



Fungal disease cluster in tropical terrestrial frogs predicted by low rainfall

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ABSTRACT

Anthropogenic forces are increasing climate anomalies and disease pressure in tropical forests. Terrestrial-breeding amphibians, a diverse group of highly endemic tropical frogs, have been experiencing cryptic population declines and extinctions, most of which have been retrospectively linked to climate anomalies and the fungal pathogen *Batrachochytrium dendrobatidis* (Bd). However, the spatiotemporal drivers of Bd infection in these species are unresolved. We tracked microhabitat conditions and Bd dynamics in terrestrial-breeding frogs in Brazil's Atlantic Forest over an annual cycle that coincided with a period of low rainfall compared to historical averages. An increase in Bd prevalence during the warm/wet season was attributable to pathogen spillover from co-occurring aquatic-breeding frogs. The deficit in rainfall compared to historical trends was the best predictor of spikes in Bd infection loads one month later and mortality among heavily infected frogs two months later. We suggest that hydrological stress may intensify seasonal pathogen amplification in direct-developing frogs, to an extent that may trigger localized disease clusters or potentially shift disease dynamics from enzootic to epizootic, even in areas with a relatively long history of host-pathogen coexistence.

1. Introduction

A growing number of wildlife and human diseases have been linked to anthropogenic climate change (Epstein, 2001). Most of these studies focus on the effects of temperature on disease emergence and transmission (Altizer et al., 2006), but shifts in rainfall regimes may also trigger outbreaks (Kohli et al., 2019; Rohr and Palmer, 2013). Shifts in rainfall and hydrology that create suboptimal microhabitat conditions may lead to stress that lowers host immune capacity (Kiesecker, 2010; Kohli et al., 2019; Rohr and Palmer, 2013; Rollins-Smith, 2017). In addition, altered hydrology may influence animal habitat selection, with downstream effects on direct transmission and encounters with environmental disease reservoirs (Burrowes et al., 2004). Drying trends in moist tropical forests are expected to intensify (Dai, 2013; Fu et al., 2013), with negative outcomes predicted for a large fraction of global biodiversity (Archaux and Wolters, 2006; Clark et al., 2016). However, the complex interplay between shifts in rainfall regimes and disease dynamics is still not well understood for many taxa, especially those that

exhibit a wide range of life history strategies.

Batrachochytrium dendrobatidis (Bd) is a waterborne pathogen of amphibians, and thus, rainfall variability is expected to affect host disease dynamics (Li et al., 2013; Ruggeri et al., 2018; Walls et al., 2013). The majority of frog population declines across the global tropics have been linked to the emergence of this chytrid fungus (Scheele et al., 2019). For example, temperature shifts from warm El Niño years to cool La Niña years triggered Bd outbreaks in Neotropical harlequin frogs (Rohr and Raffel, 2010), a trend attributed to suboptimal immunity in response to temperature variability (Greenspan et al., 2017; Raffel et al., 2015). These findings suggest that climate anomalies may be strong drivers of Bd outbreaks in amphibian populations.

Epidemiological studies of Bd have primarily focused on chytrid-related population declines and extinctions of aquatic-breeding amphibian species (Scheele et al., 2019). Yet, terrestrial-breeding frog species that complete their life cycle in the forest leaf litter (i.e., direct developers) have also experienced Bd-related population declines and extinctions (Burrowes et al., 2004; Carvalho et al., 2017; Catenazzi et al.,

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2017; Hirschfeld et al., 2016; Longo et al., 2013; Longo and Burrowes, 2010). This difference in research effort stems from observations of several high-visibility, Bd-linked amphibian population declines and extinctions of pond- and stream-associated species and the rationale that terrestrial hosts may be less threatened by a waterborne pathogen. However, the difficulty of detecting population fluctuations of less visible terrestrial breeders may be masking pathogen effects in these species.

The limited studies on Bd in terrestrial breeders suggest that this pathogen interacts with terrestrial breeders differently than with aquatic breeders. Terrestrial-breeding species are rarely exposed to water bodies, which may function as an environmental Bd reservoir (Wells, 2010). However, Bd is capable of surviving and spreading in moist terrestrial environments (Kolby et al., 2015; Raffel et al., 2015). This irregular exposure to the pathogen in terrestrial environments can translate to low Bd prevalence in wild populations of terrestrial-breeding species (Mesquita et al., 2017). Irregular exposure to Bd may also preclude terrestrial-breeding species from developing immune responses, both at the temporal scales of individuals (i.e., across life cycles) and populations (i.e., evolutionary time) (Ruggeri et al., 2018). Correspondingly, direct-developing amphibian species from Brazil experienced higher mortality rates than aquatic-breeding species when experimentally exposed to Bd (Mesquita et al., 2017). There is also evidence that terrestrial-breeding frogs are markedly sensitive to subtle shifts in precipitation, restricting feeding and reproductive activities in response to even a few consecutive days without rain (Stewart, 1995), which could influence immune mechanisms against pathogens (Rohr and Palmer, 2013; Rollins-Smith, 2017). These findings indicate that direct-developing amphibians may be particularly vulnerable to both Bd infection and shifts in rainfall.

To date, population collapses of terrestrial-breeding species have been retrospectively attributed to dry periods (Burrowes et al., 2004) and experimental work shows the potential for seasonal increases in Bd transmission in terrestrial-breeding frogs through pathogen spillover events from other species (Becker et al., 2019) and when terrestrial-breeding frogs clump together to conserve moisture in dry conditions (Longo et al., 2010). However, few long-term field studies have simultaneously tracked Bd infection patterns and climatic variables to resolve climate-disease relationships of terrestrial-breeding amphibian species (Longo et al., 2010).

Here, we tested for spatiotemporal drivers of Bd infection in direct-developing species, including rainfall, leaf litter moisture, temperature, and microhabitat structure. We recorded Bd prevalence and infection load in direct-developing frog species of two genera, *Brachycephalus* and *Ischnocnema*, over a complete annual cycle in an upland area of Brazil's Atlantic Forest. Given growing evidence that Bd spillover from aquatic-breeding frogs may drive Bd dynamics in direct-developing species, we predicted an upsurge in Bd infection prevalence and loads during the warm/wet (breeding) season, when frogs are most active. Since Bd is a waterborne pathogen, we also expected to find high Bd prevalence and infection intensity closer to streams. Our surveys overlapped with a period of exceptionally low rainfall in the region compared to historical averages, allowing for a particularly in-depth analysis of the possible links between rainfall anomalies and Bd infection dynamics in our focal direct-developing species. Collectively, our results shed light on the disease dynamics of enzootic host-generalist pathogens under projected climate change.

2. Materials and methods

2.1. Study site and species

We conducted our study at the Reserva Biológica Municipal Serra do Japi, a 20712-km² municipal biological reserve in the Atlantic Forest in the state of São Paulo, Brazil (−23.23°S, −46.96°W, 600–1200 m a.s.l.). Due to its relatively high elevation, it is one of the largest forest

fragments in the state of São Paulo. Serra do Japi is a semi-deciduous tropical forest with well-defined seasonality: a cool, dry period (April to September) with an average temperature of 17 °C and average rainfall of 72 mm/month and a warm, wet period (October to March) with an average temperature of 21 °C and average rainfall of 194 mm/month (Leitão-Filho, 1992). During the one-year sampling period, recorded leaf litter temperature ranged from 5 °C (cool/dry season) to 36 °C (warm/wet season) and averaged 14 °C (cool/dry season) to 21 °C (warm/wet season; Fig. S1a). Rainfall during the sampling period generally followed historical trends except in December (warm/wet season), when the recorded rainfall fell to less than half of the monthly average for the last six decades (59-year mean = 209 mm; 2018 mean = 101 mm; Fig. S1b) (Abatzoglou et al., 2018).

The five species of direct-developing frogs (Brachycephalidae and Craugastoridae) that occur at Serra do Japi are the pumpkin toadlet *Brachycephalus rotenbergae*, three species of the genus *Ischnocnema* (*Ischnocnema* sp. [aff. *guentheri*], *Ischnocnema* sp. [aff. *parva*], and *I. juipoca*), and *Haddadus binotatus*, the least abundant of the five species (Ribeiro et al., 2005). All of these species inhabit the forest leaf litter. *B. rotenbergae* is conspicuous (bright orange coloration) and diurnal. In contrast, the genera *Ischnocnema* and *Haddadus* contain cryptic (well-camouflaged) species that share similar crepuscular-nocturnal habits, reproductive season and environmental preferences (Ribeiro et al., 2005). The amphibian community at Serra do Japi also includes 26 aquatic breeding species. Many of these species, including *Rhinella ornata*, *Rhinella icterica*, *Proceratophrys boiei*, *Bokermannohyla luctuosa*, *Hylodes japi*, and *Scinax hiemalis*, occupy the same terrestrial microhabitats as the direct-developing species and congregate in humid areas and water bodies for reproduction and hydration (Ribeiro et al., 2005).

2.2. Study design

We established 25 sampling plots spaced at least 100 m apart in primary and mature secondary forest. Each plot was a circular area of 15-m diameter totalling 700 m². Twelve plots were adjacent to streams (first or second order) and 13 plots were at least 70 m away from streams. Plots were established along an elevation gradient (Gründler et al., 2012); thus, elevation accounted for spatial autocorrelation in our statistical analyses. We surveyed all plots approximately every two months from May 2018 to May 2019, totalling six 10-d field campaigns. Our sampling began in the cool/dry season and extended through a warm/wet season and into the beginning of the next cool/dry season. To account for imperfect host detection (see below and supplementary methods), we also selected 10 of the 25 plots with variable vegetation cover and high frog density (five plots adjacent to streams and five distant from streams) to survey on three consecutive days within field campaigns two through six (Dail and Madsen, 2011; DiRenzo et al., 2018; Zipkin et al., 2017).

2.3. Sampling protocol

To sample each plot, two observers searched for direct-developing frogs in the forest leaf litter for 1 h or until seven frogs were captured. We gently probed the leaf litter in order to find hidden animals. We temporarily placed each frog in a sterile plastic bag before processing. We rinsed each frog with 15 mL of distilled water to remove debris and swabbed the skin following a standard protocol (Hyatt et al., 2007). We released all frogs at the point of capture. Swabs were stored on ice and transferred to −20 °C within 4 h. We followed the same swabbing protocol for frogs that were found dead. We calculated the capture rate for each plot by dividing the number of animals captured by the survey duration in minutes.

2.4. Abiotic predictors of disease

We recorded daily rainfall from a rain gauge located 3 km from the

centre of the study area (Centro Integrado de Informações Agrometeorológicas), and calculated accumulated rainfall for each month of the study. For each month, we calculated deviation from historical rainfall based on the mean of monthly accumulated rainfall for the previous six decades (Abatzoglou et al., 2018). Specifically, for each month, we subtracted the historical mean accumulated rainfall from the accumulated rainfall of the sampled month, yielding negative values for dryer-than-average months and positive values for wetter-than-average months. Because Bd typically exhibits an incubation period of a few weeks to months (Berger et al., 2005), which has been documented in direct-developing species (Greenspan et al., 2018; Mesquita et al., 2017; Ribeiro et al., 2019), we also calculated two 'lagged' rainfall deviation metrics for each sampling month based on total rainfall deviation in the preceding one month and two months.

We used HOBO data loggers deployed at the centre of each plot to record temperature of the litter environment every 6 h for the study duration. We placed loggers 10 cm below the leaf litter surface to avoid direct sunlight. We used the recorded temperatures to calculate average daily and monthly temperatures for each plot. For average monthly temperatures, we calculated deviation from historical temperature using the mean of monthly average temperatures for the previous six decades (Abatzoglou et al., 2018).

During the last field campaign (May 2019), we measured leaf litter depth at 10 haphazardly selected points in each plot using a ruler. To estimate variation in leaf litter moisture among plots, we collected 1 L of leaf litter at the centre of each plot during the last sampling campaign. We then recorded leaf litter mass before and after dehydrating in a drying oven and subtracted the dry mass from the original mass. Measurements of leaf litter moisture and depth during the last sampling campaign allowed for a comparison of variation among plots. We obtained elevation data for each plot using a Garmin 62 s GPS unit.

2.5. Pathogen quantification

To extract DNA from skin swabs, we used the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA). To quantify Bd infection load, we used Taqman quantitative PCR assays (Boyle et al., 2004) with synthetic standards ranging from 10^2 to 10^6 gene copies (Integrated DNA Technologies, Coralville, Iowa, USA). Our metric of Bd infection load is thus based on number of ITS copies rather than number of zoospore genomic equivalents. We ran all samples in duplicate and mismatching samples in triplicate. We considered samples to be Bd-positive (Bd⁺) if >0 ITS copies were detected in two replicate reactions. Bd loads represent the average of the two replicate reactions.

2.6. Statistical analyses and modelling

We analysed temporal Bd infection data for 523 pumpkin toadlets (*B. rotenbergae*) and 225 *Ischnocnema* spp. (147 *Ischnocnema* sp. [aff. *guentheri*], 59 *Ischnocnema* sp. [aff. *parva*], and 19 *I. juipoca*). We grouped the *Ischnocnema* spp. for analyses because they exhibit similar environmental preferences and activity patterns relevant to disease dynamics. Furthermore, Bd prevalence and infection loads did not differ among the three *Ischnocnema* species (prevalence [logistic]: $\chi^2 = 2.889$, d.f. = 2, $P = 0.258$; infection loads [Gaussian]: $F_{[2,32]} = 0.460$, $r^2 = 0.027$, $P = 0.635$). We excluded *Haddadus binotatus* from analyses because they were spatially clustered, encountered infrequently (41 captures), and were rarely infected with Bd ($n = 2$).

We used two statistical approaches for our data analysis: generalized linear mixed models (GLMM) and a disease-structured generalized N-mixture model to account for imperfect host detection (DiRenzo et al., 2019). A detailed description of the N-mixture model can be found in the supplementary material. For the GLMMs, we used data from all 25 sampling plots. We used a binomial GLMM (logit link) with Bd prevalence as the response and a Gaussian GLMM (log link) with Bd infection loads (log₁₀-transformed ITS copy number; Bd⁺ individuals only) as the

response. We included the following explanatory variables in each GLMM and trimmed each model using a backward stepwise procedure based on AICc: elevation, stream proximity (adjacent vs. distant), accumulated monthly rainfall, monthly mean leaf litter temperature, monthly rainfall and temperature deviation (including 1-month and 2-month lagged deviations), leaf litter moisture, leaf litter depth, capture rate, and genus. For model reduction, we sequentially removed non-significant variables with the highest P -values until Delta AICc stopped dropping by the order of $\Delta AICc = 2$ (Burnham and Anderson, 2004; Hocking, 1976). For each GLMM, we included plot as a random effect and accounted for temporal autocorrelation (AR1 structure; package and function glmmTMB in Program R) (Brooks et al., 2017; R Core Team, 2019). For analysis of infection loads, we elected against zero-inflated models that include Bd-negative individuals because Bd-exposed and Bd-unexposed individuals are indistinguishable in field surveys. For comparative purposes, we also ran GLMMs with host species in place of genus, but species was not a significant predictor of Bd infection prevalence or loads and GLMM results remained unaltered (not shown).

We compared infection loads of dead vs. live Bd⁺ individuals of *B. rotenbergae* for the field campaign when we recorded the highest number of dead animals using a non-parametric Wilcoxon test. Lastly, we tested for independent effects of Bd infection load and rainfall deviation (including 1-month and 2-month lagged deviations) on the proportion of dead *B. rotenbergae* during the course of field sampling while accounting for temporal autocorrelation (AR1) and plot (random effect) using a GLMM with binomial family.

3. Results

The trimmed model for Bd prevalence included leaf litter moisture, genus, and elevation as predictors. In both *B. rotenbergae* and *Ischnocnema* spp., Bd prevalence primarily increased in the warm/wet season (Fig. 1a, b), consistent with our GLMM showing leaf litter moisture as a driver of Bd occurrence (Table 1). In addition, Bd prevalence was higher in *Ischnocnema* spp. compared to *B. rotenbergae* and higher at lower elevations (Table 1). However, we consider elevation to be a less important predictor of Bd prevalence because our study area covers a relatively narrow elevation gradient (738–1080 m). The N-mixture model revealed that pathogen prevalence was similar adjacent to and distant from streams, except for *B. rotenbergae* in December, when Bd prevalence adjacent to streams was higher than distant from streams (Fig. 1a; Table S1). Notably, rainfall in December was strikingly low compared to the historical monthly average (Fig. S1). Amphibian abundance was consistently higher adjacent to streams compared to distant from streams (Fig. 1e, f; Table S1). Detection probability was similar across disease states, regardless of stream proximity (Fig. S2; Tables S1, S2).

The trimmed model for Bd infection load included rainfall deviation and proximity to streams as predictors. Higher infection loads were best predicted by larger rainfall deficits during the month preceding sampling and closer proximity to streams (adjacent: mean \pm SD = 31 889 175 \pm 179 027 932 ITS copies; distant: mean \pm SD = 322 668 \pm 1 590 603 ITS copies; Table 1; Figs. S3, S4). Genus was not a significant predictor of Bd infection load, but when included in the best model to control for host effects, effects of rainfall deviation and proximity to streams remained statistically significant (Table 1).

We recorded nine dead ($n = 4$) or dying ($n = 5$) individuals of *B. rotenbergae* concentrated over four days during the fifth campaign (February 2019), the end of the warm/wet season (Fig. S5). Three carcasses were fresh and one carcass was in early stages of decomposition. Two dead individuals and the five dying individuals presented signs of chytridiomycosis including heavily sloughing skin, redness and anorexia, and the ill individuals died soon after swabbing procedures. Bd infection load was a strong negative predictor of survival ($\beta = -0.977$, $Z = -2.755$, $P = 0.006$; Fig. S6). Bd infection loads of dead individuals of

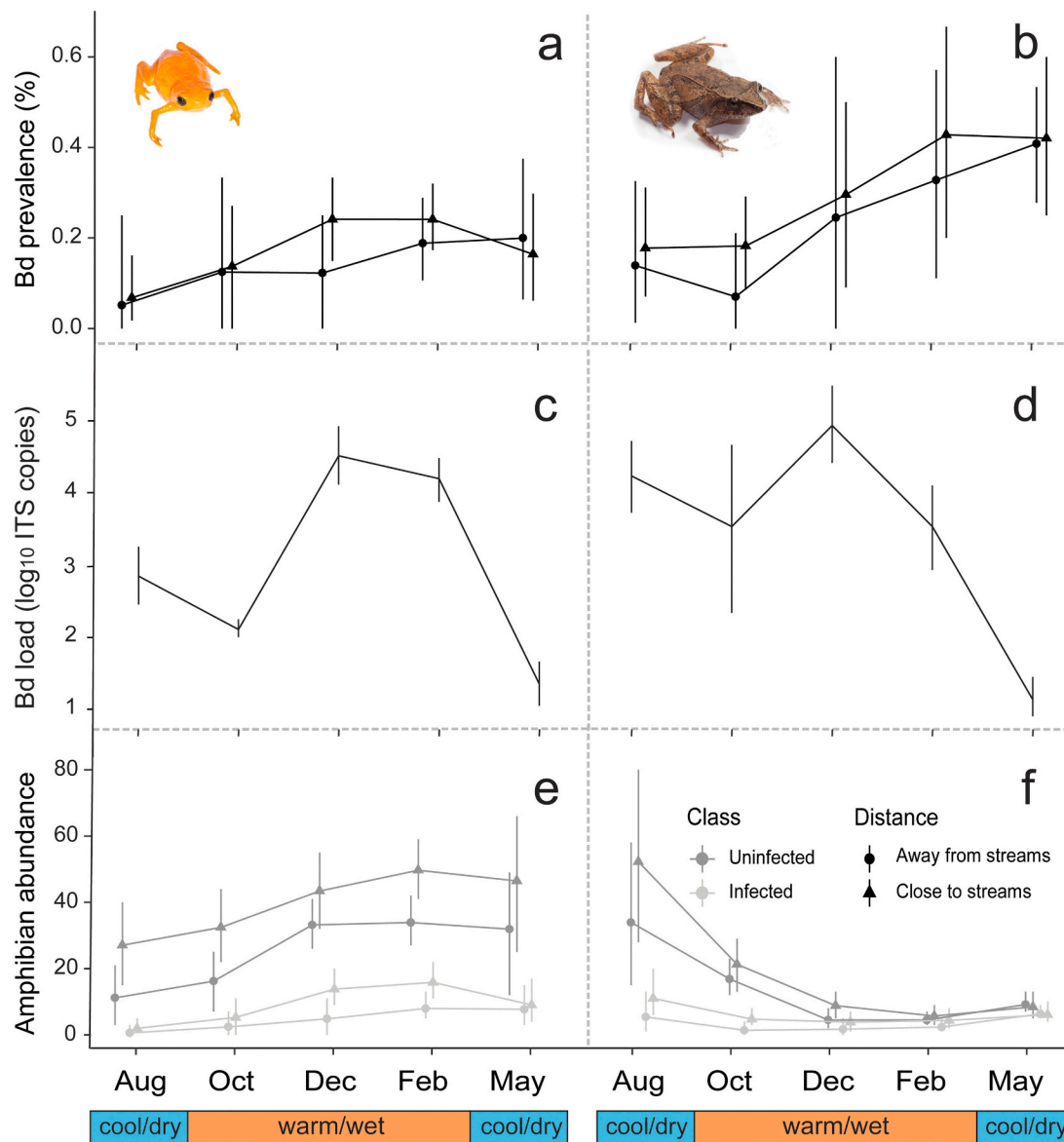


Fig. 1. Seasonal patterns in Bd infection and amphibian abundance. Average Bd prevalence (a, b), infection load (c, d), and amphibian abundance (e, f) for *B. rotenbergae* (left panels) and *Ischnocnema* spp. (right panels). Bd prevalence and amphibian abundance were estimated using a disease-structured generalized N-mixture modelling approach accounting for imperfect host detection. Error bars are 95% credible intervals (a, b, e, f) and standard errors (c, d). The first field campaign was excluded from the figure because imperfect host detection was not measured for this time point.

Table 1

Generalized Linear Mixed Models testing for environmental drivers of Bd prevalence and infection load in two genera of tropical direct developing anurans.

Predictors	Estimate	Std. Error	Z	p
Bd prevalence				
(Intercept)	8.432	2.965	2.844	0.004
Genus (<i>Ischnocnema</i>)	0.705	0.312	2.259	0.024
Elevation	-0.014	0.003	-4.167	<0.001
Leaf litter moisture	0.031	0.015	2.080	0.037
Bd infection load				
(Intercept)	2.628	0.533	4.928	<0.001
Rainfall deviation (1-month lagged)	-0.013	0.005	-2.443	0.014
Proximity to stream	0.742	0.375	1.980	0.047

Prevalence: Sampling area: Variance = 0.065, Times series: Variance = 1.966, Corr(ar1) = 0.79, N = 748, d.f. = 741; Bd infection load: Variance = 0.040, Times series: Variance = 0.561, Corr(ar1) = 0.81, N = 114, d.f. = 107.

B. rotenbergae were higher ($n = 7$; median = 1 389 181 ITS copies) than loads of asymptomatic Bd^+ individuals ($n = 27$; median = 2 175 ITS copies) during the field campaign (February 2019) when we recorded the highest number of dead animals ($\chi^2 = 9.274$, d.f. = 32, $P = 0.002$).

2-month lagged rainfall deviation was a predictor of survival (GLMM: $\beta = -0.015$, $Z = 1.909$, $P = 0.056$; N-mixture model: $\text{Pr}(\eta_1 > 0) = 0.98$, Figs. 2, S5, Table S2), although the intercept value was imprecise in the latter model (i.e., large variation in y-intercept values). 1-month lagged rainfall deviation did not predict survival.

4. Discussion

4.1. Pathogen spillover explains increased Bd prevalence in direct-developing frogs

Our focal direct-developing species were more likely to be infected with Bd in plots with high leaf litter moisture. In addition, amphibian abundance was higher adjacent to streams than further away. Direct-

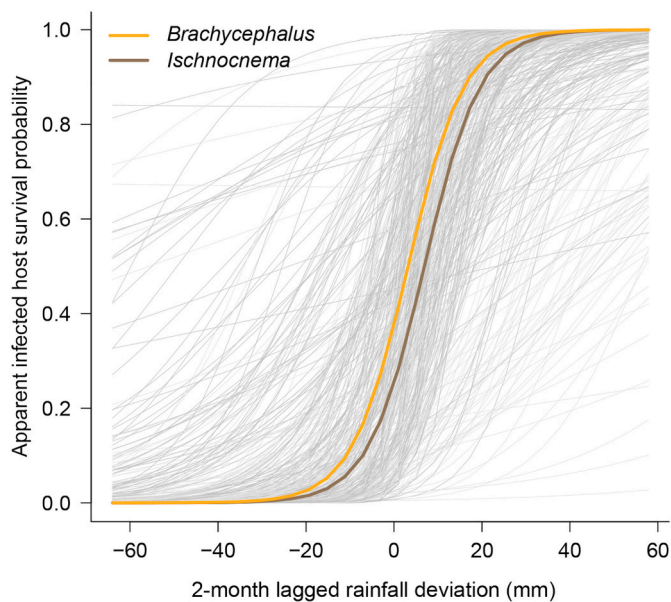


Fig. 2. Relationship between rainfall deviation lagged by two months and apparent infected host survival probability in *B. rotenbergae* (orange) and *Ischnocnema* spp. (brown). Thick coloured lines represent the predicted model mean for the relationship between apparent survival probability and rainfall deficit. For parameter estimates, see Table S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

developing species with fully terrestrial life cycles are infrequently in contact with water bodies. Thus, Bd exposure depends on transmission from moist leaf litter or direct contact with infected individuals (Kolby et al., 2015; Longo et al., 2010). Direct developers are likely to be more active under moist conditions when desiccation risk is relatively low (Stewart, 1995). Aquatic-breeding frogs are also most active under moist conditions during the warm/wet season, when they migrate to streams for reproduction and congregate at high densities (Becker et al., 2007). Because Bd is a generalist pathogen, broadly infecting both terrestrial and aquatic amphibian species (Valencia-Aguilar et al., 2015), these aquatic-breeding frogs are likely to be infected with Bd, with breeding aggregations representing a large source of infective zoospores (Becker et al., 2016; Longo et al., 2010). In addition to adult aquatic-breeding frogs foraging and migrating under these moist microclimatic conditions, recently metamorphosed individuals of aquatic-breeding species that emerge from streams may carry high Bd loads and shed zoospores into terrestrial habitats when dispersing (Kolby et al., 2015). We suggest that this heightened level of amphibian activity under moist conditions increased Bd prevalence in direct-developing species through pathogen spillover, referring to the direct or indirect transmission of Bd from one infected frog species to another amphibian species. Similarly, the high Bd loads observed in terrestrial frogs near streams could have been driven by pathogen amplification through spillover from nearby aquatic-breeding frogs. This is consistent with the finding that terrestrial-breeding *Brachycephalus pitanga* acquired lethal Bd infections from neighbouring aquatic-breeding species with mild Bd infections (Becker et al., 2019). Our study represents only one year of sampling coinciding with an anomalous period of low rainfall and leaf litter moisture was measured only once during the study. However, it is plausible that direct-developing frogs experience seasonal increases in Bd prevalence annually, coinciding with the warm/wet season when leaf litter moisture is most suitable for amphibian activity. A useful avenue for future research would be to quantify seasonal Bd abundance and viability in terrestrial environmental reservoirs.

While not directly related to our main results, we also found that direct-developing frogs were more likely to be infected with Bd at lower

elevations. Our study landscape of Serra do Japi is a relatively high elevation area (738–1018 m), where average daily leaf litter temperatures (14–21 °C) consistently varied within the optimal growth range for Bd (Muletz-Wolz et al., 2019; Piotrowski et al., 2004; Stevenson et al., 2013; Voyles et al., 2017), which could explain why micro-environmental temperature was not a strong correlate of disease. While Bd infection is typically positively associated with elevation, a pattern usually driven by temperature differences between warm lowlands and cool uplands, our study sites spanned a relatively narrow elevation gradient. Thus, the effects of elevation in our study were probably not driven by temperature. An alternative explanation is that drift of zoospores downstream created larger zoospore source pools at our lower elevation sites compared to our higher elevation sites (Sapsford et al., 2013). Another possibility is that the thermal optima of our focal direct-developing species are relatively low and that temperatures at our lower elevation sites vary farther outside this optimum range compared to temperatures at our higher elevation sites, which could give the pathogen a competitive edge at warmer low elevations (Cohen et al., 2017).

4.2. Infection spike in direct-developing frogs linked to anomalous rainfall deficit

Brachycephalus rotenbergae and *Ischnocnema* spp. were more likely to carry higher Bd loads when rainfall in the previous month fell below historical averages. Direct-developing frogs are sensitive to changes in rainfall and humidity because they largely depend on small volumes of leaf litter moisture (not water bodies) for hydration (Wells, 2010). Thus, dry periods may rapidly impose physiological stress directly or indirectly through changes in behaviour, such as reduced foraging to conserve moisture (Rohr and Palmer, 2013). Stress from dehydration may subsequently weaken immune responses of direct-developing frogs. For instance, environmental stressors including dehydration have been found to compromise the amphibian immune system by increasing secretion of corticosterone, which may alter patterns of white blood cell circulation in ways that are immunosuppressive (Kiesecker, 2010; Rollins-Smith, 2017). These changes could increase the likelihood that Bd will become established after initial infection and promote high rates of infection build-up (Kiesecker, 2010; Kohli et al., 2019; Rollins-Smith, 2017; Withers et al., 1988). Moreover, Bd infection may exacerbate water stress by interfering with water absorption through the skin, potentially setting off a positive feedback loop of Bd infection and dehydration stress that further destabilizes the immune system (Bovo et al., 2016; Savage et al., 2016; Voyles et al., 2012). We exercise some caution in attributing Bd infection spikes solely to anomalous rainfall patterns given the one-year time scale of our study but urge continued effort in this research direction in light of converging correlations detected with two robust statistical approaches.

Infection loads in direct developers could also increase in response to low rainfall if frogs move to moist refugia to avoid desiccation. This could increase frog density in retreat sites and subsequently trigger areas of high Bd infection or outbreaks through increased rates of direct pathogen transmission (Adams et al., 2017; Longo et al., 2010). The N-mixture model tested for changes in frog abundance over the annual cycle but found little support for links between rainfall and altered host spatial distribution. Rather, the model showed consistently higher frog abundance at stream-adjacent plots throughout the year. Direct-developing species are likely non-migratory and usually move relatively small distances during their life cycle (Donnelly, 1989). Thus, they may lack traits needed to locate and move to humid retreat sites that are distributed at spatial scales larger than their home ranges, especially when they are experiencing stress from dehydration. Instead, they may be adapted to remain relatively stationary even when environmental conditions within the home range become temporarily suboptimal (Rohr and Palmer, 2013), leaving them particularly vulnerable to other threats, including pathogens.

In addition to finding higher Bd infection loads associated with low rainfall in the previous month, the disease-structured generalized N-mixture model also detected an increase in Bd prevalence among *B. rosenbergae* at stream-adjacent plots in December, a month when rainfall fell to less than half of the monthly average for the last six decades, a pattern that was not detected in the generalized linear mixed models. Previous surveys have frequently documented relatively low Bd prevalence among direct-developing frogs when infection loads were relatively high (Gründler et al., 2012; Ruggeri et al., 2018). This pattern could arise if a proportion of diseased frogs spend more time in hidden refugia and less time foraging, making them more difficult to detect through visual surveys. Alternatively, we would also observe this pattern if infection is extreme and most exposed individuals die quickly from chytridiomycosis (Longo et al., 2010). Our results suggest that the sensitivity of disease-structured generalized N-mixture models may hold promise for detecting early stages of Bd outbreaks in the most vulnerable host species. This method is specifically relevant for cryptic species with low Bd prevalence in the wild.

4.3. Mortality of direct-developing frogs followed infection spike

We observed morbidity and mortality in nine heavily infected *B. rosenbergae* individuals concentrated over four days at the end of the warm/wet season. This finding, combined with recent experimental work focusing on Bd infection dynamics in *Brachycephalus pitanga* (Becker et al., 2019), strongly suggests that the observed mortality was driven by chytridiomycosis (Van Rooij et al., 2015). Both linear and N-mixture models suggest that survival was strongly correlated with rainfall anomalies two months earlier. Based on our results, we suggest that Bd infections increased over the course of several weeks, to an extent that caused mortality one month after the spike in infection loads, consistent with the typical incubation period for Bd in *Brachycephalus* spp. and *Ischnocnema* spp. (Becker et al., 2019; Greenspan et al., 2018; Mesquita et al., 2017; Ribeiro et al., 2019). Recording Bd-induced amphibian mortality in real time is extremely rare because mortality usually follows soon after disease signs are exhibited, and dead frogs decompose or are consumed by scavengers quickly, especially in tropical climates. *Ischnocnema* spp. showed higher Bd prevalence than *B. rosenbergae* and the N-mixture model detected a decrease in *Ischnocnema* spp. abundance matching an increase in Bd prevalence. While we only directly observed mortality of *B. rosenbergae*, a visually conspicuous, bright orange species, *Ischnocnema* spp. are camouflaged, which would dramatically reduce the likelihood of finding dead individuals in the wild. Thus, it is likely that the low number of dead individuals that we recorded represents only a small fraction of the actual number of animals affected by chytridiomycosis.

4.4. Population stability of direct-developing frogs under global change

Our findings suggest that direct-developing species may be particularly vulnerable to synergistic interactions between Bd and rainfall anomalies. Specifically, both Bd infection intensity and mortality were linked to a rainfall deficit. Collectively, these results raise the possibility that synergistic interactions between altered hydrology and Bd infection may be a dominant driver of the population crashes among direct-developing frogs that have occurred across disparate geographic areas (Burrowes et al., 2004; Carvalho et al., 2017; Catenazzi et al., 2017; Hirschfeld et al., 2016; Longo et al., 2013). We suggest that anthropogenic climate change may accelerate disease-linked population declines of Brachycephaloidea, a Bd-susceptible (Mesquita et al., 2017) and biodiverse amphibian clade that contains over 1100 described species, of which the vast majority are highly endemic, cryptic and understudied species that could be suffering silent population declines and extinctions in the wild (Frost, 2020).

Anthropogenic pressures across the planet are undeniably altering Earth's climate (Narisma et al., 2007). In Brazil, rapid deforestation in

the Amazon rainforest is directly modifying rainfall patterns, including in the Atlantic rainforest (Boers et al., 2017; Leite-Filho et al., 2019). Retrospective studies have linked several historical amphibian declines in the Atlantic forest to Bd, seven of which were recorded in *Brachycephalus* and *Ischnocnema* populations (Carvalho et al., 2017; Rebouças et al., 2021). Similarly, another population of *B. rosenbergae* has not been observed since the mid-1990's at Pico das Cabras, in the municipality of Campinas, about 40 km from Serra do Japi, in spite of directed searches, although it is debatable if that population (from Pico das Cabras) declined due to Bd (Nunes et al., 2021). In addition, the direct-developing Atlantic forest species *Holoaden bradei* was last seen in the wild in the late 1970s, coinciding with an upsurge of Bd in aquatic breeders (Carvalho et al., 2017) in the same mountain corridor during a period of global climatic variability (Malhi and Wright, 2004). Our study includes the first record of clinical chytridiomycosis in a natural environment in Brazil and raises the possibility that certain environmental conditions may shift Bd dynamics in the Atlantic forest toward localized but potentially lethal spikes in Bd infection, despite the relatively long history of host-pathogen coexistence in this region (Becker et al., 2014; Carvalho et al., 2017).

Our results are consistent with a scenario in which increases in Bd prevalence among direct-developing frogs may be a relatively stable, seasonal phenomenon attributable to a tolerable level of pathogen spillover. In contrast, the observed deficit in rainfall compared to historical trends appeared to function as a stochastic event that pushed the population beyond a tolerance threshold. Multi-year studies comparing infection patterns over several annual climatic cycles are needed to confirm these inferences (Catenazzi et al., 2011; Clare et al., 2016; Knapp et al., 2011; Rohr and Raffel, 2010; Zumbado-Ulate et al., 2014). However, single-year studies have offered compelling data on climate-Bd relationships (Whitfield et al., 2012) and may serve as critical calls to action when disease-induced population crashes can occur on a timescale of months (Channing et al., 2006; Weldon et al., 2020).

Our findings suggest that low rainfall may intensify seasonal infection spikes in direct-developing frogs, to an extent that may trigger localized areas with higher rates of disease or even Bd-induced die-offs. Susceptible animals, such as direct-developing species, may be on the front line of outbreaks, since they experience more intense disease burdens than other species. The combination of year-round Bd pressure and low resistance to the fungus (Mesquita et al., 2017) suggests that climatic conditions in high-elevation areas may be hovering just below a tipping point for Bd outbreaks among direct-developing anurans. We reinforce the need to further study disease dynamics of tropical terrestrial-breeding amphibian species, including long-term field surveillance, their role in community-level disease dynamics and impacts of climatic variability on persistence of this neglected and hyper-diverse amphibian clade.

CRedit authorship contribution statement

DMC and CGB designed the study. All authors carried out the study. DMC, SEG, GVD, and CGB analysed the data. DMC drafted the manuscript. All authors critically revised the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data accessibility

Data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics

All experimental procedures were approved by the University of Campinas Animal Ethics Committee (CEUA #4744-1/2017, #5440-1/2019), the Instituto Chico Mendes de Conservação da Biodiversidade (SISBio #61220-2, #27745-17), Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SISGen #A0B0E76), and Fundação Serra do Japi.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biocon.2021.109246>.

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