended the trade, because it was no longer economically viable. All international routes we mapped represent the transport of live animals. In addition, we recorded the international trade of about 70 bullfrog skins from Brazil to Austria in 2004 and 2005 (CITES, 2021).

The legal or illegal trade of live animals represents one of the main paths of pathogens transport and introduction around the world (Fisher and Garner, 2020; Martel et al., 2014; O'Hanlon et al., 2018; Rodgers et al., 2011). The main pathogens threatening amphibians in the world (chytrid fungi and ranaviruses) have been found in Brazilian bullfrog farms (Mazzoni et al., 2009; Oliveira et al., 2020; Ribeiro et al., 2019; Santos et al., 2020), and in internationally traded bullfrogs (Brunner et al., 2019; O'Hanlon et al., 2018; Schloegel et al., 2012, 2009). While the batrachochytrid fungus is restricted to amphibians (Fisher and Garner,

2020; Scheele et al., 2019), ranaviruses are also pathogenic to other ectothermic vertebrates, fish and reptiles (Duffus et al., 2015).

The Brazilian interstate commercial routes are mostly represented by the trade of live bullfrogs, usually from one farm to another or to bullfrog slaughterhouses. This frequent exchange of individuals among farms can also imply in pathogens exchange through infected bullfrogs. New pathogens may infect not only production bullfrogs, but also native populations in the surroundings areas, through the waste water or frogs escape (Borges et al., 2012; Ribeiro et al., 2019). A large portion of sales is made to slaughterhouses, since the certification is centered on a few Brazilian establishments, which will slaughter and give a destination to the products (for example, to restaurants and other food industries). Meat and co-products represent more than 20 % of the interstate trade. In addition, the bullfrog meat market supplies the states where there is no production (**Fig. 4, Fig. 5, Supplementary Table S3**).

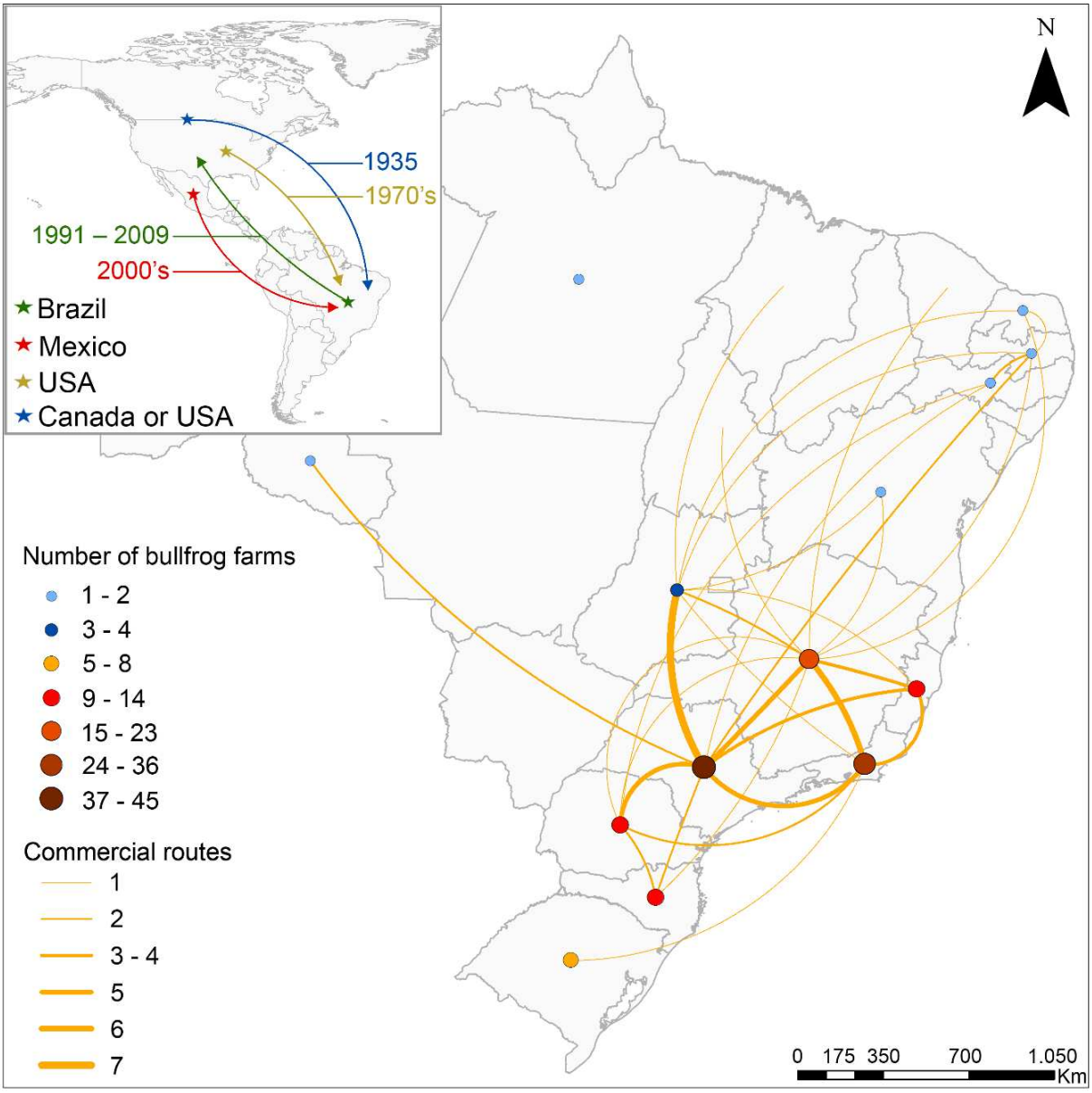

Fig 4. Commercial routes of bullfrogs (alive or meat) in the national and international trade, also indicating the number of farms in Brazilian states.

We divided states into immediate geographic regions to map the intrastate trade. This regions were established by taking into account the commercial connection of nearby cities and the relationships of dependence and displacement of the population in the search for goods (IBGE, 2017). Intrastate routes were numerous, and the state of São Paulo was the one with the largest number of routes (**Fig. 6**), followed by Rio de Janeiro, Minas Gerais, and Espírito Santo, all in the SE region, and Paraná and Santa Catarina, in the S region (**Supplementary Table S3, Supplementary Figs. S2 and S3**). It was in the intrastate scale that we observed more instances

of meat and co-products sales, with three states (Paraíba, Pernambuco, and Rondônia) selling only those. Anyway, the trade of live individuals was still relevant (**Fig. 5**).

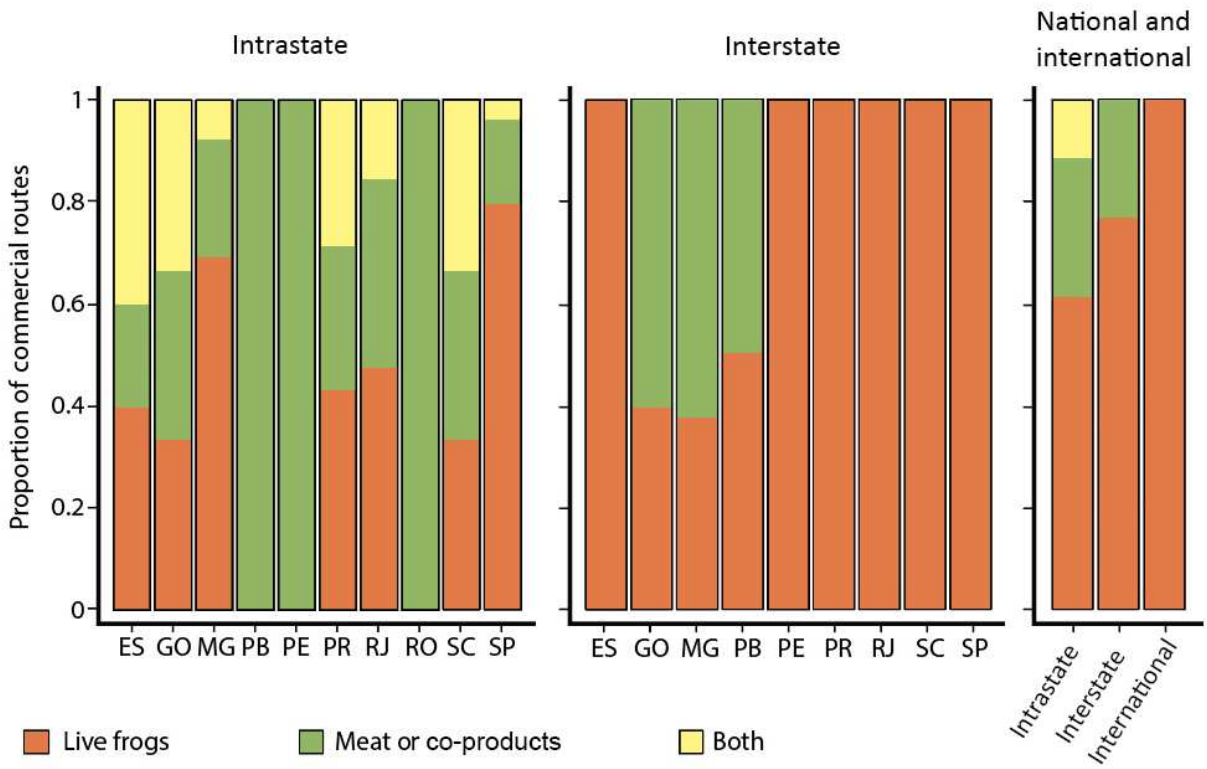

Fig 5. Proportion of marketed products (live bullfrogs, meat or co-products, and both) on the different routes, both in the national and international scales. Brazilian states are Espírito Santo (ES), Goiás (GO), Minas Gerais (MG), Paraíba (PB), Pernambuco (PE), Paraná (PR), Rio de Janeiro (RJ), Rondônia (RO), Santa Catarina (SC), and São Paulo (SP).

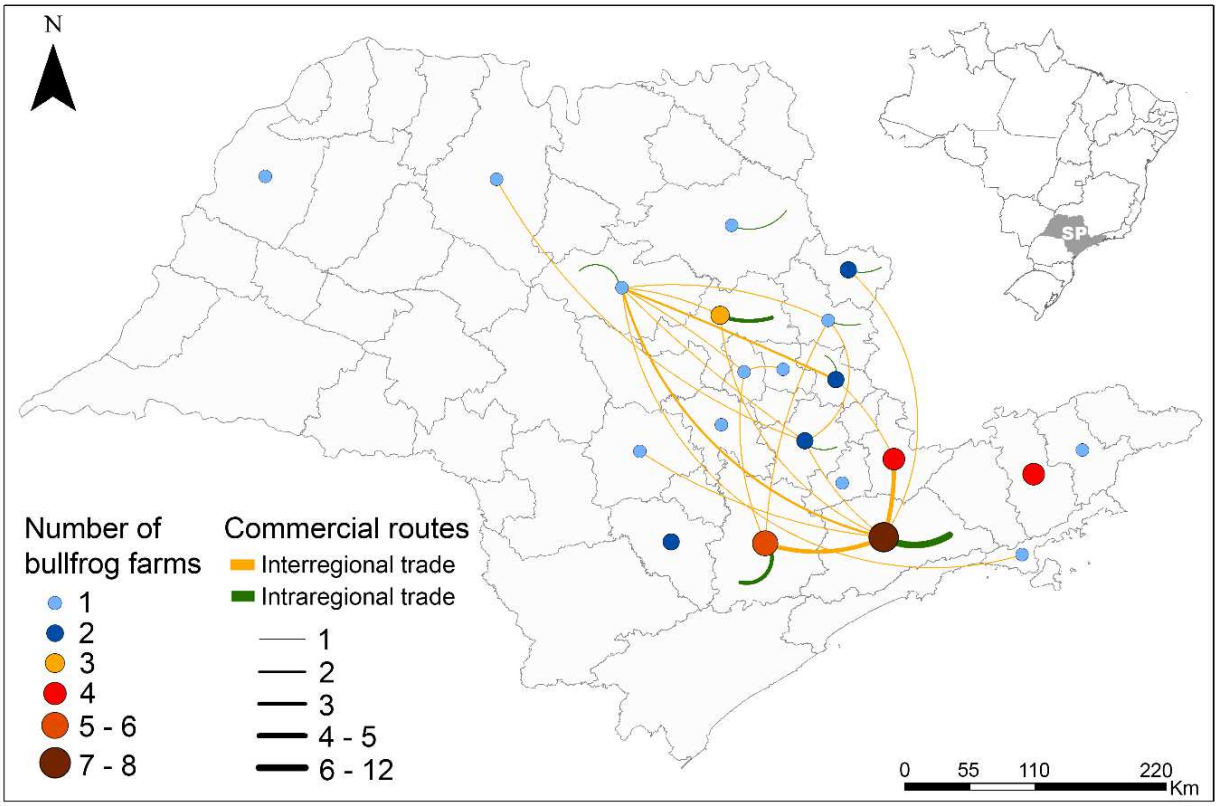

Fig 6. Commercial routes of bullfrogs (alive or meat) and number of farms in the immediate regions of the state of São Paulo.

The low and sporadic consumption of bullfrog meat by Brazilians is a reflection of the lack of knowledge about the product on the market, irregular supply, lack of consumption habits, high retail price, and unsatisfactory appearance of the product (Carraro, 2008; Costa et al., 2017; Cribb et al., 2009) (**Supplementary Fig. S4**). The main products marketed in Brazil are the whole carcasses and bullfrog legs. The former is associated with general aversion due to its appearance, while the latter presents high prices and is preferred in the international market (Cribb et al., 2009; Ramos et al., 2004). While many countries are limited to the consumption of the bullfrog noble parts, the co-products market is developing in Brazil (Furtado, 2006). Products such as frog's pâté, hamburgers, flour, shredded meat, and preserves based on parts that are usually discarded, like the back, forelegs, bones, viscera, and even the tadpoles' tail, have high acceptability of purchase intent (Afonso et al., 2017; Cribb et al., 2009; Furtado, 2006; Gonçalves and Otta, 2008; Seixas Filho et al., 2020). It is possible to add value to bullfrog meat, providing products with nutritional quality and low market price, making the most of all parts of an individual (Afonso et al., 2017; Rodrigues et al., 2014).

Food items that are not commonly consumed by the public are usually rejected for reasons other than their taste, even though many consumers are willing to try a novelty. Still,

the positive sensory experience is not enough for acceptance and the incorporation of foods considered culturally-inappropriate (Tan et al., 2016). Thus, future efforts to introduce unusual foods should focus mainly on marketing and disclosure, highlighting all the benefits of their consumption. This would encourage the market, in a way that both bullfrog meat and co-products could be placed in the shelves regularly, accompanied by an enhanced acceptance by consumers (Cribb et al., 2009; Oliveira et al., 2017; Tan et al., 2016).

8. Concluding remarks

A worldwide growth of aquaculture could be beneficial to the environment, with Brazil representing the country that would spare the greater area of land by reducing its dependency on livestock (Froehlich et al., 2018b). Brazil has great potential for expanding frog farming, not only because of the infrastructure and techniques employed by the farmers (Pahor-Filho et al., 2019), but also due to the adaptation of the bullfrog to Brazilian climatic conditions (Both et al., 2011; Rodrigues et al., 2010). Most of the farms are located in the south and southeast regions, but the north and northeast regions also have productive potential, where higher temperatures would favor the growth and weight gain of frogs (Giovanelli et al., 2008; Seixas Filho et al., 2017). However, the lack of instruction and planning when starting a frog farm enterprise generates a de-standardization of the productive systems and management by Brazilian producers, which has hampered the success of the activity (Pahor-Filho et al., 2019). Here, we proposed crucial actions for expanding the market for bullfrog meat and co-products and the rise of production activity. Among them, we highlighted the need for improvement in three segments: marketing, production, and frog farming chain.

For many years, Brazilian frog farming has been experiencing the so-called vicious cycles of supply and demand. The main products sold, the meat of the whole bullfrog or its legs, represent expensive goods, resulting in a niche market. This causes low demand, which in turn will repress production, making it low and costly (Lima, 2005; Ostrensky et al., 2008). Product presentation and the population's lack of knowledge about dishes made of bullfrog are challenges to overcome. Working on the consumer acceptance, in addition to offering more options of food based on frogs, as well as to improve the dissemination and marketing of products are fundamental actions for increasing demand.

With high demand, production needs to increase and supply products regularly, a situation that does not currently occur in Brazil (Cribb et al., 2009). The development of techniques for bullfrog genetic improvement can maximize spawning numbers and fertility rates, maintaining a regular supply of frog meat in the market (Marcantonio et al., 2002; Pahor-

Filho et al., 2019). Another difficulty faced by the producer is the high cost of the ration and the lack of specialized technical support (Ostrensky et al., 2008). As the small volume of bullfrog production in Brazil is a reflection of the low amount of bullfrog meat consumption, consequently, there is an apparent industry's lack of interest in developing specific feed for bullfrogs (Casali et al., 2005a). Besides, there is a lack of standardized research focused on nutrition, genetics, and animal management in frog farming, which are key aspects for reducing costs in this production.

We observed a large representation of small producers composing the Brazilian frog farming, which often end up developing all stages of the bullfrog production, including slaughter and commercialization of the products. The integration model and the producers' specialization could improve the activity, both economically and in relation to management techniques (Moreira, 2011). Collective thinking and creation of associations would also assist producers, especially the beginners or small ones. These associations would build a network including producers, technicians and scientists, exchanging information on various productive and commercial aspects (Almeida et al., 2017). In addition, many of the proposed improvements are associated with the main gap in Brazilian frog farming: the lack of the industry records and data on bullfrog production. The lack of information does not generate visibility, hindering several actions and external (and monetary) interests.

Finally, we encourage government to support the development of economically and environmentally coherent policies, and the compliance with current legislation. Currently, one of the main challenges in animal production is linked to environmental sustainability (Salter, 2017). Although at first glance contradictory, we believe that stimulating the frog market is a way to increase its regulation and surveillance. That would reduce the risk of new frog escapes, less untreated waste water in the environment, and the beginning of a national system of disease monitoring, especially those that most threaten wild amphibians, the chytrid fungus and ranaviruses, which are of obligatory notification to the OIE (Schloegel et al., 2010a).

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SUPPLEMENTARY DATA

Table S1. Number and percentage of farms that present each type of production as their main activity, and for each type of production, the percentage of those that perform their own slaughtering.

Type of production	Number of farms (%)	% of slaughtering
Complete	62 (81.6)	72.6
First-stage	2 (2.6)	0
Growth	6 (7.9)	16.6
Slaughtering	1 (1.3)	100
Scientific	5 (6.6)	60

Table S2. Bullfrog farms active in Brazil organized by region, showing the percentage of active farms, and in parentheses: the absolute number of active farms and the total absolute number of farms in each state. Unknown refers to the farms for which there is evidence of existence, but information on its location is missing.

Region	State	Percentage of active farms (active/total)
North	Acre	0 (0/0)
North	Amapá	0 (0/0)
North	Amazonas	0 (0/0)
North	Pará	0 (0/1)
North	Rondônia	100 (2/2)
North	Roraima	0 (0/0)
North	Tocantins	0 (0/0)
Northeast	Alagoas	0 (0/0)
Northeast	Bahia	0 (0/1)
Northeast	Ceará	0 (0/0)
Northeast	Maranhão	0 (0/0)
Northeast	Paraíba	100 (1/1)
Northeast	Pernambuco	100 (1/1)
Northeast	Piauí	0 (0/0)
Northeast	Rio Grande do Norte	0 (0/1)
Northeast	Sergipe	0 (0/0)
Midwest	Goiás	100 (4/4)
Midwest	Mato Grosso	0 (0/0)
Midwest	Mato Grosso do Sul	0 (0/0)
Southeast	Espírito Santo	61.5 (8/13)
Southeast	Minas Gerais	60 (12/20)
Southeast	Rio de Janeiro	60 (18/30)
Southeast	São Paulo	64.4 (29/45)
South	Paraná	78.6 (11/14)
South	Santa Catarina	44.4 (4/9)
South	Rio Grande do Sul	20 (1/5)
Unknown	Unknown	25 (1/4)

Table S3. Bullfrog commercial routes. Origin and destination, types of routes, period of trade, and marketed products (available as an .xlsx file).

https://drive.google.com/drive/folders/1udGeg2RUEHp-dijqzRVbttJwPWBM-6CG?usp=drive_link

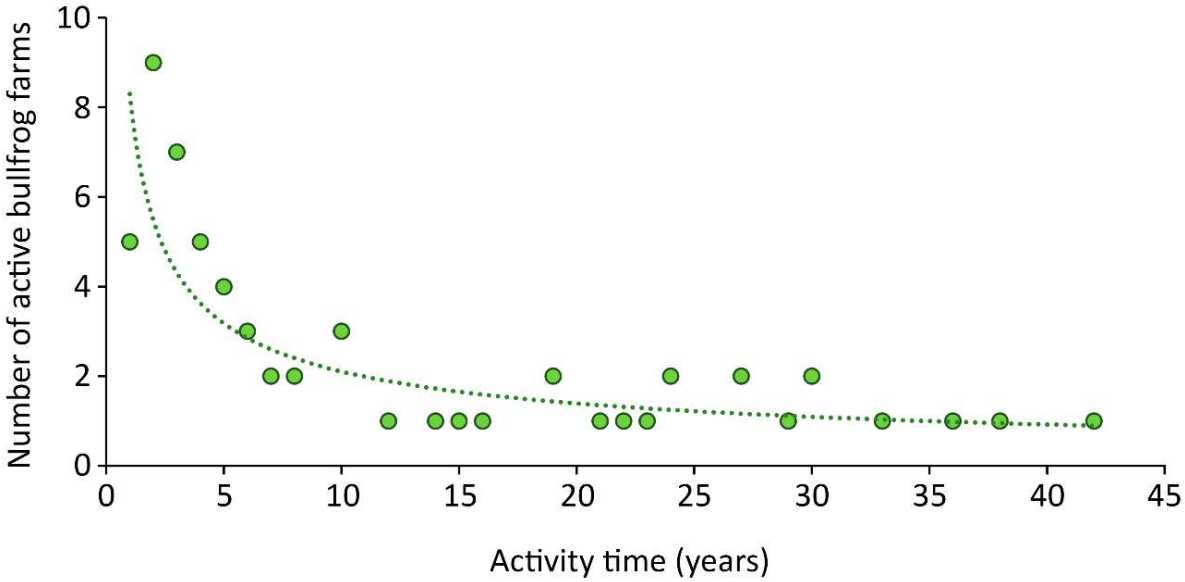

Fig S1. Bullfrog farms operating time and number of farms that have remained active until 2020.

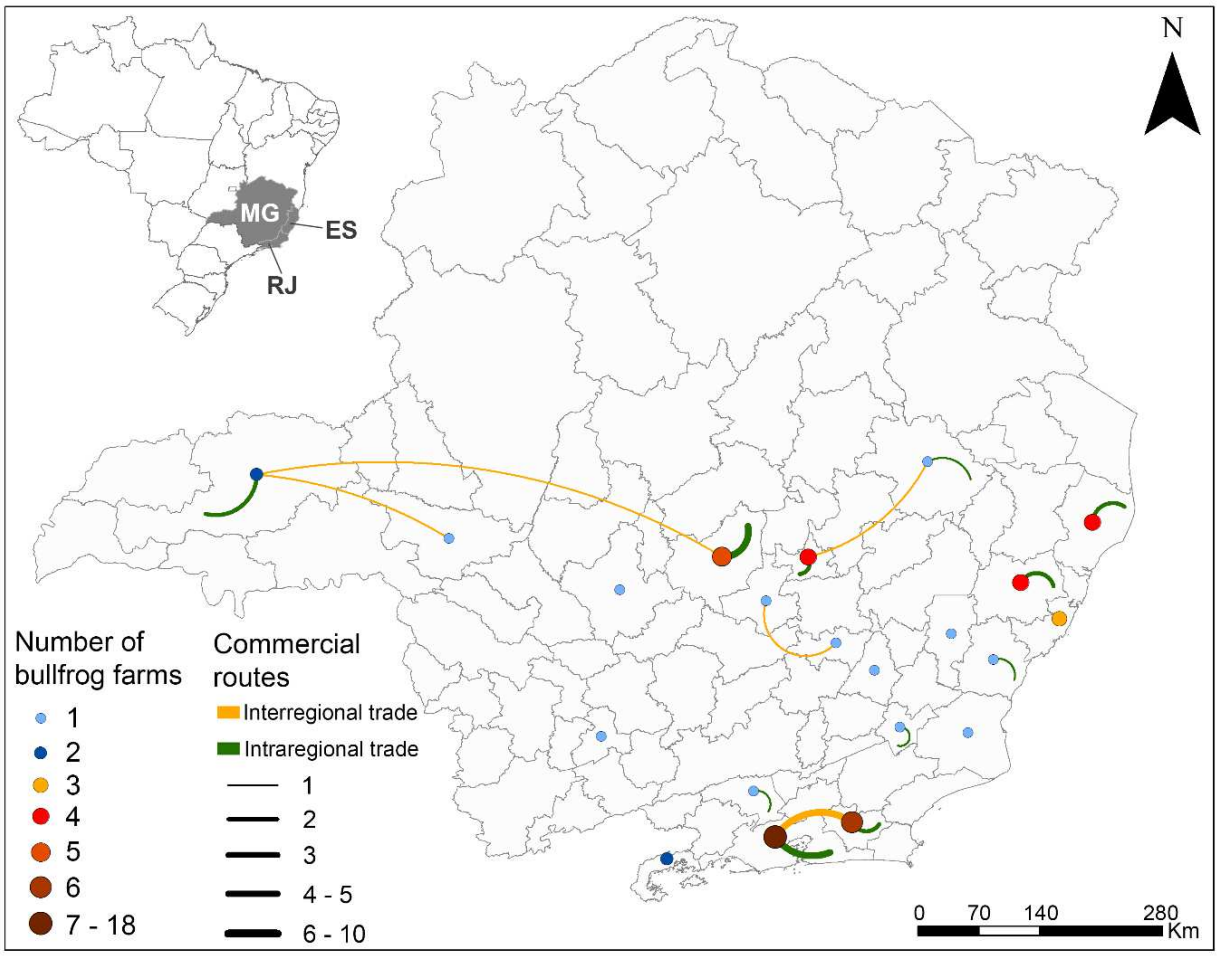

Fig. S2. Commercial routes of bullfrogs (alive or meat) and number of farms in the immediate regions of the states of Minas Gerais (MG), Rio de Janeiro (RJ), and Espírito Santo (ES) in the Southeast region of Brazil.

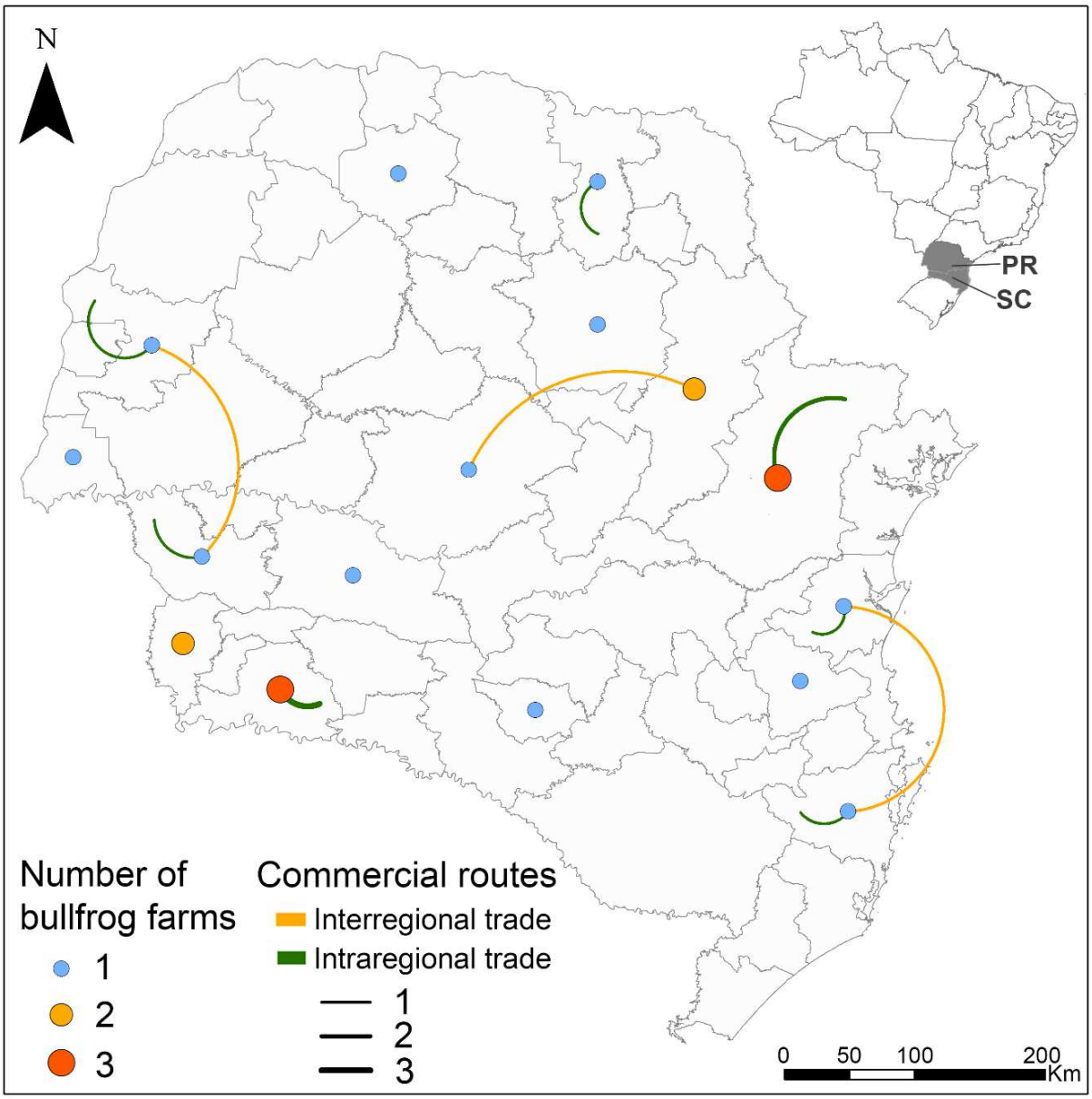

Fig. S3. Commercial routes of bullfrogs (alive or meat) and number of farms in the immediate regions of the states of Paraná (PR) and Santa Catarina (SC) in the South region of Brazil.

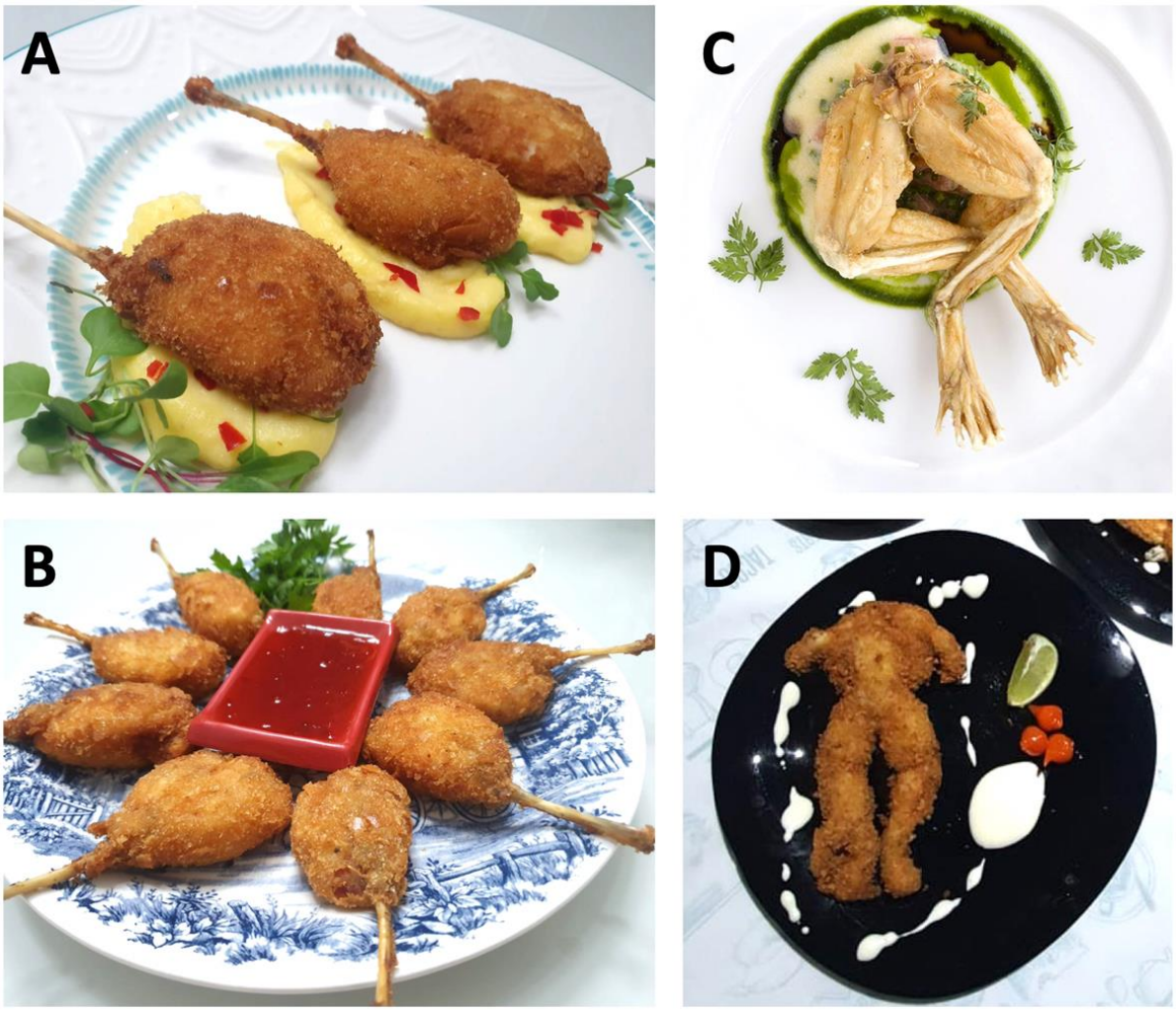

Fig. S4. Presentation of bullfrog meat dishes, exemplifying appetizing appearance that stimulates public consumption (**A** and **B**), and unsatisfactory presentation for consumption (**C** and **D**).

CHAPTER II

GENOTYPIC DISCRIMINATION OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN BULLFROG FARMS

Discriminação genotípica de *Batrachochytrium dendrobatidis* em ranários brasileiros

Luisa P. Ribeiro, David Rodriguez, Roseli Coelho dos Santos, Elaine M. Lucas, Luís Felipe Toledo

This chapter adheres to the guidelines outlined by the journal *Molecular Ecology*, to which it is intended for submission.

Genotypic discrimination of *Batrachochytrium dendrobatidis* in bullfrog farms

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Abstract

The international trade of amphibians raises concerns regarding its impact on native populations, potentially introducing distinct lineages of the chytrid fungus (*Batrachochytrium dendrobatidis* or Bd) into new environments. The widely used qPCR diagnostic method lacks the capacity to differentiate between lineages, emphasizing the necessity for more accurate genotyping approaches. Here we propose the application of TaqMan SNP genotyping assays to effectively discriminate Bd genotypes (GPL, Brazil, and Hybrid) in samples from Brazilian frog farms, aiming to enhance understanding and support conservation efforts in the context of the global amphibian trade. Samples, including skin swabs, tadpole mouth swabs, and pure Bd cultures, were collected from bullfrog farms spanning 2,215 km in Brazil. Two assays were employed to identify the presence of genotypes using both qPCR and Digital PCR (dPCR), analyzing the relationship between Bd load and genotype determination. The genotyping assay, utilizing both qPCR and dPCR, achieved an efficiency of 56.6 % (336 out of 593 samples), with a notable increase in the probability of success of genotyping with higher Bd loads. Samples with loads ranging from 10 to 1,000 g.e. showed a success rate of approximately 58 %, while those with over 1,000 g.e. had an 81.8 %. Culture samples achieved a 100 % success rate. All Bd genotypes were identified in the frog farms, with Bd-BRAZIL being the most prevalent. Additionally, we highlighted the issue of coinfections and hybrids in bullfrog farms where animals are raised at high densities. Our study presents the efficient TaqMan SNP genotyping for discrimination Bd genotypes, providing a highly effective method for determining Bd genotypes from both pure cultures and field samples with low Bd loads. We emphasize the need for in-depth investigations into the dynamics of infection by different Bd lineages, underscoring the importance of comprehensive genetic studies and advanced methods for genotype discrimination in the context of the global amphibian trade.

Keywords: Amphibian trade, Bd genotypes, chytrid fungus, TaqMan SNP genotyping, *Aquarana catesbeiana*.

1. Introduction

The chytrid fungus *Batrachochytrium dendrobatidis* (hereafter Bd) poses a significant threat to amphibian populations on a global scale, recognized as a causative agent of population declines and extinctions in several species (Luedtke et al., 2023; Scheele et al., 2019). Responsible for the chytridiomycosis, Bd exhibits a broad global distribution and is characterized by at least six phylogenetically distinct lineages: Bd-GPL, Bd-BRAZIL, Bd-CH, Bd-ASIA1, Bd-ASIA3, Bd-CAPE (Byrne et al., 2019; O'Hanlon et al., 2018). Bd-GPL is known for its extensive geographical distribution and has been directly linked to events of declines and mass extinctions of amphibians in various regions worldwide (Farrer et al., 2011; O'Hanlon et al., 2018). In contrast, the other lineages show a more restricted geographic distribution, being limited to two or three specific regions (Byrne et al., 2019; O'Hanlon et al., 2018).

The expansion of international amphibian trade has raised significant concerns regarding the potential introduction of novel lineages of Bd, particularly those endemic and with more restricted distributions (O'Hanlon et al., 2018; Schloegel et al., 2012). Recent research underscores the crucial importance of actively monitoring and regulating amphibian trade as a precautionary measure to prevent the dissemination of Bd (Borzée et al., 2021; Hughes et al., 2021). Beyond worries about the introduction of new lineages to naïve populations, there is concerns about the emergence of Bd-recombinants (hybrids) (Byrne et al., 2019; Schloegel et al., 2012), which could display heightened virulence (Greenspan et al., 2018), and the coinfection of individuals with different fungal lineages (Carvalho et al., 2023; Jenkinson et al., 2018). Few recent studies have extensively employed molecular tools to discriminate among Bd lineages (Byrne et al., 2019; O'Hanlon et al., 2018), contributing to a limited understanding of the geographic distribution and origin of these lineages.

Currently, the prevailing method for diagnosing Bd involves amplifying the fungus via real-time Polymerase Chain Reaction (qPCR) from samples of amphibian skin swabs (Boyle et al., 2004). DNA samples extracted from tadpole mouths are also employed for diagnosis using this qPCR method, as the fungus primarily infects tadpoles' mouthparts (Knapp & Morgan, 2006). Despite being a non-invasive and easily applicable method, this technique only enables the detection and quantification of the Bd fungus without the ability to discriminate the infecting lineage. For a more precise characterization of the fungal genotype, traditional methods such as whole genome sequencing (WGS) of pure Bd cultures have been utilized (Ghosh et al., 2021). However, due to the challenges in isolating pure cultures and the high associated costs of sequencing, new genotyping approaches have emerged, including the

custom Bd genotyping assay using the Fluidigm Access Array platform (Byrne et al., 2017), and a specific TaqMan qPCR assay for lineage differentiation (Ghosh et al., 2021).

Another frequently employed approach for lineage determination is genotyping of Single Nucleotide Polymorphisms (SNPs), entailing the identification of variations in DNA sequences at distinct bases within the genome. This method plays a pivotal role in comprehending the genetic foundation of both common and complex diseases (Shen et al., 2009). Recently, two SNP genotyping assays, using qPCR, were employed to discriminate Bd genotypes in mixed samples from an infection experiment (Carvalho et al., 2023). TaqMan assays, renowned for their sensitivity, cost-effectiveness, and ease in precisely identifying SNPs, enable the detection of subtle genetic variations among different Bd genotypes, even when applied to digital droplet PCR (dPCR). This method creates microdroplets from samples before traditional PCR amplification, allowing the individual detection of each droplet to obtain the initial concentration or copy number of the target molecule, demonstrating high accuracy in detecting rare alleles (Li et al., 2023).

In this context, our study aims to assess the effectiveness of a Bd genotyping method using TaqMan SNP Genotyping assays in qPCR and proposes a new SNP genotyping approach in dPCR. The goal is to efficiently discriminate between genotypes (GPL, Brazil, and Hybrid) in a manner feasible for the comprehensive monitoring of Bd lineages. By employing these new Bd fungal genotyping tools, we investigated the lineages present in Brazilian bullfrog farms (*Aquarana catesbeiana*), recognizing the importance of these environments in the global spread of the fungus. This information is crucial for enhancing the understanding of the geographic distribution of Bd lineages and providing support for conservation and management strategies in the face of the growing amphibian trade.

2. Materials and Methods

2.1. Sampling

In February 2020, we acquired tadpoles from 21 bullfrog farms located across a span of approximately 2,215 Km from the northernmost to the southernmost farm in Brazil (**Figure 1**). When permissible under the farm owners' consent, we conducted visual inspections of the tadpoles' mouthparts to identify individuals exhibiting dekeratinization, a clinical sign utilized in diagnosing Bd infection (Carvalho et al., 2017; Ribeiro et al., 2019). To ensure ethical practices, we killed tadpoles by decapitating and pithing. Subsequently, employing sterile surgical instruments sterilized via flame between each dissection, we aseptically removed the

mouthparts of the tadpoles. These collected mouthparts were individually stored in 1.5 mL microtubes containing absolute alcohol and maintained in a freezer at -20 °C.

We extracted the DNA from the tadpoles' mouthparts using the PrepMan Ultra extraction method (Applied Biosystems® by Life Technologies). We completely removed the alcohol and added 100 µL of reagent to each sample (Hyatt et al., 2007; Lambertini et al., 2013). For the detection of Bd, we conducted qPCR assays adhering to the outlined protocol by Lambertini et al. (2013), modifying it to exclude the use of Bovine Serum Albumin (BSA). To establish quantification standards for Bd, DNA dilutions from the Bd-GPL lineage (isolate CLFT 159) were prepared, comprising 1000, 100, 10, 1, and 0.1 zoospore genomic equivalents (g.e.). Additionally, two no-template controls (NTC) were included for each plate. The extracted DNA samples were diluted in 1:10. We considered Bd-positive (Bd^{+}) samples if they presented zoospore genomic equivalents ≥ 1 (Kriger et al., 2007).

To expand our sampling, we isolated Bd pure cultures from a bullfrog farm located in the Brazilian state of São Paulo (SP). These cultures were derived from bullfrog tadpoles exhibiting mouthpart dekeratinization, in accordance with protocols outlined by Fisher et al. (2018) and Vieira & Toledo (2012). Post-isolation, the Bd cultures were transferred into Petri dishes containing 1 % tryptone agar and incubated for one week. We then extracted DNA from the culture, following the protocol by James et al. (2008). Finally, we completed our sampling by including swab samples from bullfrog farms sampled in SP by Ribeiro et al. (2019), and those from the states of Paraná (PR) and Santa Catarina (SC) by Santos et al. (2020). In total, our sampling encompassed 36 Brazilian bullfrog farms, located in the states of Espírito Santo, Goiás, Minas Gerais, Paraíba, Paraná, Pernambuco, Rio de Janeiro, Santa Catarina, and São Paulo (**Figure 1**).

2.2. Bd genotyping assay design (qPCR)

We employed a genotyping assay to differentiate Bd genotypes within samples obtained from Brazilian bullfrog farms, where the presence of Bd had been previously confirmed. To determine the genotypes, we employed TaqMan SNP Genotyping assays designed specifically to identify the presence or absence of Bd-BRAZIL, Bd-GPL, or Hybrid genotypes, and identify potential coinfections within each sample. These assays leverage the Applied Biosystems TaqMan Real-Time PCR technology, utilizing target-specific primers and probes optimized for precise measurements.

We utilized two distinct genotyping assays to identify the presence or absence of Bd genotypes. The Bdmt26360 assay focuses on a mitochondrial SNP and effectively

distinguishes between Bd-Brazil (allele A, labeled with 6-FAM) and Bd-GPL or Hybrid (allele G, labeled with VIC) (Jenkinson et al., 2018). If allele G was identified by the mtDNA assay, a subsequent genotyping reaction was conducted using the BdSC9621917 assay, targeting a SNP in the nuclear genome. This secondary analysis determined the presence or absence of the Hybrid genotype (allele C, 6-FAM). The absence of allele C would indicate solely the presence of Bd-GPL in the sample (Carvalho et al., 2023).

For each genotyping reaction, we used DNA extractions (skin swab, tadpole mouth, or Bd culture) as input for qPCRs in 20 µL volumes composed of 5 µL of the sample template, 10 µL of TaqMan Fast Advanced 2X Master Mix (Applied Biosystems, Inc.), 1 µL of the SNP Assay (Applied Biosystems, Inc.) at 20X concentration (18 µM forward primer, 18 µM reverse primer, 4 µM FAM probe, and 4 µM VIC probe), and 4 µL of nuclease-free water. Furthermore, each run included two no-template controls (NTC) and one control for each Bd genotype. The cycling conditions were as follows: 30 sec at 60 °C, initial denaturation at 95 °C for 2 min, followed by 50 cycles of denaturation at 95 °C for 1 sec, annealing, and extension at 60 °C for 20 sec, with a final extension at 60 °C for 30 sec. All genotyping reactions were conducted using the QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems).

We analyzed allelic discrimination plots using TaqMan® SNP Genotyping Assays within the Thermo Fisher Connect cloud-based software. Utilizing this software enabled us to visualize and analyze raw data from all genotyping experiments, enhancing the precision of allelic discrimination results. We utilized the autocalling method, which entails cluster normalization and upheld a quality control threshold of 95 %. This threshold indicated the probability of assigning a genotype call to a well. Quality values below this established threshold were classified as undetermined.

For the first assay, alleles clustered along the x-axis (VIC, allele 1) represented homozygotes for allele 1, *i.e.*, Bd-GPL or Hybrid. Alleles clustered along the y-axis (FAM allele 2) indicated homozygotes for allele 2, *i.e.*, Bd-Brazil. Alleles clustered between the x-axis and the y-axis indicated heterozygotes, indicating a coinfection. In the second assay, alleles clustered along the x-axis (VIC allele 1) represented homozygotes for allele 1, specifically Bd-GPL. Alleles clustered along the y-axis (FAM allele 2) indicated homozygotes for allele 2, *i.e.*, Bd-Brazil. Alleles clustered between the x-axis and the y-axis represented heterozygotes, signifying a Hybrid.

2.3. Bd genotyping assay design (dPCR)

We selected samples in which we had not discriminated Bd genotypes in the qPCR assay and performed a genotyping assay using digital PCR technology. To determine the genotypes, we utilized the same TaqMan SNP Genotyping assays designed to detect the presence or absence of Bd-BRAZIL, Bd-GPL, or Hybrid, as previously mentioned (Carvalho et al., 2023; Jenkinson et al., 2018).

Each genotyping reaction comprised a final volume of 10 µL, consisting of 2 µL of Absolute Q dPCR Master Mix (5X) (Applied Biosystems, Inc.), 0.5 µL of the TaqMan SNP Assay (Applied Biosystems, Inc.) at a 20X concentration (18 µM forward primer, 18 µM reverse primer, 4 µM FAM probe, and 4 µM VIC probe), 6.4 µL of nuclease-free water, and 1.1 µL of DNA sample template. We loaded 9 µL into each well and supplemented it with 15 µL of QuantStudio™ Absolute Q™ Isolation Buffer. The cycling parameters were set as follows: initial denaturation at 96 °C for 10 min, followed by 50 cycles comprising denaturation at 96 °C for 5 sec, annealing, and extension at 60 °C for 15 sec. Controls for the three genotypes, as well as NTC were included. All genotyping reactions were conducted using the QuantStudio Absolute Q Digital PCR System (Applied Biosystems), and subsequent data analysis was performed using the QuantStudio Absolute Q Digital PCR Software.

2.4. Statistical analysis

To assess the relationship between Bd load and genotype determination, we fit a generalized linear model using the glm function from the stats package (R Core Team, 2023). We considered as the response variable whether a genotype was determined (1 = yes, 0 = no), while the predictor variable was the log-10 transformed Bd load. In this model, we set the parameter "family=binomial."

To determine the proportion of samples genotyped based on Bd load, we categorized samples into four groups: 1 to 10, 10.01 to 100, 100.01 to 1,000, and above 1,000 zoospores (g.e.). This categorization facilitated a more detailed analysis of the various Bd load ranges. We also fit a generalized linear model to investigate the association between genotype determination and our predictor variables, which were sample type (swab or mouth), and the previously mentioned Bd load categories.

To assess how Bd load differed among our predictor variables, we conducted a post-hoc Tukey test using the “emmeans” function from the “emmeans” package (Lenth, 2023). To evaluate model fit, we used the “checkresiduals” function from the “forecast” package (Hyndman & Khandakar, 2008). We created all figures using the “ggplot2” package. All

analyses were performed in RStudio (version 4.2.2; R Core Team, 2023) with a significance level of 0.05.

Finally, we mapped and determined the proportion of each Bd genotype in different bullfrog farms, categorizing the results by respective Brazilian states. The map was created in ArcGIS Pro (version 3.0.3) to illustrate the geographic distribution of Bd genotypes.

3. Results

3.1. Sampling

We sampled 21 bullfrog farms located in the states of Espírito Santo (ES: 4), Goiás (GO: 2), Minas Gerais (MG: 6), Paraíba (PB: 1), Pernambuco (PE: 1), Rio de Janeiro (RJ: 5), and São Paulo (SP: 2). Out of the 1028 mouthparts samples analyzed, we obtained 153 positive samples for Bd. All the sampled states presented at least one frog farm with Bd^+ samples. The states SP and GO presented proportionally greater quantities of Bd^+ samples, while PE and PB included farms with fewer Bd^+ samples (**Figure 1**, **Table S1**). We isolated 22 cultures of Bd from the one frog farm in SP. Additionally, we included swab samples of post-metamorphic bullfrog skin previously collected (Ribeiro et al. 2019, Santos et al. 2020) in bullfrog farms in SP (11), Paraná (PR: 3), and Santa Catarina (SC: 3). Finally, we selected all Bd^+ samples from Brazilian bullfrog farms to determine Bd genotypes (**Figure 1**, **Table S1**).

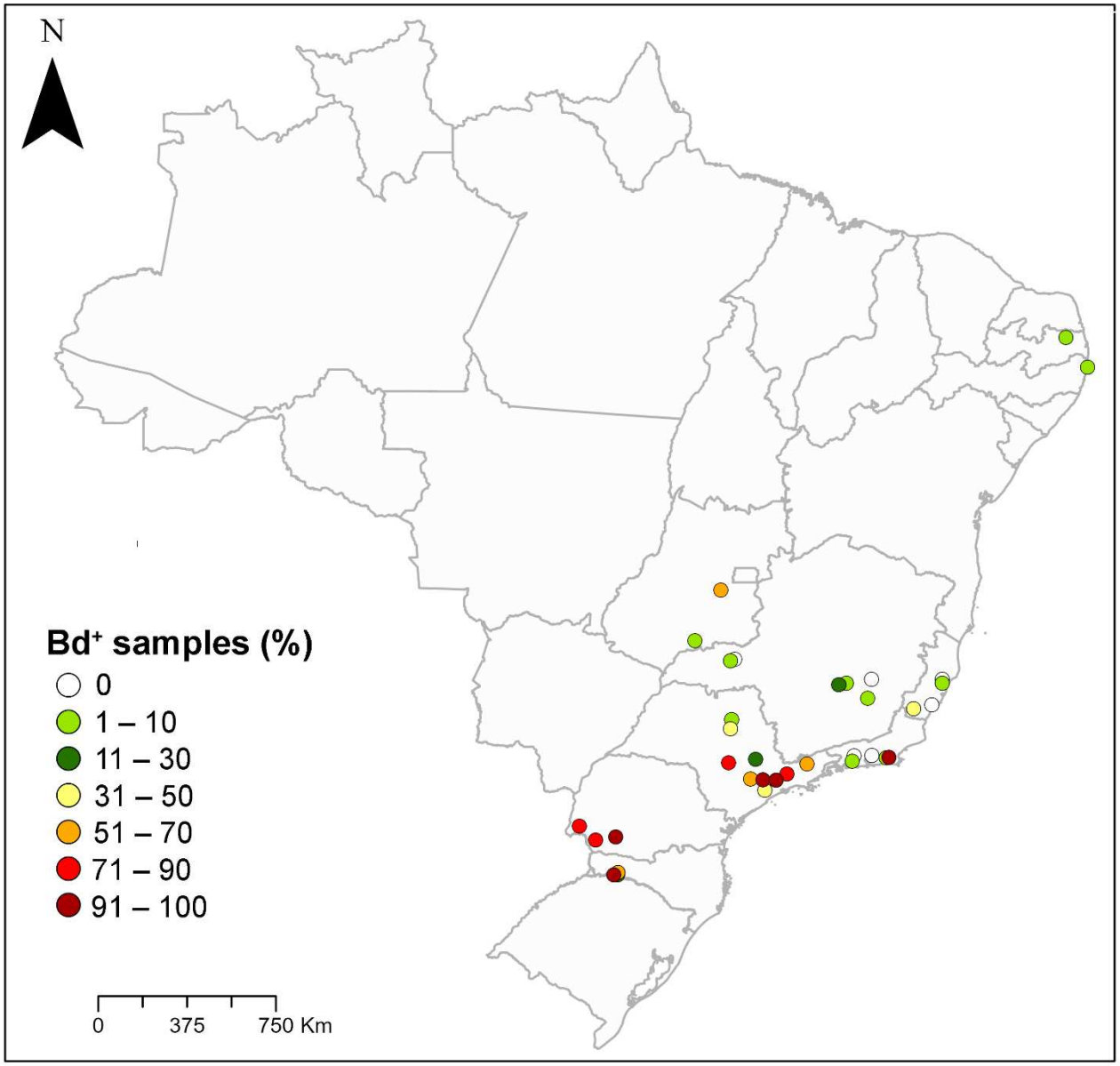

Figure 1. Bd negative (Bd^-; white circles) and percentage of Bd positive (Bd^+; colored circles) samples in Brazilian bullfrog farms that were sampled.

3.2. Bd genotyping analysis

In total, we conducted genotyping on 593 Bd^+ samples, regardless of load or sample type (swabs, mouthparts, or cultures). These samples included post-metamorphic bullfrog skin swabs (n = 419), tadpole mouthparts (n = 152), and pure Bd cultures (n = 22), collected from 30 bullfrog farms in nine Brazilian states (**Figure 1**, **Table S1**). The average Bd zoospore load in the samples was 863 zoospores g.e., ranging from 1 to 71030 (swabs mean of 717 ± 4587 SD g.e., mouth mean of 1265 ± 2845 SD g.e.).

Our genotyping assay, encompassing both qPCR and dPCR, revealed an overall genotype determination efficiency of 56.6 % (336 out of 593). Specifically, in the qPCR-based

genotyping assay, we attained an identification rate of 48.8 % (289 out of 592) for the mitochondrial assay and 32.3 % (107 out of 331) for the nuclear assay, showcasing the success in genotype discrimination. In instances where genotype determination using the qPCR assay was unsuccessful, we employed dPCR to enhance genotyping prospects. In the mitochondrial assay, we analyzed 148 samples, achieving a genotyping success rate of 41 %, while in the nuclear assay, we examined 23 samples, reaching a genotyping success rate of 78.2 % (**Table S2**).

3.3. Statistical analysis

We found a positive relationship between Bd load and the likelihood of genotype determination (Estimate *Bd load log* = 0.61, z = 6.6, $p < 0.001$); higher Bd loads corresponded to increased probability of a sample being genotyped (**Figure 2**).

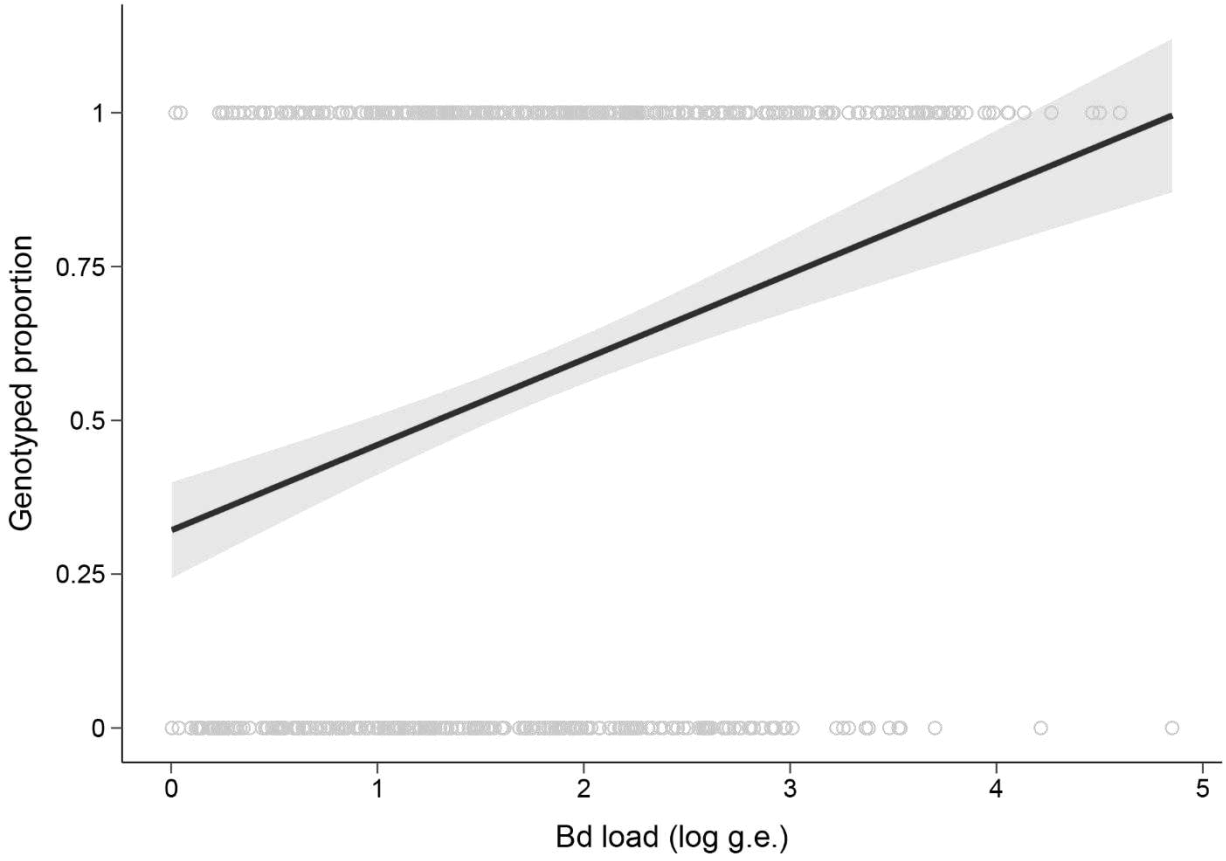

Figure 2. Positive relationship between the probability of genotype determination and Bd load. An increase in Bd load corresponds to a greater probability of genotyping a sample. Jittered dots indicate individual values that represent whether a genotype was determined (1 = yes, 0 = no). The solid red line was fitted with a generalized linear model (logistic link).

Samples from tadpole mouthparts and samples from post-metamorphic skin swabs had similar genotyping success probabilities (Estimate *type* = 0.37, z = 1.82, $p = 0.065$; **Figure S1**). However, for culture samples, we achieved a 100 % success rate in genotype discrimination (**Table S2**).

The proportion of genotyped samples differed among all categories of Bd load (post-hoc Tukey $p < 0.01$; **Figure 3 and Table S3**), except between the categories of 10 – 100 and 100.01 – 1,000 g.e. (post-hoc Tukey $p = 0.7$; **Figure 3 and Table S3**). Samples ranging from 1 – 10 g.e. had an average genotyping success rate of 37.2 %, while samples with over 1,000 g.e. had an 81.8 % probability of successful genotyping (**Table 2**).

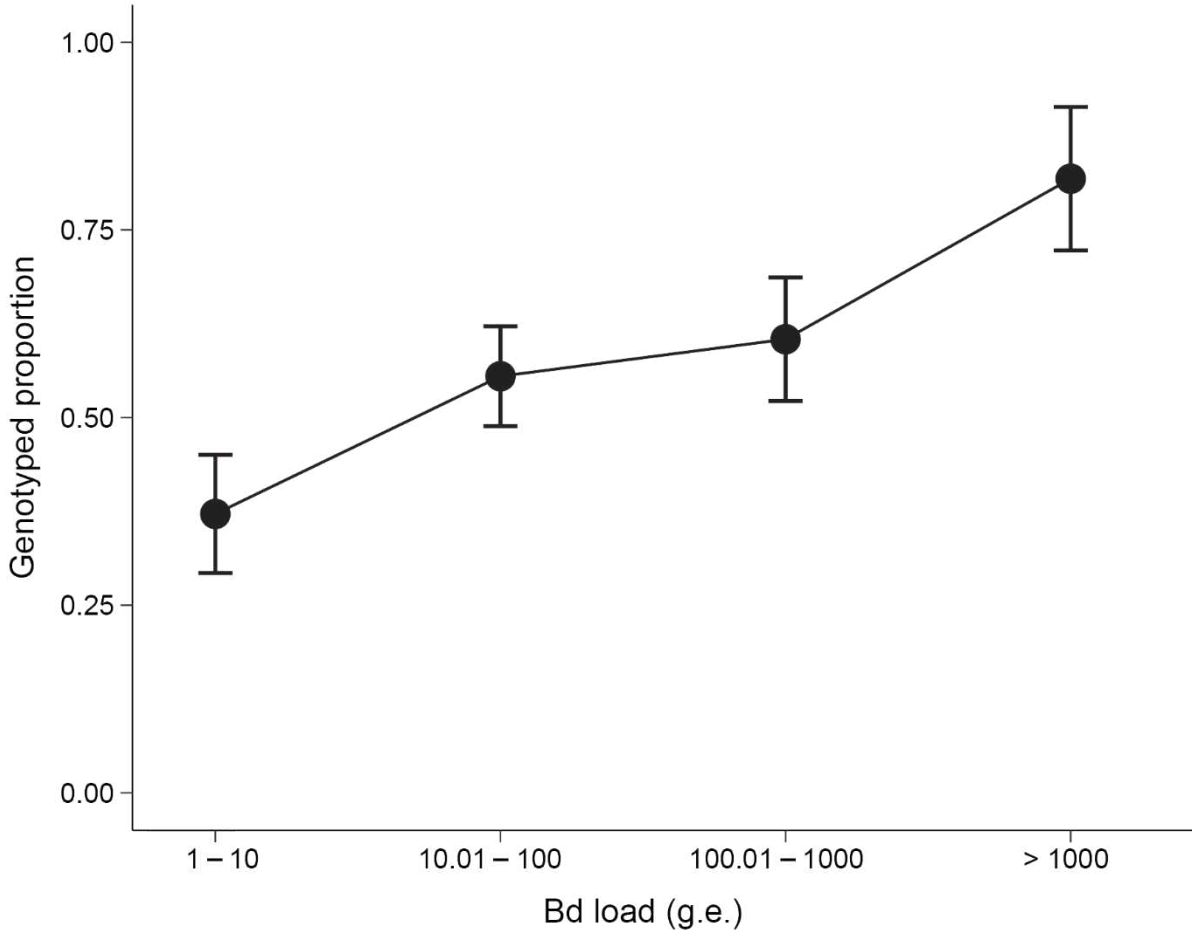

Figure 3. Proportion of successfully genotyped samples (including mouthparts and skin swabs) as a function of Bd load (genomic equivalents; g.e.). Dots represent average values, and bars denote the standard error.

Table 2. Genotyping success percentage across different Bd loads. the Bd loads are categorized based on the number of Bd zoospore (g.e.). The number of tested samples within each category, along with their respective means ± standard deviations (min – max), and the proportion of samples successfully genotyped is shown.

Bd load category (Bd g.e.)	Number of tested sample	Mean ± standard deviation	Genotyped sample (%)
1 – 10	148	4.5 ± 2.6 (1.01 – 10)	37.2
10.01 – 100	218	38 ± 26 (10.09 – 97.7)	55.5
100.01 – 1000	139	339 ± 235 (103.2 – 985.9)	60.4
> 1000	66	6621 ± 10783 (1009.5 – 71029.8)	81.8

Finally, we identified and mapped all genotypes present in Brazilian bullfrog farms (n = 336), including Bd-GPL (22 %), Bd-BRAZIL (27.4 %), coinfection between Bd-GPL or Hybrid and Bd-BRAZIL (14.6 %), Hybrid (1.2 %), and Bd-GPL or Hybrid (34.8 %) for samples whose genotype identification was possible through the nuclear SNP assay (**Table 3**, **Table S2**). The number of genotyped samples varied by state, with São Paulo having 232 genotyped samples, while Minas Gerais had none (**Figure 4**, **Table 3**, **Table S2**).

Table 3. Genotyped samples of Bd in bullfrog farms, categorized by Brazilian states: displaying the number of tested samples, the number of successfully genotyped samples, and the corresponding percentage distribution of each genotype type. Brazilian states are listed as follows: ES = Espírito Santo, GO = Goiás, MG = Minas Gerais, PB = Paraíba, PR = Paraná, PE = Pernambuco, RJ = Rio de Janeiro, SC = Santa Catarina, and SP = São Paulo.

State	Number of tested sample	Number of genotype sample	Bd-BRAZIL (%)	Bd-GPL (%)	Bd-GPL or Hybrid (%)	Coinfection (%)	Hybrid (%)
ES	19	16	25	31	44	0	0
GO	35	18	0	39	50	11	0
MG	12	0	0	0	0	0	0

PB	1	1	0	0	100	0	0
PE	1	1	0	0	100	0	0
PR	49	20	5	5	80	10	0
RJ	31	28	0	79	18	4	0
SC	38	20	45	15	20	15	5
SP	407	232	34	16	32	18	1
Total	593	336	-	-	-	-	-

We detected Bd-GPL or Hybrid genotypes in bullfrog farms in all surveyed states, while Bd-BRAZIL was found in four. Additionally, we confirmed the presence of Hybrids in bullfrog farms in two states (**Figure 4**, **Table 3**, **Table S2**).

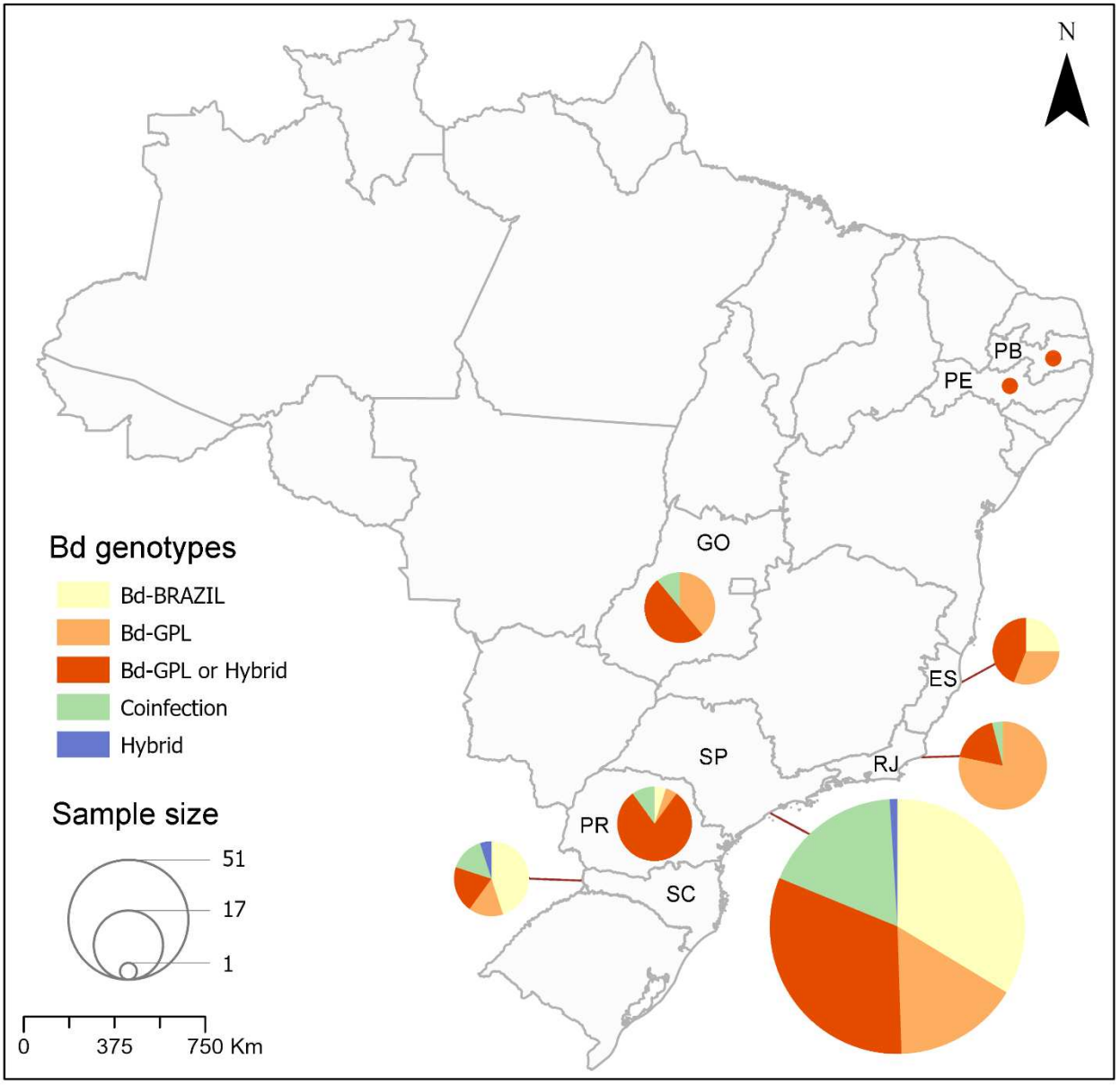

Figure 4. Distribution of Bd genotypes in Brazilian bullfrog farms. Each genotype is represented by a different color, and circle's size denotes the number of genotyped samples for each state. Brazilian states: ES = Espírito Santo, GO = Goiás, PB = Paraíba, PR = Paraná, PE = Pernambuco, RJ = Rio de Janeiro, SC = Santa Catarina, and SP = São Paulo.

4. Discussion

The investigation into the geographical distribution of distinct lineages of the Bd fungus is imperative for a comprehensive understanding of its genetic diversity and ecological implications. The scarcity of research in this area can be attributed to challenging methodologies associated with effective discrimination between lineages, emphasizing the need for advances in more sophisticated and accessible genetic approaches that encompass the major

Bd lineages known to date (Ghosh et al., 2021). Our study addresses this gap by proposing a novel method for genotyping Bd samples from Brazilian bullfrog farms, aiming to facilitate research on the ecology and distribution of Bd genotypes.

Our genotyping assay, employing both qPCR and dPCR, exhibited a genotype determination efficiency of 56.6 %. However, we observed that as Bd loads increased, the likelihood of successful genotyping also increased. While we identified a low success rate in genotyping samples with low loads (1 to 10 g.e.), those with loads ranging from 10 to 1000 g.e. showed an approximately 58 % success rate in determination. Samples with over 1000 g.e. had an 81.8 % probability of successful genotyping. This finding aligns with previous observations suggesting varying success rates in genotyping based on pathogen load (Byrne et al. 2017). Our method demonstrated higher efficiency in genotyping samples with as few as 10 g.e., compared to other methods that require samples with over 150 g.e. (Byrne et al., 2017).

In research projects entailing a large sample size and a small number of SNPs, the TaqMan assay emerges as a preferred technology due to its throughput, precision, and cost-effectiveness (Shen et al., 2009). Our study corroborates the versatility of this method across different sample types, highlighting its efficacy in both tadpole mouth and post-metamorphic skin swab samples. Moreover, we achieved a 100 % success rate in genotype discrimination from culture samples. Similarly, Ghosh et al. (2021) reported high genotyping success in pure Bd cultures. We thus highlight another highly effective, feasible, and accurate method for determining the Bd genotype through pure cultures.

The identification of various Bd genotypes, including Bd-GPL, Bd-BRAZIL, and hybrids, in bullfrog farms highlights the widespread occurrence and potential dissemination of these lineages. Bd-BRAZIL lineage, previously believed to be endemic to Brazil (Schloegel et al., 2012), has emerged as the most prevalent genotype, detected in four distinct Brazilian states. Recent findings have also revealed its presence in other countries, such as the USA and the Korean Peninsula, albeit in limited and recent records (Goka et al., 2021; O'Hanlon et al., 2018; Schloegel et al., 2012). This discovery prompts inquiries into the environmental and local factors contributing to the predominance of this lineage in specific regions. Furthermore, it raises concerns about the role of amphibian trade in facilitating the spread of potentially endemic genotypes across regions.

We highlight the southern and southeastern regions of Brazil as potential high-risk areas for the spread of Bd lineages, warranting increased attention for conservation efforts. These regions harbor all Bd lineages present in Brazil, including hybrids, which may be particularly virulent (Greenspan et al., 2018). Additionally, they are regions with the highest

number of bullfrog producers (Ribeiro & Toledo, 2022), thereby increasing the risk of global spread of different Bd lineages. Internal bullfrog trade within Brazil may also contribute to the spread of these lineages to other regions and states. Concern extends to the threat this poses to native Brazilian amphibians, which possess the world's highest biodiversity (Frost, 2024). The escape of bullfrogs from farms and water contamination by Bd could result in the introduction of these lineages into natural water bodies, further exacerbating the situation (Both et al., 2011; Ribeiro et al., 2019; Ribeiro & Toledo, 2022).

The occurrence of coinfections and hybridization represents a complex aspect of the dynamics of Bd infections, necessitating in-depth investigations into their implications for host health and population dynamics (Carvalho et al., 2024; Greenspan et al., 2018; Medina et al., 2021). While coinfections are anticipated in environments like farms, where high-density rearing is common (Ribeiro et al., 2019; Ribeiro & Toledo, 2022), their presence adds complexity to the understanding of Bd epidemiology in the wild. Similarly, the probability of generating hybrids in bullfrog farms is significant. We found hybrids in two bullfrog farms from different states, while in the wild, the distribution of this genotype remains restricted, with few confirmed records to date (Byrne et al., 2019; Carvalho et al., 2023; Jenkinson et al., 2018).

The presence of diverse Bd lineages, including a geographically limited lineage, coinfections, and potential hybridization, creates an opportune environment for thorough investigation into the dynamics of fungal infection by various lineages (*e.g.*, Carvalho et al., 2023; Jenkinson et al., 2018; Mesquita et al., 2017). Conversely, our findings underscore the urgent need for regulatory measures in both national and international amphibian trade to mitigate the risk of spreading different Bd lineages and potential impacts on biodiversity (Ribeiro & Toledo, 2022; Schloegel et al., 2010). The pressing need for regulatory measures and control protocols in amphibian trade is essential to mitigate potential negative impacts on biodiversity (Borzée et al., 2021). Additionally, investments in advanced genotyping methods and comprehensive genetic studies are essential for informing targeted management strategies to mitigate the impacts of Bd on amphibian populations.

Here, we present, for the first time, a highly effective method for determining Bd genotypes (Bd-GPL, Bd-BRAZIL, and Hybrid) from field samples with low Bd loads. This will enable the rapid generation of crucial data from field samples, allowing for a much more precise delineation of the global distributions of the Bd genotype. These investigations are crucial for the development of more effective and targeted management strategies, especially in populations where more virulent genotypes may pose a greater threat. We emphasize the need for investments in comprehensive genetic studies, effective legislation in international

amphibian trade, and the implementation of more advanced and efficient methods for genotype discrimination to understand and mitigate the impacts of Bd on amphibian populations.

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SUPPORTING INFORMATION

Table S1. Samples for Bd genotyping collected in Brazilian bullfrog farms. Location of sampled bullfrog farms, type of sample (post-metamorphic skin swab, tadpole mouthpart or pure cultures of Bd), total number of samples, and number of Bd^+ samples. Brazilian states are listed as follows: ES = Espírito Santo, GO = Goiás, MG = Minas Gerais, PB = Paraíba, PR = Paraná, PE = Pernambuco, RJ = Rio de Janeiro, SC = Santa Catarina, and SP = São Paulo.

State	Municipality	Lat	Long	Sample type	Collected sample	Bd^+ sample	Bd^+ sample (%)	Reference
ES	Anchieta	-20.699917	-40.727556	Mouth	51	0	0	This study
ES	João Neiva	-19.729722	-40.344778	Mouth	24	0	0	This study
ES	Ibiraçu	-19.864861	-40.336167	Mouth	49	1	2	This study
ES	Jerônimo Monteiro	-20.832278	-41.417972	Mouth	55	18	32.7	This study
GO	Bom Jesus de Goiás	-18.259909	-49.71621	Mouth	51	1	2	This study
GO	Guameleira de Goiás	-16.341448	-48.738519	Mouth	51	34	66.7	This study
MG	Nova Era	-19.720111	-43.012627	Mouth	50	0	0	This study
MG	Uberlândia	-18.961627	-48.204467	Mouth	52	0	0	This study
MG	Belo Horizonte	-19.87124	-43.970388	Mouth	52	1	1.9	This study
MG	Uberlândia	-19.016116	-48.365853	Mouth	53	2	3.8	This study
MG	Mariana	-20.446659	-43.162143	Mouth	52	2	3.8	This study
MG	Betim	-19.925883	-44.271619	Mouth	60	7	11.7	This study
PB	Bananeiras	-6.752211	-35.649614	Mouth	50	1	2	This study

PE	Paulista	-7.889347	-34.828122	Mouth	68	1	1.5	This study
PR	Santa Izabel do Oeste	-25.822131	-53.480656	Swab	21	15	71.4	Santos et al. 2020
PR	Medianeira	-25.295278	-54.093223	Swab	20	16	80	Santos et al. 2020
PR	Sulina	-25.700706	-52.721769	Swab	20	18	95	Santos et al. 2020
RJ	Guapimirim	-22.611799	-43.007107	Mouth	52	0	0	This study
RJ	Paracambi	-22.623945	-43.685402	Mouth	9	0	0	This study
RJ	Itaguaí	-22.837265	-43.756133	Mouth	48	1	2.1	This study
RJ	Silva Jardim	-22.698256	-42.469168	Mouth	50	2	4	This study
RJ	Silva Jardim	-22.680424	-42.354887	Mouth	31	28	90.3	This study
SC	Chapecó	-27.1409	-52.642556	Swab	20	5	20	Santos et al. 2020
SC	Chapecó	-27.048858	-52.639353	Swab	20	13	65	Santos et al. 2020
SC	Guatambu	-27.134588	-52.789161	Swab	20	20	100	Santos et al. 2020
SP	Santa Bárbara d'Oeste	-22.755742	-47.414759	Mouth	60	0	0	This study
SP	Jaboticabal	-21.252514	-48.325676	Swab	70	3	4.3	Ribeiro et al. 2019
SP	Pindamonhangaba	-22.928396	-45.459602	Swab	18	2	11.1	Ribeiro et al. 2019
SP	Santa Bárbara d'Oeste	-22.755742	-47.414759	Swab	70	10	14.3	Ribeiro et al. 2019
SP	Juquitiba	-23.928916	-47.071492	Swab	69	25	36.2	Ribeiro et al. 2019
SP	Matão	-21.601268	-48.361357	Swab	70	32	45.7	Ribeiro et al. 2019
SP	Araçoiaba da Serra	-23.508648	-47.60857	Swab	70	44	62.9	Ribeiro et al. 2019
SP	Pindamonhangaba	-22.928396	-45.459602	Swab	70	47	67.1	Ribeiro et al. 2019
SP	São Paulo	-23.55052	-46.633309	Swab	70	52	74.3	Ribeiro et al. 2019

SP	Santa Isabel	-23.315837	-46.225362	Swab	35	29	82.9	Ribeiro et al. 2019
SP	Botucatu	-22.884181	-48.444165	Swab	41	35	85.4	Ribeiro et al. 2019
SP	São Paulo	-23.55052	-46.633309	Mouth	60	57	95	This study
SP	São Roque	-23.529816	-47.1374	Swab	50	49	98	Ribeiro et al. 2019
SP	São Paulo	-23.55052	-46.633309	Culture	22	22	100	This study
Total					**1804**	**593**		

Table S2. Details related to samples undergoing Bd genotyping analyses through qPCR and dPCR.

https://drive.google.com/drive/folders/1udGeg2RUEHp-dijqzRVbttJwPWBM-6CG?usp=drive_link

Table S3. Results of the general linear model comparing Bd genotyped proportion among four distinct Bd load categories: 1 – 10, 10.01 – 100, 100.01 – 1,000, and above 1,000. The bold values are significant.

Category Bd load	**Estimate**	**z ratio**	***p* value**
1 – 10 and 10.01 – 100	-0.77	-3.54	**0.0022**
1 – 10 and 100.01 – 1,000	-1.01	-4.1	**0.0002**
1 – 10 and > 1,000	-2.17	-5.83	**<0.0001**
10.01 – 100 and 100.01 – 1,000	-0.23	-1.05	0.7207
10.01 – 100 and > 1,000	-1.4	-3.94	**0.0005**
100.01 – 1,000 and > 1,000	-1.16	-3.17	**0.0082**

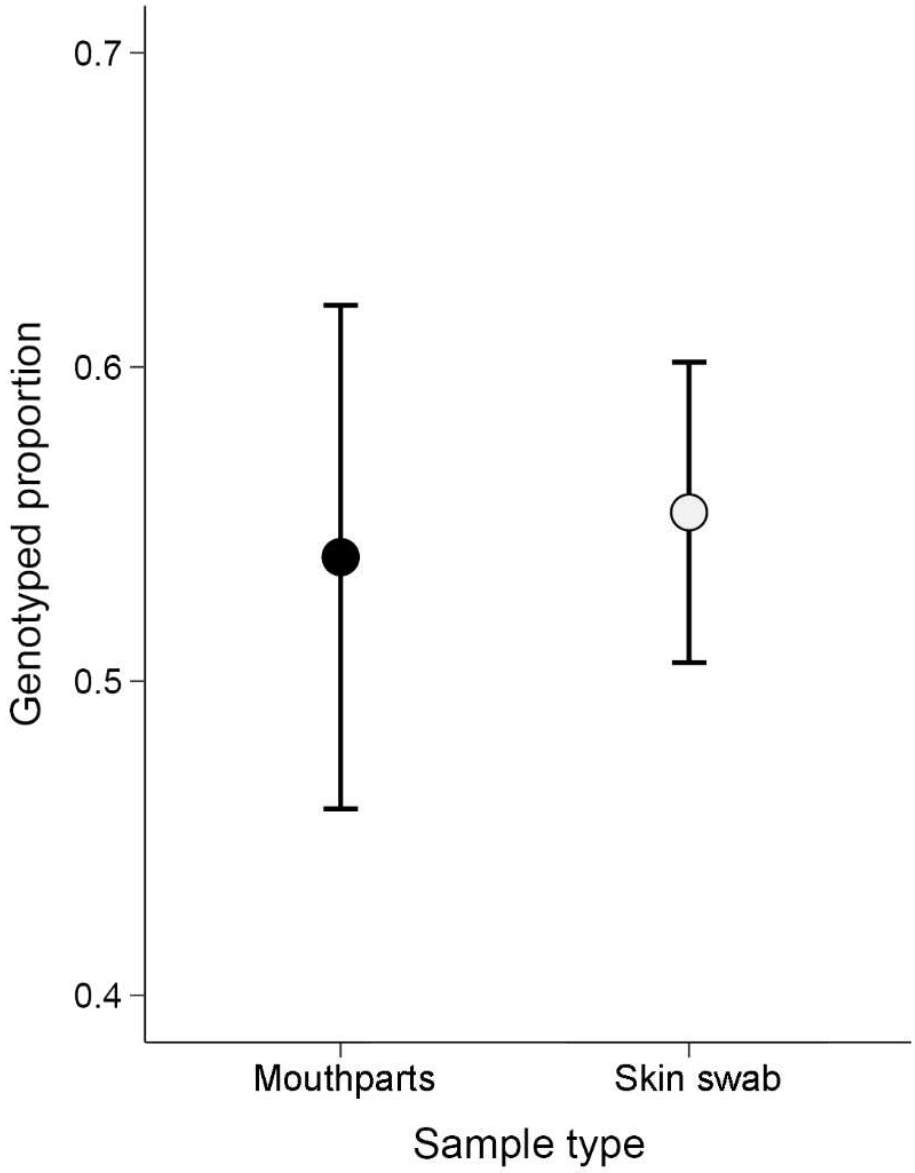

Figure S1. Proportion of samples successfully genotyped according to sample type (mouthparts or swab). Dots represent average values, and bars denote the standard error.

CHAPTER III

ANALYSIS OF GLOBAL BULLFROG TRADE AND HISTORICAL SPECIMENS SUGGESTS A NEW HYPOTHESIS FOR THE ORIGIN AND SPREAD OF AN AMPHIBIAN-KILLING FUNGUS LINEAGE

Análise do comércio global de rãs-touro e de espécimes históricos sugere uma nova hipótese para a origem e dispersão de uma linhagem do fungo quitrídio

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Analysis of global bullfrog trade and historical specimens suggests a new hypothesis for the origin and spread of an amphibian-killing fungus lineage

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Abstract

The genetic diversity of the amphibian-killing fungus *Batrachochytrium dendrobatidis* (Bd) has been extensively investigated, supporting the emergence of multiple lineages, including the Global Panzootic Lineage (Bd-GPL) linked to amphibian decline worldwide. Recent genetic analyses support the 'out-of-Asia' hypothesis, suggesting Bd-GPL originated in Asia during the early 20th century. Here, aimed to shed light on the temporal and geographic origins of Bd and the distribution of the more geographically restricted lineage, Bd-BRAZIL (= Bd-Asia-2/Bd-BRAZIL), by compiling historical records and analyzing global bullfrog trade routes. Bullfrogs were selected due to their pivotal role in the amphibian trade. Using non-invasive genetic techniques, we sampled museum frog specimens, complemented by a comprehensive review of global Bd records. Through genotypic analysis of bullfrog samples, phylogenetic assessments of traded bullfrogs, and examination of historical bullfrog trade connections, we propose an 'out-of-Brazil' hypothesis for the emergence and spread of the Bd-BRAZIL lineage. We suggest that Bd-BRAZIL likely originated in Brazil and disseminated to other regions, supported by i) bullfrog trade patterns from Brazil to East Asia, ii) genetic analyses indicating bullfrog and Bd-Brazil exportation from South America to the USA, and iii) a prevalence of Bd-BRAZIL on wild Brazilian frogs (in five Brazilian states) and Brazilian bullfrog farms (51 %), heightening the potential for international spread. Future research should expand data coverage and employ advanced genotyping methods for museum specimens. Implementing import control and pathological assessment are crucial steps in mitigating inadvertent pathogen transmissions within the global amphibian trade.

Keywords: Bd lineages, bullfrog trade, historical samples, Bd-BRAZIL, 'out-of-Brazil' hypothesis.

Significance Statement: Extensive research on the genetic diversity of *Batrachochytrium dendrobatidis* (Bd), an amphibian-killing fungus, reveals multiple lineages. Recent genetic analyses support an 'out-of-Asia' hypothesis, suggesting Bd-GPL's emergence in early 20th century Asia. Here, we aimed to elucidate the origins and the distribution of Bd-BRAZIL, exploring historical records and global bullfrog trade routes. Using non-invasive genetic methods, museum frog samples were analyzed alongside global Bd data. Our findings corroborate with an 'out-of-Brazil' scenario for Bd-BRAZIL, likely originating in Brazil and spreading, evidenced by trade patterns and prevalence of this lineage on Brazilian bullfrog farms and in wild amphibians. Enhanced research strategies and import control are vital in safeguarding global amphibian trade from inadvertent pathogen spread.

Introduction

Batrachochytrium dendrobatidis (Bd) is a pathogenic fungus distributed worldwide, causing chytridiomycosis, an infectious disease in amphibians (1, 2). This disease has led to the decline of over 500 species, with at least 90 of them already extinct across the globe (2, 3). Understanding its origin is critical to comprehending how this disease swiftly escalated into a global crisis. Initially, the 'out-of-Africa' hypothesis posited the African continent as the fungus's likely origin (4). This hypothesis was founded in museum specimens of amphibians and identified the earliest Bd-positive sample at the time, a histological sample from a *Xenopus laevis* host dating back to 1938. Therefore, it was suggested that Bd might have spread through the international trade of this frog species (4).

Two additional hypotheses have been proposed to explain the dissemination of chytridiomycosis. The "endemic pathogen hypothesis" (EPH), or enzootic hypothesis, suggested that the fungus has been globally distributed since its origin, with amphibian extinction events triggered recently due to increased pathogenicity linked to environmental changes, possibly tied to climatic conditions (5, 6). On the other hand, the "novel pathogen hypothesis" (NPH), or epizootic hypothesis, proposed that the fungus, initially thought to have originated in the African continent (4), has spread worldwide (7). When it reached more susceptible amphibian populations, it resulted in rapid and unprecedented declines (8–10). However, more recent articles presented data with older Bd records from various regions worldwide, such as Japan (1902) (11), the USA (1888) (12), and Brazil (1894) (13). These older Bd records from different continents challenged the 'out-of-Africa' hypothesis.

Investigations into the origin of Bd took a new direction with advancements in Bd genotyping and the study of its complex genetic diversity (14–18). Besides the widely distributed Bd-GPL, known as the 'global panzootic lineage', four other main Bd lineages have been identified (15, 19). These include Bd-CAPE, found in South Africa, West Africa, locally in Europe (16), and more recently in Central America (15). Bd-Asia-1 found in the Korean Peninsula, while Bd-Asia-2/Bd-BRAZIL (hereafter Bd-BRAZIL) is present in Brazil and Korea (13, 19), and likely in Japan (20). Finally, Bd-Asia-3 was found in Southeast Asia (15). Therefore, Eastern Asia houses the highest Bd lineage diversity, where all lineages are present, except for Bd-CAPE. This genetic diversity, coupled with recent molecular evidence (19) and the fact that Bd sister species, *B. salamandrivorans* (Bsal), originated in Asia (21), has led to the proposal of the 'out-of-Asia' hypothesis (19).

This hypothesis, supported by data from both nuclear and mitochondrial genomes, posits that the Bd-GPL lineage, linked to declines and mass extinctions (15, 16, 19), emerged in the mid-20th century, in the Asian continent (19). These authors employed three different dating methods to estimate the origin of Bd, which resulted in: a nuclear molecular clock indicating an origin in 1898 (with a range of HPD 95 % from 1809 to 1941), a mitochondrial clock suggesting 1962 (with a range of HPD 95 % from 1859 to 1988), and a mitochondrial clock using empirical dates of first Bd detection as calibration proposing 1975 (with an interval of HPD 95 % from 1939 to 1989). Nevertheless, the origins of endemic or geographically restricted lineages remain elusive. Concurrently, numerous studies have investigated historical infection patterns across diverse global regions employing museum specimens (22) and novel methods of genotyping lineages (23, 24). Moreover, these studies have probed the relation between the

global amphibian trade and pathogen transmission (25, 26), providing crucial historical data for reevaluating the origins of Bd lineages.

The North American bullfrog (*Aquarana catesbeiana*, hereafter bullfrog), which represents the most widely traded amphibian species on a global scale (27), plays a central role in the Bd dispersal puzzle due to its active farming and invasions into numerous global regions (28). Emerging in the 1920s, the bullfrog trade involved the introduction of bullfrogs to various regions for cultivation and frog leg consumption (29, 30). Despite being legally sanctioned for commercial purposes in many countries, concerns have steadily increased regarding potential adverse effects on native species (27). Bullfrogs have a high invasive potential, and their tolerance to chytridiomycosis, combined with intense global trade, makes them potential vectors of Bd (3, 28). The Bd lineage previously considered endemic to Brazil, Bd-BRAZIL, is frequently associated with bullfrogs sourced from commercial markets and farms (18, 31). As of this point, its origin remains unknown.

Given the occurrence of Bd-BRAZIL in regions where the bullfrog trade is an established economic activity, including Brazil, the USA, the Korean Peninsula, and Japan, we compiled historical records of Bd occurrences, shedding light on the historical distribution of Bd across various regions. We also investigated the role of the bullfrog trade, which represents the most widely traded amphibian species on a global scale (27), to elucidate the origin and direction of the intercontinental spread of the Bd-BRAZIL lineage. Our investigation delved into how the Bd-BRAZIL lineage might have spread via bullfrog trade, employing Bd genotyping and sequencing of traded bullfrogs, in conjunction with a comprehensive dataset on international bullfrog trade. Our findings shed light on a new hypothesis for the origin and spread of this lineage of the Bd fungus.

Results

Museum frog samples and literature review

We examined 2,280 amphibian specimens (Anura = 1,524; Caudata = 756) from 16 families, collected between 1815 and 2014, representing different regions of the world (*SI Appendix*, Table S1). The oldest sampled specimen we analyzed was an individual of *Strongylopus grayii* collected in 1815 from Cape Town, South Africa. In Asia, the oldest sample was from *Pelophylax nigromaculatus*, collected in 1884 in the Korean Peninsula. For Europe, we sampled individuals of *Alytes obstetricans* from France, collected in 1854. In the Americas, we sampled a bullfrog individual collected in the USA, and individuals of *Leptodactylus latrans* collected in Brazil, both in 1818 (Table 1 and Fig. 1). All museum specimens collected in North America, Portugal, Italy, South Africa, and the Korean Peninsula tested

negative for Bd. Nevertheless, we detected Bd in five alytid individual frogs collected in France (Pyrenees) in 1915, which was the oldest Bd-positive in our samples.

Table 1. Sampling period, oldest dates of Bd-positive samples, and number of examined specimens.

Continent	Country	Sampled period	Oldest Bd positive record	Specimens examined	Reference
America	Canada	1917–2001	1961	959	(61)
America	Canada	1892	-	4	This study
America	USA	1888–1989	1888	1028	(12)
America	USA	1887	-	2	This study
America	North America	1818–1858	-	3	This study
America	Mexico	1964–2009	1972	537	(49)
America	Guatemala	1980–1981	1980	114	(62)
America	Guatemala	1969–2009	1994	582	(49)
America	Costa Rica	1961–2011	1964	1016	(63)
America	Panama	1987–1997	1994	52	(64)
America	Puerto Rico	1961–2000	1976	106	(65)
America	Bolivia	1863–2016	1863	599	(32)
America	Venezuela	1895–2014	1895	1391	(34)
America	Brazil	1894–2010	1894	2799	(13)
America	Brazil	1818–1979	1964	91	This study
Europe	Switzerland	1853–2009	1901	788	(66)
Europe	France	1875–2008	2007	575	(67)
Europe	France	1854	-	2	This study
Europe	France (Pyrenees)	1879–2014	1915	1951	This study
Europe	Italy	1886	-	1	This study
Europe	Portugal	1859–1898	-	3	This study
Europe	Denmark	1888	-	1	This study
Europe	Germany	1888	-	1	This study
Europe	Spain	1881	-	4	This study
Africa	West Africa	1993–2011	-	793	(68)
Africa	West Africa	1971	-	5	(61)
Africa	Cameroon	1852–1994	1933	620	(41)
Africa	Kenya	1871–2000	1934	178	(69)
Africa	South Africa	1879–1999	1938	697	(4)
Africa	South Africa	1815–1893	-	15	This study
Asia	China	1933–2009	1933	1007	(70)
Asia	Korean Peninsula	1911–2004	1911	244	(44)
Asia	Korean Peninsula	1884–1889	-	4	This study
Asia	Japan	1902	1902	-	(11)
Asia	Japan	1890–1993	1909	591	(71)
Asia	Taiwan	1981–2009	1990	198	(45), This study
Oceania	Australia	1956–2007	1978	10183	(72)
Oceania	New Zealand	1999	1999	5	(73)
Oceania	New Zealand	1930–2010	2001	704	(74)

We identified 86 publications (including scientific articles, notes, and thesis) published between 1998 and 2023 that provided relevant information on historical analyses of Bd, ranging from 1835 to 2018 (Table 1 and *SI Appendix*, Table S2). Worldwide, the oldest Bd record dates back to 1863,

in the Bolivian Andes (32), followed by 1888 in the USA (12), 1894 in Mexico (33) and Brazil, in the Atlantic Forest (13), and in 1895 in Venezuelan Amazonia (34). Notably, all these records are from the Americas (Fig. 1).

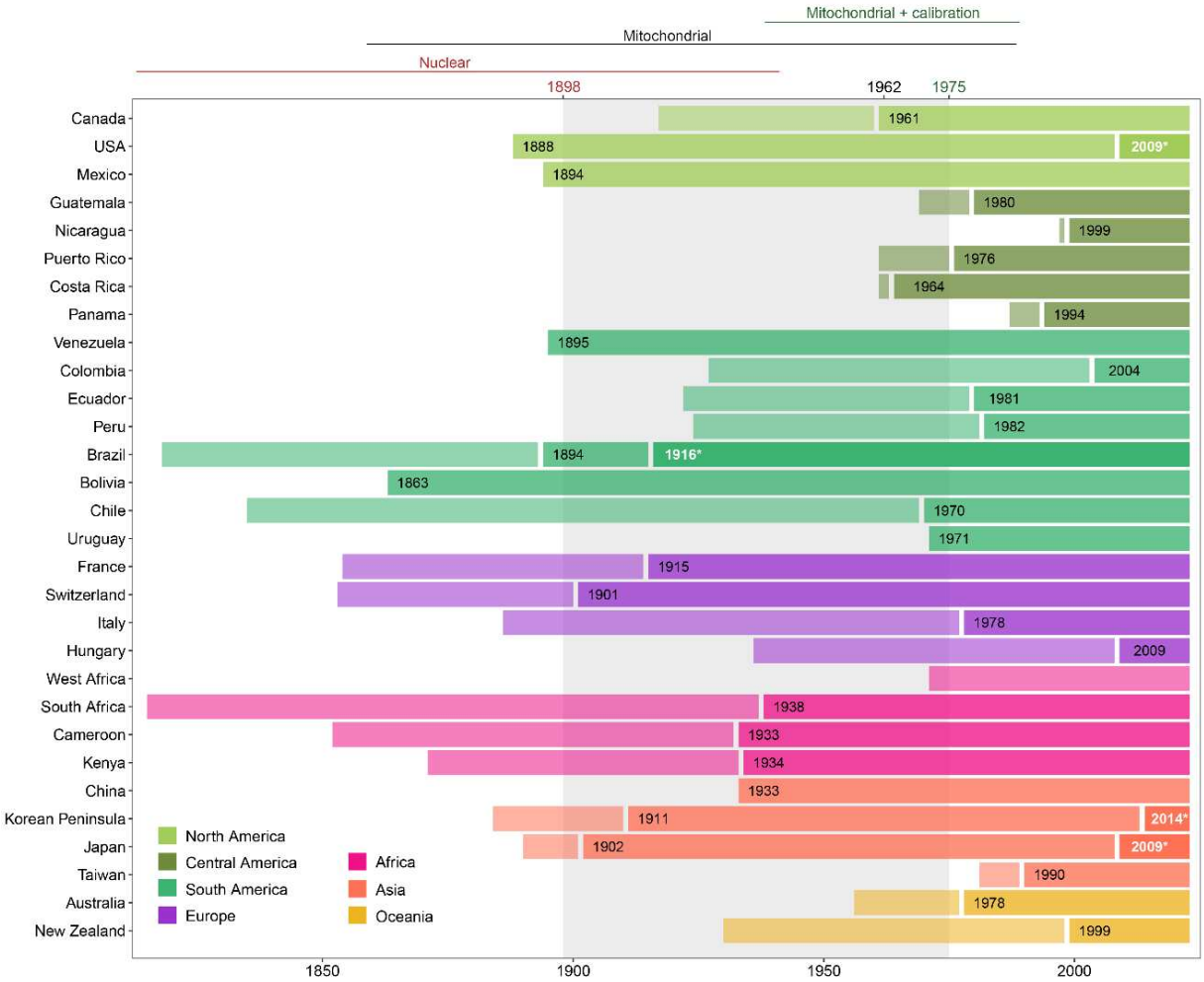

Fig. 1. Historical records of Bd detection in different countries or regions. Solid bars represent positive records, followed by the first year of detection. Dates with an asterisk represent the first records of Bd-BRAZIL lineage. The gray shaded area represents the estimated origin of Bd-GPL, based on nuclear and mitochondrial (with or without calibration) DNA dating analyses, which is also highlighted on the top of the graph (based on (19)).

There is a large proportion of historical investigations carried out from samples collected in the American continent (69 %), while key areas to elucidate the origin of Bd, such as Asia, remain under sampled. Among the articles examined, only six of them provided genotyping information for the Bd lineages associated with historical records (*SI Appendix*, Table S3). The oldest genotyped record was a Bd-GPL dated from Brazil in 1897 followed by Bd-BRAZIL in 1916 (13) (*SI Appendix*, Table S3). So far, no other lineages have been identified in museum specimens.

Bd-BRAZIL has been isolated from 10 Brazilian native amphibian species and a bullfrog from a frog market in the US, in 2010 and 2009, respectively (18) (Fig. 2 and *SI Appendix*, Table S4).

Subsequently, Bd-BRAZIL was also detected in Brazilian museum specimens collected between 1916 and 1982 (13). Additional isolates from various wild species were then recorded in Brazil (35, 36), expanding the distribution of this Bd lineage within the Brazilian Atlantic Forest. In 2014, four bullfrogs infected with Bd-BRAZIL were also recorded in South Korea (19), and a few individuals in Japan, including bullfrogs and native species (20) (Fig. 2 and *SI Appendix*, Table S4).

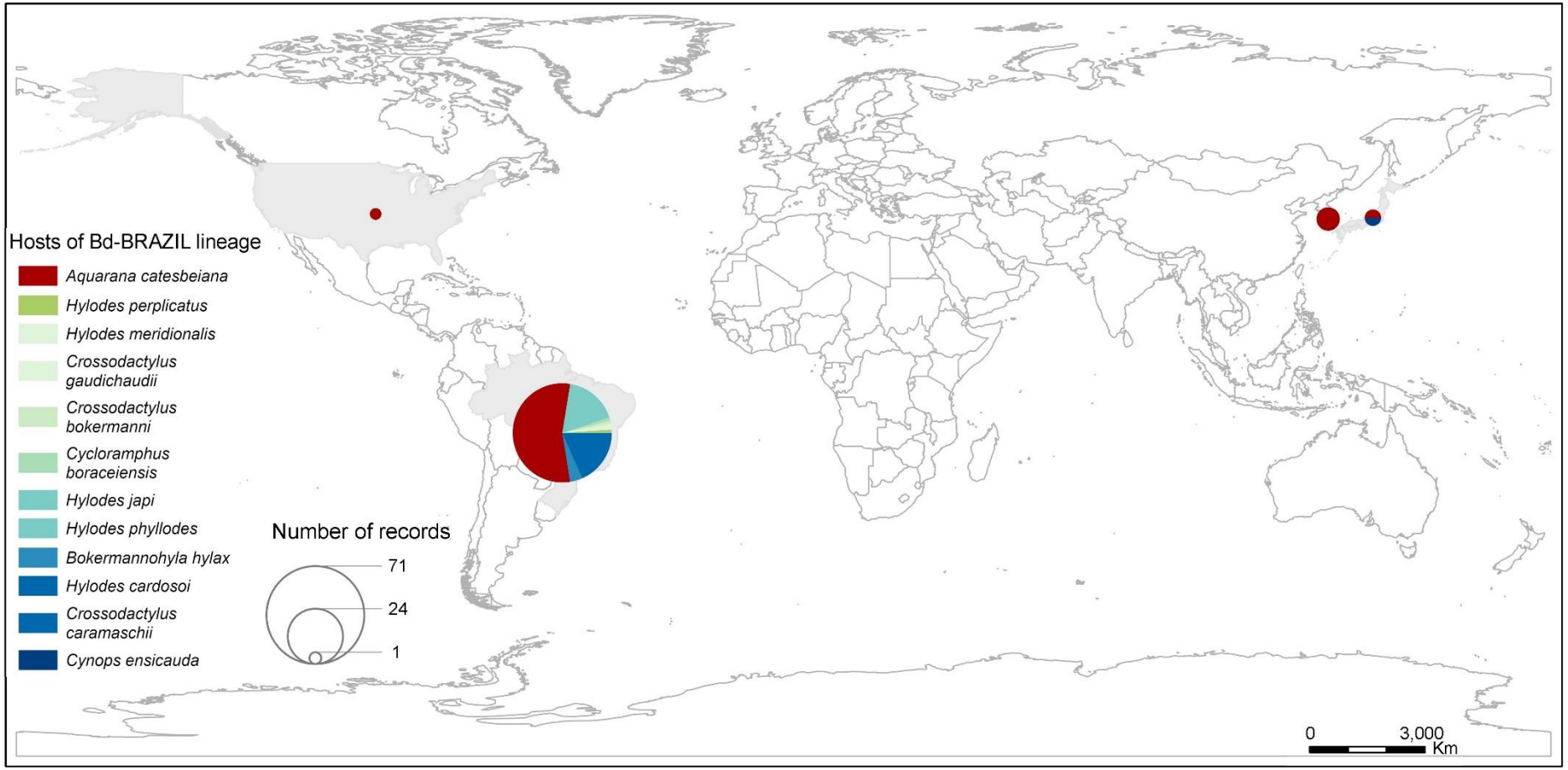

Fig. 2. Geographic distribution of the Bd-BRAZIL lineage records is presented alongside the associated host amphibian species. The proportional size of the pie chart corresponds to the number of collected samples, while the colors represent distinct species of amphibians.

Chytrid genotyping and bullfrog sequencing

Out of the 53 skin swab samples from Brazilian bullfrog farms that we genotyped, we found that almost half of the samples (51 %) belonged to the Bd-BRAZIL lineage, followed by the global pandemic lineage Bd-GPL (37.7 %), and 11.3 % of the samples were categorized as undetermined, meaning that there was insufficient evidence to classify them under any of the mentioned clades (*SI Appendix*, Fig. S1 and Table S4).

Our dendrogram recovered two major lineages within bullfrogs as was previously described (Fig. 3) (37). The samples collected from the invasive range and from trade generally grouped together. Two genotypes from farms were placed in the Western Lineage. Genotypes of bullfrogs from the Taiwan farms were identical to those from many invasive populations in Asia as well as many shops in the USA. Another cluster in the Western Lineage grouped together a single Brazilian bullfrog farm, markets in Miami and New York, USA, and invasive populations in Brazil, Cuba, China, and the Korean Peninsula. The first record of Bd-BRAZIL was isolated from a frog from Ypsilanti, MI, USA (18). This frog Cyt-b genotype was part of a large cluster of sequences from Brazilian bullfrog farms, a New York shop, and invasive bullfrogs from Brazil and Ecuador.

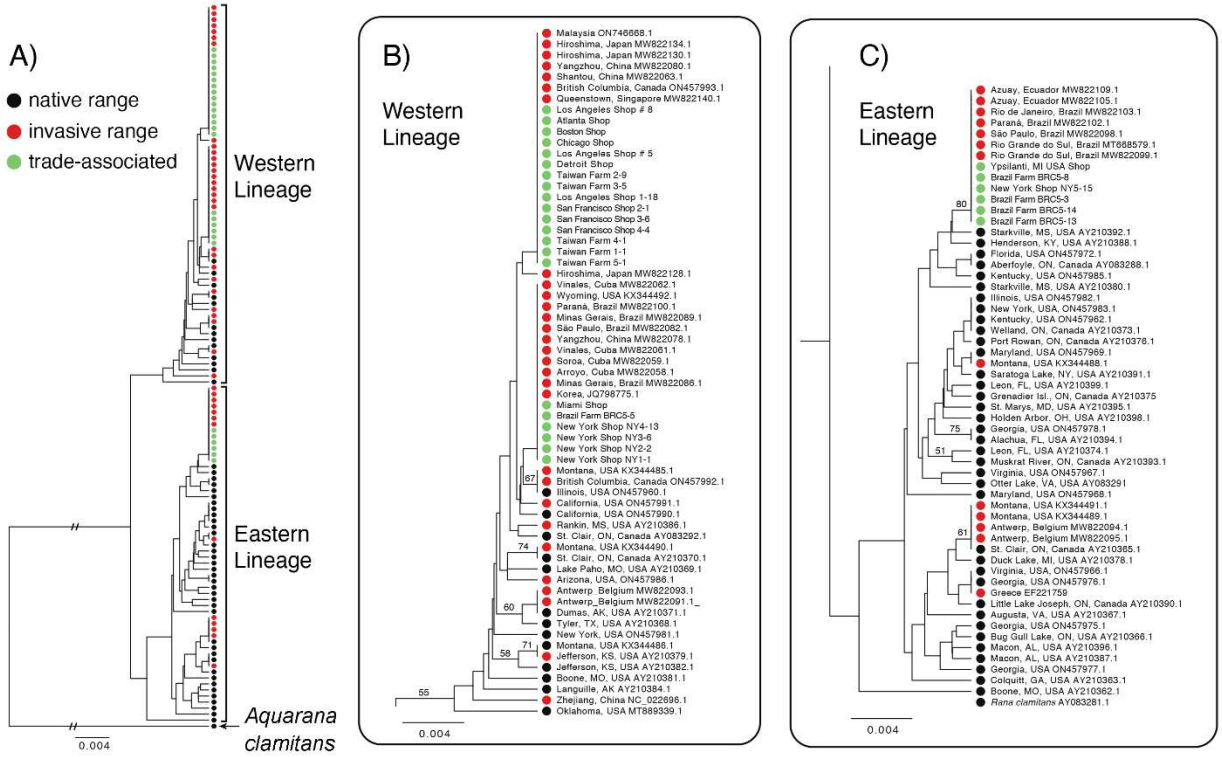

Fig. 3. Cytochrome-b dendrogram of sequences from bullfrogs (*Aquarana catesbeiana*). The eastern and western lineages correspond to designations from (37). Only bootstrap values > 50 % for non-zero branch lengths are shown. *Aquarana clamitans* is presented as an outgroup.

Bullfrog trade

We plotted a network graph illustrating the bullfrog trade between countries and its intensity (Fig. 4). A total of 48 countries participated in the international bullfrog trade as exporters, importers, or both. However, most of the trade is concentrated among few countries, with the highest levels of intensity observed in the USA, Taiwan, the Dominican Republic, Ecuador, Brazil, China, Canada, and Mexico. The USA stands out as the main importer, while Taiwan emerges as the main exporter. Moreover, the Dominican Republic, Ecuador, the USA, Brazil, and China are renowned exporters. In contrast, several other countries have export records that are either insignificant ($n < 10$) or non-existent (Fig. 4 and *SI Appendix*, Table S5). As for the importations, there is a more balanced distribution among countries. Canada and Japan are the main importers in terms of the number of importations, but Germany, France, China, and Taiwan also engage in significant bullfrog trade. We also found records of imports that have no associated countries. The strongest commercial interactions occur between the

USA and Taiwan, the Dominican Republic, Brazil, Ecuador, and China (Fig. 4 and *SI Appendix*, Table S5).

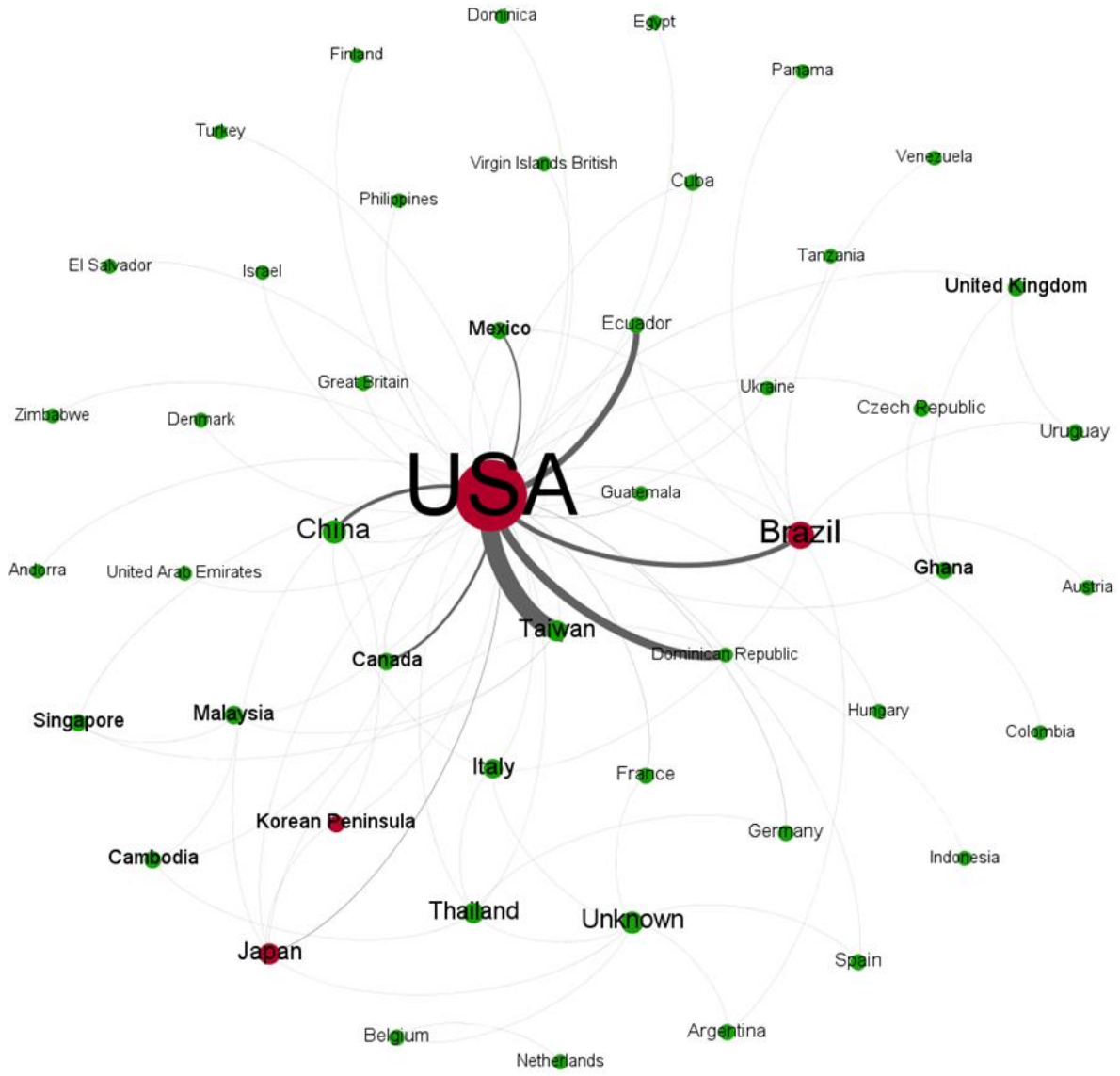

Fig. 4. Network of the international bullfrog commercial trade. The size of nodes indicates the degree (i.e., the number of bullfrog sales records, whether import or export). The width of the connections indicates the intensity (number of times that there was a purchase/sale of bullfrog) of the interaction between two countries. Red nodes represent the presence of Bd-BRAZIL lineage.

We have also mapped the commercial routes connecting all countries with confirmed Bd-BRAZIL lineage records, whether through direct or indirect connections. Among these routes, we highlight those that, based on the lineage records' dates in all countries where it has been observed, could provide insights into the direction of lineage spread (Fig. 5A and *SI Appendix*, Table S5). Concerning the hypothesis 'out-of-Brazil', we have identified eight routes. Brazil had direct exports to

the USA between 1991 and 2009, while the USA, in turn, exported to the Korean Peninsula in 2004 and 2008. Although we do not have specific records of Bd-BRAZIL in some countries like Ecuador, Italy, China, and Thailand, it is plausible that they may have acted as intermediaries, thus contributing to the spread of this Bd lineage out of Brazil (Fig. 5A and SI Appendix, Table S5). Furthermore, we have mapped three routes that could explain the Bd-BRAZIL spread, corroborating the 'out-of-Asia' hypothesis (Fig. 5B and SI Appendix, Table S5). These paths trace back to Japan since we did not find any exportation route from the Korean Peninsula (SI Appendix, Table S5).

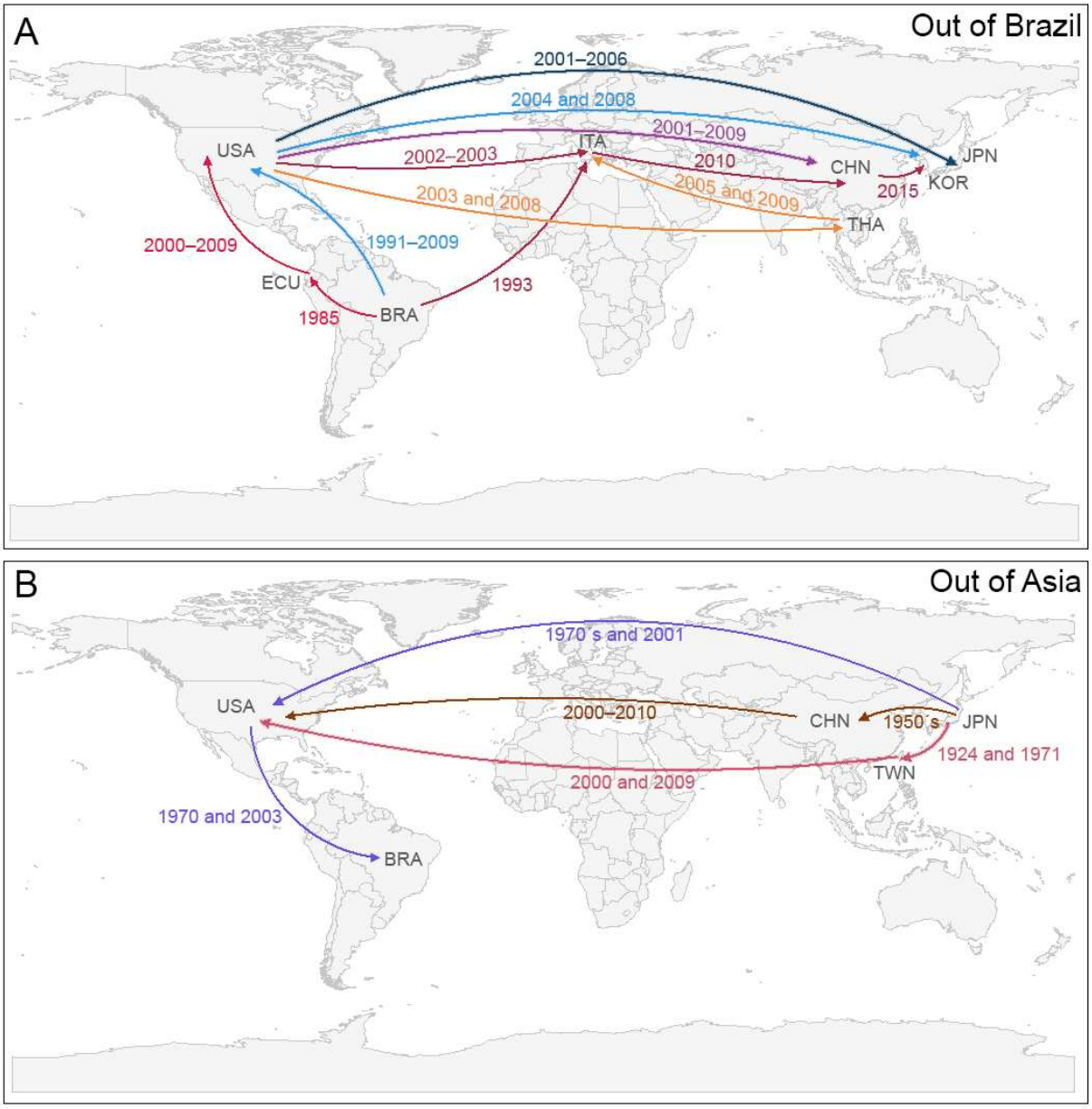

Fig. 5. Dated bullfrog commercial routes that may explain the spread of the Bd-BRAZIL lineage from Brazil (A) and Asia (B) to other regions of the world. Same colors represent possible sequences of routes.

Discussion

Our combined data provides new evidence that may elucidate the origin of the Bd-BRAZIL lineage in Brazil, as previously supposed. Here, we argue for the 'out-of-Brazil' hypothesis to explain for the current sampled diversity of Bd-BRAZIL. More specifically: i) The oldest records of Bd-BRAZIL, as well as Bd-GPL, from museum frogs are from the Americas rather than East Asia; ii) Bd-BRAZIL was detected in wild frogs from the Brazilian Atlantic Forest at least 10 years before the presence of bullfrogs and their trade in Brazil; iii) the directions and dates of bullfrog trade align with a Brazil-to-East Asia

direction rather than the opposite; iv) a genetic analysis of bullfrogs in US markets supports the export of South American bullfrogs to the US rather than the reverse; and v) Bd-BRAZIL is prevalent in bullfrog farms in Brazil compared to other regions globally, thereby increasing the likelihood of this lineage being exported; and vi) the prevalence of the Bd-Brazil lineage is notable within the Brazilian native population, whereas all additional instances of this lineage are linked to the bullfrog, save for a solitary occurrence identified within a species indigenous to Japan.

The Bd-BRAZIL lineage, initially believed to be restricted to the Brazilian Atlantic Forest, has been discovered in a bullfrog from the United States frog-leg trade (18). More recently, it has also been detected in South Korea (19) and Japan (20). However, the number of records of this lineage in these regions remains limited, predominantly associated with a single host species, the bullfrog (18–20). In contrast, Brazil stands out with the highest number of recorded Bd-BRAZIL cases (n = 71), occurring between 1916 and 2016 in both farmed and wild environments across an expansive geographic area covering approximately 1,400 km^2. Moreover, within Brazil, there exist at least 10 native species acting as hosts for this lineage. High prevalence of Bd-BRAZIL has also been observed in bullfrog farms in Brazil. The intriguing association between Bd-BRAZIL and bullfrogs in Brazil suggests that conditions within Brazilian bullfrog farms (27) might confer a selective advantage to this lineage compared to other genotypes, such as Bd-GPL. Further investigations are warranted to explore this occurrence in frog farms across multiple continents and to scrutinize the dynamic of different lineages during the intercontinental transportation of traded animals.

Brazil ranks among the leading global producers of bullfrogs, exporting a substantial number of individuals over the past few decades (27). The use of natural pond water in farming presents opportunities for cross-contamination between these amphibians and the natural environment, potentially fostering the exchange of Bd lineages between farms and the wild (27, 31). Despite the limited regulation of the global bullfrog trade, we have established a network of interactions among the countries engaged in this practice. While only a few countries, such as the USA, Taiwan, and Brazil, actively participate in bullfrog production and trade, most countries exhibit low production levels and limited involvement in the global market. However, there is a strong trade connection between countries harboring the Bd-BRAZIL lineage. The chronological order and direction of trade routes suggest the lineage's origins in Brazil, followed by subsequent dissemination to other regions. We highlight a plausible route for the spread of Bd-BRAZIL from Brazil to other parts of the world, potentially involving transit through the USA starting in 1991 and later reaching the Korean Peninsula around 2004. It is noteworthy that the only authorized trade in Brazil involves bullfrogs (38), while records of illegal trade mostly consist of pets, primarily exported from Brazil to Europe (39, 40). Notably, no Bd-infected animals have been identified in this trade so far (39). Moreover, there are records of an Asian individual recently found in Bahia, a Brazilian state (39). Thus, in addition to the robust bullfrog trade potentially explaining the spread of Bd-BRAZIL, the pet market reinforces this hypothesis.

Advancements in genetic refinement, historical sampling of Bd, and understanding amphibian trade are crucial to piece together the puzzle of the origin and spread of Bd lineages. Our extensive global historical sampling, employing museum specimens and literature review, played a key role in revising Africa's oldest recorded Bd presence from 1844 (41) to 1815. Despite mostly negative

results from museum samples, our method significantly expanded the historical records of Bd sampling. The oldest Bd-positive sample in our museum collection originated in France's Pyrenees region in 1915. Our literature review revealed the oldest known Bd occurrences in Bolivia (1863) (32), the USA (1888) (12), Brazil and Mexico (1894) (13, 33), and Venezuela (1895) (34), all traced back to the Americas. Recent research suggested the emergence of the Bd-GPL lineage in East Asia during the 20th century, employing three dating methods (19). However, our review uncovers Bd-GPL records dating to 1897 (13, 33), preceding the proposed timeline and raising doubts about its origin. Additionally, wide HPD 95 % intervals in dating methods and recent calibration dates in mitochondrial analysis pose challenges. While the oldest Australian record utilized by(19) aligns with ours, we have discovered older records for other regions. Our findings indicate that Bd was first observed in Central America in 1964 instead of 1972, in Europe in 1901 instead of 1997, and in the Pyrenees in 1915 instead of 2000.

While studies have contributed to our understanding of these lineages and their distribution, shedding light on their endemicity (19), an important challenge lies in genotyping museum samples due to difficulties in preserving samples intact and the possible contamination among co-stored individuals (42). To date, most swabs genotyped the ribosomal ITS region (18, 20). Recent advances make it possible to use numerous single copy markers for swabs, which would be enlightening for applying in museum specimens (15, 33). However, a limitation of this method is the requirement of high Bd load on the swab, which may be challenging in enzootic areas. Knowledge about other lineages like Bd-CAPE, Bd-Asia-1, Bd-Asia-3, and Bd-BRAZIL remains limited due to their lower prevalence and restricted distributions. Sampling can be further extended and encourage future studies to expand the geographical and temporal data coverage, especially in Asia. It is crucial to acknowledge a sampling bias as Asia is underrepresented in our studies, as well as in previous research (43). Most museum samples do not originate from Asia, significantly reducing the likelihood of detecting Bd-positive samples in older periods for this region. However, our detection expectations remain low due to apparent animal tolerance and the anticipation of low load and prevalence (43–45).

Despite significant strides in comprehending Bd's evolutionary narrative, notable avenues for investigation persist. Our research introduces the 'out-of-Brazil' hypothesis, proposing that Bd-BRAZIL lineage originated within Brazil and disseminated to other regions through international bullfrog trade. This hypothesis supports the concept of prolonged endemicity in the Brazilian Atlantic Forest rather than in Asia. Augmenting historical Bd sampling in Asia and characterizing lineages from presumed origin areas are crucial. Refining genetic information, identifying lineages, and conserving museum samples and data aid in historical inferences. Leveraging Bd-positive records from antiquated museum specimens offers valuable benchmarks for future investigations to elucidate the pathogen' distribution and diversification patterns. Challenges endure in global amphibian trade, necessitating measures such as import controls, pathogen assessments, and quarantine protocols to protect native wildlife (46). Non-invasive tools like eDNA protocols play a fundamental role in pathogen identification and advocating for "clean trade" practices (47). Prompt implementation of comprehensive protocols for production, trade regulation, and inspection is essential to forestall inadvertent pathogen dissemination.

Materials and Methods

Assessing Bd in museum samples

We analyzed a total of 2280 museum specimens of amphibians collected from different regions worldwide between 1815 and 2014, including Africa (n = 15), Asia (n = 202), Europe (n = 1963) (48), North America (n = 9), and South America (n = 91). Most specimens were fixed in formalin, stored in 70 % ethanol, and deposited in collections worldwide (*SI Appendix*, Table S1).

We used noninvasive genetic methods to avoid damaging museum specimens, especially older ones. Each specimen was rinsed with clean 70 % ethanol to eliminate any superficial contaminants from storage. After a brief drying period (13), we swabbed the specimens using Medical Wire swabs (MW113), following the standard Bd sampling procedures for preserved specimens (49, 50). We extracted DNA from each swab using 50 µl of PrepMan Ultra (Applied Biosystems), and performed qPCR to detect the presence of Bd, regardless of genotype, following established protocols (51–53). We diagnosed samples as Bd-positive all individuals with at least one zoospore genomic equivalent (g.e.) (50).

Literature review

We have reviewed literature concerning published data that explores historical Bd occurrences worldwide, published up to 2023. We classify data as historical when publications include samples predating the description of Bd, specifically before 1999 (1). Our approach involved compiling a list of literature through searches on the Google Scholar and Web of Science databases. We employed the following keywords: '*Batrachochytrium dendrobatidis*' or 'chytridiomycosis' along with 'museum specimens', 'retrospective studies', 'historical analyses', and 'historic occurrence'. We have incorporated all available records related to amphibians (Anura, Caudata, or Gymnophiona), carefully noting the sampling period and the oldest Bd records at each location. These records were derived from various sample materials and Bd detection methods. Furthermore, whenever available, we recorded the identification of the Bd lineage.

Chytrid genotyping of infected bullfrogs

We completed our sampling by genotyping previously collected post-metamorphic bullfrog skin swab samples (31). We extracted DNA and performed qPCR to detect the presence of Bd, as previously described in the 'Assessing Bd in Museum Specimens' section. Thus, in total, we randomly selected 53 samples that tested positive for Bd for the purpose of determining Bd genotypes. These samples were collected in 2013 and 2016 from six Brazilian bullfrog farms in the state of São Paulo. Genotype determinations were made using the Fluidigm Access Array microfluidic PCR platform following the methods described by (15, 24). The chip-based, multiplex PCR assay is designed to amplify 192 informative marker loci specific to *Batrachochytrium* from multiple samples in a single run. Among these marker loci, 191 are specific to Bd, while one marker is specific to its sister species, Bsal. However, this marker was not detected in any of our samples. For the 191 Bd markers, each locus is approximately 150 – 200 base pairs long, and the markers are distributed throughout the Bd genome.

The Fluidigm chip performs multiple PCR reactions in parallel for the 192 total loci across multiple samples. During this multiplexed PCR step, samples are dual barcoded for later identification, and sequencing adapters are added for high throughput sequencing by Illumina MiSeq.

DNA extractions from our swabs were precipitated with sodium acetate and isopropanol to purify the DNA in each sample. Before microfluidic PCR, all precipitated samples were pre-amplified in standard PCR reactions with primer pools composed of the 192 primer pairs at 500 µM for each marker. Pre-amplification PCR was performed with the FastStart High Fidelity PCR System (Roche). Individual reaction conditions follow those described in (15). Pre-amplification products were treated with 4µl ExoSAP-it (Affymetrix Inc.) then diluted 1:5 in molecular grade water. The diluted pre-amplification products were used for the microfluidic PCR and barcoding steps described above on a Fluidigm Access Array IFC at the University of Idaho IBEST Genomics Resources Core. The barcoded amplicons were pooled for sequencing on an Illumina MiSeq using 300 bp paired-end kits also at the University of Idaho IBEST Genomics Resources Core.

The sequencing results were demultiplexed and pre-processed as described in (24). We then used the bioinformatic R script published in (15) (Supporting Information) to quality filter and determine variants in our results. Briefly, we filtered out samples with more than 50 % missing data and loci for which more than 66 % of samples were missing data. After filtering, we generated contigs for each locus by alignment using the MUSCLE package in R. We visually confirmed alignments using Geneious and generated concatenated alignment sequences for each sample.

Phylogenetic analyses of our concatenated gene alignments were conducted using RAxML with a GTR substitution model. Our final concatenated phylogeny included known representative marker sequences of Bd-GPL, Bd-BRAZIL, and Bd-Cape isolates downloaded from previous genomics studies. We assessed statistical support for phylogenetic relationships by rapid bootstrapping in RAxML over 100 replicates. Finally, we used newick utils to collapse all phylogeny branches with less than 50 % bootstrap support. Swab samples within the 50 % support cutoff forming a clade with a known genomic sequence were determined to be either Bd-GPL or Bd-BRAZIL. Samples that did not form 50 % bootstrap supported clades with known sequences were undetermined. We did not observe Bd-CAPE genotypes in our samples.

Cyt-b sequencing of trade-associated bullfrog samples

We analyzed the genetic origins of bullfrog samples associated with the bullfrog trade (n = 27) by sequencing the cyt-b mitochondrial locus from swabbed frogs. Samples came from Brazilian bullfrog farms (54), shops in the USA (18, 55), and Taiwan bullfrog farms, the latter reported here for the first time. Farms were in the south of Taiwan, Pingtung County, and housed only bullfrog individuals. Bullfrogs were swabbed using a MW100 dry swab (Medical Wire and Equipment Company), and DNA extraction was performed as previously described. The swabs were analyzed using conventional PCR to detect Bd infection (56). Partial cytochrome-b (cyt-b) sequences were obtained from swab DNA extracts using the primers and protocol of (57) with the PCR mix GoTaq Green (Promega). These new sequences were aligned with sequences from native populations identified by (37) as well as more recent sequences in GenBank from native and invasive ranges using Geneious Prime. *Rana clamitans*

cyt-b was used as an outgroup sequence. We then used the unweighted pair group method with arithmetic mean to cluster the sequences into groups, using the HKY model of sequence evolution and 100 bootstrap pseudo-replicates as a measure of support.

Understanding the scope and directionality of the global bullfrog trade

Given that the sole amphibian permitted for international trade in Brazil is the bullfrog, our primary focus revolved around collating and mapping data pertaining to the trade of this species. The bullfrog holds significance not only as the most widely commercialized amphibian globally but also due to the availability of comprehensive data. Therefore, we conducted a comprehensive literature review to analyze the historical bullfrog trade worldwide. The bullfrog trade started to the 1930s, but the first officially documented export was recorded in the 1970s (27). Consequently, our research was centered on data collected from the year 1970 onward, with less emphasis on current dates.

We obtained bullfrog trade data from the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) for the period of 1975 to 2021 (58). The CITES database provided information on the number of specimens, year of trade, and the importing and exporting countries. Despite our exploration of historical data, it's important to highlight that the Bd-BRAZIL lineage was identified in countries beyond Brazil, specifically in the years 2009 and 2014. Therefore, our interest extends to trade data encompassing this period. We included import and export data for live amphibians in the USA from the U.S. Fish & Wildlife Service for the years 2001 to 2010 (55, 59). To enhance our analysis, we incorporated recent data reported by (60) covering the period from 2010 to 2020.

Leveraging this extensive dataset on the international bullfrog trade, we crafted an interaction network. This network served as a tool to identify the country's most active in this trade and the structure of their interactions. Finally, to provide a visual representation, we utilized ArcGIS® to map specific bullfrog routes that could explain the spread of the Bd-BRAZIL over the globe. These routes potentially hold the key to explaining the dispersal of the Bd-BRAZIL lineage across the global landscape. Our focus was on the oldest records of this lineage in each country, relative to the bullfrog trade routes.

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SUPPORTING INFORMATION

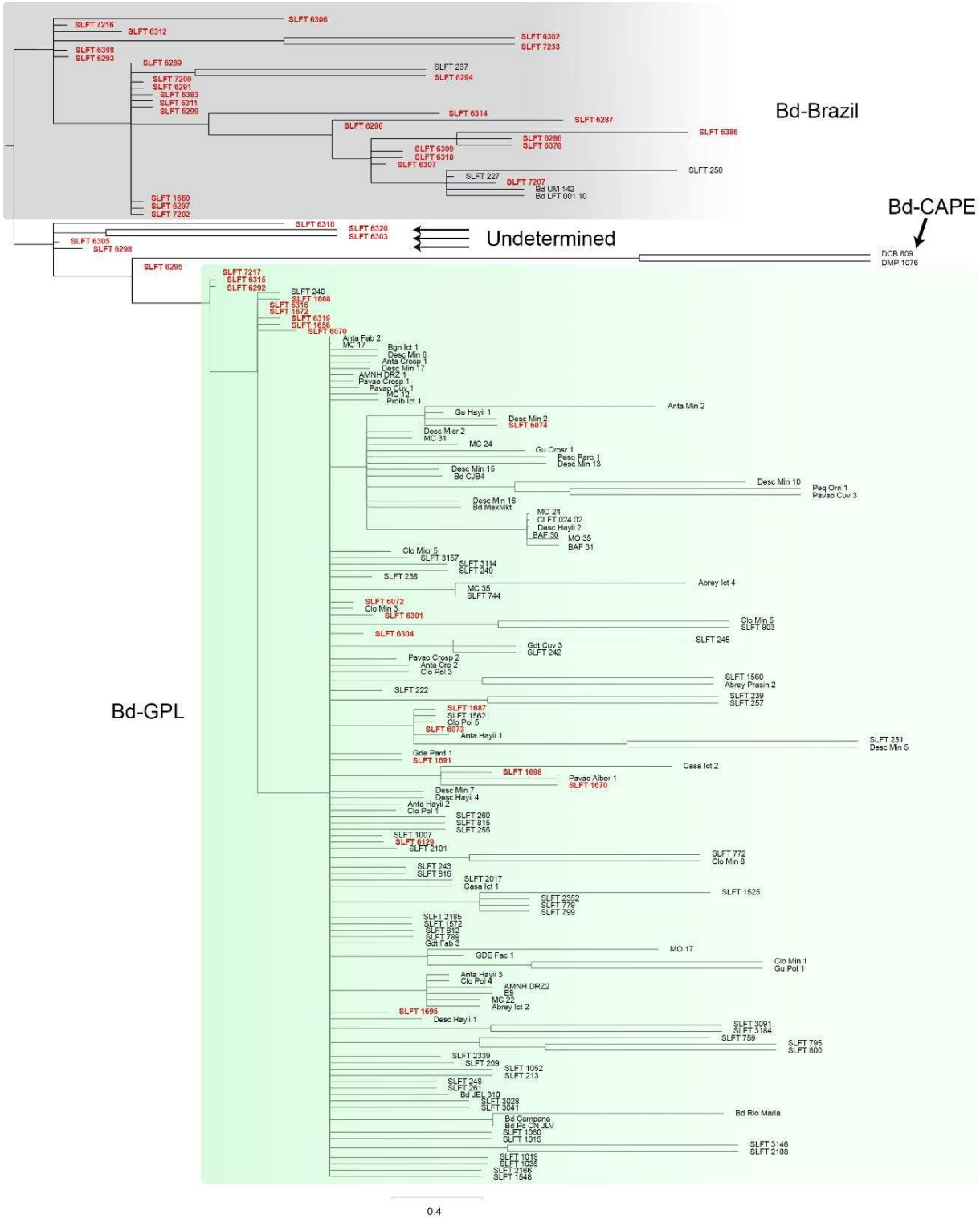

Fig. S1. Best scoring unrooted maximum-likelihood tree estimated from 192 concatenated nuclear loci and 100 bootstrap replicates performed in RAxML. The samples from Brazilian bullfrog farms are highlighted in red (n = 53). Major Bd clades are indicated by color (gray = Bd-GPL and blue = Bd-BRAZIL). Undetermined samples are meaning that there was insufficient evidence to classify them under any of the mentioned clades.

Table S1. Museum specimens. Museum voucher and ID, locality, year, species, and Bd result.

https://drive.google.com/drive/folders/1jNTMA3-09aPQKMdBrSNOIa9AZQIjdfJ2?usp=drive_link

Table S2. Literature review showing historical records for *Batrachochytrium dendrobatidis* worldwide before its discovery. NP indicates that information was not provided in the original publication.

https://drive.google.com/drive/folders/1jNTMA3-09aPQKMdBrSNOIa9AZQIjdfJ2?usp=drive_link

Table S5. Commercial bullfrog routes, with data on the exporting and importing country, year of trade, and references.

https://drive.google.com/drive/folders/1jNTMA3-09aPQKMdBrSNOIa9AZQIjdfJ2?usp=drive_link

Table S3. Determination of Bd lineages from historic samples in the literature. Lineage, oldest genotyping record, continent, country, and reference.

Lineage	Oldest genotyping record	Continent	Country	Reference
Bd-GPL	1897	South America	Brazil	(1)
Bd-BRAZIL	1916	South America	Brazil	(1)
Bd-GPL	1971	North America	USA	(2)
Bd-GPL	1975	North America	Mexico	(3)
Bd-GPL	1972	Central America	NA	(4)
Bd-GPL	1978	Oceania	Australia	(4)
Bd-GPL	1984	South America	Ecuador	(5)
Bd-GPL	1993	North America	USA	(5)
Bd-GPL	1997	Europe	Spain	(4)
Bd-GPL	1998	Africa	Equatorial Guinea	(5)
Bd-GPL	1998	Africa	Gulf of Guinea	(6)

Table S4. Records of the Bd-BRAZIL lineage. Sample number, locality, year, host species, and reference. Brazilian states are Minas Gerais (MG), Paraná (PR), Rio de Janeiro (RJ), Santa Catarina (SC), and São Paulo (SP), and USA state is Michigan (MI).

Sample number	Country	Locality	Year	Host	Reference
MNRJ005592	Brazil	SC	1916	*Hylodes perplicatus*	(7)
MNRJALMN 0480	Brazil	RJ	1923	*Crossodactylus gaudichaudii*	(7)
MZUSP056836	Brazil	MG	1979	*Crossodactylus bokermanni*	(7)
MZUSP058744	Brazil	SP	1982	*Cycloramphus boraceiensis*	(7)
UM 142	USA	MI	2009	*Aquarana catesbeiana*	(4, 8)
No information	Japan	Amami and Okinawa Is.	2009	*Cynops ensicauda* and *Aquarana catesbeiana*	(9)
CLFT 001	Brazil	SP	2010	*Hylodes japi*	(4, 10, 11)
JEL 648	Brazil	SP	2010	*Hylodes japi*	(8)
JEL 649	Brazil	SP	2010	*Hylodes japi*	(8)
SLFT 227	Brazil	SP	2012	*Hylodes phyllodes*	This study
SLFT 237	Brazil	SP	2012	*Hylodes phyllodes*	This study
SLFT 250	Brazil	SP	2012	*Hylodes phyllodes*	This study
CLFT 040	Brazil	PR	2013	*Bokermannohyla hylax*	(12)
CLFT 041	Brazil	PR	2013	*Bokermannohyla hylax*	(12)
CLFT 044	Brazil	PR	2013	*Hylodes cardosoi*	(12)
CLFT 061	Brazil	SC	2013	*Hylodes meridionalis*	(4, 12)
CLFT 065	Brazil	SP	2013	*Hylodes japi*	(4, 12)
CLFT 066	Brazil	SP	2013	*Hylodes japi*	(12)
CLFT 067	Brazil	SP	2013	*Hylodes japi*	(4, 12)
CLFT 068	Brazil	SP	2013	*Hylodes japi*	(12)
CLFT 070	Brazil	SP	2013	*Hylodes japi*	(12)
CLFT 071	Brazil	SP	2013	*Hylodes japi*	(4, 12)
SLFT 1660	Brazil	SP	2013	*Aquarana catesbeiana*	This study
KB 23	South Korea	n/a	2014	*Aquarana catesbeiana*	(4)
KB 72	South Korea	n/a	2014	*Aquarana catesbeiana*	(4)
KB 108	South Korea	Hwaseong-si	2014	*Aquarana catesbeiana*	(4)
KB 45	South Korea	n/a	2014	*Aquarana catesbeiana*	(4)
CLFT 136	Brazil	PR	2014	*Bokermannohyla hylax*	(4, 12)
CLFT 139	Brazil	PR	2014	*Hylodes cardosoi*	(12)
CLFT 141	Brazil	PR	2014	*Hylodes cardosoi*	(12)
CLFT 142	Brazil	PR	2014	*Crossodactylus caramaschii*	(12)
CLFT 143	Brazil	PR	2014	*Hylodes cardosoi*	(12)
CLFT 144	Brazil	PR	2014	*Hylodes cardosoi*	(4, 12)
CLFT 145	Brazil	PR	2014	*Hylodes cardosoi*	(12)
CLFT 146	Brazil	PR	2014	*Hylodes cardosoi*	(12)
CLFT 148	Brazil	PR	2014	*Hylodes cardosoi*	(12)
CLFT 149	Brazil	PR	2014	*Hylodes cardosoi*	(12)

CLFT 150	Brazil	PR	2014	*Hylodes cardosoi*	(12)
CLFT 151	Brazil	PR	2014	*Hylodes cardosoi*	(12)
CLFT 153	Brazil	PR	2014	*Hylodes cardosoi*	(12)
CLFT 170	Brazil	SP	2016	*Aquarana catesbeiana*	(13)
CLFT 171	Brazil	SP	2016	*Aquarana catesbeiana*	(13)
CLFT 172	Brazil	SP	2016	*Aquarana catesbeiana*	(13)
CLFT 175	Brazil	SP	2016	*Aquarana catesbeiana*	(13)
CLFT 183	Brazil	SP	2016	*Aquarana catesbeiana*	(13)
CLFT 202	Brazil	SP	2016	*Aquarana catesbeiana*	(13)
CLFT 203	Brazil	SP	2016	*Aquarana catesbeiana*	(13)
CLFT 204	Brazil	SP	2016	*Aquarana catesbeiana*	(13)
CLFT 205	Brazil	SP	2016	*Aquarana catesbeiana*	(13)
CLFT 207	Brazil	SP	2016	*Aquarana catesbeiana*	(13)
CLFT 209	Brazil	SP	2016	*Aquarana catesbeiana*	(13)
SLFT 6286	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6287	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6289	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6290	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6291	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6293	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6294	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6297	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6299	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6302	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6306	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6307	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6308	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6309	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6311	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6312	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6314	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6318	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6378	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6383	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 6386	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 7200	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 7202	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 7207	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 7216	Brazil	SP	2016	*Aquarana catesbeiana*	This study
SLFT 7233	Brazil	SP	2016	*Aquarana catesbeiana*	This study

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CHAPTER IV

HOW ARTIFICIAL SELECTION INFLUENCES IMMUNOLOGY: INSIGHTS FROM FARMED AND WILD POPULATIONS OF A CHYTRID FUNGUS SUPERSHEDDER

Como a seleção artificial influencia a imunologia: insights de populações cativas e selvagens de um superdispersor do fungo quitrídio

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This chapter adheres to the guidelines outlined by the journal *Journal of Applied Ecology*, to which it is intended for submission.

How artificial selection influences immunology: insights from farmed and wild populations of a chytrid fungus supershedder

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Abstract

The decline of amphibians due to the *Batrachochytrium dendrobatidis* (Bd) fungus poses a significant threat to biodiversity. Although some species, *i.e*. *Aquarana catesbeiana* (hereafter bullfrogs), present defense mechanisms to Bd infection, these defenses are unclear. The global introduction of bullfrogs for aquaculture raises concerns due to the high prevalence of Bd in these farms. Continuous exposure of high-density farmed bullfrogs to Bd can enhance immune responses, benefiting resistant and/or tolerant individuals and aiding in host-pathogen studies. Our study aimed to assess the impact of mass-produced bullfrogs on Bd infection, immunity, and zoospore release in aquatic ecosystems. We exposed both farmed and wild bullfrogs to high Bd loads from two lineages, hypothesizing higher mortality and reduced leukocyte production in wild bullfrogs, and potentially heavier Bd loads in farmed bullfrogs, which might release more Bd zoospores into the environment. Our analyses included individual monitoring, weekly swab skin and blood collection, and Bd quantification in water. Although one Bd lineage produced more zoospores in culture, loads in individuals did not vary. Likewise, we did not observe differences in mortality rates among experimental groups. Contrary to prior studies, Bd load in bullfrogs increased over time. Farmed bullfrogs, potentially due to stress and confinement, exhibited higher Bd loads and zoospore release, along with potential immunosuppression, indicated by a greater reduction in lymphocyte production compared to their wild counterparts. Our results suggest lower resistance and tolerance to Bd infection, especially in farmed bullfrogs, with the potential for them to act as supershedders of Bd. By exploring how human-animal interactions impact defense mechanisms against pathogens, we can gain valuable insights into disease ecology and the environmental consequences of large-scale production. We emphasize the ecological impacts of mass production on Bd infection and underscore the importance of sustainable production practices in reducing disease risks and conserving biodiversity.

Keywords: *Batrachochytrium dendrobatidis*, Bd infection dynamics, bullfrog, high-density farming, resistance and tolerance.

1. Introduction

The global decline in amphibian populations caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd) poses a critical concern for biodiversity (Scheele et al. 2019, Luedtke et al. 2023). Chytridiomycosis, the disease which results from Bd infection, triggers amphibian epidermal proliferation, hyperkeratosis, and cell death, while disrupting vital functions like osmoregulation and cardiac activity (Pessier et al. 1999, Voyles et al. 2011, Salla et al. 2015, Brannelly et al. 2017). Disease-causing abilities may differ among Bd lineages (Greenspan et al. 2018, Ribeiro et al. 2019, Carvalho et al. 2023), with some producing toxins that impair immune responses in the host and exacerbate the severity of the disease (Brutyn et al. 2012, Fites et al. 2013, 2014). As such, understanding Bd infection dynamics across frog species is crucial for the implementation of effective conservation strategies.

Amphibians species may differ in susceptibility to Bd infections due to their lifestyle, level of pathogen exposure, and environmental conditions (Gervasi et al. 2013a, Savage et al. 2015, Mesquita et al. 2017). Species like *Atelopus* spp. and *Dendrobates* spp. are notably susceptible to Bd in their natural habitats (Nichols et al. 2001, Lips et al. 2008). Similarly, species within the Brachycephaloidea clade, known for their direct development, often exhibit vulnerability to Bd infections (Mesquita et al. 2017, Moura-Campos et al. 2021). Conversely, certain species may exhibit tolerance or even resistance to Bd infections, as recurrently shown in the *Aquarana catesbeiana* (hereafter bullfrog) (Daszak et al. 2004, Hanselmann et al. 2004, Schloegel et al. 2010, Eskew et al. 2015). However, despite the evidence in support of Bd resistance and tolerance in bullfrogs, the defense mechanisms that underlie their ability to withstand Bd infections, particularly regarding immune responses, remain unclear.

Developing resistance or tolerance to Bd infection is pivotal for managing amphibian populations, yet the diverse ways that species respond to Bd complicate these endeavors. Resistant hosts are those able to reduce pathogen load and replication, whereas tolerant hosts are those able to limit the damage caused by the pathogen (Råberg et al. 2009, Grogan et al. 2023). In this context, leukocyte recruitment to the site of infection plays an important role in innate immune response (Grogan et al. 2018). Evidence suggests, however, that the response of leukocytes to chytridiomycosis is inconsistent across species (Young et al. 2014), even in congeners. Some Bd-infected species had increased basophils, and decreased eosinophils and neutrophils relative to control individuals (Woodhams et al. 2007). By contrast, Bd-infected Australian green tree frogs had increased neutrophils, but decreased eosinophils compared to control individuals (Peterson et al. 2013). Better understanding amphibian immune

responses to chytridiomycosis holds promise in not only revealing existing knowledge gaps, but also in pinpointing treatment targets. Ultimately, this information may be used to devise immune-based strategies, as well as predict the decline of at-risk species for timely mitigation (Grogan et al. 2018).

The bullfrog is a species native to North America that has been globally introduced for farming and is now part of significant international trade (Sales et al. 2021, Ribeiro & Toledo 2022, Frost 2024). This global trade, however, poses significant threats to amphibians worldwide (Kats & Ferrer 2003, Laufer et al. 2008, Schloegel et al. 2009, Carpenter et al. 2014, O'Hanlon et al. 2018), including, but not limited to, high Bd prevalence observed in bullfrog farms (Schloegel et al. 2009, Ribeiro et al. 2019, Santos et al. 2020). Repeated infections in farmed bullfrogs might have led to a robust acquired immune response to Bd in this species (Fu & Waldman 2017), with Bd-resistant and/or tolerant individuals being favored over generations that were raised in high-density farm environments.

Artificial selection in animal production aims to bolster traits like productivity and disease resistance through strategies such as vaccinations, hygiene practices, and biosafety measures (Hulst et al. 2022). Nevertheless, ongoing pathogen evolution can inadvertently lead to the emergence of new pathogens and the development of resistance among hosts (Hulst et al. 2022, Dong et al. 2023). Selective breeding, while enhancing desired traits in animal production, often reduces genetic diversity, particularly in high-density systems (Mennerat et al. 2010, Espinosa et al. 2020). Intensive farming thus offers a valuable setting to examine host-pathogen interactions, considering that bullfrogs undergo continuous exposure to the pathogen (Kriger & Hero 2009, Schloegel et al. 2012, O'Hanlon et al. 2018).

Concerning chytridiomycosis, the bullfrog is unique in that it influences Bd transmission dynamics by serving as both a reservoir (Daszak et al. 2004, Hanselmann et al. 2004, Schloegel et al. 2010) and an international vector for this pathogen (O'Hanlon et al. 2018, Fisher & Garner 2020). In this study, we assessed the effects of artificial selective pressure on mass bullfrog production by investigating Bd infection patterns, immune responses, and the release of infectious zoospores into aquatic ecosystems. Specifically, we examined whether mass bullfrog production induced artificial selective pressures that resulted in heightened resistance and tolerance to chytridiomycosis in farmed bullfrogs compared to wild counterparts. Additionally, we explored the potential for bullfrogs, owing to their immune defense, to serve as supershedders of Bd. We hypothesized that wild bullfrogs would have higher mortality rates and lower leukocyte production than their farmed counterparts. Moreover, we anticipated that

farmed bullfrogs would carry higher pathogenic loads, potentially releasing larger quantities of Bd zoospores compared to wild bullfrogs.

2. Materials and methods

2.1. Bullfrog collection

To test the defense mechanisms to chytridiomycosis attributed to the bullfrog and its possible role as a supershedder, we used two experimental groups: wild and farmed bullfrogs. We collected adult bullfrogs in September 2022 from the state of Hidalgo, Mexico, where native populations of this species occur, using the active search method. We captured bullfrogs using dipnets and placed them in individual sterile bags, using a new pair of nitrile gloves for each individual. We categorized post-metamorphic individuals based on body size into three groups: small (5–19 g; 30 – 59 mm; n = 12), medium (20–80 g; 60 – 85 mm; n = 2), and large (over 80 g and 85 mm; n = 46).

Once we collected all the individuals, we swabbed them following a standard protocol (see "Bd quantification in bullfrogs"). After swabbing, we placed bullfrogs individually into aerated plastic bags filled with 100 mL of water from their site of capture. We packed the bags in coolers containing ice and transported the individuals to the laboratory. Swabs were stored on ice and transferred to a -20 °C refrigerator.

We also 30 purchased adult bullfrogs from a commercial farm in Michoacán, Mexico, in October 2022. These individuals were selected to match the body size and sample size of those collected from the wild. We transported farmed bullfrogs individually, within aerated plastic bags filled with 100 mL of farm water, to the Universidad Autónoma Nacional de México, Ciudad de México. The bags were packed in coolers with ice and transported by car. We swabbed these individuals as soon as they arrived in the lab, once more following a standardized protocol.

2.2. Animal husbandry

To minimize the stress inherent to our experimental procedure, we allowed all individuals to be acclimated to controlled lab conditions for at least two weeks before exposure to Bd. Importantly, we kept bullfrogs under the same conditions during both the acclimation and exposure periods. We housed bullfrogs individually in appropriately sized containers. Small bullfrogs were placed in 5-liter aquariums (24 cm long × 12 cm wide × 14 cm tall), medium-sized ones were housed in 10-liter aquariums (30 cm long × 15 cm wide × 18 cm tall), and large ones were kept in 16-liter plastic boxes (35 cm long × 27 cm wide × 18 cm tall).

We covered the containers with mesh nets that were secured with elastic bands, and pierced holes in the lids of the plastic boxes facilitate gas exchange. To minimize external disturbances during the experiment, we covered the sides of all containers with white paper. We filled the containers with dechlorinated autoclaved water in amounts appropriate to their respective sizes: 150 mL for small bullfrogs, 200 mL for medium-sized bullfrogs, and 400 mL for large bullfrogs. We maintained the containers at an angle to allow bullfrogs to move between the water and dry areas without restrictions (**Fig. 1**).

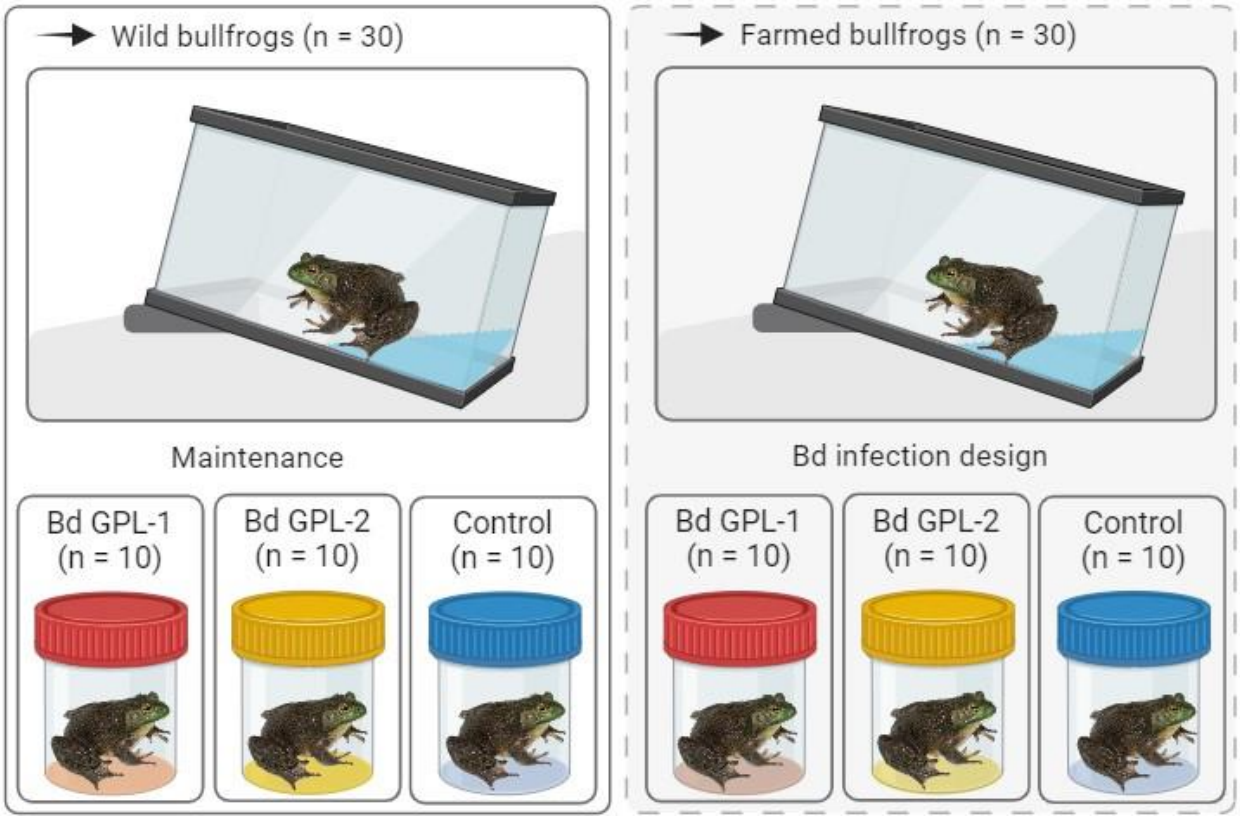

Fig. 1. Experimental design for infection assay and maintenance conditions. Experimental assays are color-coded, with red indicating the GPL-1 lineage, yellow denoting the GPL-2 lineage, and blue indicating the control. We kept bullfrogs under the same housing conditions during both the acclimation and infection phases of the study.

We conducted our experiment in a room with an average temperature of 20 °C (± 1 °C) and a photoperiod of 12:12 h light:dark cycle throughout the study period (ASTM 2002). We monitored the bullfrogs daily for any clinical signs of infection or death. We fed bullfrogs with commercially bred crickets three times a week, with the number of crickets based on the size of the bullfrogs: three crickets for small bullfrogs, four crickets for medium-sized

bullfrogs, and six crickets for large bullfrogs. After feeding, we removed all leftover food, feces, and water from the tanks, and refilled the tanks with dechlorinated autoclaved water. We sterilized any waste with 12 % aqueous hypochlorite solution for 1 hour before discarding it (Cashins et al. 2008). We also monitored physical and chemical water parameters daily, using waterproof pH, EC/TDS & Temperature Testers (Hanna® Instruments), to ensure that conditions remained at acceptable levels, following the guidelines set by the American Society for Testing and Materials (ASTM 2002).

2.3. Rate of Bd zoospore production

We assessed the growth rate of Bd by quantifying the number of active zoospores produced by each culture over time. To this end, we obtained a 15-mL sample of pure cultures grown in 1 % tryptone broth for each of the two lineages used in the experimental infection: GPL-1 and GPL-2 (**Table S1**). From each sample, we counted the number of active zoospores using a Neubauer Chamber three times, taking approximately 10 μL each time. Additionally, we prepared 50 tryptone-agar plates of each strain, using 1 mL of pure culture in 1 % tryptone per plate and kept them at 12 °C. After 6 days, we randomly selected five plates for each strain, filled them with 10 mL of sterile distilled water, and waited for five minutes to allow for zoospore release into the suspension. We collected the suspension from the five plates (50 mL) and counted the number of active zoospores three times using a Neubauer Chamber. We repeated the collection and counting of zoospores on days 7 to 15 (Urbina et al. 2018).

2.4. Experimental design and Bd inoculation

After acclimation, we randomly assigned individuals from farmed and wild populations to one of three treatments: control (n = 10; 2 small, 1 medium, and 7 large bullfrogs), exposed to Bd lineage GPL-1 (n = 10; 2 small and 8 large bullfrogs), or exposed to Bd lineage GPL-2 (n = 10; 2 small and 8 large bullfrogs) (**Fig. 1, Tables S1** and **S2**). To infect the animals, we used Bd strains derived from pure cultures grown in 1 % tryptone broth at 4 °C. We propagated 1 mL of each strain in Petri dishes containing 1 % tryptone agar (Longcore et al. 1999) and kept the cultures at 12 °C for 12 days. Afterwards, we filled the Petri dishes with sterile distilled water and waited for approximately 30 minutes for the zoosporangia to release zoospores (Langhammer et al. 2013). We then passed the Bd suspension through a sterile filter to obtain the infecting phase, which contained only zoospores. An aliquot of the zoospore suspension was checked on a Petri dish for contamination and kept for seven days at 12 °C.

We performed the infection by keeping each bullfrog in a plastic container that limited its movement. We kept its venter completely in contact with 30 mL of Bd inoculum for 24 hours, at a concentration of 1 x 10^6 zoospores/mL (total inoculum dose of 3 x 10^7 zoospores). This inoculum concentration was greater than that used in most studies investigating chytrid infection under laboratory conditions (Gervasi et al. 2013b, Langhammer et al. 2013, Eskew et al. 2015, Salla et al. 2015, DiRenzo et al. 2018, Urbina et al. 2018). The control treatment (Bd^-) was exposed to the same volume of distilled water, which was kept in tryptone-agar plates, similar to the Bd^+ groups, but without the addition of the pathogen inoculum. After the initial 24-hour period, the Bd inoculum was discarded, and replaced with a new solution of the same volume, at a concentration of 7.3 x 10^5 zoospores/mL (total inoculum dose of 2.1 x 10^7 zoospores), to ensure zoospore viability. Individuals were then exposed to this new inoculum for an additional 24 hours.

We checked Bd activity under an optical microscope to confirm the viability of the zoospores after both infections (*i.e.*, GPL-1 and GPL-2). After the infections, the individuals were placed back in their respective containers with the infection solution for 35 days. At the end of this period, the individuals were euthanized with 2 % lidocaine hydrochloride, following the American Veterinary Medical Association guidelines (AVMA 2020). The individuals were fixed in 10 % buffered formalin and transferred to 70 % ethanol for permanent storage.

2.5. Bd quantification in bullfrogs

We measured the body mass (g), snout-to-vent length (SVL; mm) and monitored individuals for signs of infection or injuries on a weekly basis, from day 0 to day 35. We also swabbed each individual before infection and then at seven-day intervals following the infection for 35 days, which is sufficient time for multiple Bd cycles (Longcore et al. 1999), or when individuals died during the experiment. For each individual, we used a pair of gloves and swabbed them using sterile swabs (MW113, Medical Wire & Equipment). We swabbed the underside of each foot for five times, and five times on each side of the venter, accounting for a total of 30 swabs (Hyatt et al. 2007). We dry-stored swabs in sterile tubes and immediately stored them in a - 20 °C freezer, where they were kept for DNA extraction.

We performed DNA extraction using Prepman® Ultra (Applied Biosystems), and we employed qPCR for Bd quantification. Real-time Taqman PCR assays were conducted using a StepOnePlus qPCR system (Applied Biosystems). Each assay was performed in 15 µL reaction volume (12.5 µL master mix and 2.5 µL template DNA). We included in each 48-well plate standards serial dilutions that ranged from 1000 to 1 Bd genome equivalents (g.e.), no DNA

template, and samples diluted 1:10. All samples were run in duplicates. To adjust the fungal quantitation curve, qPCR standard curves were constructed for each strain used for the infection. The cycling conditions matched those of the Bd diagnostic method by Boyle et al. (2004). We considered samples with zoospore g.e. ≥ 1 to be Bd^+ and we expressed the results as the average of zoospores g.e.

2.6. Immune responses

We always collected blood samples after swabbing individuals. To evaluate immune responses, we made a quick puncture through the musculo-cutaneous vein using a small-caliber needle (27G) and collected approximately 10 μL of blood from each individual, which we applied to a slide to make a smear (Forzán et al. 2012). The slides were fixed in pure cold methanol (4 °C) for 20 minutes and stained with 15 % Giemsa for 12 minutes. Entellan and coverslips were used to seal the slides. For the analysis of immune responses over time, we selected five individuals from each treatment and group. We analyzed these slides under a light microscope and, for each individual, estimated the total count of white blood cells (following Heatley & Johnson 2009). Additionally, we counted and differentiated 100 of these cells to estimate the percentage of each leukocyte type, which encompassed lymphocytes, neutrophils, eosinophils, basophils, monocytes, and thrombocytes (Fanali et al. 2018).

2.7. Bd quantification in water

To test whether bullfrogs acted as fungus supershedders, we collected water samples on four days (day 10, 17, 24, and 31), which represented three days following the collection of swabs. Each bullfrog was individually placed in a 500-mL container filled with 50 mL of autoclaved water for 30 minutes to measure the shedding rate of Bd zoospores (DiRenzo et al. 2018). To collect water samples, we used a vacuum pump and filtered the water through permeable membranes with a pore size of 0.45 μm. Prior to filtering the samples, we filtered autoclaved distilled water (hereafter control water) to ensure that there was no cross-contamination. This step was implemented to serve as a contamination control for the filtration system. After filtration, the membranes were stored in their own dry packaging at -20 °C until they could be processed in the laboratory. Following this, we extracted DNA from membranes and quantified Bd zoospores using qPCR as described above (Boyle et al. 2004, Lambertini et al. 2013, Ribeiro et al. 2019). The results are expressed as the load of Bd zoospores (g.e.) per mL.

2.8. Statistical analyzes

All analyses were performed in RStudio (version 1.4.1106) using R (version 4.2.2; Team 2023) with a significance level of 0.05. We used the “fitdist” function from the “fitdistrplus” package (Delignette-Muller & Dutang 2015) to visually inspect residuals and Q-Q plots in search for obvious deviations from homoscedasticity and normality. To evaluate model fit for all models, we employed the “checkresiduals” function from the “forecast” package (Hyndman & Khandakar 2008). We generated all figures using the “ggplot2” package.

Before conducting our analyses, we log10 transformed both body mass and SVL (hereafter “logBody Mass” and “logSVL”, respectively). To assess the allometric relationship between body mass and SVL, we performed a type II regression with logBody Mass being the response variable and logSVL being the predictor. We obtained estimates of body condition by calculating the 'scaled mass index' (SMI) following Peig & Green (2009). Due to the significant differences in body mass and SVL across different developmental stages (juveniles and adults), we calculated the SMI for each stage separately. This approach also enabled us to assess how body condition changed over time for each individual, allowing us to analyze temporal variations in SMI across treatments for both farmed and wild bullfrogs.

To assess the difference in zoospore production potential between the two Bd lineages (GPL-1 and GPL-2), we used the “aov” function from the “stats” package (Team 2023). In this test, the number of active zoospores (log) was the response variable, while Bd lineage was the predictor. To assess variations in Bd load over time, we first log-10 transformed Bd load data with an offset of 1 (*i.e.*, log10 + 1). To mitigate the influence of limited data from days when animals died, we excluded those records from the dataset. Thus, we focused on days 7, 14, 21, 28, and 35, which corresponded to days in which observations were not interrupted by mortality events.

To assess variations in Bd load over time, we used the load value on day 0 as a reference point, facilitating control and comparison of fluctuations relative to the initial load value. We fitted a generalized mixed-effect model (GLMM) using the “glmer” function from the “lme4” package (Bates et al. 2015). In this model, log Bd load was the response variable, and group (farm or wild) and treatment (control, GPL-1, or GPL-2) were the predictor variables. We included time (sampling day: 7, 14, 21, 28 and 35), SMI, and log Bd load day 0 as covariates, and individual ID as a random factor, to account for repeated measurements across days. To select the model that best explained the variation in our data, we used the corrected Akaike information criterion corrected for small sample sizes (AICc) through the “dredge” function from the “MuMIn” package (Bartoń 2023). Finally, we performed a Tukey post-hoc test to

evaluate differences in Bd load relative to our predictor variables with the “emmeans” function from the “emmeans” package (Lenth 2023).

Using survival probability as a proxy for pathogen virulence, we built survival curves to test the effect of group (farm or wild) and treatment (control, GPL-1 or GPL-2) on host mortality. To do so, we created a survival object and fitted a Cox regression model (Cox proportional hazards) that had survival probability as the response variable, and group and treatment as predictors. We created survival curves following the Kaplan-Meier method, implemented through the “survfit” function from the “survival” package (Therneau 2023). Subsequently, we conducted comparisons of the survival curves through log-rank tests employing the “survdiff” function, also from the “survival” package (Therneau 2023).

We followed the same workflow described for Bd load data from bullfrogs to analyze Bd load data obtained from water. We first log-10 transformed water Bd load data with an offset of 1 (*i.e.*, log10 + 1). Then, we fitted a GLMM with the “glmer” function from the “lme4” package (Bates et al. 2015). This model had log Bd load (water) as the response variable, group and treatment were the response variables, Bd load (control water) and SMI were covariates, and day of sampling was a cofactor. To account for pseudo-replication, we included individual ID as a random factor. Model selection was performed with AICC as previously described. Finally, we performed a Tukey post-hoc test to discern significant differences between groups.

We used GLMMs to analyze hematological samples fit with the “glmer” function from the “lme4” package (Bates et al. 2015). We conducted two sets of tests: one that had the log of total leukocyte count as the response variable, and another with the proportion of each leukocyte as the response variable. On all models, we included group, treatment, and log Bd load as predictor variables. We also considered cell count on day 0 as a covariate, and we included individual ID as a random effect to control for pseudo-replication. All GLMM models were fit with the parameter “family=gaussian”, except for those analyzing monocytes and basophils, which were set with the parameter “family=poisson”.

3. Results

3.1. Body condition: scaled mass index (SMI)

We calculated SMI for each individual with body mass and SVL information obtained over the length of the experiment. Juveniles had an average body mass of 18.8 g and an average SVL of 49.1 mm, while adults had an average body mass of 388.4 g and an average SVL of 133 mm (**Table 1**).

Table 1. Mean ± standard deviation and range of body mass (g) and snout-vent length (SVL; mm) for juvenile and adult bullfrogs (*Aquarana catesbeiana*). Data are shown relative to experimental treatments (control, GPL-1, and GPL-2), and whether animals belonged to the farm or wild groups.

Treatment	Group	Stage	N	Body mass	SVL
Control	Farm	Juvenile	3	30.9 ± 28.5 (12 – 69)	58.6 ± 16.3 (40.9 – 83.7)
Control	Wild	Juvenile	3	22.2 ± 14.1 (12 – 40)	51.1 ± 9.75 (39.5 – 67.7)
GPL-1	Farm	Juvenile	2	12.4 ± 1.06 (9 – 15)	45.3 ± 0.226 (36.2 – 50.2)
GPL-1	Wild	Juvenile	2	14.1 ± 2.71 (8 – 21)	45.8 ± 4.29 (39.1 – 54)
GPL-2	Farm	Juvenile	2	10.3 ± 0.236 (8 – 14)	41.4 ± 1.20 (33.8 – 46)
GPL-2	Wild	Juvenile	2	15.3 ± 2.12 (8 – 20)	46.5 ± 4.21 (21.2 – 54.4)
Control	Farm	Adult	7	386.6 ± 182.1 (195 – 507)	133.8 ± 12.3 (94.2 – 302)
Control	Wild	Adult	7	335.5 ± 182 (77 – 527)	123.9 ± 19.4 (75.6 – 147.8)
GPL-1	Farm	Adult	8	399.3 ± 182.1 (267 – 519)	131.5 ± 6.92 (63.5 – 151.8)
GPL-1	Wild	Adult	8	372.2 ± 181.7 (241 – 684)	140 ± 11.1 (115 – 170.8)
GPL-2	Farm	Adult	8	471.8 ± 182.1 (336 – 575)	138 ± 6.17 (108.5 – 156.4)
GPL-2	Wild	Adult	8	351.2 ± 181.3 (175 – 520)	133 ± 9.39 (108.3 – 229.2)

3.2. Bd zoospore production rate

Initially, we quantified active Bd zoospores in samples taken from pure cultures grown in 1 % tryptone broth, prior to transferring them to growth plates. On the first assessment, zoospore counts were 3.0 x 10^6 for GPL-1 and 3.7 x 10^6 for GPL-2. Between days 6 to 15, we conducted daily assessments of active zoospores, with an average count of 4.3 x 10^5 for GPL-1 and 9.8 x 10^5 for GPL-2 in this period. Both strains showed their highest counts of active zoospores (GPL-1: 7.2 x 10^5, GPL-2: 1.8 x 10^6) 12 days after being cultured on the plates. This indicated that bullfrogs should become infected after at least 12 days of Bd cultivation at 12 ºC. We also found that the GPL-2 lineage produced more active zoospores than the GPL-1 lineage ($F_{1,18}$ = 5.918, P = 0.02; **Fig. 2A**).

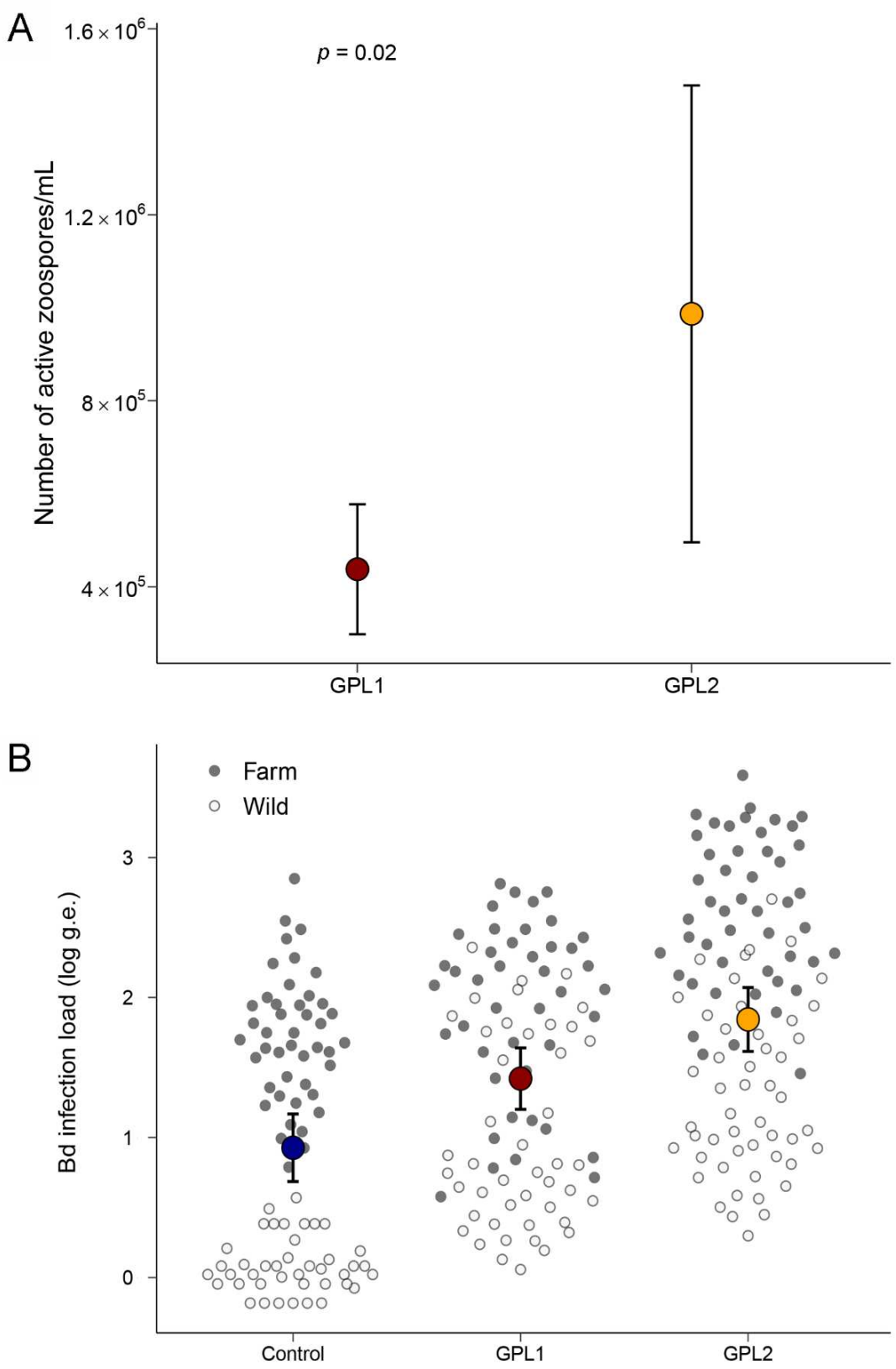

Fig. 2. Comparison of Bd zoospore production between GPL-1 and GPL-2 lineages, presented as the number of active zoospores/mL (**A**); and Bd infection load among control, GPL-1, and GPL-2 treatments (**B**). Bd lineages are color-coded, with GPL-1 shown in red, GPL-2 shown in yellow, and control shown in blue. On both panels, dots represent average values and bars

denote the corresponding confidence limits. Jittered dots represent individual values that make up the averages.

3.3. Bd infection load

The best candidate model (*weight* = 0.513) had day, treatment, and group as predictor variables, while SMI and Load0 did not predict zoospore load in bullfrogs. Below, we present our results by providing an average estimate followed by the lower and upper 95 % confidence intervals [95 % CI]. Our analyses revealed that Bd load differed between the control group and GPL1 (0.56 [0.10, 1.01], $P = 0.016$), as well as between the control group and GPL2 (1.05 [0.61, 1.50], $P < 0.001$); we found similar Bd loads between GPL1 and GPL2 (**Fig. 2B**). Bd load increased over time between days 7 and 35 (0.43 [0.17, 0.69], $P = 0.001$; **Fig. 3A**). Our model also indicated that farmed bullfrogs exhibited higher Bd loads than their wild counterparts (-1.32 [-1.68, -0.95], $P < 0.001$; **Fig. 3B**).

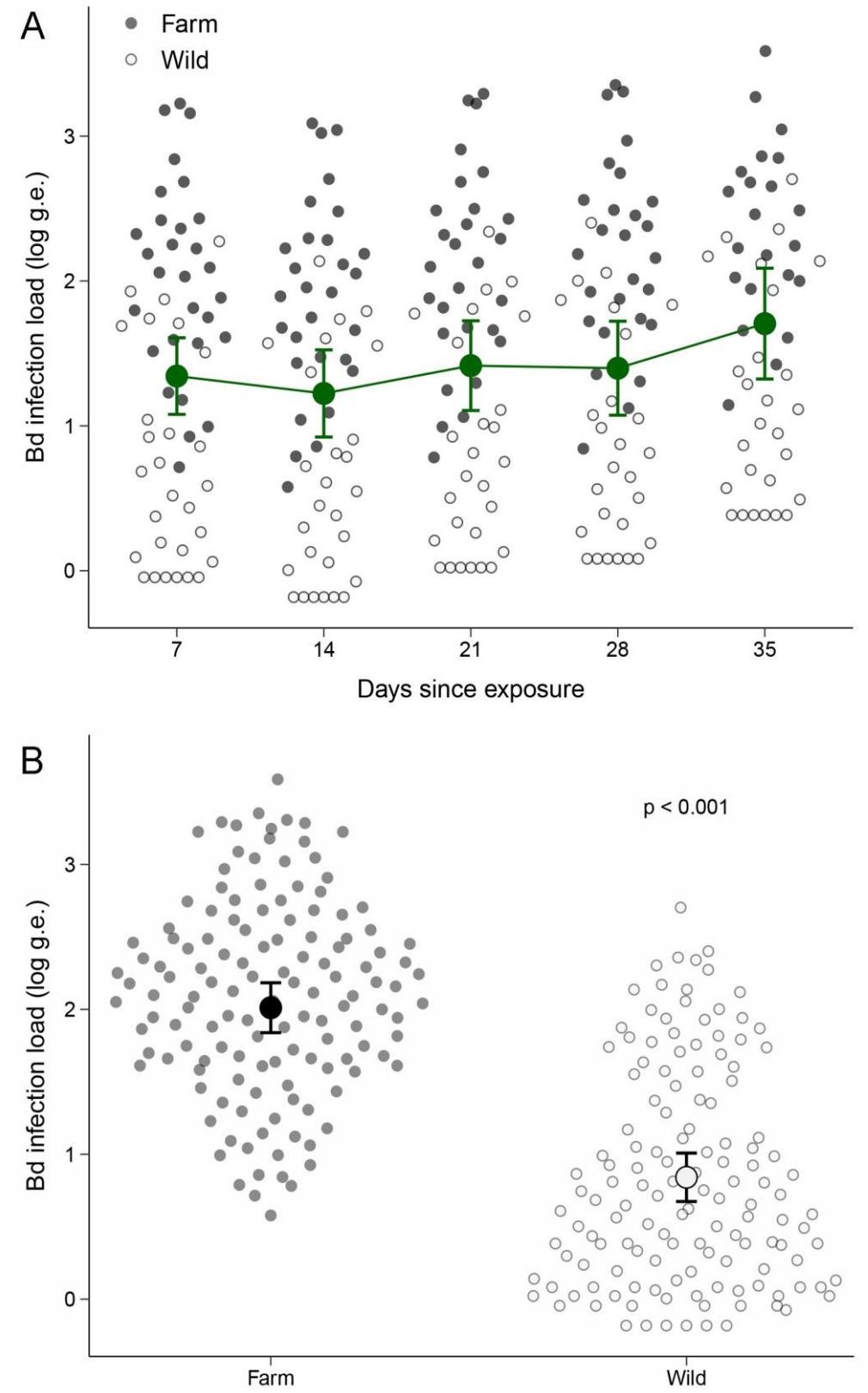

Fig. 3. Temporal variation in Bd infection load (log g.e. zoospore) over the course of the experiment (**A**); and Bd infection load between experimental groups (farm and wild) (**B**). On both panels, dots represent average values and bars indicate confidence limits and jittered dots represent individual values that make up the averages.

3.4. Survival curves

Survival rates were similar between farmed and wild bullfrogs ($P = 0.12$; **Fig. S1**). Similarly, neither experimental treatment (control, GPL-1, and GPL-2) predict survival rates in bullfrogs ($P = 0.33$).

3.5. Immune responses

The total leukocyte production varied over the course of our experiment. Lymphocytes were generally the predominant cell type in relation to other blood cell types in both farmed (**Fig. S2A**) and wild bullfrogs (**Fig. S2B**). Our analyses revealed a decrease in total leukocytes across days (post-hoc Tukey $P\ \text{day}_{7–35} = 0.04$, post-hoc $P\ \text{day}_{14–35} = 0.03$, post-hoc $P\ \text{day}_{21–35} = 0.01$; **Fig. 4A**). Farmed bullfrogs exhibited a higher leukocyte production compared to the wild ones (post-hoc $P = 0.01$; **Fig. 4B**).

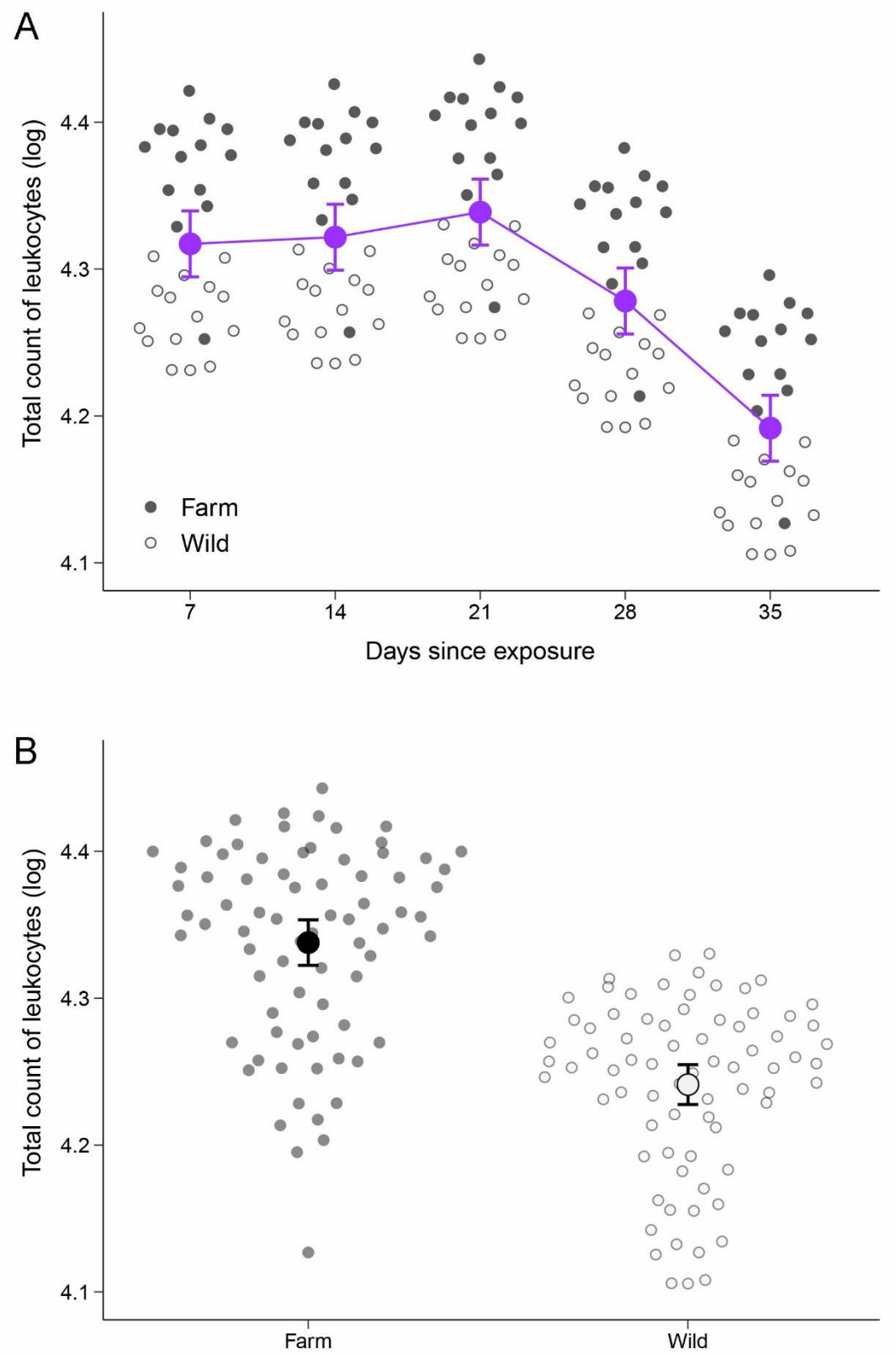

Fig. 4. Variation in total leukocyte count across days (**A**) and between farmed and wild bullfrogs (**B**). On both panels, dots show average values and bars indicate confidence limits. Jittered dots indicate individual values.

Infected bullfrogs, whether farmed or wild, showed lower lymphocyte production compared to the control ($P = 0.003$; **Fig. 5A**). Farmed bullfrogs produced fewer lymphocytes than wild ones ($P = 0.03$; **Fig. 5B**). We did not find any relationship between monocytes, eosinophils, basophils, thrombocytes, and our predictor variables.

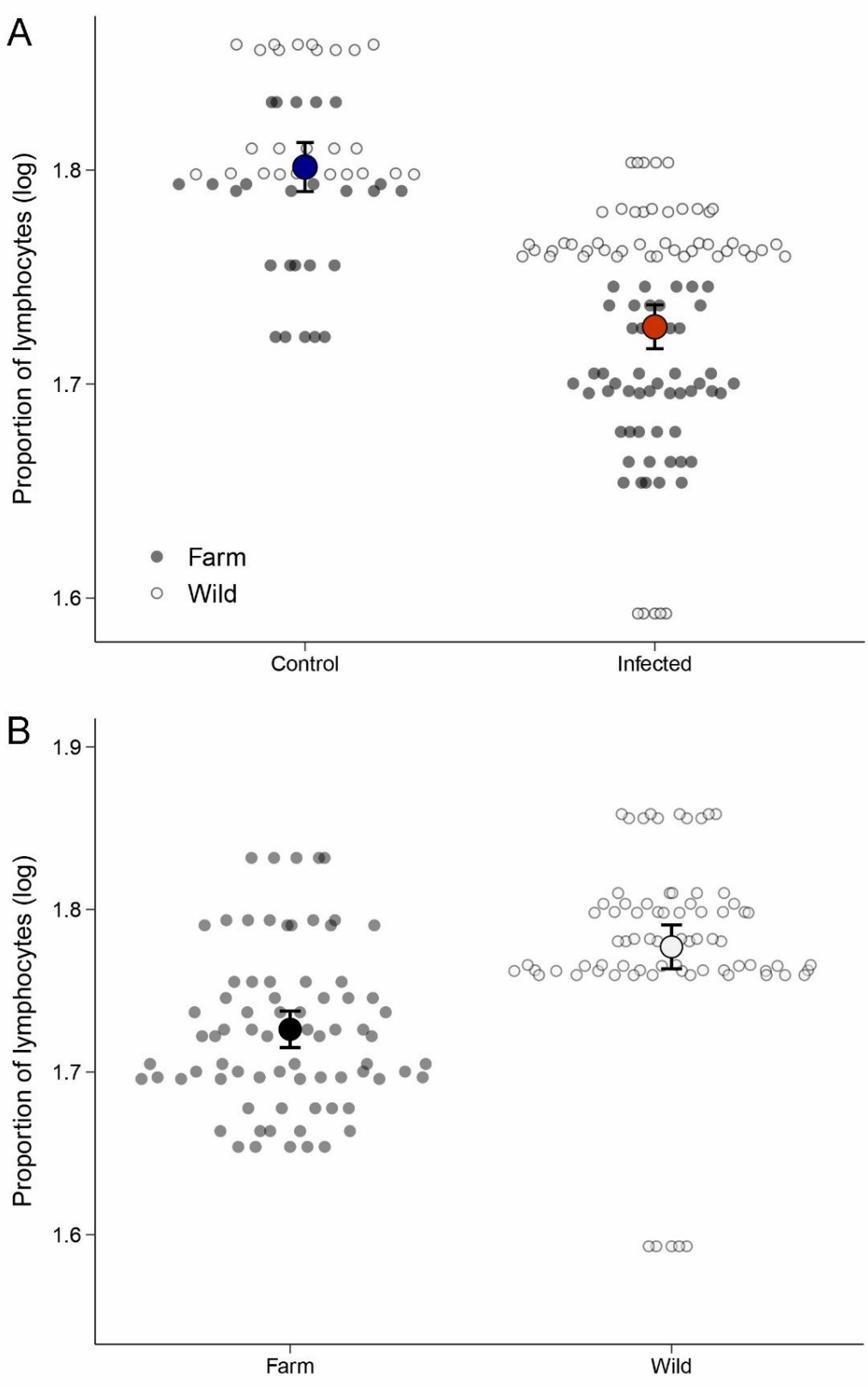

Fig. 5. Proportion of lymphocytes between control and infected groups (**A**) and between farm and wild treatments (**B**). On panel **A**, blue indicates control individuals, whereas orange represent infected individuals with either GPL-1 or GPL-2. On both panels, dots represent averages and bars indicate confidence limits. Jittered dots show individual values.

3.6. Bd quantification in water

The best candidate model (*weight* = 0.607) suggested that group explained the variation in Bd load in the water. Our analysis revealed that farmed bullfrogs released greater amounts of Bd zoospores in the water compared to wild bullfrogs ($P < 0.0001$; **Fig. 6**).

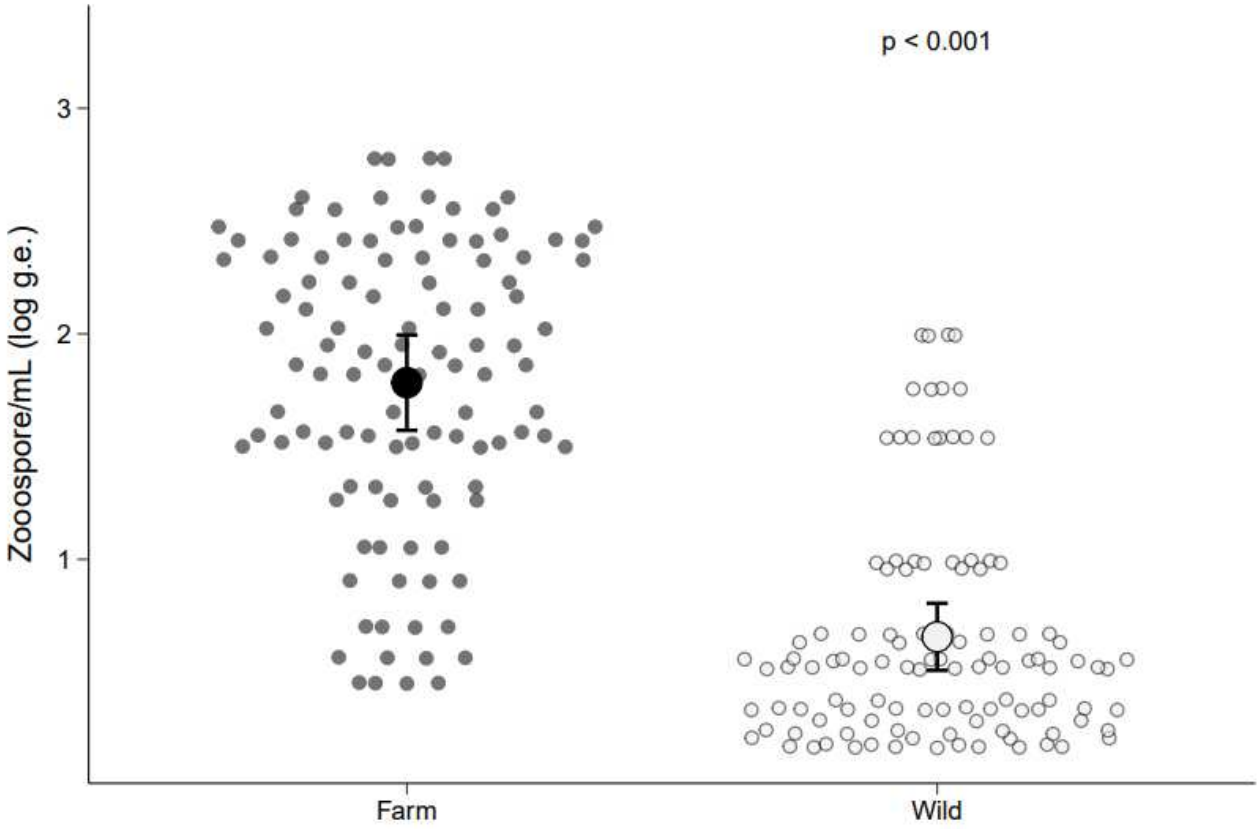

Fig. 6. Quantification of Bd zoospores (log g.e.) released into the water by farmed and wild bullfrogs. Dots indicate average values and bars denote confidence limits. Jittered dots represent individual values.

4. Discussion

Human-animal interactions in production activities constitute potential pathways for the transmission, evolution, and emergence of diseases (Nghiem et al. 2023). Continuous confinement of individuals in artificial environments can impact their well-being and how they respond to pathogens, ultimately affecting their adaptability and resilience (Barnett 2007, Jovanovic et al. 2009). Importantly, chronic stress associated with confinement and intensive handling can disrupt the immune system, reducing animals' natural defenses (Inbaraj et al. 2019). Farmed bullfrogs are known for their high prevalence of the Bd fungus (Ribeiro et al.

2019, Santos et al. 2020), thus being a relevant study system to further our understanding of immune responses in amphibians. In this study, we showed that susceptibility to Bd infection differs between farmed and wild bullfrogs, while highlighting that immune responses and the ability to spread pathogens also differs between conspecifics raised in contrasting environments.

We measured the rate of zoospore production of two Bd lineages raised in cultures to gauge whether virulence differed between lineages (Langhammer et al. 2013). Although we found that the GPL-2 lineage had higher growth in cultures than the GPL-1, we noted that bullfrogs exposed to both lineages showed similar infection loads, which was also corroborated by Urbina et al. (2018). Pathogen growth in cultures does not always translate to observed behaviors in susceptible hosts (Piovia-Scott et al. 2015), as the infection load of a pathogen may vary among individuals due to host susceptibility and the pathogen's own virulence (Grogan et al. 2018). We also found that farmed bullfrogs carried a higher load of Bd than wild frogs. In nature, the average infection load in bullfrogs has been reported to show low levels of Bd infection, both in terms of prevalence and infection load (Gervasi et al. 2013b, Walke et al. 2015, Neely et al. 2021). Furthermore, invasive bullfrog populations exhibited considerably higher prevalence and intensity of Bd compared to native populations (Lafond et al. 2022).

In our study, observed instances of death were not explained by Bd lineage in neither wild nor farmed bullfrogs. These findings are aligned with other research on the survival of Bd-infected bullfrogs (Daszak et al. 2004, Hanselmann et al. 2004, Gahl et al. 2012, Eskew et al. 2015). Previous studies emphasized that bullfrogs showed resistance to Bd infection (*e.g.*, Gervasi et al. 2013b, Eskew et al. 2015). Our results, however, showed that Bd load increased over time in both farmed and wild bullfrog. Thus, this finding challenges the hypothesis that bullfrogs can actively limit Bd load, *i.e.*, showing resistance to Bd infection. Further research may test whether bullfrogs are indeed unable to limit Bd load, clarifying if their main defense mechanism against chytridiomycosis is tolerance, rather than resistance.

Hosts vary in their ability to handle pathogens; some remain healthy despite high loads, while others suffer even with relatively low burdens (Råberg et al. 2009). Although the nonspecific immune system is crucial in pathogen defense, the effects of Bd on white blood cell populations in amphibians have been understudied (Davis et al. 2010, Grogan et al. 2023). Our study revealed that the immunological responses of bullfrogs do not seem to play a central role in resistance to Bd infection, with leukocytes reducing over time, which indicates the immunosuppressive potential of chytridiomycosis, irrespective of developmental stage. We observed a decrease in the total number of leukocytes over time in infected individuals, along with a reduction in lymphocyte production, regardless of the bullfrogs' origin (*i.e.*, nature of

frog farm). In vitro studies showed that Bd can impair lymphocyte proliferation and induce apoptosis, while fungal recognition and phagocytosis by macrophages and neutrophils remain unaffected (Fites et al. 2013). Previous studies also noted multiple defects in the immune function of the host (Young et al. 2014). For instance, Bd-infected bullfrogs had altered leukocyte profiles, with tadpoles showing significantly more neutrophils and fewer eosinophils (Davis et al. 2010), and post-metamorphic stages exhibiting increased neutrophils and decreased lymphocytes (Peterson et al. 2013).

Our results also showed a reduction in lymphocytes, which may be caused by an increase in stress hormones due to an infection (Peterson et al. 2013). The finding that farmed bullfrogs produced fewer lymphocytes than their wild counterparts suggests that mass production environments may have an effect over immune response. Mass production, by definition, comprises a range of influencing factors, including stress from infection, confinement stress and limited dietary composition. Together, these factors may further limit the proper development of the immune system, rendering farmed frogs more susceptible to infections (Assis et al. 2023). These aspects may act as a selective pressure over the host's immune system, and over generations, result in individuals with increased tolerance to Bd, or individuals that are able to reallocate resources to alternative infection-fighting mechanisms (Råberg et al. 2007, Young et al. 2014). Although the adaptive immune system of amphibians is responsive to Bd, the factors that influence lymphocyte immunosuppression remain unclear. Understanding such factors is relevant, given that as the infection persists, an ineffective immune response may lead to immunopathology, contributing to morbidity (Grogan et al. 2020).

Individuals in a pre-morbid state typically exhibit higher tolerance and lower resistance to chytridiomycosis, meaning that they have a great capacity to withstand high pathogen loads and likely function as supershedders of Bd (Grogan et al. 2023). In this context, farmed bullfrogs meet all the requirements to be supershedders; this is reflected in our findings of increased release of Bd zoospores into the water in farmed compared to wild frogs. The lack of regulation in frog farming, coupled with the absence of protocols for treating residual water, brings ecological and epidemiological implications for amphibian conservation (Ribeiro & Toledo 2022). For example, species that are highly susceptible to chytridiomycosis may become supershedders of the pathogen by rapidly carrying high Bd loads (DiRenzo et al. 2014). In systems in which a supershedder contributes disproportionately to the maintenance of pathogen persistence and infection intensity, strategies that target population exclusion or reduction may be an efficient immediate approach (Grogan et al. 2023). Indeed, a recent study consisting of a

spatially replicated experimental approach revealed that eradicating bullfrogs reduced the risk of chytridiomycosis for native species (Hossack et al. 2023).

Our results offer a comprehensive view of Bd fungal infection dynamics, integrating immune system responses with environmental influences. In the context of animal production environments, the pressure of mass production is manifested in immunosuppressed populations through decreased resistance to infection and increased potential to become Bd supershedders. Understanding the evolution of host-pathogen interactions is vital for managing amphibian populations, and immunological research remains pivotal in achieving this goal (Grogan et al. 2018). However, assessing tolerance in animals remains a significant challenge due to the complex parameters involved in evaluating individual fitness in response to infection load. Moreover, tolerance and resistance are not mutually exclusive and may interact in complex ways (Råberg et al. 2007).

We emphasize the need for further studies assessing resistance and tolerance to chytridiomycosis in different species and environmental contexts. Understanding interactions between farmed animals and pathogens is crucial, especially if one aims to prevent pathogen spread in productive systems. While bullfrog farms emerged as a solution to excessive exploitation of native amphibians for food (Jennings & Hayes 1985, Oza 1990, Schlaepfer et al. 2005, Carpenter et al. 2014), global concerns have arisen due to the lack of biosafety protocols in bullfrog farming practices (Ribeiro & Toledo 2022). Comprehending how large-scale animal production and global trade act as disease amplifiers is essential for conservation strategies, especially for native species. Transitioning to more sustainable and agroecological animal production models that prioritize biodiversity, natural selection, and ecological balance may help reduce risks associated with intensive artificial selection. By integrating quantitative assessments with host characteristics, infection dynamics, and environmental impacts, our study offers pertinent insights for the scientific community, conservation practitioners, and policymakers.

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SUPPORTING INFORMATION

Table S1. *Batrachochytrium dendrobatidis* (Bd) strains used in the experimental inoculations. Lineage, host species, locality, year of isolation, and approximate number of passages are given for each strain.

Strain	Lineage	Host species	Locality	Year	Passage
TPZ24A	GPL-1	*Aquarana catesbeiana*	Tepetzintla, Veracruz, Mexico	2018	11
TAC04B	GPL-2	*Plectrohyla sagorum*	Talquián, Chiapas, Mexico	2018	10

Table S2. Number of hosts per treatment (control, GPL-1, and GPL-2) from the two groups (wild and farmed) evaluated in the infection assay.

	Wild		Farm		Total
Stage	**Adult**	**Juvenile**	**Adult**	**Juvenile**	
Control	7	3	7	3	20
GPL-1	8	2	8	2	20
GPL-2	8	2	8	2	20
Total	23	7	23	7	60

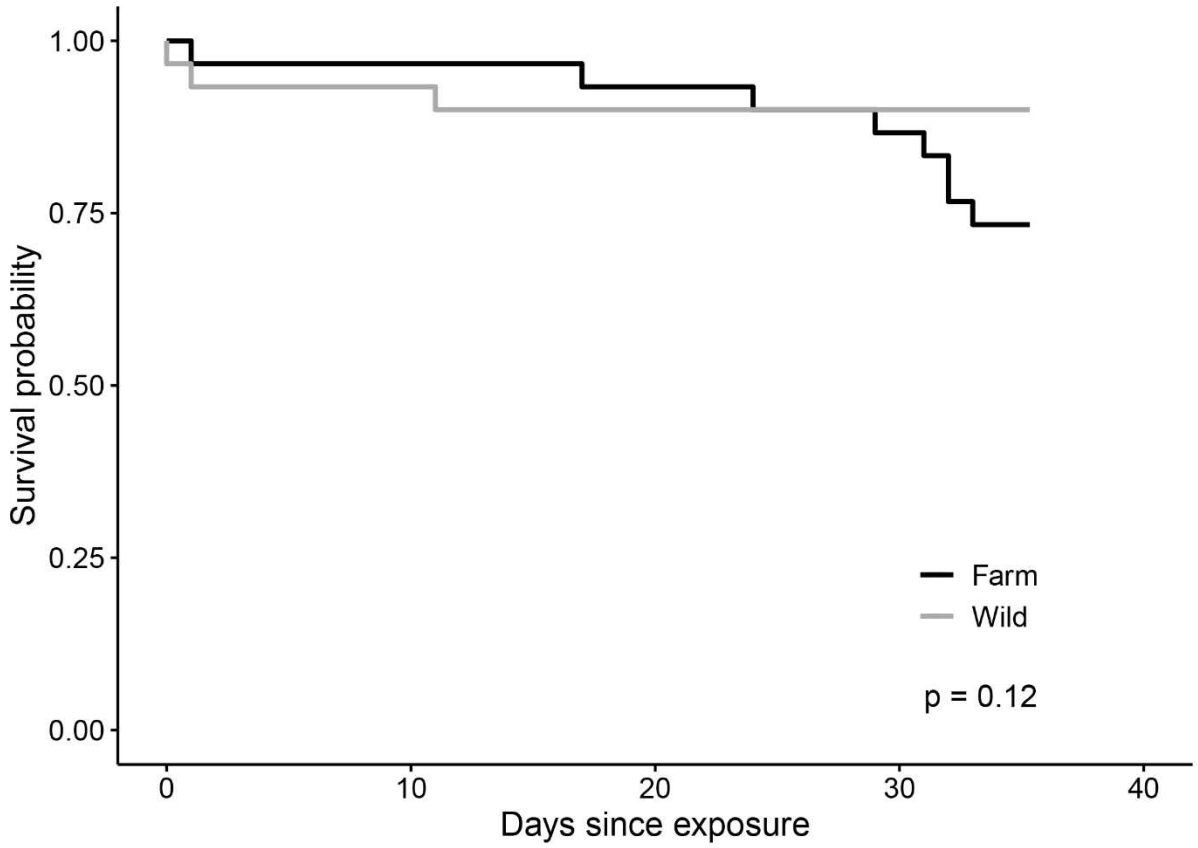

Fig. S1. Survival curves for the two experimental groups: farmed and wild bullfrogs.

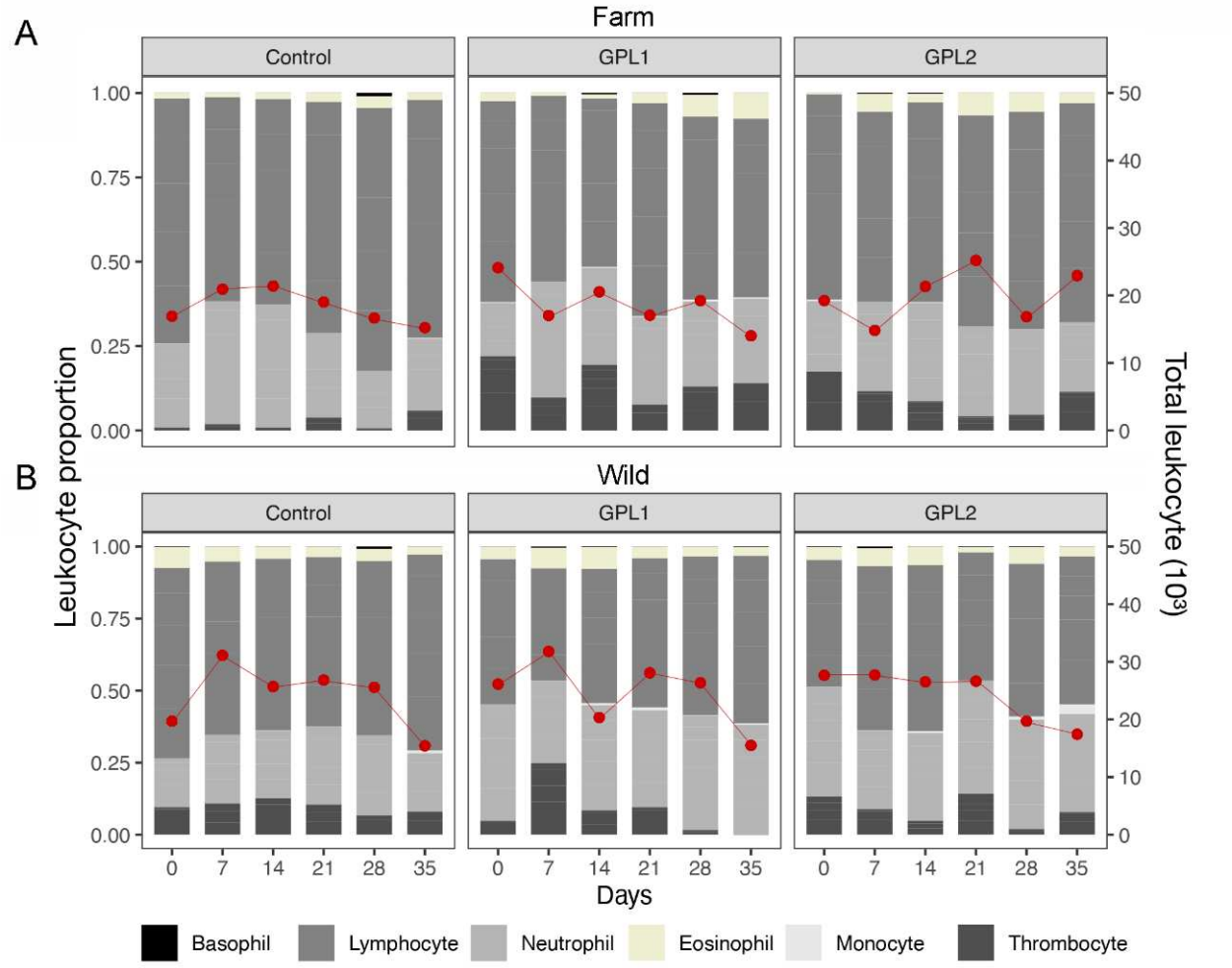

Fig. S2. Temporal variation in leukocyte proportion and total leukocytes counts (10^3) across experimental treatments for farmed (**A**) and wild (**B**) bullfrogs. On both panels, leukocyte types are color-coded. The red dots and lines represent the change in total leukocyte count over time.

CAPÍTULO V

APRIMORAMENTO DE POLÍTICAS PÚBLICAS SOBRE A RANICULTURA BRASILEIRA

Enhancing public policies for Brazilian bullfrog farming

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Aprimoramento de políticas públicas sobre a ranicultura brasileira

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Resumo

A aquicultura é uma indústria global importante na prática do cultivo de organismos aquáticos para fornecer alimentos à população e impulsionar a economia dos países produtores. O Brasil se destaca na produção global de rãs-touro, mas essa atividade tem gerado problemas ecológicos e econômicos. A falta de regulamentação e controle pode ameaçar a biodiversidade, já que essas rãs atuam como vetores de patógenos como o fungo quitrídio (Bd) e o Ranavirus (Rv). Tanto o Bd, quanto o Rv, estão associados a declínios e extinções em massa de anfíbios em todo o mundo. Estudos no Brasil identificaram alta prevalência desses patógenos em ranários, representando ameaças à indústria e ao ambiente. A ausência de regulamentação eficaz na ranicultura é um desafio, já que a quitridiomicose e a ranavirose são doenças de notificação obrigatória à Organização Mundial de Saúde Animal, exigindo gestão dos impactos na produção de rã-touro. Portanto, nosso objetivo foi promover a articulação entre pesquisadores, órgãos públicos e líderes do setor, visando revisar as legislações existentes e propor medidas eficazes de biosseguridade. Realizamos extensivas revisões sobre a ranicultura no Brasil, participamos e promovemos reuniões com representantes governamentais e produtivos e nos engajamos ativamente nos Planos de Ação para a conservação de espécies. Nesse contexto, esforços foram feitos para mapear ranários e desenvolver propostas de biosseguridade visando o controle de patógenos. Concluímos que a colaboração entre setores é crucial para elaborar e implementar políticas eficazes. A implementação e manutenção de regulamentações mais abrangentes e a adoção de práticas sustentáveis na ranicultura são essenciais para garantir a sustentabilidade da indústria e a conservação da biodiversidade.

Palavras-Chave: Aquicultura, *Aquarana catesbeiana*, colaboração inter-setorial, conservação da biodiversidade, práticas sustentáveis, quitridiomicose, ranavirose.

Enhancing public policies for Brazilian bullfrog farming

Abstract

Aquaculture is a globally significant industry involved in the cultivation of aquatic organisms to provide food for the population and boost the economies of producing countries. Brazil stands out in the global production of bullfrogs, but this activity has generated ecological and economic problems. The lack of regulation and control can threaten biodiversity, as these bullfrog's act as vectors for pathogens such as chytrid fungus (Bd) and Ranavirus (Rv). Both Bd and Rv are associated with declines and mass extinctions of amphibians worldwide. Studies in Brazil have identified a high prevalence of these pathogens in bullfrog farms, posing threats to both the industry and the environment. The absence of effective regulation in bullfrog farming is a challenge, as chytridiomycosis and ranaviruses are diseases that must be reported to the World Organisation for Animal Health, requiring management of impacts on bullfrog production. Therefore, our goal was to promote collaboration among researchers, public entities, and industry leaders, aiming to review existing legislation and propose effective biosecurity measures. We conducted extensive reviews on bullfrog farming in Brazil, participated in and organized meetings with government and industry representatives, and actively engaged in Species Conservation Action Plans. In this context, efforts were made to map bullfrog farms and develop biosecurity proposals for pathogen control. We conclude that sectoral collaboration is crucial for formulating and implementing effective policies. The implementation and maintenance of comprehensive regulations and the adoption of sustainable practices in frog farming are essential to ensure the industry's sustainability and biodiversity conservation.

Keywords: Aquaculture, *Aquarana catesbeiana*, inter-sectoral collaboration, biodiversity conservation, sustainable practices, chytridiomycosis, ranaviruses.

1. Introdução

A aquicultura, que envolve o cultivo de organismos aquáticos em condições controladas, destaca-se como uma indústria de relevante impacto global na oferta de alimentos e na economia (Garlock et al. 2019). Segundo a Organização das Nações Unidas para Agricultura e Alimentação (FAO), a produção global aquícola alcançou um marco histórico de 114,5 milhões de toneladas em 2020, com um valor estimado em US$ 263,6 bilhões (FAO 2024). Peixes, crustáceos (especialmente camarões) e moluscos, como ostras e mexilhões, são organismos comumente cultivados nesse contexto. Além disso, a prática da ranicultura, voltada principalmente para a criação da espécie *Aquarana catesbeiana*, conhecida como rã-touro, também integra o campo da aquicultura e tem experimentado um notável aumento de popularidade, principalmente na Ásia e na América do Sul (Ribeiro & Toledo 2022).

A rã-touro, nativa da América do Norte, foi introduzida em diversas regiões do mundo com fins comerciais para consumo de sua carne, tornando-se uma das espécies invasoras mais amplamente distribuída, com impactos ecológicos e econômicos significativos (Falaschi et al. 2020). Esse cenário se deve, em grande parte, às fugas ou solturas intencionais durante o processo de criação da espécie (Both et al. 2011). O Brasil se destaca como um dos pioneiros e maiores produtores de rã-touro globalmente (Ribeiro & Toledo 2022) e como o país de maior diversidade de anfíbios do mundo (Frost 2024). Entretanto, a ausência de regulamentação e controle na criação e no comércio de rãs está emergindo como uma preocupação crescente para a conservação da biodiversidade.

As rãs-touro são conhecidas por carregar e servir como vetores de diversos patógenos, como o fungo quitrídio (*Batrachochytrium dendrobatidis* ou Bd), causador da quitridiomicose (Longcore et al. 1999), e o Ranavirus (*Ranavirus* spp. ou Rv), responsável pela ranavirose (Mazzoni et al. 2009; Schloegel et al. 2010a; O'Hanlon et al. 2018). Esses patógenos têm sido associados a eventos de mortalidade em massa e extinções em escala global. Enquanto o Bd está amplamente disseminado pelo mundo, causando declínios e extinções de diversas espécies de anfíbios (Scheele et al. 2019), o Rv possui uma distribuição geográfica mais restrita, mas é capaz de infectar uma maior diversidade de organismos, incluindo peixes, répteis e anfíbios (Brunner et al. 2015).

No Brasil, a ranavirose tem sido responsável por eventos de mortalidade em massa entre anfíbios e peixes (Ruggeri et al. 2019, 2023), enquanto o Bd tem sido associado a diversos declínios populacionais de espécies nativas de anfíbios (Carvalho et al. 2017). Ambos os patógenos foram encontrados em ranários brasileiros. Estudos realizados nos estados de São Paulo, Paraná e Santa Catarina relataram uma alta prevalência do fungo (Ribeiro et al. 2019;

Santos et al. 2020), além da detecção de Bd em elevadas cargas nas águas residuais que alcançam corpos d'água naturais (Ribeiro et al. 2019). Similarmente, o Rv foi detectado em ranários brasileiros causando mortalidade em massa em rãs-touro (Mazzoni et al. 2009; Candido et al. 2019). A presença e evolução desses patógenos em ambientes de criação representam uma ameaça significativa tanto para a indústria de rãs quanto para o meio ambiente (Ribeiro & Toledo 2022).

Tanto a quitridiomicose quanto a ranavirose são doenças de notificação obrigatória pela Organização Mundial de Saúde Animal (OMSA) (Schloegel et al. 2010b), o que demanda a gestão dos impactos provenientes da produção de rã-touro. Isso vai além das preocupações com a invasão da rã-touro e inclui a disseminação desses patógenos. A introdução e a expansão geográfica de espécies exóticas invasoras, juntamente com disseminação de patógenos e a gestão inadequada de resíduos provenientes da produção de rãs, representam ameaças de grande relevância que requerem atenção imediata. Identificar e o controlar esses patógenos são aspectos cruciais para assegurar a continuidade bem-sucedida e sustentável da indústria de criação de rã-touro.

A falta de informações e de articulação entre setores envolvidos na ranicultura tem representado um desafio para a criação de regulamentações eficazes. Embora haja controvérsias sobre a regulamentação ambiental, reconhece-se sua importância na proteção da saúde e do ambiente (Clausen et al. 2023). O modelo da "hélice tripla" ressalta a colaboração entre indústria, reguladores e acadêmicos para promover a inovação regulatória, enfatizando a necessidade de cooperação na formulação e implementação de regulamentações (Zhou & Etzkowitz 2021).

Dessa forma, nosso objetivo foi promover a articulação entre pesquisadores, órgãos públicos e líderes do setor, visando a revisão das legislações existentes, a proposta de medidas eficazes de biosseguridade, a monitoramento regular de patógenos, tratamento de indivíduos e águas contaminadas, além de discutir soluções específicas para cada área prioritária. Ao adotar práticas responsáveis e esforços de conservação, almejamos garantir a sustentabilidade e rentabilidade a longo prazo na ranicultura, assegurando a sanidade animal e a qualidade dos ecossistemas.

2. Métodos

Realizamos uma revisão do histórico da ranicultura no Brasil, identificando a legislação associada e os órgãos responsáveis por essa atividade em cada estado onde atuamos nos Planos de Ação para conservação de espécies. Promovemos reuniões reunindo

representantes do setor público, nas esferas estaduais e federais, setor privado e pesquisadores para abordar a lacuna na regulamentação da ranicultura, visando reduzir os impactos negativos da produção sobre os anfíbios nativos. Ademais, buscamos engajamento ativo em Planos de Ação Nacionais (PANs) e Planos de Ação Territoriais (PATs) coordenados por órgãos governamentais, propondo e nos responsabilizando por ações alinhadas aos objetivos de conservação de espécies e à prática responsável da ranicultura.

3. Resultados e discussão

3.1. Um breve panorama da ranicultura brasileira

No contexto da aquicultura, que engloba a criação e cultivo de organismos aquáticos, encontra-se a ranicultura, uma prática especializada na produção de rãs, frequentemente realizada para fins comerciais. No Brasil, esta atividade teve início na década de 1930 com a importação das primeiras rãs-touro (*Aquarana catesbeiana*) da América do Norte (Ferreira et al. 2002). Em 1935, estabeleceu-se o primeiro ranário comercial no Brasil, localizado no estado do Rio de Janeiro. A ranicultura em São Paulo teve seu início em 1939 (Silva et al. 2013). O mercado brasileiro de rãs começou a ganhar destaque no início da década de 1980. No entanto, muitos produtores abandonaram a atividade devido à inadequação das instalações para a criação de rãs e das técnicas de manejo (Braz Filho 2001; Feix et al. 2004).

Em 2009, o Brasil foi classificado como o segundo maior produtor de rãs, ficando atrás apenas de Taiwan (Embrapa 2015). A ranicultura apresenta infraestrutura, condições ambientais e potencial de mercado promissores em várias regiões do país. Estima-se que o Brasil conte com cerca de 600 ranários estabelecidos, além de 15 indústrias de abate e processamento (Lima et al. 1999). Na região Sudeste, há um total de 144 ranários, distribuídos em 60 municípios no estado de São Paulo, 16 no Rio de Janeiro, 10 em Minas Gerais e 3 no Espírito Santo (Rodrigues et al. 2010). Atualmente, foram mapeados 151 ranários, distribuídos principalmente no sudeste do Brasil, com uma produção estimada de cerca de 400 toneladas de rã-touro (Ribeiro & Toledo 2022).

O Brasil possui condições favoráveis para a criação de rãs-touro, especialmente devido ao clima propício à atividade. Observamos um grande interesse e entusiasmo financeiro para iniciar criação de rãs, porém, a cadeia produtiva da ranicultura, ainda pouco organizada, gera vários desafios. Estes incluem a lentidão no processo de licenciamento ambiental, a burocracia no cadastramento do produtor e dificuldades no escoamento dos produtos (Cribb et al. 2013). Com frequência, vemos projetos na ranicultura sendo iniciados sem um planejamento

prévio adequado, levando a um número considerável de produtores frustrados, que entram e saem da atividade ano após ano (Cribb et al. 2013).

De acordo com o decreto nº 62.243, de novembro de 2016, referente ao licenciamento ambiental da aquicultura no estado de São Paulo, toda atividade de cultivo ou criação de organismos com ciclo de vida que ocorre total ou parcialmente em meio aquático é considerada aquicultura (ver **Tabela 1** para revisão de bases legais para ranicultura). Assim, como um segmento da aquicultura, a ranicultura requer que os produtores que ingressam nesse setor realizem seu registro como aquicultores junto ao Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA). Além disso, a produção também deve ser registrada a cargo de um produtor rural através do órgão competente de cada região. Apesar da existência dessas legislações, a unificação desses cadastros ou mesmo o acesso a eles é inviável, dificultando a realização de estudos de pesquisa e a elaboração de planos de ação para atender às leis em vigor.

Um desafio presente na ranicultura que pode impactar diretamente a conservação dos anfíbios nativos está relacionado a doenças infecciosas, especialmente a quitridiomicose e a ranavirose (Mazzoni et al. 2009; Ribeiro et al. 2019; Santos et al. 2020; Ruggeri et al. 2023). Nesse contexto, destaca-se um programa de aquicultura do Ministério da Agricultura e Pecuária (MAPA) voltado para a saúde das rãs produzidas e comercializadas. A Instrução Normativa nº 4, de fevereiro de 2015, que abrange todos os estabelecimentos envolvidos no cultivo ou manutenção de animais aquáticos no território nacional, entrou em vigor somente em setembro de 2017 (BRASIL 2015) (**Tabela 1**). Seu propósito é fomentar a sustentabilidade dos sistemas de produção de animais aquáticos e assegurar a sanidade da matéria-prima proveniente dos cultivos nacionais. Esta instrução estabelece diretrizes sobre normas de registro, boas práticas de produção, medidas profiláticas, biosseguridade, transporte nacional e internacional, critérios para quarentena e abate. Os objetivos da Instrução Normativa, bem como todos os seus aspectos contemplados, são de extrema importância para aprimorar a regulamentação da ranicultura. Apesar de ter sido implementada recentemente, será crucial um esforço de promoção e divulgação desta instrução para que esta seja adotada e viável em toda a cadeia produtiva da ranicultura.

Tabela 1. Compilado da legislação e regulamentação pertinente para a ranicultura no âmbito federal e nos estados onde atuamos.

Identificação da base legal	Data da publicação	Informações mais relevantes	Responsável	Âmbito
Lei nº 6.938/1981	31 de agosto de 1981	Dispõe sobre a Política Nacional do Meio Ambiente, seus fins e mecanismos de formulação e aplicação	Ministério do Meio Ambiente (MMA)	Federal
Lei nº 9.433/1997	8 de janeiro de 1997	Institui a Política Nacional de Recursos Hídricos, cria o Sistema Nacional de Gerenciamento de Recursos Hídricos	Ministério do Meio Ambiente (MMA)	Federal
Portaria IBAMA nº 102/98	15 de julho de 1998	Normatiza os criadores comerciais de fauna silvestre exótica	Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA)	Federal
Lei de Crimes Ambientais nº 9.605/1998	12 de fevereiro de 1998	Estabelece as sanções penais e administrativas derivadas de condutas e atividades lesivas ao meio ambiente. Práticas relacionadas à ranicultura devem ser realizadas de acordo com as diretrizes estabelecidas nesta lei.	Presidente da República do Brasil	Federal
Lei Estadual nº 3.239/1999	2 de agosto de 1999	Institui a Política Estadual de Recursos Hídricos, cria o Sistema Estadual de Gerenciamento de Recursos Hídricos e regulamenta a Constituição Estadual	Governador do Estado do Rio de Janeiro	Estadual: RJ
Lei nº 11.959/2009	26 de junho de 2009	Dispõe sobre a Política Nacional de Desenvolvimento Sustentável da Aquicultura e da Pesca	Ministério da Pesca e Aquicultura (MPA)	Federal
Resolução CONAMA nº 413/2009	26 de junho de 2009	Dispõe sobre o licenciamento ambiental da aquicultura	Conselho Nacional do Meio Ambiente (CONAMA)	Federal

Resolução INEA nº 78/2013	4 de outubro de 2013	Estabelece procedimentos a serem adotados no licenciamento ambiental de empreendimentos de aquicultura continental em operação no Estado do Rio de Janeiro	Instituto Estadual do Ambiente (INEA)	Estadual: RJ
Instrução Normativa nº 23/2014	11 de setembro de 2014	Determina a obrigatoriedade da Guia de Trânsito Animal (GTA) para amparar o transporte de animais aquáticos vivos e matéria-prima de animais aquáticos provenientes de estabelecimentos de aquicultura e destinados a estabelecimentos registrados em órgão oficial de inspeção	Ministério da Pesca e Aquicultura (MPA)	Federal
Decreto nº 3831-R/2015	09 de julho de 2015	Dispõe sobre o licenciamento ambiental da aquicultura no Estado do Espírito Santo	Governadoria do Estado	Estadual: ES
Instrução Normativa nº 4/2015	04 de fevereiro de 2015	Institui o Programa Nacional de Sanidade de Animais Aquáticos de Cultivo - "Aquicultura com Sanidade"	Ministério da Pesca e Aquicultura (MPA)	Federal
Portaria nº 20/2015	04 de fevereiro de 2015	Designa os laboratórios como instituições capacitadas e autorizadas pelo Ministério da Pesca e Aquicultura a ministrar treinamento de coleta e remessa de amostras oficiais, com a finalidade de habilitação de profissional legalmente habilitado para fins de	Ministério da Pesca e Aquicultura (MPA)	Federal

		execução de atividade de defesa sanitária de animais aquáticos		
Portaria nº 19/2015	04 de fevereiro de 2015	Define a lista de doenças de notificação obrigatória de animais aquáticos ao Serviço Veterinário Oficial (SVO)	Ministério da Pesca e Aquicultura (MPA)	Federal
Lei n° 13.123/2015	20 de maio de 2015	Dispõe sobre o acesso ao patrimônio genético, sobre a proteção e o acesso ao conhecimento tradicional associado e sobre a repartição de benefícios para conservação e uso sustentável da biodiversidade	Presidente da República do Brasil	Federal
Decreto n° 8.772/2016	11 de maio de 2016	Regulamenta a Lei nº 13.123/2015	Presidente da República do Brasil	Federal
Decreto nº 62.243/2016	01 de novembro de 2016	Dispõe sobre as regras e procedimentos para o licenciamento ambiental da aquicultura, no Estado de São Paulo	Assembleia Legislativa do Estado de São Paulo	Estadual: SP
Nota técnica nº 9/2017/CAQ/DSA/CGSA/DSA	NA	Detalha a condição sanitária para os anfíbios do Brasil na WAHIS/OIE	Ministério da Agricultura e Pecuária (MAPA)	Federal
Portaria SMC nº 103/2018	04 de maio de 2018	Aprova lista de referência de espécies animais aquáticas que foram introduzidas no território nacional, utilizadas nas atividades agrícolas	Ministério da Agricultura e Pecuária (MAPA)	Federal
Instrução Normativa nº 2/2018	27 de setembro de 2018	Dispõe sobre a análise de risco de importação de organismos aquáticos e seus derivados	Ministério da Pesca e Aquicultura (MPA)	Federal

Norma nº 17/2020	16 de janeiro de 2020	Estabelece procedimentos para o manejo, o uso e a criação da rã-touro em Santa Catarina	Instituto do Meio Ambiente (IMA)	Estadual: SC
Resolução SEDEST nº 14/2020	05 de março de 2020	Estabelece normas e critérios para o licenciamento ambiental de empreendimentos e atividades de aquicultura	Secretaria de Estado do Desenvolvimento Sustentável (SEDEST)	Estadual: PR

Os desafios enfrentados pela ranicultura, seja devido a lacunas na legislação, falta de estímulo, divulgação, investimento ou organização da cadeia, têm resultado em uma instabilidade no setor, refletida na escassez de dados sobre produção e comércio, além da dificuldade de acesso a essas informações. Assim, é crucial contribuir para a melhoria da regulamentação da ranicultura no Brasil, não apenas para tornar as leis mais aplicáveis e abrangentes para os produtores rurais, mas também, e sobretudo, para que o acesso à informação seja facilitado, visando a conservação os anfíbios nativos.

3.2. Implicações de decisões políticas relativas à rã-touro

A rã-touro, introduzida no Brasil em 1935 para fins comerciais, atualmente está amplamente distribuída em todo o território brasileiro (Ferreira et al. 2002; Melo-Dias et al. 2023). Considerada uma das espécies invasoras mais impactantes para a biodiversidade, ações de controle e mitigação são essenciais, especialmente à luz das Metas de Biodiversidade de Aichi, estabelecidas internacionalmente pela Convenção sobre Diversidade Biológica (CDB).

A rápida e massiva perda de biodiversidade global demanda uma resposta ágil da pesquisa científica para orientar políticas eficazes. No entanto, líderes políticos brasileiros têm dificultado esse objetivo. Em 2009, o Congresso Brasileiro propôs uma lei que visava "naturalizar por decreto" várias espécies de peixes não nativos, com o objetivo de promover o desenvolvimento da aquicultura (Pelicice et al. 2014). Anos depois, foi promulgada a Nova Lei da Biodiversidade (Lei n° 13.123/2015; Decreto n° 8.772/2016; **Tabela 1**). Esta legislação, embora apresentada como avanço científico, impõe exigências burocráticas e punições severas, o que tem dificultado significativamente a pesquisa sobre biodiversidade no Brasil (Bockmann et al. 2018).

No contexto da ranicultura, a controvérsia se intensifica com a divulgação, em 2018, de uma portaria pelo MAPA que propõe que as espécies aquáticas introduzidas e estabelecidas no Brasil, incluindo a rã-touro, sejam consideradas "nativas" (Portaria SMC n° 103/2018; **Tabela 1**). Isso significa que aprovação desta nova portaria permitiria o livre comércio e criação dessas espécies em todo o Brasil. Tal medida pode intensificar a introdução de novas populações e levar à perda de serviços ecossistêmicos, além do conhecimento tradicional sobre as espécies nativas (Speziale et al. 2012). Além disso, o Brasil compartilha grandes bacias hidrográficas com outros países sul-americanos, o que poderia torná-lo uma fonte importante de espécies não nativas para outros países (Brito et al. 2018).

Esse retrocesso entra em conflito com várias Metas de Biodiversidade de Aichi, especialmente aquela relacionada com a prevenção, controle ou erradicação de espécies não

nativas (Lima Junior et al. 2018). Como o Brasil abriga a fauna aquática mais diversificada do mundo e representa a maior diversidade de anfíbios (Padial et al. 2017; Frost 2024), é crucial que as autoridades adotem medidas adequadas para valorizar e conservar a biodiversidade nativa. Caso contrário, a taxa de introduções no Brasil provavelmente superará as pesquisas que investigam seus efeitos negativos (Brito et al. 2018). Para evitar um impacto devastador, os cientistas devem pressionar por leis que facilitem a colaboração internacional e incentivem a pesquisa, garantindo que a biodiversidade não seja sufocada por barreiras burocráticas (Bockmann et al. 2018).

3.3. Promovendo o engajamento dos setores envolvidos na ranicultura: estratégias e discussões iniciais para formulação de políticas públicas

Em dezembro de 2018, ocorreu nosso primeiro encontro visando solidificar parcerias que pudessem impulsionar os objetivos delineados neste estudo. A Coordenação de Animais Aquáticos (CAQ) do MAPA promoveu uma reunião técnica dedicada à discussão sobre a sanidade dos anfíbios. O objetivo principal foi discutir a organização das ações de defesa sanitária relacionadas à sanidade desses animais no Brasil. O encontro reuniu membros de diferentes divisões do MAPA, pesquisadores ativos na área de ranicultura, doenças e conservação de anfíbios, além de representantes que desempenham funções técnicas nesse setor. Durante essa reunião, direcionamos nossa atenção para os avanços e desafios da ranicultura no Brasil, discutindo as principais doenças que afetam os anfíbios e delineando estratégias para fortalecer as políticas públicas relacionadas à sanidade animal no segmento da ranicultura.

Durante o IX Congresso Brasileiro de Herpetologia em 2019, o principal evento para estudos de anfíbios e répteis no Brasil, organizamos um workshop para avançar na discussão sobre a regulamentação da ranicultura no país, com ênfase na consolidação de propostas como o mapeamento dos ranários (**Figura 1**). Este encontro reuniu pesquisadores de diversas universidades, incluindo a Universidade Estadual de Campinas (UNICAMP), Universidade Estadual Júlio de Mesquita Filho (UNESP), Universidade de São Paulo (USP), Universidade Federal Rural de Pernambuco (UFRPE) e Universidade Federal do Paraná (UFPR), bem como representantes de importantes órgãos regulamentadores como MAPA, Ministério do Meio Ambiente (MMA) e Defesa Agropecuária do estado de São Paulo. Essa diversidade de atores estimulou debates construtivos para aprimorar nossos planos.

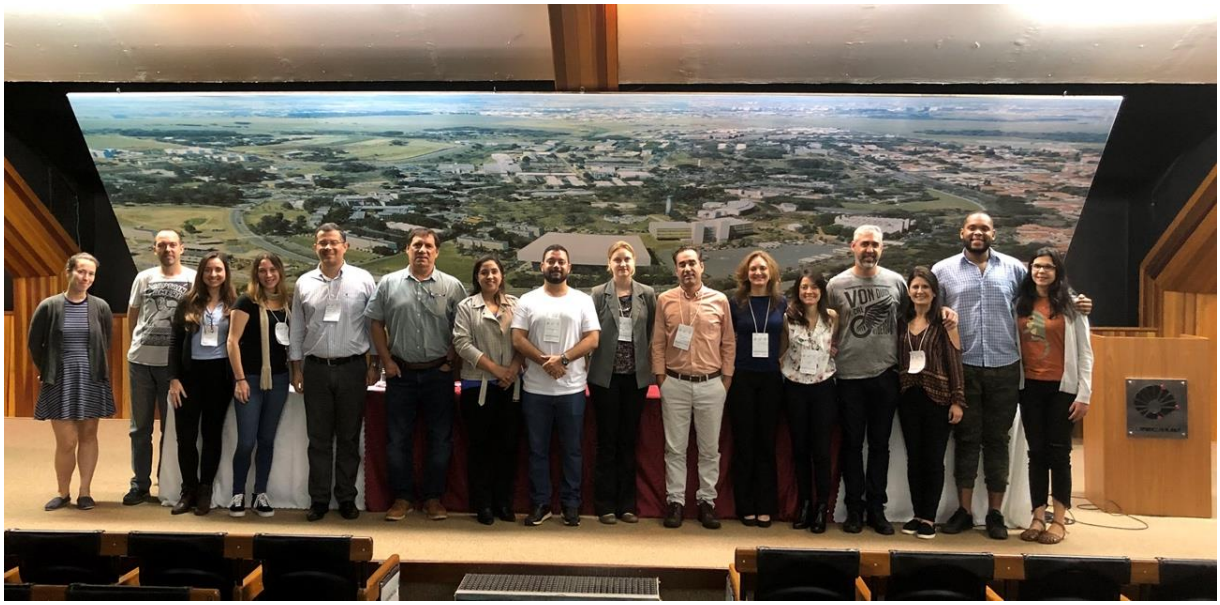

Figura 1. Registro oficial dos participantes do workshop dedicado à discussão sobre a regulamentação da ranicultura brasileira. Na ordem da esquerda para a direita: Lauren Ash (University of Vermont/EUA), Rodrigo Lingnau (Universidade Tecnológica Federal do Paraná - UTFPR), Raquel F. Salla (University of Houston/EUA), Joice Ruggeri (Universidad Nacional de Misiones/Argentina), Ricardo Luiz Moro de Sousa (Universidade de São Paulo - USP), Cláudio Regis Depes (Secretaria de Agricultura e Abastecimento do Estado de São Paulo - SAA-SP), Rita Coelho Gonçalves (SAA-SP), Ivan Nunes (Universidade Estadual Paulista - UNESP), Tatiani Elisa Chapla (Ministério do Meio Ambiente - MMA), André Carneiro (Ministério da Agricultura e Pecuária - MAPA), Isabella Fontana (MAPA), Luisa P. Ribeiro (Universidade Estadual de Campinas - UNICAMP), Luís Felipe Toledo (UNICAMP), Elaine M. Lucas (Universidade Federal de Santa Maria - UFSM), Ricardo Santos Nagô (Universidade Federal Rural de Pernambuco - UFRPE) e Karla M. Campião (Universidade Federal do Paraná - UFPR).

Durante as conversas, consideramos a viabilidade de estabelecer um cadastro oficial de produtores, inicialmente proposto em São Paulo. Embora crucial para a conservação dos anfíbios, a concretização dessas propostas exige prudência e tempo, evidenciando a importância da cooperação entre pesquisadores, órgãos regulamentadores e produtores para a implementação efetiva das políticas de regulamentação da ranicultura no Brasil.

A ranicultura no Brasil enfrenta desafios decorrentes da ineficácia das políticas públicas e da falta de integração entre os setores envolvidos. Identificamos lacunas cruciais durante nossas discussões, incluindo a falta de informações precisas sobre os ranários, como sua quantidade, localização e dados de produção. Esses dados são fundamentais para

compreender o impacto da produção de rãs e sugerir melhorias na atividade. Nesse contexto, nosso primeiro passo foi realizar uma revisão sobre os ranários brasileiros, detalhando os processos de produção e comercialização, e destacando melhorias necessárias para garantir a sustentabilidade da atividade (ver Ribeiro & Toledo 2022).

Considerando que tanto as rãs-touro quanto a água residual da produção liberada em ambientes naturais carregam altas cargas de Bd (Ribeiro et al. 2019; Santos et al. 2020) e que não há tratamento para o fungo, ressaltamos a urgência na elaboração de protocolos de biosseguridade para infraestrutura e comercialização desses animais. A implementação de medidas de controle em todas as fases de produção e venda, associada à inclusão de diretrizes em um manual de boas práticas para a criação de rãs, é essencial para conscientizar os produtores sobre os riscos potenciais da produção de rãs-touro na disseminação de patógenos. Essas medidas não só ajudarão a orientar os produtores na implementação de estratégias eficazes de controle de animais e patógenos, mas também são cruciais para minimizar os riscos para a saúde dos anfíbios e ecossistemas.

Todos os locais que mantêm ou criam animais aquáticos para qualquer finalidade devem registrar-se no Órgão Estadual de Defesa Sanitária Animal (OESA) ou na Secretaria de Agricultura do Estado, conforme estipulado pela Instrução Normativa nº 04/2015 (BRASIL 2015). Desde então, o Brasil mantém uma lista de doenças de animais aquáticos que requerem notificação obrigatória ao Serviço Veterinário Oficial (SVO) (Portaria MPA nº 19/2015) (MPA 2015). Atualmente, as doenças de notificação para anfíbios incluem a quitridiomicose e a ranavirose. Isso significa que, ao suspeitar ou confirmar qualquer uma dessas doenças, o SVO deve ser informado para tomar medidas no controle ou erradicação do problema.

Qualquer cidadão pode notificar suspeitas de doenças por meio de um formulário de investigação inicial. Após a notificação, o MAPA aciona o SVO, responsável pela investigação epidemiológica, incluindo coleta de dados e amostras, como estabelecido na Instrução Normativa nº 04/2015 (BRASIL 2015). A análise das amostras é conduzida por laboratórios credenciados pelo MAPA. Embora tenha alta demanda das análises, são escassos os laboratórios credenciados para realizá-las. A fim de agilizar o processo de análise, em 2013 foi desenvolvido um manual padronizado para coleta de amostras para diagnóstico de doenças em peixes, camarão e molusco, mas não para anfíbios (MPA 2013). Apenas em 2022 foi publicado um instrutivo de coleta de amostra para este grupo de animais (MAPA 2022).

A Organização Mundial de Saúde Animal (OMSA) realiza avaliações semestrais das condições sanitárias, as quais são classificadas em falta de informação, informação qualitativa e informação quantitativa. A primeira notificação oficial de doenças em anfíbios no

Brasil ocorreu em 2018. Embora o Rv tenha sido identificado em locais de criação de rãs (Mazzoni et al. 2003), não havia registros desse patógeno em anfíbios selvagens. Em relação ao fungo Bd, não havia registros oficiais de sua presença em anfíbios de cativeiro, contudo, estudos apontaram a presença desse patógeno em diversos ranários no Brasil (Ribeiro et al. 2019; Santos et al. 2020). Consequentemente, nós notificamos a presença do patógeno nos ranários, e essa informação foi considerada na avaliação da OMSA. No que diz respeito aos anfíbios selvagens, a presença do fungo foi documentada oficialmente em todo o território nacional.

Devido à natureza das notificações, frequentemente restritas a ocorrências em animais domésticos, é vital estabelecer uma integração entre diversas instituições para identificar ameaças, avaliar e reduzir os riscos de propagação de patógenos. Reforçar a vigilância epidemiológica e zoossanitária envolve a integração de diferentes setores, incluindo o SVO, entidades de pesquisa e o setor privado. A existência de um canal de comunicação para notificar prontamente suspeitas ou casos confirmados de doenças é crucial nesse cenário. Portanto, foi criada a "Rede Fauna", na qual participamos representando o setor acadêmico. Trata-se de um manual elaborado para facilitar a comunicação eficiente entre as principais instituições relacionadas à saúde animal. Essa iniciativa visa compreender a situação atual do Brasil em relação à presença e distribuição das doenças de notificação obrigatória, mantendo informações transparentes e atualizadas por meio do compartilhamento com a OMSA, promovendo, assim, uma cooperação internacional mais ampla em prol da sanidade animal e da conservação da biodiversidade.

O futuro da ranicultura está intrinsecamente ligado à manutenção da saúde dos animais cultivados. Assim, ressaltamos a importância do conhecimento e divulgação do Programa Nacional de Sanidade de Animais Aquáticos de Cultivo, conhecido como "Aquicultura com Sanidade" (Instrução Normativa MPA nº 04/2015, atualizada pela Instrução Normativa MAPA nº 04/2019) (MAPA 2019). Esse programa nacional, em vigor desde 2017, visa promover a sustentabilidade dos sistemas de produção de animais aquáticos, prevenindo, controlando e/ou erradicando doenças nos sistemas de produção. O programa capacita o Serviço Veterinário Oficial (SVO) para responder rapidamente a surtos de doenças, certificar sanitariamente os estabelecimentos e regular o serviço de quarentena de indivíduos.

Em síntese, as discussões abordaram os possíveis impactos dos ranários na conservação dos anfíbios nativos e as estratégias fundamentais para proteger a saúde desses animais e dos ecossistemas. Destacou-se a necessidade da elaboração e ampla divulgação de um manual de boas práticas específico para a criação rãs-touro, além da elaboração de um plano

de ação para garantir a saúde dos anfíbios. Com a parceria estabelecida com o MAPA e uma rede de contatos envolvida no projeto, acreditamos que a regulamentação da ranicultura no Brasil se torna mais viável.

3.4. Importância de Planos de Ação na construção e implementação de políticas públicas para a ranicultura

3.4.1. Âmbito estadual – Plano de Ação Territorial

Um Plano de Ação, seja ele Nacional (PAN) ou Territorial (PAT), assume a função crucial de ser uma ferramenta estratégica de gestão e de políticas públicas destinada a consolidação de medidas prioritárias focadas na conservação de espécies. Esse processo é delineado de maneira colaborativa, envolvendo representantes do setor acadêmico, do governo, de organizações não governamentais e do setor produtivo, com o objetivo de alcançar consenso e estabelecer diretrizes precisas para aprimorar o estado de conservação das espécies em questão. O plano é composto por objetivos e ações estruturados sob a lógica de projetos, incluindo prazos definidos para sua execução e produtos a serem entregues ao longo desse processo. Ao contrário dos PANs, de escala federal e focados em espécies, os PATs são concentram-se em territórios-alvo e possuem escala estadual.

Com o intuito de reduzir as ameaças aos anfíbios, participamos ativamente das oficinas de planejamento e das reuniões temáticas para o desenvolvimento do PAT Caminho das Tropas Paraná - São Paulo, iniciadas no ano de 2020. O Ministério do Meio Ambiente (MMA), em colaboração com agências e parceiros, concebeu o Projeto “Pró-Espécies”. Coordenado pelo Governo Federal através do MMA e financiado pelo *Global Environment Facility* (GEF), com o Fundo Brasileiro para a Biodiversidade (FUNBIO) atuando como agência implementadora e o WWF-Brasil como executor, o projeto inclui a elaboração e implementação de Planos de Ação Territoriais (PATs) como uma das atividades centrais. A área abrangida pelo PAT em questão compreende 12.474.067 hectares, englobando 163 municípios nos estados do Paraná e São Paulo (**Figura 2**). O principal objetivo desse PAT é promover ações específicas para reduzir as ameaças enfrentadas pelas espécies, ao mesmo tempo em que estimula a conservação integrada da fauna e flora.

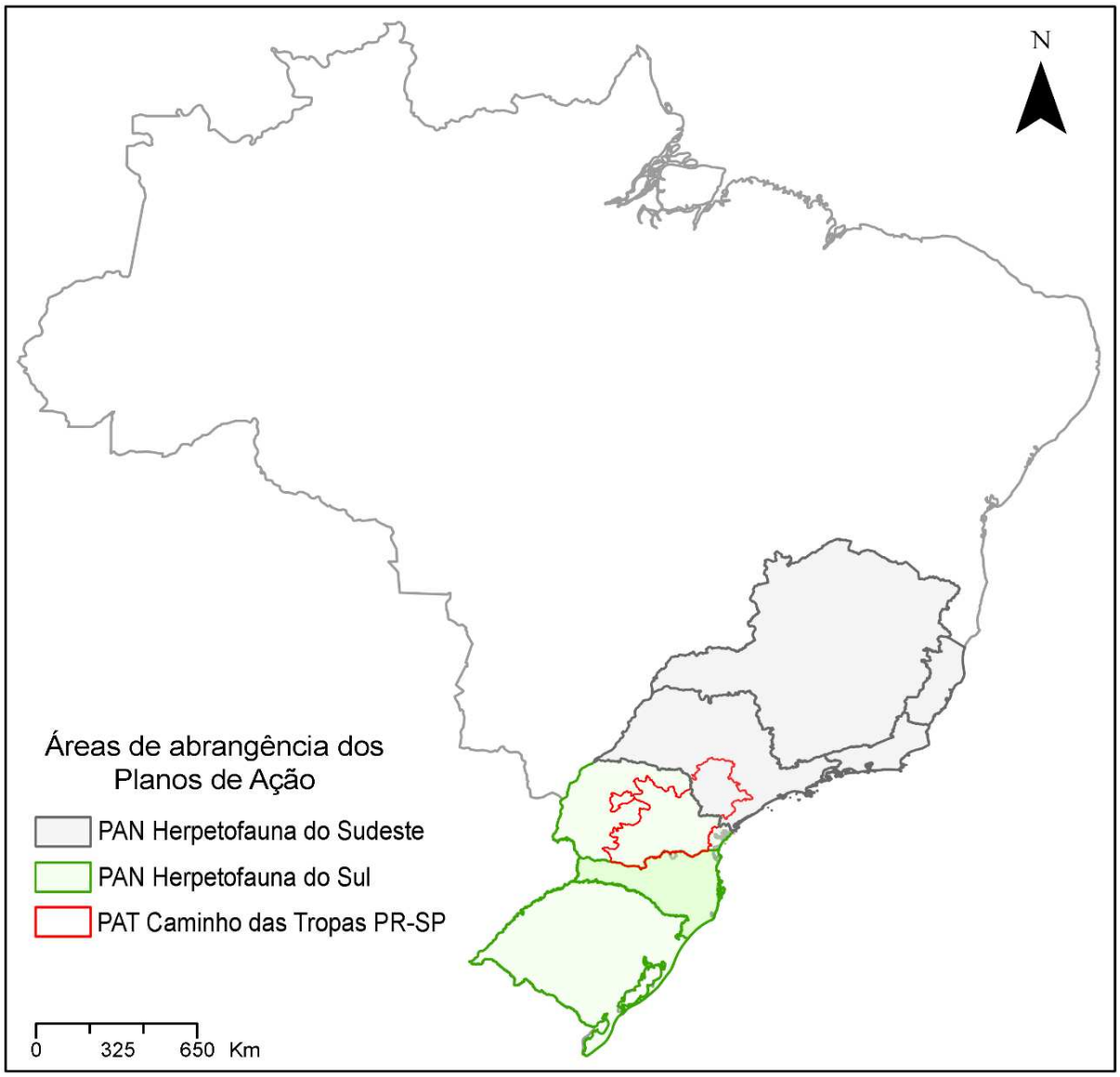

Figura 2. Áreas abrangidas pelos Planos de Ação Nacional e Territorial nos quais estamos envolvidos em ações para conservação de anfíbios.

O PAT está estruturado em seis objetivos específicos, dos quais estamos concentrados e contribuindo ativamente para dois deles: (i) Estabelecer áreas prioritárias para ações de prevenção, detecção precoce, resposta rápida, controle, erradicação e boas práticas no cultivo/criação relativos a espécies exóticas invasoras e (ii) Planejar e propiciar ações formativas sob a perspectiva da Saúde Única. Em relação ao primeiro objetivo, nosso enfoque é na bioinvasão pela rã-touro (*Aquarana catesbeiana*), propondo ações para identificar seus locais de criação e avaliar os impactos, buscando prevenir, controlar ou interromper essa invasão. Paralelamente, estamos engajados na elaboração de um manual de boas práticas para a ranicultura. No que diz respeito ao segundo objetivo, nossa prioridade é entender os impactos

da criação da rã-touro na disseminação de patógenos, particularmente o Bd e o Rv. Para lidar com essa questão, estamos focados em identificar e avaliar os protocolos sanitários existentes, propondo ajustes quando necessário, e integrando esses protocolos às atividades dos órgãos estaduais responsáveis pelo monitoramento sanitário.

Até o momento, concluímos o mapeamento abrangente dos locais de criação de rãs-touro e de suas populações selvagens, não apenas no âmbito do PAT, mas em toda a extensão dos estados de São Paulo, Paraná e Santa Catarina. Esse amplo levantamento dos locais de criação e das populações dessa espécie nos estados mencionados é de suma importância, dado sua proximidade com o território definido pelo PAT, podendo representar potenciais fontes de bioinvasão. Em março de 2021, encaminhamos todos os dados relativos à distribuição dessas espécies à coordenação do PAT para análise e consideração nas futuras ações planejadas. Diante dos preocupantes índices de prevalência de Bd e Rv nos ranários brasileiros e dos incidentes de escapes de rãs-touro, perseveramos na execução das medidas propostas para reduzir os impactos negativos sobre as populações de anfíbios nativos. Reforçamos a importância da colaboração entre setores governamentais, pesquisadores e produtores para estabelecer políticas públicas efetivas que regulamentem a prática da ranicultura no Brasil.

3.4.2. Âmbito Federal – Planos de Ação Nacionais

Em um escopo mais abrangente, as iniciativas para a conservação dos anfíbios expandiram-se para o âmbito federal, onde estamos envolvidos em dois Planos Nacionais de Conservação (PANs): o PAN da Herpetofauna do Sul e o PAN da Herpetofauna do Sudeste (**Figura 2**). Ambos PANs encontram-se no segundo ciclo de implementação e têm como objetivo principal a manutenção da diversidade da fauna de anfíbios e répteis das regiões Sul e Sudeste, respectivamente.

Em novembro de 2020, participamos de uma reunião técnica para atualização de uma norma para a ranicultura do estado de Santa Catarina. A norma de número 17/2020, estabelecida em 16 de janeiro do mesmo ano, tem a finalidade de regulamentar o manejo, uso e criação da rã-touro (*Aquarana catesbeiana*) no estado (FATMA 2020). Essa atualização normativa é uma ação específica delineada dentro de um dos objetivos do PAN Herpetofauna do Sul, com o propósito de controlar a bioinvasão das rãs-touro e minimizar a disseminação de patógenos no ambiente. O objetivo principal é aprimorar essa norma já existente em Santa Catarina, assegurando que ela englobe todos os elementos necessários para reduzir as ameaças da ranicultura sobre as populações nativas. Além disso, a intenção é que esta sirva como um

modelo replicável para outros estados brasileiros, garantindo a redução dos impactos negativos dessa prática sobre a biodiversidade.

Esta ação conta com a colaboração de representantes de órgãos governamentais, incluindo o Instituto do Meio Ambiente de Santa Catarina (IMA) e o Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), bem como pesquisadores de instituições públicas, como Universidade Federal de Santa Maria (UFSM), Universidade Estadual de Campinas (UNICAMP) e Universidade Federal do Amazonas (UFAM). Diante do impacto das espécies exóticas invasoras na biodiversidade global, focamos nossas discussões na identificação de melhorias e adições à norma. Destacamos aspectos relativos à autorização e registro para o manejo e uso da espécie em cativeiro, que devem aderir a critérios específicos para atividades como comércio, aquisição, transporte, cultivo, distribuição e propagação. No que tange ao controle da rã-touro e seus patógenos, delineamos medidas fundamentais, como a implementação de telas para evitar fugas ou entrada de outras espécies no ranário, o uso de malhas mais finas em tubulações para evitar a fuga de girinos, e a adoção de portas duplas com fechamento automático para prevenir a passagem de animais.

Além disso, discutimos a necessidade de controle e tratamento de animais infectados por Bd ou Rv, bem como o tratamento adequado dos efluentes. Por fim, debatemos sobre as ações necessárias para avaliar as condições sanitárias nos ranários, os protocolos viáveis e os órgãos responsáveis por essas medidas. Até o momento, contribuímos significativamente para a reestruturação, revisão e sugestão de melhorias na norma, a qual está em processo de aprovação em diferentes setores do IMA antes de sua publicação final.

Em março de 2023, participamos de uma oficina de planejamento do segundo ciclo do PAN Herpetofauna do Sudeste (**Figura 3**), presencialmente no Centro de Formação em Conservação da Biodiversidade (ACADEBio). Esse Plano abrange os estados de São Paulo, Rio de Janeiro, Minas Gerais e Espírito Santo. Durante essa oficina, concentramo-nos nas fases iniciais do planejamento do PAN, realizando uma análise das ameaças aos anfíbios, delineando o objetivo geral do PAN e trabalhando na definição dos objetivos específicos, além de criar a matriz de planejamento para as ações. Utilizando nossa experiência prévia com políticas públicas, trouxemos à tona a preocupação com os impactos da ranicultura na fauna de anfíbios, propondo ações alinhadas com as estratégias de outros Planos de Ação, visando reduzir o impacto da produção de rãs-touro na fauna de anfíbios nativos.

Figura 3. Registro oficial dos participantes da oficina de planejamento do 2º ciclo do Plano de Ação Nacional (PAN) Herpetofauna do Sudeste.

Nossa experiência no contexto das políticas públicas, a qual detalhamos anteriormente, voltadas para a regulamentação da ranicultura no Brasil, com foco na conservação das espécies nativas, destaca a importância crucial de coordenar estratégias alinhadas com outras políticas de conservação. A concentração de esforços na definição de objetivos específicos e na elaboração de ações concretas torna-se crucial não apenas para sua implementação, mas para assegurar a continuidade da ação em prol biodiversidade nessas áreas. A colaboração entre diversos agentes e a adoção de abordagens integradas são fundamentais para o sucesso das políticas de conservação, delineando um caminho promissor rumo à efetiva conservação da herpetofauna brasileira.

4. Considerações finais

Corroborando os princípios da Triple Helix (Zhou & Etzkowitz 2021), nossas experiências ressaltam que a colaboração entre os diferentes setores envolvidos na regulamentação de uma atividade é fundamental para o sucesso das políticas regulatórias. Nossa vivência direta com políticas públicas destaca que atuar em esferas menores, como as estaduais, pode agilizar e facilitar a implementação de ações, comparativamente a contextos mais amplos, como os federais. Além disso, observamos a consistência das origens dos problemas e das

soluções em todos os estados brasileiros. Portanto, as estratégias propostas para resolver a problemática da rã-touro, tanto no caso da bioinvasão quanto na disseminação de patógenos, podem ser aplicáveis em todo o Brasil. Embora nossa ênfase de trabalho tenha sido nas regiões Sul e Sudeste, mais impactadas pela ranicultura, é conhecido que essa espécie é produzida e comercializada em todo o país (Ribeiro & Toledo 2022), com populações estabelecidas em todo o território nacional (Melo-Dias et al. 2023). Portanto, ações para reduzir os impactos dessa espécie invasora devem ser implementadas em todo o território brasileiro.

O compartilhamento de ideias e discussões entre diferentes perspectivas são fundamentais para aprimorar a elaboração e execução das ações voltadas à conservação de espécies. Portanto, reforçamos a importância crucial da cooperação entre diversos setores do poder público, pesquisadores e produtores para concretizar políticas efetivas relacionadas à regulamentação da ranicultura no Brasil. É evidente a existência do distanciamento entre os setores governamentais, acadêmicos e produtivos que necessitam ser superadas. Da mesma forma, o compartilhamento de nossos resultados de pesquisa e a divulgação de publicações científicas desempenham um papel significativo ao servirem de referência e orientação para as iniciativas de conservação de espécies. A regulamentação da ranicultura no Brasil e a conservação dos anfíbios são temas que demandam atenção e requerem um esforço colaborativo entre os setores envolvidos para implementar e manter a prática da ranicultura de forma sustentável, tanto economicamente quanto ambientalmente.

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CONSIDERAÇÕES FINAIS

Os anfíbios, a classe de vertebrados mais ameaçado da atualidade, enfrentam diversas ameaças, e entre elas, destacamos a produção comercial de rã-touro (*Aquarana catesbeiana*), atuando na disseminação do fungo quitrídio, *Batrachochytrium dendrobatidis* (Bd), causador da quitridiomicose. A falta de informação sobre a presença e localização dos ranários, bem como das rotas comerciais, cria lacunas que podem dificultar a tomada de decisões em prol da conservação. Portanto, buscamos preencher essas lacunas, cruciais para orientar nossas estratégias e planos de conservação, incluindo a regulamentação da produção e comércio de rã-touro. Isso é fundamental para minimizar as ameaças enfrentadas pelos anfíbios nativos em todo o mundo.

Esta tese reúne os resultados obtidos ao longo dos anos dedicados ao doutorado, em que concentrei minha pesquisa na ecologia de doenças no contexto de produção animal. Dentro das várias possibilidades de estudo nesse campo, busquei empregar diversas abordagens para coletar dados, incluindo revisões bibliográficas, coletas de campo, desenvolvimento de métodos moleculares, experimentação em laboratório e contribuições para políticas públicas. Nosso objetivo foi explorar desde a produção e comércio da rã-touro até a identificação das linhagens de Bd em ranários brasileiros, investigar suas origens e avaliar os impactos nas respostas das rãs-touro à quitridiomicose, além de propor estratégias para regulamentar a ranicultura no país.

Em nossa revisão do capítulo I, concluímos que o Brasil possui um considerável potencial de crescimento na ranicultura, apesar de enfrentar desafios como a ausência de padrões consistentes na produção, a demanda limitada por produtos derivados de rã-touro, a carência de avanços técnicos e os elevados custos de produção. A escassez de dados sobre a produção afeta diretamente a capacidade de implementar melhorias eficazes, tornando os dados compilados nesta tese essenciais para dar visibilidade à essa atividade. É crucial fomentar a colaboração entre produtores e obter o respaldo governamental para estabelecer políticas que promovam a sustentabilidade ambiental e assegurem o controle de doenças que representam ameaças aos anfíbios.

Nos capítulos II e III, nós enfatizamos a relevância de explorar a distribuição das diversas linhagens do fungo Bd, frequentemente negligenciadas. Apresentamos um novo método de genotipagem eficiente e sensível que tem potencial para uma aplicação abrangente, auxiliando em futuros estudos na identificação de linhagens em escala global. Além disso, investigamos a provável origem da linhagem Bd-BRAZIL no Brasil, apontando sua disseminação por meio do do comércio de rã-touro. Destacamos disparidades geográficas nas

amostragens históricas de Bd e a necessidade de identificar linhagens, incentivando pesquisas futuras que empreguem métodos de genotipagem e amostragem em museus de regiões pouco exploradas, como o continente asiático.

No contexto da ecologia de doenças, que analisa como as interações entre organismos, ambiente e doenças impactam sua prevalência e disseminação, a compreensão dessas complexas interações é crucial para elaborar estratégias eficazes de prevenção e controle. A tríade epidemiológica convencional, a qual considera as relações entre hospedeiro, patógeno e ambiente no desenvolvimento de doenças (Snieszko 1974), subestima a influência humana, especialmente em ambientes de produção animal. Para suprir essa lacuna, foi proposta uma tétrade epidemiológica que separa fatores humanos e ambientais (Shields 2013), oferecendo uma visão mais ampla e aprimorada para investigar e reduzir doenças em ambientes de criação (Dong et al. 2023).

No capítulo IV incorporamos os aspectos da influência humana na quitridiomicose, e destacamos as alterações nos mecanismos de defesa contra a infecção por Bd em populações de rãs-touro. A pressão exercida durante o processo de criação resultou em indivíduos menos resistentes à infecção, proporcionando condições favoráveis para que esses animais atuem como superdispersores do fungo para os ambientes aquáticos. Sugerimos, portanto, a necessidade de estudos adicionais sobre o impacto da produção na resposta imunológica dos hospedeiros, bem como a viabilidade de aplicação de tratamentos em animais e efluentes, buscando soluções eficazes, economicamente viáveis e ecologicamente sustentáveis.

Finalmente, incentivamos estudos integrativos na ecologia de doenças, que possam fundamentar políticas públicas direcionadas à conservação de anfíbios, conforme delineado no capítulo V desta tese. Aprimorar as estratégias de investigação e controle de patógenos ao longo dos processos de produção e comercialização é crucial para salvaguardar o comércio global de anfíbios contra a disseminação de patógenos. Ressaltamos a importância crítica de integrar e unir diferentes setores envolvidos na atividade da ranicultura, desde a comunidade acadêmica até os setores produtivo, privado e órgãos governamentais, a fim de implementar políticas regulatórias efetivas para a ranicultura. Esperamos que os dados aqui apresentados possam fomentar discussões sobre esses temas, contribuir para o avanço científico e político, oferecer base para novas pesquisas e despertar o interesse pela conservação dos anfíbios.

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ANEXOS

Anexo I – Registro no Sistema Nacional de Gestão do Patrimônio Genético (SISGen #AEB9E4D)

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO
SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Certidão
Cadastro nº AEB9E4D

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

Número do cadastro:	**AEB9E4D**
Usuário:	**UNICAMP**
CPF/CNPJ:	**46.068.425/0001-33**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**
Espécie	
Batrachochytrium dendrobatidis	
Lithobates catesbeianus	
Título da Atividade:	**Implicações do comércio nacional e internacional de rã-touro na dispersão e tolerância adquirida ao fungo quitrídeo e medidas para conservação de anuros**
Equipe	
Luisa de Pontes Ribeiro	**UNICAMP**
Luís Felipe de Toledo Ramos Pereira	**UNICAMP**

Data do Cadastro:	**30/11/2020 18:21:26**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **9:25** de **08/02/2024**.

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

Anexo II – Declaração de Bioética e Biossegurança

UNICAMP
COORDENADORIA DE PÓS-GRADUAÇÃO
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IB

DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Tese de Doutorado, intitulada "***Implicações do comércio nacional e internacional de rã-touro na dispersão e tolerância adquirida ao fungo quitrídio e medidas para conservação de anuros***", desenvolvida no Programa de Pós-Graduação em do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

Assinatura: Luisa Ribeiro
Nome do(a) aluno(a): Luisa de Pontes Ribeiro

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

Data: 10 de dezembro de 2023

Anexo III – Declaração de Direitos Autorais

Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **Implicações do comércio nacional e internacional de rã-touro na dispersão e tolerância adquirida ao fungo quitrídio e medidas para conservação de anuros**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 10 de dezembro de 2023

Assinatura : Luisa Ribeiro
Nome do(a) autor(a): **Luisa de Pontes Ribeiro**
RG n.° 46.822.713-1

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