Chytrid fungus infection in Neotropical tadpoles and across life stages

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ABSTRACT

Amphibian species are rapidly declining at a global scale, largely due to the devastating impacts of emerging infectious diseases. *Batrachochytrium dendrobatidis* (*Bd*), is a pathogenic chytrid fungus that causes chytridiomycosis, a highly infectious disease that infects the keratinized tissues of amphibians. Anurans are especially vulnerable to *Bd*; however, response to infection differs among species and within life stages. The tadpole life stage often harbours low level, nonlethal infections and thus can contribute to the spread and maintenance of the pathogen within ecosystems by serving as disease reservoirs. Many studies have investigated the role of species persisting in the wild as potential reservoirs at the postmetamorphic stage, but few have directly assessed infection in wild tadpole hosts, especially in Neotropical regions. Moreover, studies analyzing infection in tadpoles often employ different methods for detection and diagnosis, which can complicate accurate disease assessments. The objective of my thesis was to investigate *Bd* infection in Neotropical frog species, highlighting the tadpole life stage while also comparing infection in adults for a more robust understanding of disease landscapes. My research was conducted in Panamá, a country with high biodiversity that has been undergoing severe declines in frog populations and species due to chytridiomycosis. I evaluated the importance of conducting triplicate qPCR assays to detect *Bd* in tadpoles and showed that the inclusion of all three replicates is essential for diagnosing low intensity infections. I also investigated infection prevalence and severity in three sympatric species coexisting with *Bd* across the tadpole and adult life stages. My results showed that both life stages can harbour infections to varying degrees depending on the species, thus all three species are likely acting as disease reservoirs throughout their life cycle. My thesis provides insight into *Bd* infection in Neotropical tadpole species and emphasizes the significant role of tadpoles in *Bd* systems.

RÉSUMÉ

Les espèces d'amphibiens sont en déclin rapide à l'échelle globale, en grande partie à cause des effets dévastateurs de maladies infectieuses émergentes. *Batrachochytrium dendrobatidis* (*Bd*) est un champignon chytride pathogène qui provoque la chytridiomycose, une maladie hautement infectieuse qui infecte les tissus kératinisés des amphibiens. Les anoures sont particulièrement vulnérables à la *Bd*; cependant, la réponse à l'infection diffère selon les espèces et les stades de vie. Le stade du têtard héberge souvent des infections non létales de faible niveau et peut donc contribuer à la propagation et au maintien de l'agent pathogène dans les écosystèmes en servant de réservoir de maladie. De nombreuses études ont examiné l'influence des espèces persistantes au stade postmétamorphique en tant que réservoirs potentiels, mais peu d'entre elles ont évalué directement l'infection chez les têtards sauvages, en particulier dans les régions néotropicales. De plus, les études analysant l'infection chez les têtards utilisent souvent différentes méthodes de détection et de diagnostic, ce qui peut compliquer l'évaluation précise de la maladie. L'objectif de ma thèse était d'étudier l'infection par *Bd* chez les espèces de grenouilles néotropicales, en mettant l'accent sur le stade de vie du têtard tout en comparant également l'infection chez les adultes pour une compréhension plus solide des paysages de la maladie. Mes recherches ont été menées au Panamá, un pays à forte biodiversité qui a connu un déclin important des populations et des espèces de grenouilles en raison de la chytridiomycose. J'ai évalué l'importance de réaliser des essais qPCR en trois replicats pour détecter la présence de *Bd* chez les têtards et j'ai montré que l'inclusion des trois puits répétés est essentielle pour diagnostiquer les infections de faible intensité. J'ai également étudié la prévalence et l'intensité de l'infection chez trois espèces sympatriques coexistantes avec Bd aux stades du têtard et de l'adulte. Mes résultats ont montré que les deux stades de vie peuvent abriter des infections à des degrés divers en fonction de l'espèce, de sorte que les trois espèces

agissent probablement comme des réservoirs du pathogène tout au long de leur cycle de vie. Ma thèse donne un aperçu de l'infection par *Bd* chez les espèces de têtards néotropicaux et souligne le rôle important des têtards dans les systèmes de *Bd*.

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Frog swabbing team in action at Río Frijoles. March 2023. Photo by Henk van der Meulen.

CONTRIBUTION OF AUTHORS

This thesis consists of two chapters formatted as manuscripts intended for submission to peer-reviewed scientific journals. For both chapters, the candidate, Kelsey Wilson, designed the studies, conducted data collection, performed statistical and descriptive analyses, and is the lead author of the manuscripts. Dr. Virginie Millien was involved with the project development, reviewed the manuscripts and contributed to data analyses. Dr. Roberto Ibáñez participated in study designs, provided resources and funding for the projects, and reviewed the manuscripts. Dr. Millien and Dr. Ibáñez are listed as co-authors for both papers. Estefany Illueca is listed as a co-author on the first manuscript. She participated in project development and technical assistance. Chapter 1 has been formatted for submission to Herpetologica and Chapter 2 has been formatted for submission to Wildlife Research.

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INTRODUCTION

Wildlife biodiversity is declining at unprecedented rates globally. Amphibians are the vertebrate group at the highest risk, with 41% of species threatened by extinction (IUCN 2024). Anthropogenic activities such as habitat destruction, chemical pollution, and overexploitation are major drivers of amphibian declines, however, the emergence of novel threats has further accelerated biodiversity loss (Collins and Storfer 2003; Lips 2016). Among these novel threats are invasive species and infectious diseases that spread rapidly across ecosystems, disrupting community structure and increasing extinction risk in vulnerable species (Bellard et al. 2016; Scheele et al. 2019; Glassock et al. 2021). Chytridiomycosis is an amphibian skin disease caused by the waterborne pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), that is both highly invasive and infectious (Rosenblum et al. 2010; Scheele et al. 2019). *Bd* was first identified by researchers in 1998 as the cause of dramatic declines in frog populations occurring concurrently in Central America and Australia since the late 1970s (Berger et al. 1998, 2016; Longcore et al. 1999) and has subsequently been detected on every continent except Antarctica (Fisher et al. 2009). The pathogen has devastated amphibian populations at a global scale, driving the declines, regional extirpations, and extinctions of over 500 species (Burns et al. 2016; Scheele et al. 2019; Turner et al. 2022). Despite significant advances in *Bd* research over the past two decades, there are currently no universally effective strategies to mitigate its widespread impact (Garner et al. 2016, Fisher and Garner 2020).

Bd Physiology and Ecology

Bd zoospores are aquatic and motile, spreading rapidly through water and embedding in the keratinized tissues of the host (Piotrowski et al. 2004; Rosenblum et al. 2010). Here, the

encysted zoospore develops into a reproductive thallus which produces a single zoosporangium that releases new, free-swimming zoospores into the water column that can colonize the same or different individuals (Berger et al. 2005). Under laboratory conditions, the lifecycle of *Bd* from zoospore to mature zoosporangium takes 4 to 5 days, with optimal growth and reproduction occurring between 17–25°C at a pH of 6–7, slowing significantly at lower temperatures, and ceasing above 28°C or below 10°C (Piotrowski et al. 2004; Berger et al. 2005). Although transmission commonly occurs through water, *Bd* can also spread via direct contact with contaminated substrates or infected individuals (Johnson and Speare 2005; Lips et al. 2006; Rowley and Alford 2007; Kolby et al. 2015). Moreover, zoospores can survive in sterile freshwater for up to seven weeks and in moist soil for up to twelve weeks, suggesting that the pathogen can persist within ecosystems for months even in the absence of hosts (Johnson and Speare 2003, 2005).

In natural environments, *Bd* is particularly virulent in cool, moist ecosystems often found at high elevations (Skerratt et al. 2007; Brem and Lips 2008; Sapsford et al. 2013; Adams et al. 2022). Few species of non-amphibian animals, including aquatic birds, fish, decapod crustaceans (shrimp, prawn, and crayfish), and reptiles, have been found to carry *Bd* without experiencing declines, potentially contributing to its environmental prevalence in certain regions (Rowley et al. 2006; Kilburn et al. 2011; Garmyn et al. 2012; McMahon et al. 2013; Burrowes and De la Riva 2017; Liew et al. 2017; Oficialdegui et al. 2019). However, amphibians suffer the greatest impacts globally, with 90 species presumed extinct due to chytridiomycosis and with frog and toad species (Order Anura, hereafter frogs) undergoing the most severe losses (Scheele et al. 2019).

Bd in Anurans

Anuran skin is a protective, sensory organ responsible for essential physiological regulatory functions (Varga et al. 2019). When *Bd* infects the keratin in the skin cells of metamorphic and postmetamorphic (juvenile and adult) frogs, the presence of the fungus disrupts homeostasis through osmotic imbalance and causes symptoms including skin sloughing, discolouration, lethargy, weight loss, and heart failure, that often lead to mortality (Voyles et al. 2007, 2009; Ohmer et al. 2015). However, differences in habitat type, environmental conditions, immune response, and skin microbiome cause *Bd* infection susceptibility to vary significantly among and within species, populations, and life stages (Tobler and Schmidt 2010; Fisher and Garner 2020; Rebollar et al. 2020; Rollins-Smith, 2020; Burns et al. 2021; Rumschlag et al. 2022). Variation in susceptibility at different life stages, specifically, can disrupt community structures and influence disease dynamics within populations. Here, we define susceptibility as infection prevalence (i.e., the proportion of positive individuals within a species, population, or life stage) and infection intensity or severity (i.e., the number of zoospores on an individual).

Larval anurans are aquatic; therefore, the tadpole life stage experiences prolonged exposure to *Bd* in habitats where the pathogen is present. However, keratin is absent in larval skin during early developmental stages and is exclusively found in the jaw sheaths and teeth within their mouthparts which are later shed as individuals undergo metamorphosis and develop keratinized skin (Marantelli et al. 2004; Rachowicz and Vredenberg 2004; McMahon and Rohr 2015). As a result, *Bd* infection at the larval stage is mild and rarely lethal (Rachowicz and Vredenberg 2004; Bradley et al. 2019; Sauer et al. 2020). Despite this, the presence of *Bd* can damage the delicate oral structures of tadpoles, thereby reducing foraging efficiency and slowing growth rates which

can potentially have long-term population-level fitness consequences (Drake et al. 2007; Venesky et al. 2009, 2010; DeMarchi et al. 2015; Hanlon et al. 2015).

As tadpoles undergo metamorphosis and transition from larval to adult immune systems, their skin is especially vulnerable to chytrid infection (Rollins-Smith et al. 2011; Grogan et al. 2018; Humphries et al. 2022). Several studies have reported high mortality in species of newly metamorphosed frogs (Berger et al. 1998; Rachowicz et al. 2006; Garner et al. 2009; Tobler and Schmidt 2010). However, the persistence of tadpoles in ecosystems where metamorphs and postmetamorphs have experienced *Bd*-related declines remains an area of significant interest and has led to research investigating their role as potential disease reservoirs (Stockwell et al. 2016; Narayan et al. 2014; Ruggeri et al. 2018; Burns et al. 2021).

Larval Anurans as Reservoir Hosts

Reservoir hosts are disease-tolerant carriers that can amplify infection risk in more sensitive hosts without apparent fitness costs (Blaustein et al. 2005; Stockwell et al. 2016; Burns et al. 2021). Tadpoles are excellent reservoir host candidates for *Bd* since they can harbour low-intensity, nonlethal infections, are confined to their aquatic habitats, and often exist in high densities (Russell et al. 2010; Piovia-Scott et al. 2011; Ruggeri et al. 2018). Infected tadpoles can continuously shed zoospores into the water over time, increasing infection potential across different life stages both intraspecifically and interspecifically (Wilber et al. 2019). Tadpoles have been reported at outbreak sites after adults have declined, indicating their resilience to the pathogen and suggesting their ability to maintain *Bd* within ecosystems (Berger et al. 1998; Briggs et al. 2010; Catenazzi et al. 2013). Several studies have confirmed low intensity *Bd* infections in wild tadpoles, establishing their role as disease reservoirs and identifying environmental factors that

can influence their susceptibility (Russell et al. 2010; Piovia-Scott et al. 2011; Böll et al. 2012; Scheele et al. 2015). For example, infection prevalence can vary seasonally, with higher prevalence often detected in tadpoles during cold months with high precipitation rates (Valencia-Aguilar et al. 2016; Ruggeri et al. 2020). Certain species with prolonged larval stages overwinter in their aquatic habitats, maintaining infections between seasons, which can, in turn, impact infection prevalence in species that breed at different times of the year (Narayan et al. 2014; Rumschlag and Boone 2018; Blaustein et al. 2020).

Many studies focus on adults of non-declining species as potential reservoir hosts for *Bd*, yet fewer studies directly investigate the role of wild tadpoles as reservoirs. Research involving *Bd* transmission and disease prevalence in tadpoles is often conducted in controlled laboratory settings, since assessing infection dynamics in the field is challenging due to the influence of fluctuating biotic and abiotic factors on susceptibility (Rachowicz and Vredenburg 2004; Blaustein et al. 2005; Searle et al. 2011; Lambertini et al. 2021; Daversa et al. 2022; Haver et al. 2022). Furthermore, tadpoles can be difficult to identify to the species level in the field due morphological similarities at the larval stage (Grosjean et al. 2015; Fatorelli et al. 2018). Finally, the absence of standardized protocols for detecting and diagnosing *Bd* in tadpoles further complicates accurate infection prevalence estimations and may lead to underestimations of their role as reservoir hosts. More comprehensive research on *Bd* in persisting populations is required to better understand their contribution in communities affected by chytridiomycosis.

Bd in the Neotropics

Anuran species found in Neotropical regions spanning Central and South America are suffering the greatest *Bd*-related biodiversity loss (Lips et al. 2006; Arellano et al. 2017). These

regions are not only incredibly species-rich, but also have high numbers of endemic species that typically occur in the moist habitats that favour the growth and spread of the pathogen (Lips et al. 2006; Whiles et al. 2006; Kilburn et al. 2010). Consequently, *Bd* outbreaks in the Neotropics impact a wide range of hosts, and species that exclusively occupy specific geographic areas are at particularly high risk of extinction if the pathogen invades these ecosystems (Smith et al. 2009; Lewis et al. 2019; Valencia and Fonte 2021). In response to rapid declines, many studies investigating *Bd* in Neotropical anurans have focused on vulnerable populations and species, yet few have closely examined the role of persisting species that may be acting as disease reservoirs (La Marca et al. 2005; Ryan et al. 2008; Burrowes and De la Riva 2017; Carvalho et al. 2017). Furthermore, very little is known about *Bd* prevalence at the larval life stage in Neotropical tadpole assemblages and very few reports have directly compared infection between life stages of cooccurring species.

Thesis Objectives

The main objective of my thesis is to evaluate *Bd* infection prevalence and severity in wild Neotropical tadpoles and to emphasize the significant role of larvae in chytrid systems. My research was conducted across lowland streams in central Panamá, a country that has a long history of *Bd*-related declines that have been closely documented, yet little is known about infection in species at the larval life stage. This general introduction serves as a literature review of the history and ecology of *Bd* and outlines knowledge gaps in the field that I seek to address in my two manuscript chapters.

In Chapter 1, I investigate the question "What determines a positive result in tadpoles?" by examining the *Bd* loads from wild tadpole samples in triplicate quantitative polymerase chain

reaction (qPCR) assays. I discuss inconsistencies in *Bd* detection and diagnosis methods at the larval life stage and investigate the detectability of low-intensity infections often observed in wild tadpoles. Finally, I compare diagnostic methods and recommend a highly sensitive protocol inclusive of individuals with mild infections in order to achieve more accurate estimations of disease prevalence in wild populations. My results also provide valuable insight into the role of Neotropical tadpoles as disease reservoirs.

My findings from Chapter 1 inform my second chapter, wherein I evaluate infection prevalence and intensity in low-risk frog species to assess their potential as reservoir hosts across different life stages. I compare infection in species at the adult and tadpole life stage to provide a comprehensive perspective of their contribution to the persistence of the pathogen within the study ecosystems. Chapter 2 highlights the important role tadpoles play in community infection dynamics, as well as identifies tolerant species that can carry and spread the pathogen across their life cycle.

Chapter 1: Detection and Diagnosis of Low Intensity Chytrid Fungus in Tadpoles

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ABSTRACT: The amphibian disease chytridiomycosis, caused by the waterborne chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), has been linked to severe declines in anuran species and populations globally. Tadpoles can carry low intensity, nonlethal *Bd* infections and thus can play an important role in maintaining pathogen within ecosystems by serving as disease reservoirs. However, *Bd* can be challenging to detect at low levels, therefore comprehensive, standardized methods are needed to accurately detect and diagnose infections at the tadpole life stage. Although traditional visual techniques are sometimes still employed, qPCR assays have become the most common method used to identify and quantify *Bd* loads by amplifying DNA, typically in up to three replicates. Yet, studies conducting qPCR assays often utilize different sampling techniques, replicate numbers, or diagnostic criteria. These inconsistencies among studies can lead to unreliable disease prevalence estimates and to the underestimation of potential reservoir species. This study investigates the importance of conducting triplicate qPCR assays in the detection and diagnosis of low intensity *Bd* infections in tadpoles. We evaluated the relationship between *Bd* loads and the number of replicates that tested positive and assessed detection consistency by analysing samples in triplicate in two separate assays. We found that most tadpoles harboured low pathogen loads, which were primarily detected in just one out of three replicates and were rarely detected in both assays. Conversely, tadpoles with high pathogen loads yielded three positive replicates and were consistently redetected. We then compared prevalence outcomes based on whether at least one or at least two positive replicates were required for a positive diagnosis and calculated the proportion of samples misclassified as negative when single positive replicates were excluded. Our results suggest that all samples yielding one or more positive replicates should be considered *Bd*-positive and that including three replicates is necessary to account for low level infections. By applying our methods, we identified three potential reservoir species out of the

seven species we investigated. *Bd* loads differed significantly among species as well as between early and late development stage tadpoles. We recommend that future field and experimental studies assessing *Bd* infection in tadpoles apply our methods to ensure that individuals, species, and life stages with low pathogen burdens are included in disease prevalence assessments.

Key words: Amphibian; *Batrachochytrium dendrobatidis;* Chytridiomycosis; Larvae; Pathogen reservoir; qPCR

AMPHIBIAN species are currently facing the greatest documented pathogen-driven biodiversity loss due to the highly virulent, chytrid fungus, *Batrachochytrium dendrobatidis*, hereafter *Bd* (Scheele et al. 2019). *Bd* causes chytridiomycosis, a disease that can spread via infectious zoospores dispersing through water and embedding in the keratinized tissues of amphibian hosts (Berger et al. 1998; Fellers et al. 2001). In frogs, infection occurs on the skin surfaces of metamorphic and postmetamorphic individuals, whereas in tadpoles, it is found primarily in the keratinized areas of the mouthparts (Berger et al. 1998; Marantelli et al. 2004). Consequently, infection in tadpoles is restricted to a smaller surface area and is often nonlethal (Kilpatrick et al. 2010; Rachowicz and Vredenberg 2004; Sauer et al. 2020).

Due to their aquatic life history, infected tadpoles can contribute to the long-term maintenance and spread of *Bd* within ecosystems and increase infection risk in more susceptible hosts by acting as disease reservoirs (Narayan et al. 2014; Ruggeri et al 2020; das Neves-da-Silva et al. 2021). Several studies have evaluated *Bd* infection in wild tadpoles, often reporting low pathogen loads (Russell et al. 2010; Böll et al. 2012; Scheele et al. 2015; Ruggeri et al. 2018). Yet, *Bd* can be difficult to detect at low levels, which increases the potential of misclassifying infected individuals as uninfected (i.e., false negatives) should inappropriate detection and diagnosis

methods be used (Hyatt et al. 2007; Shin et al. 2014; DiRenzo et al. 2018). Accurately identifying low intensity infections in tadpoles is therefore important for determining which species may serve as reservoir hosts for *Bd*, as well as to provide a more complete understanding of chytrid disease landscapes. However, there are currently no standardized protocols to detect and diagnose *Bd* at the larval life stage.

Tadpole mouthparts are comprised of soft sensory tissues called papillae that border keratinized tooth rows and jaw sheaths (Altig 2007). As *Bd* infection progresses, individuals may exhibit oral abnormalities such as depigmentation, damage, or loss of these keratinized structures (Fellers et al. 2001; Knapp and Morgan 2006; Drake et al. 2007). Initially, infection in tadpoles was confirmed through traditional diagnostic approaches, such as histology (Berger et al. 2000; Bosch et al. 2001; Rachowicz and Vredenburg 2004) and visual identification of oral deformities (Fellers et al. 2001; Knapp and Morgan 2006; Drake et al. 2007). However, visual methods often yield false positive or false negative results, and have largely been supplemented or replaced by rapid, highly sensitive DNA-based assays, which allow more samples to be tested with greater accuracy (Annis et al. 2004; Boyle et al. 2004; Kriger et al. 2006a; Padgett-Floh and Goble 2007).

The detection of *Bd* using quantitative polymerase chain reaction (qPCR) assays, first developed by Boyle et al. (2004), is the most reliable molecular diagnostic approach to date, demonstrating the ability to detect as little as a single zoospore. This advancement in *Bd* testing introduced new methods to evaluate infection severity in tadpoles, such as zoospore quantification using nonlethal (mouthpart swabbing) and lethal (mouthpart extraction) sampling techniques. Although both methods are widely used, swabbing can damage larval mouthparts and is ultimately less sensitive and robust than excising entire oral discs for quantitative analysis (Retallick et al. 2006; Hyatt et al. 2007; Shin et al. 2014). Owing to its high precision and increased accessibility

in recent years, qPCR assays have become the preferred method for *Bd* detection in tadpoles. However, some researchers continue to employ traditional visual approaches (Arellano et al. 2017; Amorim et al. 2019; Santos et al. 2024). The use of different detection methods with varying sensitivities can lead to inconsistencies in reported infection prevalences and loads, preventing comparisons across species and habitats. Moreover, studies often apply different criteria to diagnose infections, resulting in varied interpretations of qPCR results and further complicating reliable disease estimates in wild populations with low level infections.

Triplicate qPCR assays, which analyze targeted DNA sequences in a single sample three times, are a common technique used to detect and quantify *Bd* across all life stages (Kriger et al. 2006b; Hyatt et al. 2007). While some studies suggest that duplicate or singlicate assays are sufficient for pathogen detection, running triplicates minimizes the likelihood of obtaining false negatives by increasing the detection probability in samples with low zoospore counts (Kriger et al. 2006b; Hyatt et al. 2007). Yet, studies that perform triplicate qPCR analyses on tadpole samples often consider the presence of *Bd* in just one out of three replicates as a negative diagnosis, thus potentially underestimating infection prevalence and overlooking the potential contribution of reservoir species in long-term pathogen persistence and community disease dynamics (Rachowicz and Briggs, 2007; Searle et al. 2013; Hanlon et al. 2015; Blaustein et al. 2020).

Here, we evaluated *Bd* prevalence and severity in wild-caught tadpoles of seven species of frogs breeding in central Panamá. We performed qPCR analysis in triplicate to investigate the relationship between pathogen load and the number of replicates that tested positive, assess redetection rates, and compare prevalence outcomes according to whether at least one or at least two positive replicates designate a positive infection status. Based on our results, we recommend a method for *Bd* detection and diagnosis at the larval life stage that is inclusive of low intensity

infections and can be implemented in field surveys to achieve more accurate disease assessments and identify reservoir host species.

Materials and Methods

Field Sites and Tadpole Collection

We conducted fieldwork over five weeks, from February–April 2023, at five lowland streams (30–90 meters above sea level) in Soberanía National Park, a 195 km^2 area of protected tropical rainforest in central Panamá. Although *Bd* has been previously detected at Soberanía, its occurrence in specific streams has not been assessed (Woodhams et al. 2008; Rebollar et al. 2014; Rodríguez-Brenes et al. 2016). We thus confirmed the pathogen's presence at all five streams by swabbing adult frogs for infection, yielding positive results.

We collected tadpoles along 200-m stream transects using bulb basters and aquarium nets. We focused our search efforts along stream edges and in shallow regions to include basins or pools where different species might occur. We targeted tadpoles between Gosner stages 25–40 (Gosner 1960), as this developmental range encompasses the stages where keratinized tissue is present in their mouthparts but very little is found on their newly emerged hindlimbs (Marantelli et al. 2004; McMahon and Rohr 2015). We humanely euthanized all tadpoles on-site using a buffered MS-222 solution and preserved them in tubes containing 95% ethanol. All samples were stored at –20°C in the laboratory until taken out for species identification, Gosner stage classification and mouthpart extraction. We identified species and Gosner stages using external morphology and tooth row formulae (Navarro-Lozano et al. 2018).

DNA Extraction and qPCR

We removed the entire oral disc of each tadpole, including the tooth rows, jaw sheath, and papillae, to ensure comprehensive coverage of all keratinized areas for qPCR analysis. We used new, single-use scalpel blades for each tadpole and stored the dissected mouthparts individually at –20°C. Before starting the extraction process, we vacuum-dried the samples on a medium spin cycle for 18 minutes using a Savant SpeedVac (Thermo Fisher Scientific) to remove any residual ethanol and reduce the risk of inhibition during DNA amplification.

We followed the qPCR protocol outlined by Boyle et al. (2004), with modifications introduced by Kriger et al. (2006a) and Hyatt et al. (2007). In brief, we adhered to the extraction and bead-beating procedure of Boyle et al. (2004), but we suspended mouthpart tissues in 50 µl of PrepMan Ultra (Applied Biosystems) instead of 40 µl. We prepared the reaction mix using FastStart Essential DNA Probes Master (Roche Diagnostics) and LightCycler Uracil-DNA Glycosylase (Roche Diagnostics) to prevent carryover contamination. All samples were run in triplicate on 96-well assay plates using a Roche LightCycler 96 system (Roche Diagnostics). We designated replicate wells, hereafter referred to as wells, as positive if *Bd* was detected and negative if there was no detection.

Bd Detection

We analyzed the samples in two independent runs. Run 1 consisted of all tadpole samples ($n = 409$; Supplemental Fig. S1). Assay plates included one concentration standard of 100 zoospore genomic equivalents (ZGE), one positive control, and five negative controls. Samples that tested *Bd*-positive in at least one well during the initial round of qPCR ($n = 44$) along with a subset of samples that tested negative in all three wells (n=55) were retested in triplicate to assess detection consistency (Run 2; $n = 99$). When rerunning the 44 samples that detected *Bd* in Run 1, we

employed a series of tenfold dilutions of the standards, ranging from 0.1–10,000 ZGE, along with one positive control and five negative controls. We used replicates of five for low concentration standards (0.1–100 ZGE) to enhance the detection sensitivity of low zoospore counts, while higher concentration standards (1,000 and 10,000 ZGE) were run in triplicate. All standards were prepared using the JEL423 *Bd* strain originating from Panamá.

Zoospore Quantification

We calculated *Bd* loads for all samples that were positive in at least one well using an equation derived from pooling the quantitative cycle (Cq) values we obtained for the standards run in four different plates. We used the Cq values for the 10,000 (n = 12), 1,000 (n = 12), 100 (n = 20), 10 (n = 20), 1 (n = 18) and 0.1 (n = 13) ZGE standards to obtain the linear regression equation (Supplemental Fig. S2):

$$Cq = -3.507 \times \log10(ZGE) + 34.087; (R^2 = 0.996)$$

We then estimated the ZGE in each positive well using the Cq values as:

$$ZGE = 10^{\wedge} ((Cq - 34.087) / -3.507))$$

Calculated ZGE were averaged for each sample and multiplied by 100 to compensate for the extraction and dilution of the samples. The resulting values, hereafter *Bd* loads, were rounded to the nearest integer and used as a measure of infection severity for individual tadpoles.

Data Analysis

All analyses were conducted using R software v.4.4.2 (R Core Team 2024). We compared *Bd* loads to the number of wells that tested positive in each run using a nonparametric Kruskal-Wallis test from the *stats* package in R (R Core team 2024). We then ran a post-hoc Dunn pairwise comparison test with Bonferroni correction using the *dunn.test* package (Dinno 2024) to identify

significant differences in *Bd* load between samples grouped by number of positive wells. To assess the detection and redetection rates of *Bd*, we examined the number of positive wells in individual runs and across the two runs. We ran a paired Wilcoxon signed-rank test from the *stats* package (R Core team 2024) to compare differences in *Bd* loads from samples in which we detected the pathogen in both Run 1 and Run 2. Next, we applied two distinct diagnostic criteria to the same results from each run to compare infection prevalence outcomes based on whether: (1) at least one positive well designates a positive infection status; and (2) at least two positive wells designate a positive infection status. Samples that were positive in one well and did not meet the second criterion in either run were considered false negatives. We calculated prevalences by dividing the number of positive samples by the total number of tadpoles analyzed. Finally, to investigate the differences in *Bd* loads between species, as well as across Gosner stages, we fit a generalized linear mixed model (GLMM) with a negative binomial family using the *glmmTMB* package (Brooks et al. 2017). We ran this analysis on the subset of samples that were run twice and included the run number as a random effect. Due to low sample sizes in two of the sampled species, we only included the three most abundant species in this model (n = 97 tadpoles). We grouped Gosner stages into three categories: stages 25–29, stages 30–34, and stages 35–40 and included this categorical variable as a factor in the model, together with the species.

RESULTS

Field Samples

We collected a total of 409 tadpoles belonging to seven species: *Smilisca sila* (n = 265), *Rhinella alata* (n = 94), *Silverstoneia flotator* (n = 27), *Lithobates warszewitschii* (n = 10), *Boana rosenbergi* (n = 9), *Rhinella horribilis* (n = 3), and one individual, which we were unable to

identify, was classified as an unknown species. The most abundant species was *Smilisca sila*, representing 64.8 % of all tadpoles we collected.

Bd Detection

Bd was detected in 11.7% (48/409) of all tadpoles we analyzed. Overall, most samples in which *Bd* was detected were positive in only one well (Table 1). In Run 1, 10.8% (44/409) of samples were positive in at least one well and 21.2% (21/99) were positive in at least one well in Run 2. In Run 2, *Bd* was redetected in 38.6% (17/44) of the samples that yielded one or more positive wells in Run 1. Among the 55 samples that were initially negative in all three wells, 7.3% (4) yielded either one or two positive wells when retested (Table 1). In total, 31.3% (31/99) of the samples that were analyzed in triplicate twice tested positive in only one of the two runs; the majority of which (87.1%; 27) yielded only one positive well, while 12.9% (4) yielded two positive wells. All samples that were positive in three wells had positive wells in both runs, with 90.0% (9/10) yielding three positive wells twice.

Zoospore Loads

Samples with one positive well had a mean *Bd* load of 6 ZGE ± 0.47, with a minimum of 0 ZGE (estimated ZGE of 0.4) and a maximum of 12 ZGE. For samples testing positive in two wells, the mean *Bd* load was 7 ZGE ± 0.83, with ZGE values ranging from 3–12. Samples that were positive in three wells had a mean *Bd* load of 3,706 ZGE ± 1860, with a range of 9–26,468 ZGE. Overall, *Bd* loads differed across the number of positive wells in Run 1 (Kruskal-Wallis test, $\chi^2 = 21.897$, $P < 0.0001$) and in Run 2 ($\chi^2 = 14.335$, $P < 0.001$). Both runs demonstrated significant differences in *Bd* loads between samples with one and three positive wells (Fig. 1; post-hoc Dunn test, Run 1: $Z = –4.531$, $P < 0.0001$; Run 2: $Z = –3.432$, $P < 0.001$) and between samples with two

and three positive wells (Run 1: $Z = -3.477$, $P < 0.001$; Run 2: $Z = -2.708$, $P < 0.011$). However, *Bd* loads did not vary among samples testing positive in either one or two wells (Fig. 1; Run 1: $Z = 0.231$, $P = 1.00$; Run 2: $Z = -0.795$, $P = 0.640$).

Samples that were positive in only one of the two runs yielded ≤ 12 ZGE when only one well tested positive and ≤ 8 ZGE when yielding two positive wells and there was no significant difference in *Bd* loads between runs for samples that were analyzed twice (Wilcoxon signed rank test, $V = 45.5$, $P = 0.255$).

Diagnostic Criteria

Infection prevalence was 10.8% in Run 1 when one or more positive wells were required for a positive diagnosis and 3.7% when at least two positive wells were required. In Run 2, these rates were 21.2% and 16.2%, respectively (Fig. 2). In Run 1, 65.9% (29/44) of positive tadpoles were misclassified as *Bd*-negative under the criterion of at least two positive wells, representing an overall 7.1% (29/409) false negative rate. Using this same criterion for diagnosis (at least two positive wells), 23.8% (5/21) of tadpoles were misclassified as negative in Run 2, with an overall false negative rate of 5.1% (5/99).

Species Infection Prevalence and Intensity

Bd was detected in at least one well in the three most abundant species we collected: *S. sila* (48/265; prevalence=14.3%), *R. alata* (9/94; 9.6%) and *S. flotator* (1/27; 3.7%). The model with *Bd* load as a response variable explained 68% of the variance and *Bd* loads differed significantly among species. *S. sila* carried higher loads than *R. alata*, while there was no difference in *Bd* load between *S. flotator* and *R. alata* ($Z = 2.414$, $P < 0.016$; and $Z = -0.579$, $P = 0.563$, respectively;

Supplemental Table S1). Significant differences were observed in *Bd* loads between stages 25–29 and stages 35–40 ($Z = 2.412$, $P < 0.016$; Table S1), with later stages having higher zoospore counts.

Discussion

In this study, we investigated the detection and diagnosis sensitivity of *Bd* in wild-caught tadpoles using triplicate qPCR assays. Previous studies employ different techniques for sampling and evaluating *Bd* infections in tadpoles, making comparisons between systems challenging. Since *Bd* can be difficult to detect at low levels and tadpoles can harbour mild infections, it is essential to employ comprehensive, standardized protocols to understand how tadpoles may contribute to the persistence and spread of *Bd* in amphibian populations.

Our main findings suggest that wild tadpoles can indeed harbour low intensity *Bd* infections with varying detection success, emphasizing the need for thorough measures to detect and diagnose infections at the larval life stage. We present evidence of a positive correlation between *Bd* load and the number of wells that test positive in triplicate qPCR assays and conclude that the exclusion of samples with a single positive well when diagnosing individuals overlooks low level infections, thus leading to underestimations of infection prevalence.

Bd Detection and Diagnosis

We found that *Bd* loads in tadpoles were significantly lower when one or two wells were positive in the triplicate assays, compared to when all three wells were positive. These findings align with those reported by Kriger et al. (2006b), who observed lower *Bd* loads in swab samples that had fewer than three positive wells. Previous studies evaluating the accuracy of *Bd* detection protocols suggest that, despite the high sensitivity of qPCR methods, the probability of detecting the pathogen can be reduced when a sample only has a few zoospores, as only a small amount of

genetic material is allocated into each well for analysis following sample dilution (Kriger et al. 2006a; Hyatt et al. 2007). Consequently, if the sample initially contains a low quantity of *Bd* DNA, the likelihood of its presence in multiple wells is also low and detection rates can be inconsistent (Peccoud and Jacob 1996; Kriger et al. 2006a; Hyatt et al. 2007). Our results are in line with this conclusion, since most samples in which *Bd* was detected had only one positive well (i.e., had low *Bd* loads) and we did not always detect the pathogen in both runs. We also observed inconsistent detection results from a few samples that were initially negative in all three wells but showed low *Bd* loads upon reanalysis. Conversely, we found that most samples from which we consistently detected the pathogen across both runs had three positive wells (i.e., had high *Bd* loads) and similar zoospore counts in each run. These results show that detection rates decreased when samples had lower amounts of zoospores. Specifically, *Bd* was more challenging to detect in tadpoles with low intensity infections, whereas tadpoles with severe infections carried enough *Bd* DNA to be reliably detected in nearly all replicate wells. This suggests that the pathogen's presence in just one well indicates the presence of small quantities of *Bd* zoospores and should therefore be considered when diagnosing infections given its low detection probability. Furthermore, our results suggest that negative wells may not always indicate the absence of *Bd*, but rather non-detection due to low zoospore quantities.

Our diagnostic results show that overall infection prevalences were higher when including one positive well in assessments. This outcome was expected, since the sensitivity of the test was increased by relaxing the criteria used for diagnoses. The observed differences in prevalences between runs may be attributed to the selective sampling in Run 2. That is, since Run 2 included all samples with known positive wells and a random subset of negative samples, it resulted in a higher calculated prevalence. Despite this, we found that the rate of false negative diagnoses, was

comparable between runs, as indicated by the similar differences in prevalence outcomes between diagnostic criteria. Although these rates were relatively low, at 7.1% and 5.1%, we believe they are important to be included in analyses, since low *Bd* loads are often only detected in one well, if at all, as we demonstrated. Furthermore, the exclusion of even a small number of positive cases can lead to substantially different results when infection prevalences are low in tadpole populations, causing misinterpretations of disease's severity within an ecosystem.

The question of what qualifies as a positive result has been posed in molecular-based *Bd* research for many years (Hyatt et al. 2007), but is particularly relevant when considering samples, such as tadpoles, that may have low *Bd* loads. While studies frequently use triplicate qPCR assays to detect and quantify *Bd* in tadpoles, the criteria used to designate a positive diagnosis varies widely. For example, many studies do not consider one positive well as sufficient evidence for a positive diagnosis and either immediately classify the sample as negative or rerun the samples a second time to assess whether *Bd* is detected again (Rachowicz and Briggs 2007; Venesky et al. 2012; Searle et al. 2013; Blaustein et al. 2020). Certain studies that run duplicate assays also conduct confirmatory qPCR runs if *Bd* is not initially detected in both wells (Garner et al. 2009; Navarro-Lozano et al. 2018; Cramp et al. 2022; Daversa et al. 2022). However, even when conducting two separate runs, different criteria are often applied to determine infection statuses, with some studies designating samples as *Bd*-positive if just one well returns a positive result upon retesting, and with others requiring two wells.

Singlicate assays are another diagnostic method used to evaluate *Bd* infections in tadpoles (Hanlon and Parris 2014; McMahon and Rohr 2015; Rumschlag et al. 2022; Ruggeri et al. 2024). Kriger et al. (2006b) first recommended running singlicate tests in their study investigating cost-efficiency in qPCR detection methods, suggesting that they should be used as a screening method

for *Bd,* and triplicate assays should be subsequently run for confirmation and quantification purposes. However, the singlicate assay approach does not consider the high detection variability of low intensity infections and risks nondetection by decreasing the number of wells used for analysis. While this method may be useful when pooling samples to determine the presence or absence of the pathogen in wild populations (Kriger et al. 2006b), it can likely lead to underestimations of prevalence and should not be employed for low level pathogen detection.

Few studies have used more direct and inclusive diagnostic criteria in which they consider all samples that yield either 0.1 or 1 ZGE as positive for infection, however they employ different sampling techniques, such as mouthpart swabbing (Catenazzi et al. 2013; Bosch et al. 2021; Hollanders et al. 2024) or excision (Searle et al. 2010; Venesky et al. 2011; Ruggeri et al. 2018). While these approaches both use qPCR methods that encompass low level infections, their detection sensitivities differ, compromising the reliability of cross-study comparisons and accurate disease assessment.

Implications

Our study provides insights into the challenges of accurately detecting and diagnosing wildlife pathogens and has important ecological implications in *Bd* research. As chytridiomycosis continues to impact amphibian populations globally, conducting thorough and reliable disease surveys across all life stages is necessary for prioritizing the protection of vulnerable species and for identifying reservoir hosts that sustain *Bd* within ecosystems. Our results suggest that running multiple replicates is necessary to detect low level infections in tadpoles and that all wells that detect *Bd* should be included when diagnosing infections. Classifying samples with one positive well as *Bd*-positive ensures that individuals with mild infections are accounted for, leading to more accurate estimates of infection prevalences across species and populations. Furthermore, in cases

where pathogen detection can be challenging, it is preferable to overestimate rather than underestimate infection prevalence to ensure conservation efforts are efficiently implemented to prevent and mitigate further declines or outbreaks (Smith and Weldon 2007).

Overall, we found that infection prevalence and intensity was low in tadpoles inhabiting neotropical lowland streams. Previous research has shown that stream tadpoles carry low *Bd* loads (Ruggeri et al. 2018), however estimates of prevalence vary between studies (Conradie et al. 2011; Catenazzi et al. 2013; Ruggeri et al. 2018; das Neves-da-Silva et al. 2021). While environmental variables including water temperature, flow rate, and pH may influence prevalence in stream systems (Catenazzi et al. 2013; Valencia-Aguilar et al. 2016; das Neves-da-Silva et al. 2021), our findings suggest that the low prevalences reported in previous studies may be partially due to limitations in detection and diagnostic methods, rather than from fluctuating environmental factors or actual absent hosts.

We found that infection prevalence and severity differed interspecifically, with some species harbouring higher *Bd* loads than others and certain species yielding negative infection results altogether. Variation in infection response between tadpole species has previously been established and can be influenced by differences in behaviour and natural history (Blaustein et al. 2005; Venesky et al. 2009, 2011; Valencia-Aguilar et al. 2016). Further research and larger sample sizes are needed on these species at the larval stage to determine the factors driving infection differences. However, these variations highlight the importance of employing thorough methods to identify species more likely to harbour lower pathogen burdens.

By including all wells in which *Bd* was detected, we identified three species of neotropical stream-dwelling tadpoles that carry infections, and thus may act as disease reservoirs. Had we applied the stricter criterion of requiring at least two positive wells for a positive diagnosis, *S.*

flotator would have been excluded as a potential reservoir host. These three species were also the most frequently sampled species in our study. Although more thorough surveys are required to evaluate the relationship between infection prevalence and species abundance in this region, this high encounter rate suggests that these populations should be closely monitored since they are coexisting with *Bd* in their ecosystems. Moreover, later life stages of these species may also act as reservoir hosts as *Bd* infections can transfer from the mouthparts to hind limbs as tadpoles develop (McMahon and Rohr 2015; Humphries et al. 2024). Further investigations evaluating the reservoir potential of these species across life stages are necessary to better understand their impact on the amphibian community.

We found that tadpoles in later stages of development had significantly higher loads compared to those in early stages. Older tadpoles are exposed to the pathogen for a longer period and may carry more severe infections owing to increased amounts of keratinized areas in their larger mouthparts and emerging limbs (Knapp and Morgan 2006; Vieira et al. 2013; McMahon and Rohr 2015). While some studies have reported no effect of tadpole development stage and *Bd* infection rate (Julian et al. 2016; Valencia-Aguilar et al. 2016), others reported a positive correlation between developmental stage and the prevalence and intensity of infection (Smith et al. 2007; Catenazzi et al. 2013). Our results support the latter, suggesting that infection in earlier life stages may be more challenging to detect due to lower *Bd* loads.

Recommendations

To our knowledge, this study is the first to directly investigate the relationship between *Bd* loads in tadpoles and the number of replicate wells testing positive in triplicate qPCR assays. Testing methods used to date often lead to inconsistent conclusions, particularly when infection intensity is low (Peccoud and Jacob 1996; Lachish et al. 2012; Miller et al. 2012; DiRenzo et al.

2018). We recommend future studies evaluating *Bd* infection in tadpoles run a minimum of three replicates with multiple standards and negative controls included in each plate. Should this option be inaccessible because of large sample sizes and the high cost of including standards, we suggest first conducting a qualitative triplicate run to confirm the presence or absence of the pathogen. Samples with at least one positive well can be reanalyzed in triplicate using standards, and *Bd* loads can be calculated as described here, with ZGE averaged across both runs. By applying these methods, disease assessments encompass all infected individuals, regardless of severity, and can be applied across species and ecosystems, as well as in experimental studies. Because of their aquatic life history, reliable detection and diagnosis of *Bd* infection in tadpoles are essential for understanding the complete scope of chytrid disease dynamics and are necessary to inform effective conservation efforts in the context of the rapid decline of amphibian populations globally.

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FIGURES

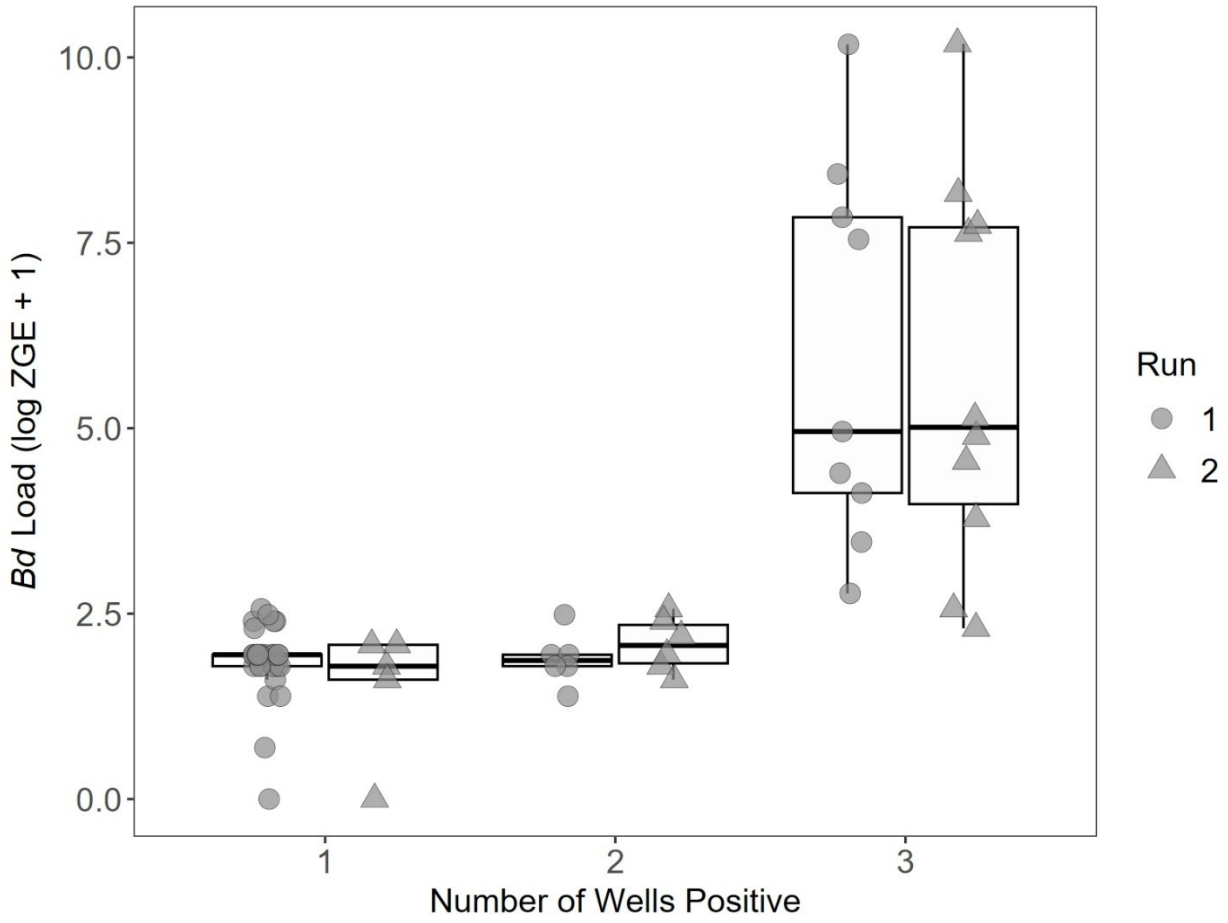

FIG. 1. Box plots with jittered data points of *Batrachochytrium dendrobatidis* loads in tadpole samples yielding one, two, or three positive replicate wells in Run 1 (n = 44) and Run 2 (n = 21).

TABLE 1 — Number of samples that tested positive in zero, one, two, or three replicate wells in each triplicate qPCR run (n = 99).

		Run 2			
		0 wells	1 well	2 wells	3 wells
Run 1	0 wells	51	2	2	0
	1 well	25	3	1	0
	2 wells	2	0	3	1
	3 wells	0	0	0	9

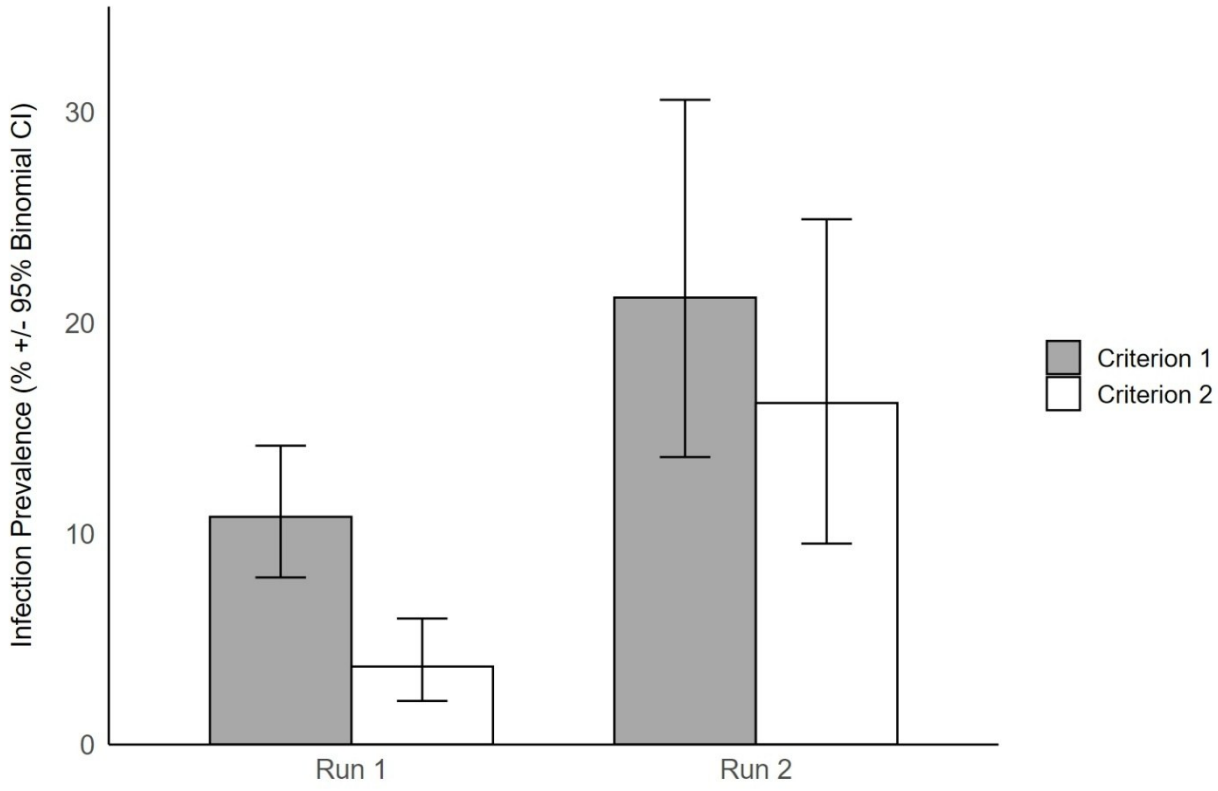

FIG. 2. *Batrachochytrium dendrobatidis* infection prevalence with 95% binomial confidence intervals for Run 1 (n = 409) and Run 2 (n = 99) according to two criteria: when at least one positive replicate well designates a positive infection status (Criterion 1), or when at least two positive replicate wells designate a positive infection status (Criterion 2).

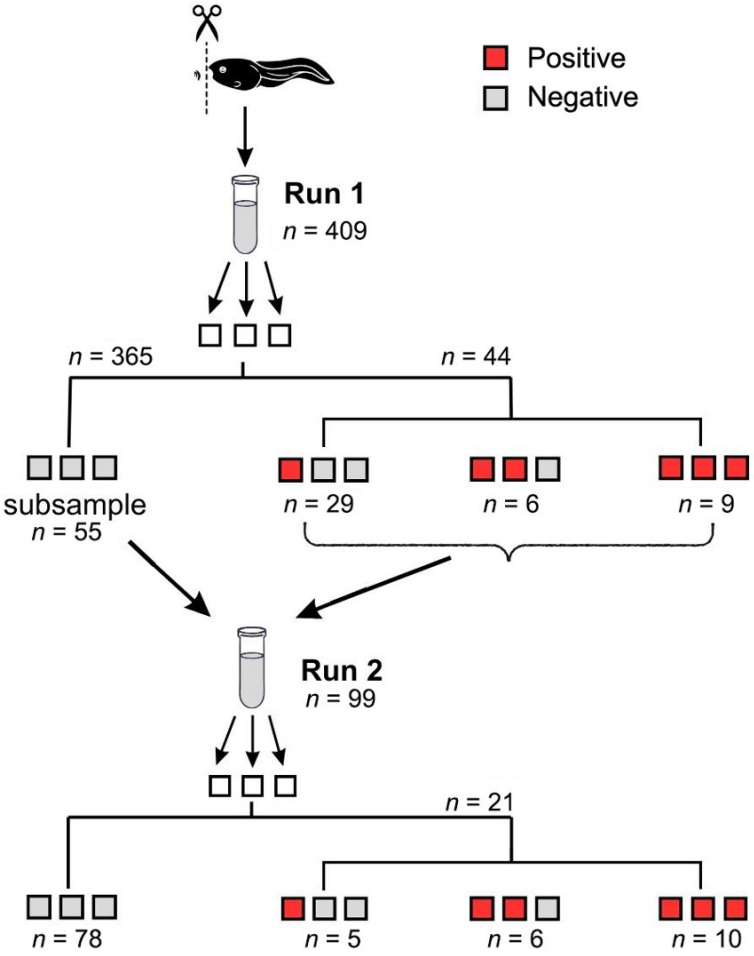

FIG. S1. Flow diagram of sampling design.

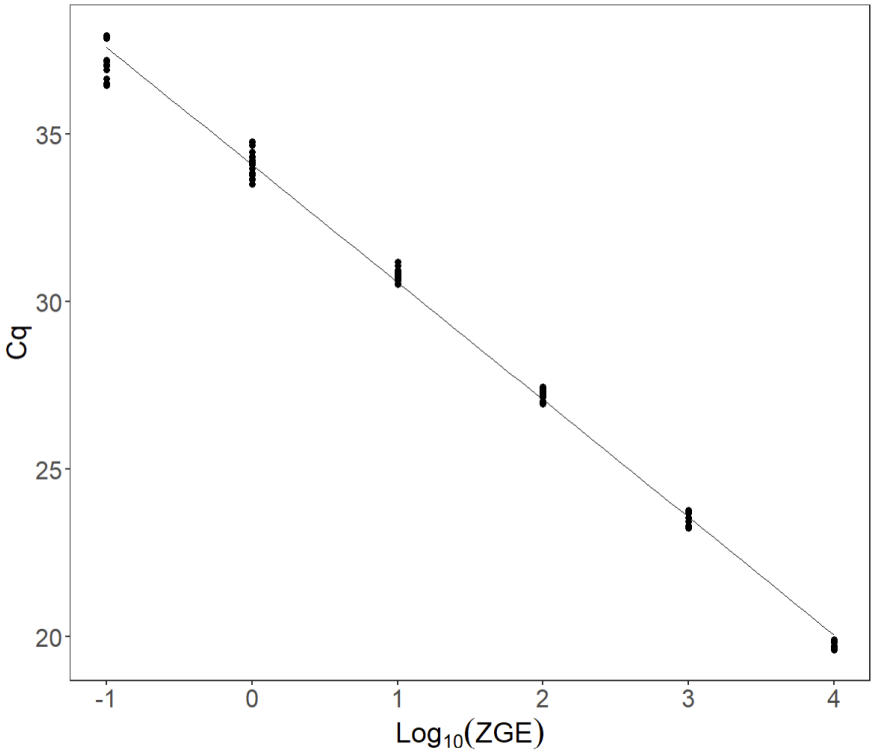

FIG. S2. Linear regression derived from pooling quantitative cycle (Cq) values obtained from standards with known concentrations of zoospore genomic equivalents (ZGE): 10,000 (n = 12), 1,000 (n = 12), 100 (n = 20), 10 (n = 20), 1 (n = 18) and 0.1 (n = 13). The linear regression equation is Cq = –3.507 x log10(ZGE) + 34.087; (R^2 = 0.996).

TABLE S1. — Model summary of the generalized linear mixed model for the effects of species and Gosner stage on *Batrachochytrium dendrobatidis* loads. Species effect is relative to *R. alata* and Gosner stage effect is relative to Stages 25–29. Conditional R^2: 0.994; Marginal R^2: 0.679

Effects	Estimate	Std. Error	*Z* Statistic	*P* Value
Intercept	4.842	0.617	7.846	4.29e–15
Species (*Silverstoneia flotator*)	–0.620	1.070	–0.579	0.5627
Species (*Smilisca sila*)	1.050	0.435	2.414	0.0158
Gosner (Stages 30–34)	0.273	0.409	0.668	0.5041
Gosner (Stages 35–40)	0.830	0.344	2.412	0.0159

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CONNECTING STATEMENT

The first chapter of my thesis addresses inconsistencies in *Bd* detection and diagnosis methods in tadpole research. I showed that tadpoles carry low level infections that are challenging to detect, therefore it is important to run multiple qPCR replicates and include all positive wells when analysing infection prevalence and intensity at this life stage. I also discussed the ecological implications of excluding samples with single positive wells and evaluated infection differences between the three tadpole species that tested positive.

In Chapter 2, I explore a knowledge gap that I identified in my first chapter. I investigate the reservoir potential of the three positive species across both the tadpole and adult life stages to gain a better understanding of how they individually contribute to the spread and maintenance of *Bd* in the ecosystem throughout their life cycle. To do this, I compare infection prevalence and intensity across species, and within each species, at both life stages. Furthermore, I apply my findings from my first chapter by including all tadpoles with positive wells in my analysis. Together, these two chapters thoroughly explore the role of tadpoles as hosts in chytrid systems, while also comprehensively assessing the reservoir potential of three common species persisting in regions with *Bd.*

Chapter 2: Chytrid fungus reservoirs across life stages in coexisting Neotropical frogs

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RHH: Wilson et al. — Chytrid reservoirs across life stages

Abstract

Context: The amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), is driving severe biodiversity loss in Neotropical anurans. Panamá has a well documented history of *Bd*-related declines, however most research focuses on long-term population monitoring and the management of high-risk species. Consequently, less is known about species that may act as reservoir hosts for *Bd* and data at the tadpole life stage is particularly limited. ***Aims:*** This study investigates the reservoir potential of three sympatric low-risk species coexisting with *Bd* at both the tadpole and adult life stages. We aim to assess differences in infection response and identify whether certain species serve as reservoirs throughout their life cycle. ***Methods:*** We sampled tadpoles and adults of *Rhinella alata*, *Silverstoneia flotator*, and *Smilisca sila* over five weeks at five lowland streams in Soberanía National Park in central Panamá. We compared *Bd* infection prevalence and intensity across and within species and life stages. ***Key results:*** We detected *Bd* in the adults and tadpoles of all three species, however infection prevalence and intensity at both life stages differed among species. Infections were significantly higher in adult *R. alata*, suggesting their role as important reservoirs for *Bd*. We found that *S. sila* tadpoles carried high pathogen loads and this species had no significant difference in infection between life stages, indicating that they may act as competent reservoir hosts across all stages of their development. Lower prevalence and *Bd* loads in both the tadpoles and adults of *S. flotator* suggest that this species may be more resistant to infection. ***Conclusions****:* Our results emphasize the importance of including multiple life stages in disease assessments. We also provide new insights into *Bd* infection in wild Neotropical species at the tadpole life stage. ***Implications:*** Understanding how coexisting species contribute to disease dynamics at both pre-and postmetamorphic stages can lead to the development of targeted management strategies aimed to prevent further declines in Neotropical amphibians.

Keywords: Amphibians; *Batrachochytrium* d*endrobatidis*; Conservation; Chytridiomycosis; Disease ecology; Panama; Reservoir host; Tadpoles

Introduction

Emerging fungal pathogens are an increasing threat to wildlife biodiversity (Al-Shorbaji et al. 2015; Fisher et al. 2020; Case et al. 2022). *Batrachochytrium dendrobatidis*, hereafter *Bd*, is a pathogenic chytrid fungus that causes the highly infectious amphibian disease, chytridiomycosis (Berger et al. 1998). *Bd* has been linked to the declines, extirpations, and extinctions of hundreds of amphibian species globally (Burns et al. 2016; Scheele et al. 2019). The effects of *Bd* have led to substantial biodiversity loss in anuran amphibians, especially in Neotropical regions, where high diversity and endemism of frog species coincides with a moist climate that supports the growth and spread of the pathogen (Fouquet et al. 2007; Pereira et al. 2013; Lips 2016; Scheele et al. 2019). Since *Bd* was first identified as the main driver of mass mortality events occurring in Panamá in the early 1990s, it has been driving anuran declines across diverse ecosystems in Central and South America, including high elevation montane habitats and lowland rainforests (Lips 1999; La Marca et al. 2005; Ryan et al. 2008; Crawford et al. 2010; Burrowes and De la Riva 2017; Russell et al. 2019; Cádiz et al. 2019; Urgiles et al. 2021). These declines in frog species richness and abundance can alter the structure of communities, leading to significant cascading effects and broader biodiversity loss (Whiles et al. 2006; Zipkin and DiRenzo 2022).

The presence of reservoir hosts within communities can increase disease prevalence and accelerate population declines (Fellers et al. 2011; Al-Shorbaji et al. 2016; Scheele et al. 2015; Brannelly et al. 2018). Reservoir hosts are disease-tolerant carriers that can amplify infection risk in more sensitive, sympatric hosts (Stockwell et al. 2016; Scheele et al. 2017; Burns et al. 2021). In *Bd*

systems, competent reservoirs are those that carry long-term infections, have high prevalence, and can spread and maintain high pathogen loads within an ecosystem with minimal fitness costs (Catenazzi et al. 2013; Brannelly et al. 2018; Burns et al. 2021). Frog species can serve as reservoirs for *Bd* across different life stages, with each stage influencing disease dynamics in distinct ways. The tadpole life stage, in particular, can be important reservoirs due to their aquatic life history. *Bd* is a waterborne pathogen, therefore tadpoles have more contact with infected habitats compared to semi-terrestrial or terrestrial postmetamorphic stages (juveniles and adults) (Berger et al. 2005; Ruggeri et al. 2018). Extended exposure to *Bd* can increase the opportunity for infection and can lead to higher pathogen loads over time (Carey et al. 2006; Smith et al. 2007; Vieira et al. 2013). Furthermore, tadpoles often exist in high densities and can continuously shed infectious zoospores into the water throughout their development, thereby contributing to the environmental persistence of the pathogen (Alford et al. 2013; Mitchell et al. 2008; Briggs et al. 2010). Tadpoles of both declining and non-declining species have been identified as *Bd* reservoirs, emphasizing the significance of the larval life stage in community infection dynamics (Woodhams and Alford 2005; McCallum 2005; Catenazzi et al. 2013; Narayan et al. 2014; Valencia-Aguilar et al. 2016; Ruggeri et al. 2020). Postmetamorphic frogs belonging to *Bd*-tolerant species are also relevant reservoir hosts. These later life stages can carry high pathogen loads (Reeder et al. 2012; Scheele et al. 2017; Brannelly et al. 2018; Hudson et al. 2019) and facilitate *Bd* dispersal between aquatic and terrestrial environments (Kolby et al. 2015; Burns et al. 2021). Therefore, the occurrence of postmetamorphic reservoirs can increase the potential for infection in vulnerable species across multiple habitat types (Kolby et al. 2015; Scheele et al. 2017; Burns et al. 2021).

Tolerant species that have high infection prevalence and intensity (i.e., high pathogen loads) throughout their life cycle are highly competent reservoirs. However, certain species or life stages

may be more competent hosts than others. Previous studies have evaluated the role of reservoir hosts in Neotropical amphibian declines (Catenazzi et al. 2013; Valencia-Aguilar et al. 2016; Arellano et al. 2017; Hudson et al. 2019), yet few have compared *Bd* infection prevalence and intensity across cooccurring reservoir species at different life stages. Understanding how hosts coexisting with *Bd* influence the spread and persistence of the pathogen is essential for guiding targeted conservation efforts in regions experiencing declines and to mitigate outbreaks in naïve communities with similar host composition.

In this study, we investigated the reservoir potential of persisting Neotropical frog species from lowland streams in Panamá at the tadpole and adult life stage. Panamá has a well-documented history of *Bd*-related amphibian declines. The pathogen first emerged in western regions of the country and has since spread southeastward, inciting the establishment of several long-term monitoring programs that survey changes in amphibian diversity, abundance, and disease prevalence across ecosystems before, during, and after outbreaks occur (Lips 1999; Brem and Lips 2008; Woodhams et al. 2008; Crawford et al. 2010; Hertz et al. 2012; McCaffery et al. 2015). Consequently, many studies investigating *Bd* in Panamá have focused on general trends of pathogen invasion in specific regions and communities or have prioritized the management of species at high extinction risk (Brem and Lips 2008; Woodhams et al. 2008; Crawford et al. 2010; Kilburn et al. 2010; Hertz et al. 2012; McCaffery et al. 2015; Gratwicke et al. 2016; Lewis et al. 2019). As such, there is a lack of research directly evaluating *Bd* infections in potential reservoir species. Furthermore, existing field research has primarily been directed toward postmetamorphic individuals and information on infection in wild tadpoles is particularly limited.

We seek to provide insight into infection prevalence and intensity across life stages in understudied species persisting in regions where *Bd* has previously been detected. We conducted our study in

Soberanía National Park, an area of protected tropical rainforest located along the eastern bank of the Panama Canal within the provinces of Panamá and Colón. *Bd* was first detected in adult frogs at Soberanía in 2008, but few comprehensive disease surveys have been conducted since (Woodhams et al. 2008; Rebollar et al. 2014; Rodríguez-Brenes et al. 2016). We suspect that common species surviving in this area may function as reservoirs for *Bd* and anticipate observing high prevalence in both tadpoles and adults. However, given that infection response can vary among anuran families and species (Blaustein et al. 2005; Corey and Waite 2008; Castro Monzon et al. 2020; Kruger 2020), we expect certain species will have higher infection rates and carry higher pathogen loads than others, thus serving as more competent reservoirs. Tadpoles in previous studies have been found to harbour low intensity infections (Chapter 1 of this thesis; Russell et al. 2010; Böll et al. 2012; Scheele et al. 2015; Ruggeri et al. 2018), therefore we expect tadpoles to have lower infection levels than adults.

Materials and methods

Study species

We focused our sampling efforts on the tadpoles and adults of three frog species belonging to distinct families: *Rhinella alata* (Bufonidae)*, Silverstoneia flotator* (Dendrobatidae), *and Smilisca sila* (Hylidae). The ranges of these species overlap in central Panamá, where they can be commonly found along lowland streams throughout the dry season. Our study species all have IUCN conservation statuses of Least Concern (IUCN 2024) and have been persisting in regions of Panamá where *Bd* has been reported (Rebollar et al. 2014; Medina et al. 2017). Very few records of *Bd* infection exist for each species at the postmetamorphic stage and, to our knowledge, there are no records of infection in tadpoles (Perez et al. 2014; Medina et al. 2017; Hertz et al. 2018; Klocke et al. 2024; but see Chapter 1 of this thesis). While all three species have aquatic larvae,

their postmetamorphic stages occupy different niches. *R. alata* and *S. flotator* are diurnal, terrestrial species, whereas *S. sila* are nocturnal tree frogs (Ibáñez et al. 1999; Grant and Meyers 2013; Dias et al. 2021; Malone 2004).

Field sites

We conducted field sampling at five streams over five weeks between February and April 2023, in Soberanía National Park (9°04'27.5"N, 79°39'35.3"W; Fig. 1). Soberanía has a low elevation (≤ 332 meters) and high amphibian biodiversity, including 66 documented species, the majority of which are anuran (MiAmbiente, 2023). Two streams, Río Masambi Grande and Río Chico Masambi, are situated along a transit road, Carretera Gamboa. The other three streams, Quebrada Juan Grande, Río Frijolito, and Río Frijoles, are located along Pipeline Road, a route often accessed on foot by researchers and tourists. All streams were sampled twice weekly, once during the day and once at night, to encompass both diurnal and nocturnal species, except for Río Chico Masambi, which was only sampled twice before it dried out.

Sampling methods

We located and captured tadpoles and frogs along 200-meter stream transects by active visual and acoustic searches. We standardized our survey efforts by searching for ten minutes in 50-meter sections and processing captured individuals between each section. All footwear and reusable equipment were cleaned with a bleach solution between streams to prevent potential cross-contamination of samples and sampling sites (Cashins et al. 2008). Since *Bd* infects keratinized tissues, which occur across the skin of postmetamorphic frogs but primarily in the mouthparts of tadpoles (Berger et al. 1998; Marantelli et al. 2004), we used different sampling methods for each life stage.

Adult frogs were captured using clean plastic bags and swabbed with sterile rayon-tipped swabs (MW113) a total of 60 times over skin surfaces known to have frequent infection: the flanks, vent, sides, and feet (Hyatt et al. 2007; Skerratt et al. 2008). We used a new pair of powder-free nitrile gloves or disposable plastic gloves for each frog handled. We released all individuals near their capture location and stored swabs dry in individual tubes.

We collected tadpoles between Gosner stages 25–40 (Gosner, 1960) along stream edges and in shallow basins or pools using bulb basters and aquarium nets. We targeted this developmental range as it includes the stages before *Bd* infections occur on the skin of emerging limbs and before adult mouthparts replace the oral disc of tadpoles (Marantelli et al. 2004; McMahon and Rohr 2015; Valencia-Aguilar et al. 2016). Accordingly, any tadpoles that had visibly developing forelimbs were released. We humanely euthanized tadpoles on-site using a buffered MS-222 solution and preserved them individually in tubes containing 95% ethanol. All samples were kept in a cooler in the field and transferred to a –20°C freezer in the laboratory within six hours of collection.

We identified tadpole species in the laboratory using external morphology and tooth row formulae and extracted whole mouthparts with single-use scalpel blades. Dissected mouthparts were vacuum-dried in a Savant Speedvac (Thermo Fisher Scientific) prior to DNA extraction to remove any residual ethanol that could inhibit *Bd* detection. All swab and mouthpart samples were stored at –20°C until taken out for quantitative PCR (qPCR) analysis.

Bd detection and quantification

To evaluate *Bd* infection prevalence and intensity, we analysed swab and mouthpart samples using the qPCR protocol developed by Boyle et al. (2004), with modifications introduced by Kriger et

al. (2006a) and Hyatt et al. (2007). We ran all samples in triplicate on 96-well assay plates using a Roche LightCycler 96 system (Roche Diagnostics). We prepared the reaction mix with FastStart Essential DNA Probes Master (Roche Diagnostics) and LightCycler Uracil-DNA Glycosylase (Roche Diagnostics) to prevent carryover contamination. Each assay plate included one concentration standard of 100 zoospore genomic equivalents (ZGE), one positive control, and five negative controls. For swab samples taken from adults, any sample in which *Bd* amplified in at least two of the three replicate wells was considered positive. For mouthpart samples of tadpoles, we increased the diagnostic sensitivity by considering *Bd* amplification in any of the three replicates as a positive result. This modification was implemented to include individuals with low intensity infections which can be challenging to detect, even when using highly sensitive qPCR methods (Chapter 1 of this thesis; Peccoud and Jacob 1996; Kriger et al. 2006b; Hyatt et al. 2007). All samples that had no amplification in any of the three replicates were considered negative.

We reanalysed positive samples in triplicate to quantify *Bd* infection loads. Assay plates included tenfold standard dilutions of the Panamanian *Bd* strain JEL423, ranging from 0.1 to 10,000 ZGE (0.1, 1, 10, 100, 1,000, 10,000), along with one positive control and five negative controls. High concentration standards (1,000 and 10,000 ZGE) were run in triplicate and low concentration standards (0.1–100 ZGE) were run in replicates of five. We report calculated *Bd* loads for swab samples as the average number of ZGE from positive replicate wells. *Bd* loads for mouthpart samples were calculated to include samples testing positive in the initial run using the calibration curve calculated in Chapter 1 of this thesis and were averaged across all positive wells. Calculated ZGE were multiplied by 100 to compensate for the extraction and dilution of the samples. We rounded all *Bd* load values up to the nearest integer.

Data analysis

We used R software v.4.4.2 to perform all analyses (R Core Team 2024). To test for the effect of species, life stage and their interaction on infection prevalence, we first fitted a generalized linear model (GLM) with a binomial family using the *stats* package (R Core Team 2024). We included all positive (infected) and negative (uninfected) individuals in this model. Next, we evaluated differences in infection intensity (*Bd* load) by fitting a second GLM with a negative binomial family function using only *Bd*-positive individuals and the *glmmTMB* package (Brooks et al. 2017). Species, life stage and their interaction were included as fixed variables. All main effects for both models had low to moderate correlations (VIF < 5) and no overdispersion was detected. Preliminary models included site as a random effect, however it was removed as it did not account for any significant variation and led to a singular fit of the model. We then used the *emmeans* package (Lenth, 2024) on both models to perform pairwise comparisons with a Tukey adjustment to assess differences across and within fixed effect levels.

Ethics statement

Field sampling and laboratory protocols were reviewed and approved by the Animal Care and Use Committee at the Smithsonian Tropical Research Institute (IACUC number: SI-22066). Research and collection permits were issued by the Panamanian Ministerio de Ambiente (ARB-0135-2022).

Results

We collected a total of 568 samples from individuals of the three species, including 65 adults and 94 tadpoles for *R. alata*, 83 adults and 27 tadpoles for *S. flotator,* and 34 adults and 265 tadpoles for *S. sila* (Table 1). Each stream had all three species present, however we did not observe both life stages of *S. flotator* or *S. sila* at all streams (Table S1).

We detected *Bd* in all three species at both the tadpole and adult life stages (Fig. 1; Table 1). Overall *Bd* prevalence was 11.4% in tadpoles and 29.1 % in adults, with an overall prevalence of 17.1% among all our study species. Our binomial model for *Bd* prevalence (infected/not infected) showed that prevalence was significantly higher in adults compared to tadpoles ($Z = – 5.126$, $P < 0.001$). Among species, *R. alata* had higher prevalence than both *S. sila* ($Z = –3.911$, $P < 0.001$) and *S. flotator* across life stages ($Z = –2.289$, $P < 0.022$). A significant interaction effect was found between life stages and species when comparing *R. alata* and *S. sila* ($Z = 2.513$, $P < 0.012$), but not when comparing *R. alata* and *S. flotator* ($Z = 0.539$, $P = 0.590$, Table S2). Pairwise comparisons revealed that among adults, *R. alata* had the highest prevalence (47.7%), which significantly differed from adult *S. flotator* (prevalence = 16.9%, *z* ratio = 3.911, $P < 0.0003$), and was marginally significant when compared to *S. sila* (prevalence = 23.5%, *z* ratio = 2.289, $P = 0.0573$; Fig. 2; Table 1; Table S3). Infection prevalence between adult *S. flotator* and adult *S. sila* did not differ (*z* ratio = –0.834, $P = 0.682$). Among tadpoles, *Bd* prevalence was highest in *S. sila* (13.2%), followed by *R. alata* (8.5%) and *S. flotator* (3.7%), however paired comparisons showed that differences between species were not significant. When comparing infection prevalence between life stages within a single species, *R. alata* was the only species that showed a significant difference between adults and tadpoles (*z* ratio =5.126, $P < 0.0001$; Table S3).

In terms of infection intensity, life stage had a significant effect on *Bd* loads ($Z = –10.984$, $P < 0.001$; Table S4). There was also significant variation across species, with *R. alata* having higher loads than both *S. flotator* ($Z = –10.438$, $P < 0.001$) and *S. sila* ($Z = –6.189$, $P < 0.001$). A significant interaction effect between life stage and species on *Bd* loads was found when comparing *R. alata* and *S. flotator* ($Z = 2.956$, $P < 0.003$), as well as *R. alata* and *S. sila* ($Z = 8.499$, $P < 0.001$). Among adults, pairwise comparisons revealed that *R. alata* had the highest *Bd* loads (mean

ZGE = 97579) compared to both adult *S. flotator* (mean ZGE = 63, *z* ratio = 10.438, $P < 0.0001$) and *S. sila* (mean ZGE = 463, *z* ratio = 6.189, $P < 0.0001$; Fig. 3; Table 1; Table S5). *Bd* loads did not differ between adults of *S. flotator* and *S. sila* (*z* ratio: –2.052, $P = 0.100$). Among tadpoles, *S. sila* carried higher loads than *R. alata* (mean ZGE *S. sila* = 1011; mean ZGE *R. alata* = 7, *z* ratio = –5.831, $P < 0.0001$), but not *S. flotator*, although marginally (mean ZGE = 6; *z* ratio = –2.280, $P = 0.0586$). *Bd* loads did not differ between tadpoles of *R. alata* and *S. flotator* (*z* ratio = 0.034, $P = 1.00$) Within a single species, only *R. alata* demonstrated significantly higher loads in adults than in tadpoles (*z* ratio = 10.984, $P < 0.0001$).

Discussion

The objective of our study was to identify and compare *Bd* infection in the tadpoles and adults of persisting species that may be serving as reservoir hosts in a region of central Panamá where chytridiomycosis has previously been detected. Our results indicate that *Bd* is widespread throughout Soberanía National Park and that three common, low-risk species in the region, *S. flotator*, *R. alata*, and *S. sila*, can carry the pathogen at both the larval and postmetamorphic life stages. These findings suggest that all three species may be serving as reservoir hosts across their life cycle. We found that infections varied across species and life stages and thus emphasize the importance of including both life stages in disease surveys to better understand how individual species may contribute to the persistence of *Bd* in ecosystems pre- and postmetamorphosis.

Our results show that overall prevalence and intensity was higher in adults compared to tadpoles, however the effect of life stage on infection outcome depended on the species. Anuran immune systems are substantially restructured during metamorphosis and the higher infection loads often found in adults may be due to differences in immune defences between life stages (Rollins-Smith

1998, Grogan et al. 2018; Ruggeri et al. 2018; Ruiz and Robert 2023). Furthermore, tadpoles possess less keratin than adults and therefore have less surface area available for *Bd* to colonize (Grogan et al. 2018; Rumschlag and Rohr 2018; Humphries et al. 2022). Despite this, few field studies have reported higher infections in tadpoles compared to adults (Woodhams et al. 2008; Hollanders et al. 2024), illustrating the complexities of multi-host pathogen interactions and supporting the interaction effects we observed between life stages and species. Experimental evidence has also shown interspecific and intraspecific variation in infection response between life stages, further supporting our results (Gervasi et al. 2013).

Overall, we found infection prevalence to be low in tadpoles. Previous studies have reported high prevalence in stream tadpoles (Catenazzi et al. 2013; Ruggeri et al. 2018; das Neves-da-Silva et al. 2021) and stream-associated species are especially vulnerable to *Bd* infections (Lips 1999; Hero et al. 2005; Kriger and Hero 2007; Ruggeri et al. 2018). It is possible that environmental factors (i.e., water temperature, stream depth, flow rate, pH) or developmental stage at the time of capture influenced prevalence results in tadpoles (Catenazzi et al. 2013; Valencia-Aguilar et al. 2016; Knapp and Morgan 2006; Vieira et al. 2013; McMahon and Rohr 2015). Alternatively, lower prevalences may be a consequence of missed detections due to the low pathogen loads carried by tadpoles in Gosner stages 25–40 (see Chapter 1). However, even with low prevalence and pathogen loads, tadpoles may still play an important role as disease reservoirs. Investigating population densities and zoospore shedding rates at this life stage could provide further insight into their influence on community infection dynamics.

Among adults, we found that *R. alata* harboured higher *Bd* loads than both *S. sila* and *S. flotator*. *R. alata* also had a higher infection rate than *S. flotator*. The high prevalence and infection load found in adult *R. alata* may be attributed to differences in reproductive strategies between our

study species. *R. alata* are explosive breeders often found calling along permanent streams and ponds at night during the wet season and early dry season (Wells 1979, Ibáñez et al. 1999; Dos Santos et al. 2015). Choruses contain several males that exhibit physically competitive mating behaviours, such as attempting to displace pairs in amplexus (Wells 1979). The high number of individuals present during breeding events, coupled with competitive behaviours, may increase the frequency of contact between conspecifics and potentially facilitate *Bd* transmission near egg laying areas. Moreover, *R. alata* pairs can be found in the water throughout the day for oviposition (Wells 1979), further increasing the opportunity for infection through waterborne transmission. In contrast, adult *S. sila* are arboreal and only descend from the canopy to breed near stream edges during the dry season nights (Malone 2004). Unlike *R. alata*, males do not aggregate, spacing themselves along the stream and adjoining low vegetation (Tuttle and Ryan 1982), and adults likely spend less time in contact with water and conspecific frogs. Previous research has shown that arboreality can lead to lower infection intensity (Burrowes et al. 2017), which may also explain the lower *Bd* loads we observed in adult *S. sila*. Finally, *S. flotator* had the lowest prevalence and *Bd* loads among adults. This species breeds terrestrially and lay their eggs in the leaf litter (Ibáñez and Smith 1995). After hatching, tadpoles are synchronically transported to the stream on the back of parental males (Ibáñez and Smith 1995; Grant and Myers 2013). Consequently, adults have low contact with water and with conspecifics throughout the breeding season and therefore may have reduced risk compared to *S. sila* and *R. alata* adults.

In addition to the difference in reproductive behaviours, factors such as the composition of skin microbial communities can also contribute to varying infection tolerances in adult anurans (Rebollar et al. 2016, 2020; Schmeller et al. 2022). Some bacteria associated with amphibian skin can produce antifungal metabolites that inhibit the growth of *Bd* (Harris et al. 2006; Becker et al.

2009; Rebollar et al. 2020). Microbial communities can differ across sympatric species, in part due to innate immune defences and microhabitat selection (Kueneman et al. 2014; Rebollar et al. 2016, 2020; Rollins-Smith 2020). Previous research has shown that the bacterial richness of *S. flotator* decreased from west to east in Panamá, a trend that is consistent with the spread of *Bd* across the country (Medina et al. 2017), and with the onset of the wet season (Varela et al. 2018). Moderate proportions of *Bd*-inhibitory bacteria are known to be present in the skin of *S. flotator* frogs at Pipeline Road during the dry and wet seasons (Varela et al. 2018). More direct analyses on the relationship between *Bd* infection and the skin microbiome are needed to understand underlying tolerance mechanisms in our study species. However, given that all three species belong to distinct families and occupy different ecological niches (i.e., terrestrial vs. arboreal, diurnal vs. nocturnal), potential differences in skin microbial communities may further explain the observed variation in infection we reported here.

We found no difference in infection prevalence among species at the tadpole stage. However, in terms of infection intensity, *S. sila* tadpoles carried higher pathogen loads than the tadpoles of *R. alata.* This finding suggests that the tadpoles of all species can become infected at similar rates, but species-specific differences can influence infection severity. The high *Bd* loads observed in larval *S. sila* may be due to the microhabitats they occupy within streams. *S. sila* tadpoles hatch in small, shallow basins where they can be found in high densities during early development (Malone 2004). Consequently, tadpoles are in close contact with each other, increasing infection risk when *Bd* is present (personal observation; Jaslow 1982; Han et al. 2008; Venesky et al. 2011). Conversely, we often found *R. alata* tadpoles aggregated in open bottom areas along the stream edge. Only one *S. flotator* tadpole was found to be *Bd*-positive with low loads (6 ZGE). Lower infection prevalence and intensity in *S. flotator* may be in part due to their terrestrial clutches and

less exposure time in infected stream water than the other species with aquatic clutches. Furthermore, we did not observe aggregations of *S. flotator* tadpoles, but rather found them individually in shallow regions of the stream, which may indicate limited contact with conspecifics and other tadpole species. Alternatively, low infections may be due to the absence of labial tooth rows in *S. flotator* mouthparts, resulting in a smaller area for *Bd* infection than other species with keratinized teeth (Ibáñez and Smith 1995; Dias et al. 2021). Unfortunately, little is known about the natural history and behaviour of the tadpoles of these three species, therefore more research is needed in order to understand interspecific differences in infection response.

When comparing infection outcomes within a single species, both prevalence and intensity were higher in *R. alata* adults compared to tadpoles. Since adult *R. alata* also had significantly higher infections than the other species, our findings suggest that adults of this species are likely to be important reservoir hosts. For *S. sila*, we did not observe a significant difference between life stages, however, on average, *Bd* loads were higher in tadpoles than in adults. This trend warrants further investigation, as *S. sila* tadpoles may be an important source for aquatic zoospores. Furthermore, when considering the interaction effects, *S. sila* showed different prevalence and intensity patterns compared to *R. alata*. This result is likely due to smaller infection differences between life stages and suggests that *S. sila* may be a competent reservoir throughout their life cycle. No differences between infection prevalence and intensity were observed across life stages of *S. flotator*. Both adults and tadpoles of this species had the lowest infection rates and *Bd* loads among all species, suggesting that *S. flotator* may have a higher overall tolerance or resistance to infection.

Infection response to *Bd* is complex and context-dependent (Daskin and Alford 2012; Gervasi et al. 2017). Our study shows that the prevalence and intensity of *Bd* infections vary interspecifically

and intraspecifically across life stages in three low-risk species coexisting with *Bd* in Panamá. Our findings provide new insights on Neotropical species at the tadpole life stage and emphasize the necessity of assessing chytrid infections in anurans at multiple life stages. Understanding *Bd* infection in these species is important because they are common, can be found at different elevations and have ranges that extend outside of Panamá (Ibáñez and Smith 1995; Malone 2004; Dos Santos et al. 2015). We recommend that future studies investigating potential reservoir hosts also sample the juvenile life stage and compare infections across different elevations and habitat types in order to achieve a more precise understanding of how specific species and life stages may contribute to pathogen persistence. While more detailed infection research is needed on both the tadpoles and adults of each of our study species (i.e., transmission potential, infection persistence, long-term fitness), we provide evidence supporting their roles as reservoirs for *Bd.* This evidence is an important preliminary step towards the development of targeted conservation efforts aimed to prevent further biodiversity loss due to chytridiomycosis in Neotropical amphibians.

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Figures

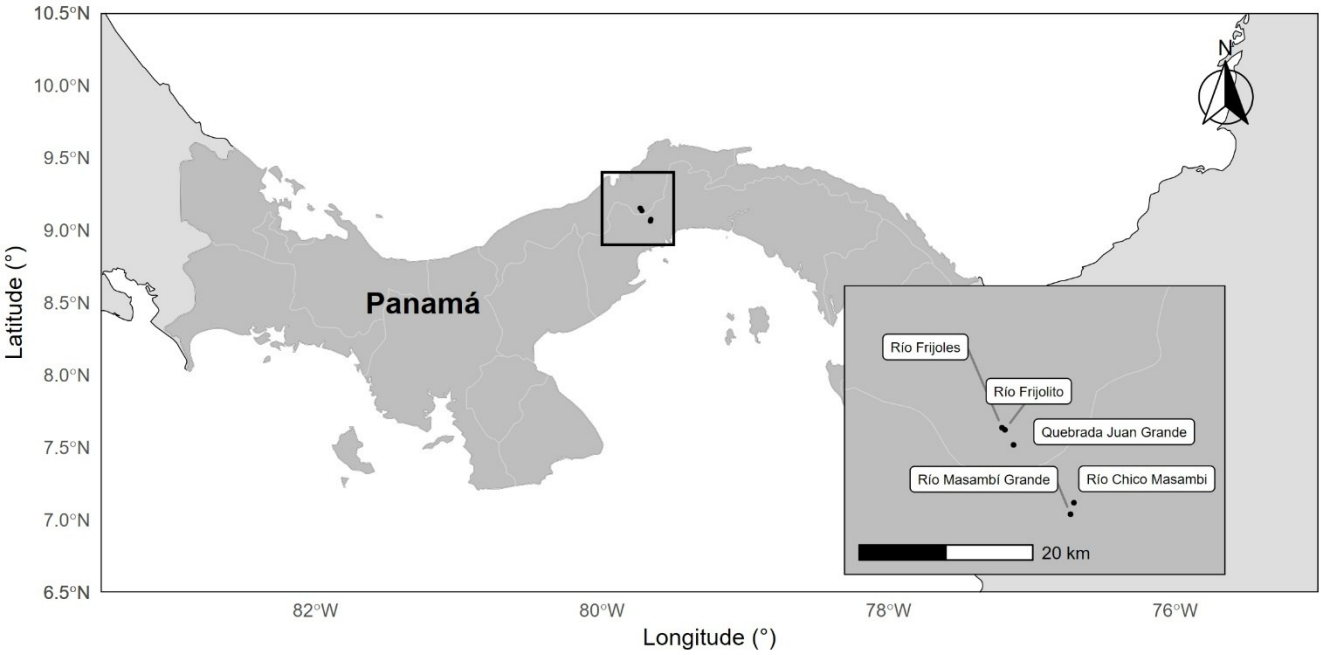

Fig. 1. Map of Panamá (indicated in dark grey) displaying the locations of five sample streams.

Table 1. *Batrachochytrium dendrobatidis* (*Bd*) infection prevalence and intensity among three common stream-associated frog species at the tadpole and adult life stages. Prevalence is reported as the number of positive individuals over the total number sampled. *Bd* loads were rounded up to the nearest integer.

Species	**Life stage**	**# Individuals (# infected)**	**Infection prevalence (%)**	**Mean (min–max) *Bd* loads**
Rhinella alata	tadpole	94 (8)	8.5	7 (3–10)
	adult	65 (31)	47.7	97579 (4–2075000)
	both	159(39)	24.5	77564 (3–2075000)
Silverstoneia flotator	tadpole	27 (1)	3.7	6
	adult	83 (14)	16.9	64 (7–439)
	both	110(15)	13.6	60 (6–439)
Smilisca sila	tadpole	265 (35)	13.2	1011 (1–26369)
	adult	34 (8)	23.5	463 (7–2762)
	both	299 (43)	14.4	909 (1–26369)
All 3 species	tadpole	386 (44)	11.4	805 (1–26369)
	adult	182 (53)	29.1	57161 (4–2075000)
	both	568 (97)	17.1	31598 (1–2075000)

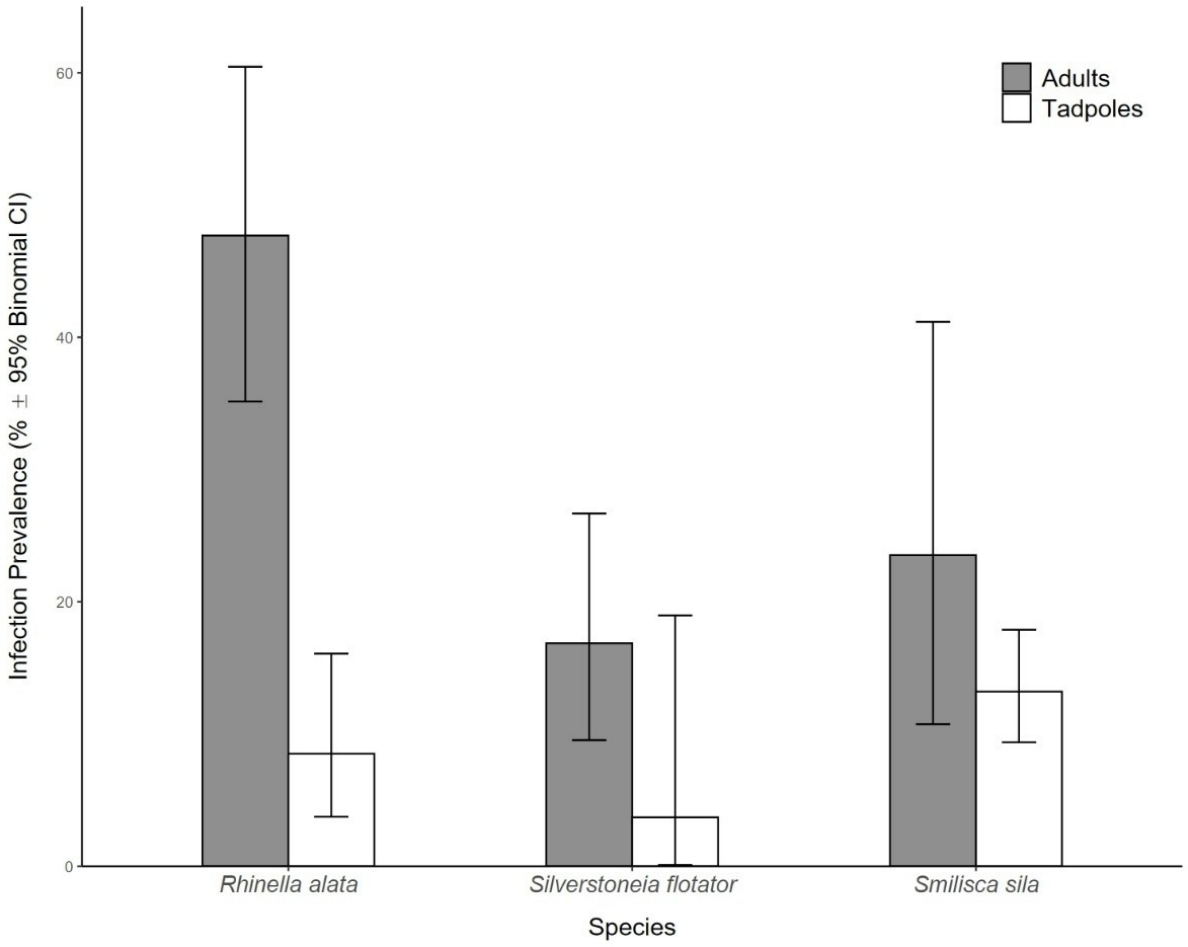

Fig. 2. *Batrachochytrium dendrobatidis* infection prevalence with 95% binomial confidence intervals in *Rhinella alata*, *Silverstoneia flotator,* and *Smilisca sila* tadpoles and adults.

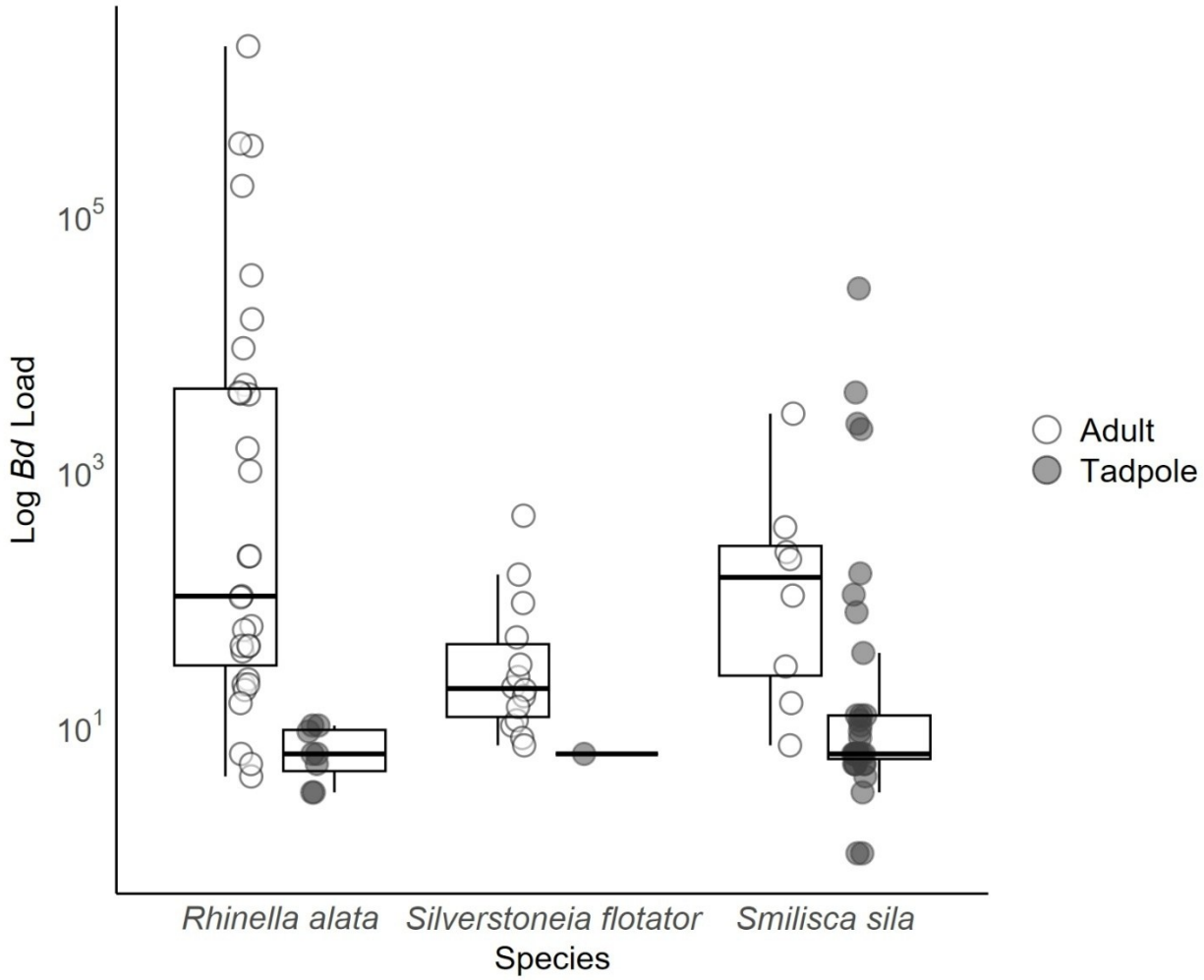

Fig. 3. Box plots with jittered data points of *Batrachochytrium dendrobatidis* loads for *Rhinella alata*, *Silverstoneia flotator,* and *Smilisca sila* tadpoles and adults.

Supplemental material

TABLE S1. *Batrachochytrium dendrobatidis* (*Bd*) infection prevalence and intensity across life stages of *R. alata*, *S. flotator*, and *S. sila* at five lowland streams in Soberanía National Park, Panamá. Prevalence is reported as number of positive individuals over the total number sampled. *Bd* loads were rounded up to the nearest integer.

Site (latitude, longitude)	Species	Life stage	# Individuals (# infected)	Infection prevalence (%)	Mean (min–max) *Bd* loads
Río Masambi Grande (9°04'26.7"N, 79°39'29.4"W)	*R. alata*	tadpole	28 (2)	7.1	7 (5–9)
		adult	11 (6)	54.5	117491 (19–359700)
		both	39 (8)	20.5	88120 (5–359700)
	S. flotator	tadpole	0	-	-
		adult	17 (1)	5.9	24
		both	17 (1)	5.9	24
	S. sila	tadpole	26 (4)	15.4	579 (1–2303)
		adult	0	-	-
		both	26 (4)	15.4	579 (1–2303)
	All 3 species	tadpole	54 (6)	11.1	389 (1–2303)
		adult	28 (7)	25.0	100710 (19–359700)
		both	82 (13)	15.9	54408 (1–359700)
Río Chico Masambi (9°04' 29.28" N, 79° 39' 32.04" W)	*R. alata*	tadpole	16 (0)	0.0	-
		adult	3 (1)	33.3	5
		both	19 (1)	5.3	5
	S. flotator	tadpole	0	-	-
		adult	5 (2)	40.0	50 (9–91)
		both	5 (2)	40.	50 (9–91)
	S. sila	tadpole	1 (0)	0.0	-
		adult	0	-	-
		both	1 (0)	0.0	-
	All 3 species	tadpole	17 (0)	0.0	-
		adult	8 (3)	37.5	36 (5–91)
		both	25 (3)	12.0	36 (5–91)
	R. alata	tadpole	29 (4)	13.8	8 (3–10)

Quebrada Juan Grande (9° 08' 4.92" N, 79° 43' 20.28" W)		adult	18 (8)	44.4	2582 (6–15160)
		both	47 (12)	25.5	1724 (3–15160)
	S. flotator	tadpole	16 (0)	0.0	-
		adult	25 (7)	28.0	96 (7–439)
		both	41 (7)	17.1	96 (7–439)
	S. sila	tadpole	84 (9)	10.7	240 (6–2092)
		adult	3 (1)	33.3	104
		both	87 (10)	11.5	226 (6–2092)
	All 3 species	tadpole	129 (13)	10.1	169 (3–2092)
		adult	46 (16)	34.8	1340 (6–15160)
		both	175 (29)	16.6	815 (3–15160)
Río Frijolito (9°09' 1.08" N, 79° 43' 52.68" W)	*R. alata*	tadpole	14 (1)	7.1	3
		adult	19 (10)	52.6	21940 (56–167500)
		both	33 (11)	33.3	19946 (3–167500)
	S. flotator	tadpole	3 (1)	33.3	6
		adult	20 (3)	15.0	27 (11–49)
		both	23 (4)	17.4	21 (6–49)
	S. sila	tadpole	74 (9)	12.2	455 (1–4045)
		adult	11 (2)	18.2	1389 (15–2762)
		both	85 (11)	12.9	625 (1–4045)
	All 3 species	tadpole	91 (11)	12.1	373 (1–4045)
		adult	50(15)	30.0	14817 (11–167500)
		both	141 (26)	18.4	8706 (1–167500)
Río Frijoles (9° 9' 9.00" N, 79° 44' 3.84" W)	*R. alata*	tadpole	7 (1)	14.3	6
		adult	14 (6)	42.9	346657 (4–2075000)
		both	21 (7)	33.3	297135 (4–2075000)
	S. flotator	tadpole	8 (0)	0.0	-
		adult	16 (1)	6.3	17
		both	24 (1)	4.2	17
	S. sila	tadpole	80 (13)	16.3	2062 (3–26369)
		adult	20 (5)	25.0	165 (7–356)
		both	100 (18)	18.0	1535 (3–26369)
	All 3 species	tadpole	95(14)	14.7	1915 (3–26369)
		adult	50(12)	24.0	173398 (4–2075000)
		both	145 (26)	17.9	81061 (3–2075000)

Table S2. Model summary of the generalized linear mixed model for the effects of species, life stage and their interaction on *Batrachochytrium dendrobatidis* infection prevalence. Species effect is relative to *R. alata* and life stage effect is relative to adults. R^2: 0.097

Effects	Estimate	Std. error	*Z* statistic	*P* value
Intercept	–0.092	0.248	–0.372	0.710
Life stage	–2.283	0.445	–5.126	2.96e–7
Species (*R. alata–S. flotator*)	–1.503	0.384	–3.911	9.18e–5
Species (*R. alata–S. sila)*	–1.086	0.474	–2.289	0.022
Lifestage: *R. alata–S. flotator*	0.619	1.150	0.539	0.590
Lifestage: *R. alata–S. sila*	1.578	0.628	2.513	0.012

Table S3. Contrast table of the estimated marginal means showing pairwise comparisons of species and life stage on *Batrachochytrium dendrobatidis* infection prevalence. *P* values are adjusted using the Tukey method.

Contrasts	Odds ratio	Std. error	*Z* ratio	*P* value
Adults				
R. alata / *S. flotator*	4.944	1.730	3.911	0.0003
R. alata / *S. sila*	2.963	1.410	2.289	0.0573
S. flotator / *S. sila*	0.659	0.329	–0.834	0.6820
Tadpoles				
R. alata / *S. flotator*	2.419	2.620	0.815	0.6939
R. alata / *S. sila*	0.611	0.252	–1.195	0.4560
S. flotator / *S. sila*	0.253	0.262	–1.329	0.3791
Adult / Tadpole				
R. alata	9.800	4.360	5.126	2.963e–7
S. flotator	5.280	5.590	1.568	0.1168
S. sila	2.020	0.896	1.589	0.1121

Table S4. Model summary of the generalized linear mixed model for the effects of species, life stage and their interaction on infection intensity (*Batrachochytrium dendrobatidis* loads). Species effect is relative to *R. alata* and life stage effect is relative to adults. R^2: 0678

Effects	Estimate	Std. error	*Z* statistic	*P* value
Intercept	11.488	0.392	29.344	2e–16
Life stage	–9.617	0.876	–10.984	2e–16
Species (*R. alata–S. flotator*)	–7.335	0.703	–10.438	2e–16
Species (*R. alata–S. sila*)	–5.351	0.865	–6.189	6.04e–10
Lifestage:*R. alata–S. flotator*	7.225	2.455	2.956	0.003
Lifestage:*R. alata–S. sila*	10.397	1.223	8.499	2e–16

Table S5. Contrast table of the estimated marginal means showing pairwise comparisons of species and life stage on infection intensity (*Batrachochytrium dendrobatidis* loads). *P* values are adjusted using the Tukey method.

Contrasts	Ratio	Std. error	*Z* ratio	*P* value
Adults				
R. alata / *S. flotator*	1533.229	1080.0	10.438	3.020e–14
R. alata / *S. sila*	210.866	182.0	6.189	1.812e–9
S. flotator / *S. sila*	0.138	0.133	–2.052	0.100
Tadpoles				
R. alata / *S. flotator*	1.083	2.550	0.034	1.000
R. alata / *S. sila*	0.006	0.006	–5.831	1.654e–8
S. flotator / *S. sila*	0.006	0.013	–2.280	0.057
Adult / Tadpole				
R. alata	15012.200	13100.0	10.948	4.543e–28
S. flotator	10.607	24.300	1.030	0.303
S. sila	0.458	0.391	–0.914	0.361

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General discussion

Chytridiomycosis has contributed to significant global biodiversity loss in amphibians for several decades. Numerous mitigation efforts have been explored, including environmental fungicides (Hanlon et al. 2015, Rohr et al. 2017), antifungal treatments (Rohr et al. 2017; Knapp et al. 2022) and the establishment of captive assurance colonies (Young et al. 2007; Zippel et al. 2011; Lewis et al. 2019). However, significant variability in infection response among hosts has complicated the development of universally effective disease management strategies. Different life stages, in particular, can have distinct roles in *Bd* infection dynamics, yet disease surveys and field studies have historically been focused on postmetamorphic stages. Consequently, less is known about many species at the tadpole life stage and their contribution to the spread and maintenance of *Bd* within ecosystems has been largely underexplored, especially in species-rich Neotropical regions. Given the ongoing amphibian crisis, it is essential to monitor populations across life stages and to identify and manage sources, such as reservoir hosts, that contribute to further declines in vulnerable species.

In this thesis, I investigated *Bd* infection in wild Neotropical anurans at the tadpole life stage and assessed their potential as reservoir hosts. I aimed to emphasize the important role of tadpoles in *Bd* systems through two interconnected manuscript chapters. In my first chapter, I showed that tadpoles commonly carry low level infections which may be undetected or overlooked due to absence of standardized detection and diagnosis protocols at the larval life stage. As such, I recommended a method to detect and diagnose *Bd* infection in tadpoles that is inclusive of individuals with low *Bd* loads and applied it to identify infection in three common species. In my second chapter, I further investigated the role of these species as potential reservoir hosts by comparing infection prevalence and intensity across life stages. I found that all three species

harboured *Bd* infections as both adults and tadpoles, however infections at each life stage differed among species. To my knowledge, my research is the first to report *Bd* infections in wild tadpoles of *R. alata*, *S. flotator* and *S. sila* and to conduct targeted disease surveys on these species.

My results have practical applications in the fields of wildlife conservation and disease management. Both *S. sila* and *S. flotator* have ranges that extend from Costa Rica to northern Colombia, while *R. alata* range from Panamá to western Colombia and Ecuador (IUCN 2024). All three species have conservation statuses of Least Concern and have stable populations, except for *S. flotator* which has been estimated as decreasing (IUCN 2024). Although these three non-endangered species likely contribute to the persistence of *Bd* across their ranges, their potential impact in Panamá is of particular interest. Panamá has high anuran species richness, with approximately 189 identified species, many of which are endemic (AmphibiaWeb 2024; Frost 2024). The effects of *Bd* have been especially pronounced in this country, with dramatic declines first observed in highland regions in the 1990s (Lips 1999; Crawford et al. 2010). The pathogen has since been detected at multiple lowland sites, however evidence of declines at low elevations is scarce and requires further investigation (Woodhams et al. 2008; Kilburn et al. 2010; Rebollar et al. 2014; Rodríguez-Brenes et al. 2016). *Bd* has driven multiple species endemic to Panamá to near extinction, notably those belonging to the highly susceptible genus *Atelopus* (La Marca et al. 2005; McCaffery et al. 2015; Gratwicke et al. 2016; Lewis et al. 2019); Lötters et al. 2023). The ranges of *R. alata*, *S. sila*, and *S. flotator* overlap in regions where *Bd*-related declines have been recorded and several critically endangered species occur, suggesting that these three species may have increased infection risk in more sensitive sympatric species (Crawford et al. 2010; Kilburn et al. 2010; Rebollar et al. 2014; Panama Amphibian Rescue and Conservation Project 2024). My findings provide important insight into the reservoir potential of *R. alata*, *S. sila*, and *S. flotator* at

two distinct life stages and can be applied to inform life stage-specific conservation actions in Panamá and in other countries across their ranges. Specifically, I show that the tadpoles of each species, especially of *S. sila*, require attention when developing management strategies and that increasing the sensitivity of detection and diagnosis methods at this life stage is necessary for thorough disease assessments. Furthermore, I show that adults of each species, particularly of *R. alata*, should also be closely monitored due to high infection prevalence and intensity.

While my research presents new data on *Bd* host species, further investigations into their natural history are needed to better understand the mechanisms driving differences in infection response across species and life stages. This includes research on factors that can influence *Bd* infection such as diet, behaviour, microhabitat occupancy, and development (Smith et al. 2007; Richards-Zawacki 2010; Venesky et al. 2012; von May et al. 2018; das Neves-da-Silva et al. 2021). For example, duration of the larval stage can influence infection in tadpoles. Previous studies have shown that slow-developing species have higher *Bd* prevalence than those with short developmental periods (Ruggeri et al. 2020; das Neves-da-Silva et al. 2021). Furthermore, stream tadpoles typically develop slower than tadpoles from temporary and ephemeral ponds, suggesting that the tadpoles of my study species may occupy streams for extended periods and thus potentially serve as a long-term source for aquatic zoospores (Wells, 2007; Ruggeri et al. 2018). Throughout five weeks of field sampling, I rarely observed late-stage tadpoles, except for few *R. alata* and *S. flotator* individuals. More evidence is needed to determine the duration of the tadpole life stages of these species; however, our findings provide a starting point for future investigations.

Further research on infected individuals is also needed to more accurately determine reservoir host competency. Once infected, hosts can have different zoospore shedding rates, with some species producing extremely high zoospore outputs (DiRenzo et al. 2014; Maguire et al.

2016; Daversa et al. 2022). Transmission and maintenance of infections is also host-specific; therefore, a positive infection status does not guarantee that one host contributes to the persistence of the pathogen as effectively as another host (Gieger et al. 2011; Brannelly et al. 2015; Daversa et al. 2022). Evaluating these fine-scale disease parameters can offer broader insights on how host identity (i.e., species and life stage) influence community-level infection dynamics.

The research methods used in both chapters of my thesis can be applied in future studies to better understand *Bd* infections in tadpoles and across life stages of persisting species. While the methodology I introduced in Chapter 1 has important implications in *Bd* research for larval anurans, it can also be adapted for use across life stages in experimental or field settings when *Bd* is presumed to be present at low prevalence. This method increases the detection sensitivity of standard qPCR testing by including multiple replicates of low concentration standards and several additional negative controls for monitoring contamination and enhancing the reliable detection of low pathogen loads. Diagnostic sensitivity is also increased, as samples yielding one positive well are considered positive for infection. Since postmetamorphic anurans typically have higher prevalence and pathogen loads than tadpoles (Chapter 2; Russell et al. 2010; Piovia-Scott et al. 2011; Scheele et al. 2015), the application of this method is more critical in tadpole studies than for those focusing on later life stages. Nevertheless, it can be modified for general use to detect early infections or to quantify infection in tolerant or resistant species with low *Bd* loads.

Throughout my thesis, I addressed the challenges faced with identifying and managing threats in taxa with biphasic life cycles. A recent study has shown that over half of described anuran species lack information on their tadpole stage (Vera Candioti et al. 2023). This finding may explain the knowledge gaps also seen in *Bd* research regarding infection in tadpoles. In Neotropical regions, many closely related species are sympatric and share similar morphologies, complicating

accurate species identification (Grosjean et al. 2015, Dubeux et al. 2022). More recently, DNA barcoding has been used to identify species at the tadpole life stage (Vences et al 2012; Grosjean et al. 2015, Dubeux et al. 2022). This advancement holds great promise for *Bd* research, as previously undescribed species can be identified and targeted research on the role of different tadpole species in *Bd* systems can be conducted.

Assessing *Bd* infections across life stages is essential for the development of comprehensive conservation strategies, yet conservation actions for amphibians are rarely designed to mitigate threats across multiple life stages (Nolan et al. 2023). This oversight is concerning, as chytridiomycosis continues to devastate amphibian populations globally and both life stages can become infected and spread the pathogen. Evaluating infection susceptibility at only one life stage in species with complex life cycles can result in substantial underestimations of infection prevalence within communities. This could potentially result in the implementation of incomplete and ineffective management efforts that overlook a key source of environmental zoospores. The results from my thesis emphasize that studies focusing on the tadpole life stage should be of equal priority to those assessing postmetamorphic stages. Based on my findings, I strongly recommend that future studies include the larval life stage in field research and disease surveys.

CONCLUSION

My thesis research contributes to the growing knowledge of chytridiomycosis in tadpoles and calls for more accurate and more frequent assessment of larval anurans in *Bd* studies. Throughout my two chapters, I identified potential reservoir species at the tadpole stage by applying a highly sensitive method to detect and diagnose low level infections. I also placed the role of tadpoles as reservoir hosts into broader ecological context by comparing infection prevalence and intensity in tadpoles to adults across and within different species. My results have important implications in *Bd* research and in the field of wildlife conservation. I showed that the tadpole stage of common, non-endangered Neotropical species can harbour infections and therefore should be included in disease management actions. Furthermore, I proposed a new method for disease assessment in tadpoles that can be used in future research to more reliably estimate infection prevalence and to identify reservoir host species. Despite increasing recognition on the important role of tadpoles in *Bd* systems, infection response remains unknown for most species at this life stage. Addressing these knowledge gaps in *Bd* research, as I have done in this thesis, is critical to achieve a more complete understanding of disease dynamics within amphibian communities globally and to inform more thorough and effective conservation efforts amid ongoing amphibian declines.

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