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Fungal pathogen infection intensity associated with reproductive mode and elevation in an afrotropical anuran community

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The effects of host ecology and environmental conditions on infection dynamics of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) have been documented in several tropical and montane regions across the globe. These interactions are often complex and unique at local scales. Data on the historical and current chytridiomycosis-amphibian system is largely lacking in Africa, especially central Africa. We conducted the first survey of *Bd* in continental Equatorial Guinea in 2019, and extensively sampled the land-bridge island, Bioko, in 2011 and 2019. Our results provide novel information on the distribution and prevalence of *Bd* in Equatorial Guinea and demonstrate the effects of host developmental mode and elevation on *Bd* infections. We found that *Bd* infection loads are positively linked with elevation and that direct-developing species have higher average *Bd* loads than aquatic breeding species at all elevations. These patterns corroborate previous findings in afrotropical amphibian assemblages. Additionally, our study confirms the presence of *Bd* in continental Equatorial Guinea, providing updated information on the distribution of *Bd* in central Africa, and confirming the need for careful monitoring of this biodiverse region.

Keywords: Bioko, Equatorial Guinea, *Bd* infection load, amphibian conservation

Los efectos de la ecología del huésped y las condiciones ambientales en la dinámica de infección del hongo quitridio anfibio *Batrachochytrium dendrobatidis* (*Bd*) se han documentado en varias regiones tropicales y montañosas de todo el mundo. Estas interacciones suelen ser complejas y únicas a escala local. Los datos sobre el sistema quitridiomycosis-anfibios histórico y actual faltan en gran medida en África, especialmente en África central. Realizamos la primera encuesta de *Bd* en Guinea Ecuatorial continental en 2019, y un muestreo extenso de la isla Bioko en 2011 y 2019. Nuestros resultados brindan información novedosa sobre la distribución y prevalencia de *Bd* en Guinea Ecuatorial y demuestran el efecto del modo de desarrollo del huésped y la elevación de las infecciones por *Bd*. Descubrimos que las cargas de infección por *Bd* están relacionadas positivamente con la elevación y que las especies en desarrollo directo tienen cargas promedio de *Bd* más altas que las especies acuáticas reproductoras a lo largo de la elevación. Estos patrones corroboran hallazgos previos en conjuntos de anfibios afrotropicales. Además, nuestro estudio confirma la presencia de *Bd* en Guinea Ecuatorial continental, proporcionando información actualizada sobre la distribución de *Bd* en África Central y confirmando la necesidad de un seguimiento cuidadoso de esta región biodiversa.

INTRODUCTION

Amphibian declines over the past three decades have impacted nearly 30 % of all amphibian species on Earth (Stuart et al., 2004). Among the leading causes for the observed amphibian declines is the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), a species of chytrid fungus that causes the infectious skin disease chytridiomycosis (Berger et al., 2005; Lips et al., 2006;

Longcore et al., 1999). A generalist pathogen, *Bd* has affected amphibians in several environments and, while the effects of *Bd* on amphibian communities have been studied intensely in the New World, Europe and Australia, data on *Bd* prevalence and the impact of chytrid on African amphibian communities is sparse (Doherty-Bone et al., 2013; Hirschfeld et al., 2016; Lips, 2016; Scheele et al., 2019; Zimkus et al., 2020). *Bd* was first reported from the African continent in the early 2000s from contemporary field survey data and from

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museum-preserved specimens originally collected in the 1930s (reviewed in Zimkus et al., 2020). *Bd* has now been documented across south, central, and east Africa, as well as Madagascar and the Gulf of Guinea Islands, but has not yet been documented in the Seychelles or West Africa (Zimkus et al., 2020). At least two lineages of *Bd*, *Bd*-CAPE and *Bd*-GPL, are present in Africa, but the current ranges of these genotypes have not been fully documented (Byrne et al., 2019). Here, we performed the first survey of *Bd* in continental Equatorial Guinea and conducted extensive sampling on Bioko Island, from which only museum specimen-based surveys of *Bd* have previously been conducted (Hydeman et al., 2017).

Equatorial Guinea is situated in the Lower Guinean rainforests, which extend into the Gulf of Guinea archipelago (Bioko, Príncipe, São Tomé, and Annobón). Mainland Equatorial Guinea (also known as Río Muni) is characterised by a tropical, hot and humid climate with fairly homogeneous lowland rainforest habitats that are home to at least 66 species of amphibians (Sánchez-Vialas et al., 2020). Bioko Island (formerly known as Fernando Po) is a land bridge island composed of three volcanic peaks reaching up to over 3000 m elevation. This complex topography, along with a precipitation gradient ranging from 1930 mm annual precipitation in the north to upwards of 10,000 mm in the south, result in a patchwork of vegetation types including lowland rainforest (up to 800 m elevation), montane forest (800–1400 m), Schefflera forest/mossy forest (1400–2600 m), and montane heath (2600–3000 m; Fa, 1991). Throughout its history, Bioko has been connected to continental Africa during glacial periods and thus shares most of its 44 reported amphibian species with the adjacent mainland (Sánchez-Vialas et al., 2020). Hydeman et al. (2017) detected *Bd* in museum specimens collected on Bioko Island between 1966 and 1998; however, they did not report infection intensity – a proxy for disease severity – because the effects of preservation on estimating pathogen loads from museum specimens are poorly understood. Consequently, variation in infection intensity across species and habitats has not yet been characterised.

The anuran communities of Río Muni and Bioko Island include representatives of ten families and both aquatic breeder (AB) and terrestrial direct-developer (DD) host life histories. Studies in other amphibian communities indicate that a given individual's response to *Bd* infection can be dependent on the ecological and/or evolutionary background of host species (Valencia-Aguilar et al., 2016; Lips, 2016; Mesquita et al., 2017). For example, neotropical studies have shown significant effects of host life history in *Bd* prevalence and patterns of infection intensity (Brem & Lips, 2008; Gründler et al., 2012; Mesquita et al., 2017). Likewise, in the Neotropics, enzootic infection patterns present as increased *Bd* prevalence and infection loads at higher elevations, leading to increased risk of anuran declines at upland sites (Lips, 1999; Lips et al., 2003; Brem & Lips, 2008; Becker & Zamudio, 2011; Gründler et al., 2012). Thus, variation in both life history and habitat

(largely structured by elevation) may also influence *Bd* prevalence and infection intensity in afrotropical amphibian communities.

Here, we performed the first survey of *Bd* in continental Equatorial Guinea and conducted comprehensive sampling on Bioko Island. Our field study included samples from Rio Campo Natural Reserve (sea level to ~60 m elevation) and Monté Alen National Park (~670 m elevation) on the mainland, and from sea level to over 1900 m elevation on Bioko Island. We tested whether *Bd* prevalence and infection loads were influenced by elevation and if they varied among host species identity and life histories. We hypothesised that aquatic-breeding (AB) species and upland amphibian communities would have higher *Bd* prevalence, and that infection loads would be higher in species with direct-development (DD) and in upland terrestrial environments. Our results provide novel information on *Bd* prevalence in Equatorial Guinea, support the effect of elevation and life history on *Bd* infection patterns (Brem & Lips, 2008; Gründler et al., 2012), and inform future studies of *Bd* in west and central Africa.

MATERIALS & METHODS

Geographic and taxonomic sampling

We conducted amphibian surveys during 19 August–12 September 2011 on Bioko Island (Bioko Sur province), and 11–30 April 2019 on Bioko Island (Bioko Sur) and two provinces in continental Equatorial Guinea (Centro Sur and Litoral; Fig. 1). Our sampling on Bioko Island included a range of habitats including lowland forest, agricultural fields, montane forest, and Schefflera forest/mossy forest, spanning elevations from sea level to > 1900 m. Our sampling in continental Equatorial Guinea included lowland forest and wetlands in the Rio Campo Nature Preserve, just above sea level, and agricultural fields at the edge of Monté Alen National Park ~650 m elevation. Sampling for Bioko Sur was more comprehensive, with a combined six weeks of surveys whereas the two continental sites were sampled for just two nights at each site. Our combined sampling includes a total of 455 individual amphibians (Bioko Island = 377, Centro Sur = 38, Litoral = 40) consisting of 43 species and 10 families of frogs. Very few amphibian surveys have been conducted in Equatorial Guinea and thus our understanding of amphibian species diversity is likely incomplete, but we estimate that our sampling represents ~60% of the Bioko amphibian community and 40% of the Río Muni amphibian community (Sánchez-Vialas et al., 2020). Species identifications were made in the field by RCB, PJM and LAS with some tentative identifications indicated by “cf.”. Based on field guides and primary literature, we classified species according to their larval life histories: aquatic breeders (AB) or terrestrial direct-developers (DD). These reproductive modes are largely fixed within the genera we sampled and therefore our tentative identifications for some specimens should not affect the AB or DD classification (Portik & Blackburn, 2016; Channing & Rödel, 2019).

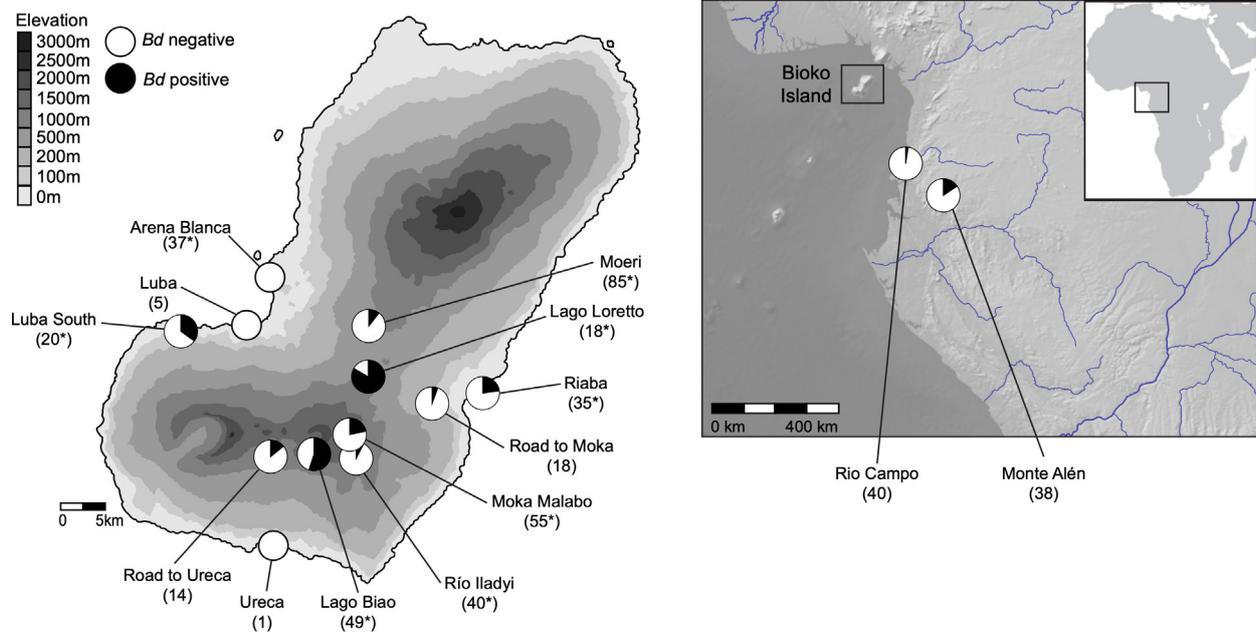


Figure 1. Sampling localities and *Bd* prevalence in Equatorial Guinea. The sample size of amphibians swabbed per site is indicated in parentheses. Asterisks denote sites sampled in both 2011 and 2019.

We conducted surveys in the evening and captured frogs by hand, placing them in individual plastic bags until processing. We collected epithelial samples from post-metamorphic individuals with sterile fine-tip swabs (Medical Wire & Equipment Co. MW113) following the methods of Hyatt et al. (2007). Swabs were stored in 95 % EtOH and kept as cool as possible in the field and then stored at -80°C until processing. The swabbed individuals were euthanised, prepared as voucher specimens (Table S1), and deposited at the California Academy of Sciences (CAS), Cornell University Museum of Vertebrates (CUMV), North Carolina Museum of Natural Sciences (NCMS), and US National Museum of Natural History (USNM). All research was conducted with IACUC approval (Cornell University 1999-0010, Drexel University 18748, Smithsonian Institution National Museum of Natural History 2016-09).

Molecular analyses

Prior to DNA extraction, we placed the sample tubes in a SpeedVac to evaporate excess ethanol. We performed DNA extractions using the PrepMan Ultra kit (Applied Biosystems). To measure *Bd* prevalence and infection loads, we used a 1:10 dilution of the extracted DNA samples for qPCR analysis in duplicate plates. Samples collected in 2011 were analysed via zoospore genomic equivalents (GE) and ViiA 7 software (Applied Biosystems). Standard curves ranging from 0.1 to 1000 zoospores were generated from templates of known zoospore concentrations of *Bd* strain JEL427 (following Hydeman et al., 2017). Analyses of samples collected in 2019 are a measure of ITS copies based on synthetic standards, not zoospore genomic equivalents. We used primers ITS-1 and 5.8s, Taqman reagents, and synthetic standards (102 to 106 ITS copies) in the QuantStudio™ 3 system to amplify rRNA regions of *Bd* (Boyle et al., 2004).

Statistical analyses

We used Generalised Linear Models (GLMs) to test whether elevation, host species identity, and life history (aquatic breeding vs. direct-developing) were significant predictors of *Bd* prevalence (GLM with binomial distribution and logit link) and log-transformed *Bd* infection loads (GLM with normal distribution and identity link; *Bd*-positive samples only). The most parsimonious model for *Bd* prevalence and *Bd* infection loads were chosen based on AICc and we confirmed normality of residuals for each of our models using the Anderson-Darling test. Due to the different methods of qPCR quantification for the 2011 and 2019 surveys, estimates of infection intensity across years were not combined in the main reported models.

RESULTS

Of the 455 frogs sampled across 2011 and 2019, 91 individuals tested positive for *Bd*, resulting in a 20.0 % (Clopper-Pearson 95 % confidence interval [hereafter CI] = 16.58–23.92 %) global prevalence for our Equatorial Guinea samples. Of the 321 aquatic breeding individuals sampled on Bioko Island, 72 tested positive for *Bd* [prevalence = 22.4 %, CI = 18.21–27.30 %]. Of the 56 direct-developing individuals sampled on Bioko Island, 12 tested positive for *Bd* (prevalence = 21.4 %, CI = 12.71–33.82 %). Of the 75 aquatic breeding individuals sampled in Rio Muni, 6 tested positive for *Bd* (prevalence = 8.0 %) and of the 3 direct-developing individuals, 1 tested positive for *Bd* (prevalence 33.3 %). For more detailed information on 2011 Bioko Island, 2019 Bioko Island, 2019 Rio Muni (mainland) and total Equatorial Guinea prevalence and load data, see Table 1.

For the 2019 Bioko sampling season, the best AICc model pointed to a significant effect of elevation linked

Table 1. Summary table of host life history (AB - aquatic breeder, DD - direct-development), sample size, *Bd* prevalence, and *Bd* infection load data for 2011 Bioko, 2019 Bioko, 2019 Rio Muni and total Equatorial Guinea data surveys. 2011 *Bd* load quantification is a measure of zoospore genomic equivalents (GE), while 2019 *Bd* load quantification is a measure of ITS copies based on synthetic standards.

	Life history	Sample size	Prevalence (%)	Mean load (positives only)	Min, max loads (positives only)	Median load (standard deviation)
2011 Bioko		187	13.9	1.76	0.04, 7.36	1.03 (1.95)
	AB	163	14.1	1.79	0.04, 7.36	0.99 (2.07)
	DD	24	12.5	1.53	0.89, 2.41	1.29 (0.79)
2019 Bioko		190	30.5	332.10	2.34, 2368.13	35.27 (617.44)
	AB	158	31.0	281.49	2.34, 2368.13	29.17 (579.48)
	DD	32	28.1	607.67	9.18, 2005.05	41.05 (774.85)
2019 Rio Muni		78	9.0	139.52	3.13, 841.35	13.12 (311.25)
	AB	75	8.0	22.55	3.13, 96.06	9.62 (36.31)
	DD	3	33.3	841.35	841.35	841.35
All		455	20.0	222.90	0.04, 2368.13	10.42 (519.86)
	AB	396	19.9	179.09	0.04, 2368.13	7.15 (476.85)
	DD	59	22.0	485.76	0.89, 693.23	31.62 (693.23)

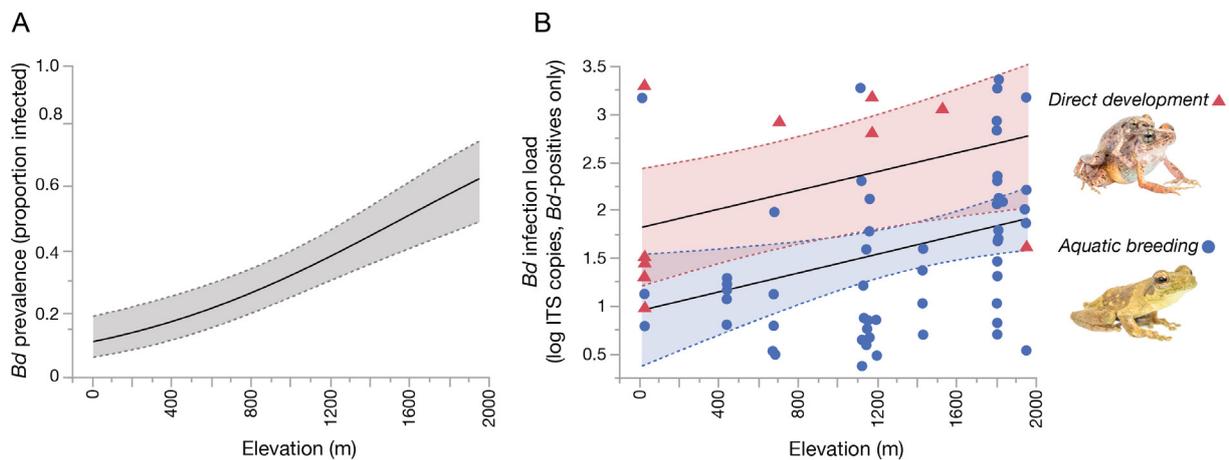


Figure 2. Effect of elevation (m) on both *Bd* prevalence (**A**; logistic fit) and infection intensity (**B**; gaussian fit) using data from the 2019 Bioko sampling season. 95 % confidence intervals are highlighted around the means (solid lines). Independent regression lines are shown for aquatic-breeding and terrestrial developing species in panel B.

to an increase in *Bd* prevalence (Whole Model Test: $\chi^2 = 31.955$, $df = 1$, $\beta = 0.001$, $AICc = 207.210$, $p < 0.0001$; Fig. 2A). Host species identity had a significant effect on *Bd* prevalence when included as a single explanatory variable ($\chi^2 = 68.845$, $df = 22$, $AIC = 220.227$, $p < 0.0001$), but this model had poorer $AICc$ fit. Elevation and host life history were included in the most parsimonious data explaining *Bd* infection loads in the 2019 Bioko sampling data (whole model test: $F_{[2,55]} = 4.614$, $r^2 = 0.143$, $AICc = 150.467$, $p = 0.014$). In this model, elevation had a positive relationship with *Bd* infection loads ($\beta = 0.001$, $t = 2.54$, $p = 0.014$; Fig. 2B), and direct-developing frogs carried higher average *Bd* infection loads than aquatic breeders (Fig. 2B). When we included host species identity in our models instead of host life history (whole model

test: $F_{[12,45]} = 2.561$, $r^2 = 0.405$, $AIC = 158.284$, $p = 0.011$), elevation remained a significant positive predictor of *Bd* loads ($\beta = 0.001$, $t = 2.89$, $p = 0.006$), but the model had poorer fit according to delta $AICc$. Although these relationships were stronger in the 2019 Bioko sampling data, the same patterns were found with combined 2019 Bioko and Rio Muni data (Prevalence: $\chi^2 = 49.172$, $df = 1$, $\beta = 0.001$, $p < 0.0001$; Infection Loads: $F_{[2,264]} = 31.891$, $r^2 = 0.195$, $p = 0.0001$). Models for 2011 Bioko sampling data did not reveal significant effects of life history, species identity or elevation on *Bd* prevalence, whereas host identity was a single strong predictor of *Bd* infection loads ($F_{[10,15]} = 3.951$, $r^2 = 0.725$, $p = 0.008$). Models using our Rio Muni data alone detected an effect of elevation on *Bd* prevalence ($\chi^2 = 4.548$, $df = 1$, $\beta = 0.002$, $p < 0.033$),

and an effect of host life history on *Bd* infection loads, with direct-developing species carrying higher infections ($F_{[5,1]} = 10.346$, $r^2 = 0.674$, $p = 0.023$), although sample size was limited.

DISCUSSION

We found a positive association between *Bd* prevalence, infection intensity, and elevation in amphibian communities on Bioko Island and mainland Equatorial Guinea. Significant effects of elevation on infection dynamics have been observed in several amphibian communities around the world. For instance, there are several documented *Bd*-driven population declines at high elevation sites in both tropical and temperate regions (Vredenburg et al., 2010; Walker et al., 2010; Catenazzi et al., 2011; Carvalho et al., 2017), and host elevational range predicted decline probability in a neotropical amphibian community (Lips et al., 2003). Likewise, several studies report increased *Bd* prevalence at high elevation sites (Brem & Lips, 2008; Kielgast et al., 2010; Piovia-Scott et al., 2011; Gründler et al., 2012) and increased *Bd* infection loads at higher elevation (Gründler et al., 2012). Our results indicate that elevation may also play an important role in *Bd* dynamics within Equatorial Guinea's amphibian communities.

One framework for explaining why amphibians at higher elevations have higher pathogen loads is the thermal mismatch hypothesis, in which hosts are predicted to be more susceptible to parasites when environmental conditions move away from the host's ideal thermal range (Cohen et al., 2017). In particular, amphibian exposure to warmer temperatures and temperature variability is known to reduce physiological fitness (Rome et al., 1992; Longhini et al., 2021), and increase disease susceptibility and mortality (Raffel et al., 2006; Rohr & Raffel, 2010; Neely et al., 2020). Tropical amphibians, which live at consistently higher temperatures than temperate amphibians, are generally more sensitive to temperature changes associated with global warming (Deutsch et al., 2008; Duarte et al., 2012) and have a narrower thermal range (Rohr et al., 2018). Additionally, several studies demonstrate that high elevation amphibians are more susceptible to temperature variability than low elevation amphibians (Kissel et al., 2019; Cohen et al., 2019). Thus, tropical, high elevation amphibian communities are particularly prone to a thermal mismatch as the climate warms. Furthermore, multiple studies have shown that as global temperatures continue to rise, *Bd*'s range will shift to cooler, higher elevations (Pounds et al., 2006; Cohen et al., 2019) that better match its optimal growth conditions (Woodhams et al., 2008; Voyles et al., 2012). Though *Bd* is less lethal under warmer environmental conditions (Fisher et al., 2009), pathogens generally have larger thermal performance curves than hosts, and thus if the temperature is under the lethal limit for the pathogen, a thermal mismatch will generally favour the pathogen over the host (Cohen et al., 2017; Rollins-Smith, 2017). Correspondingly, Neely et al. (2020) found

higher mortality of cool-adapted frogs when both broad thermal range and high elevation, cool-adapted frogs were exposed to *Bd* in a simulated warming event. The combination of more optimal *Bd* growth conditions at higher elevations and the suboptimal immunity found in hosts experiencing a thermal mismatch may explain why high elevation Equatorial Guinea amphibian communities have higher *Bd* infection loads.

Our results support a significant effect of host developmental mode on *Bd* infection patterns. We found that species with direct-development exhibit higher *Bd* infection loads (considering only infected frogs) than aquatic breeders. However, both aquatic breeders and direct-developers showed surprisingly similar *Bd* prevalence, indicating that direct-developing frogs respond differently to *Bd* once they become infected. Differing host ecology and *Bd* epizootiology are known in other tropical amphibian communities. For instance, Gründler et al. (2012) documented similar findings where infection loads were significantly higher in terrestrial breeding species than lotic breeding species, and Mesquita et al. (2017) found that direct-developers acquired higher loads and had higher mortality than aquatic breeders after exposure to *Bd* in a lab experiment. Both studies were conducted with frogs from Brazil's Atlantic Forest where *Bd* is considered enzootic (Gründler et al., 2012; Rodriguez et al., 2014). This pattern of direct-developing species afflicted with *Bd* in enzootic conditions has also been documented in other tropical communities by Burrowes et al. (2004), Catenazzi et al. (2017) and Moura-Campos et al. (2021). In epizootic stages, however, aquatic breeding rather than direct-developing species show conspicuous declines, and this pattern shifts during the subsequent enzootic stage (Lips et al., 2003; 2006; Kriger & Hero, 2007; Brem & Lips, 2008; Catenazzi et al., 2011). Surviving aquatic breeding populations may subsequently become enzootic reservoirs of *Bd* for less resistant direct-developing species (Scheele et al., 2017; Catenazzi et al., 2017).

Terrestrial direct-developing species typically do not congregate around water bodies during the breeding season (Rowley & Alford, 2007); consequently, they are in contact with water less frequently than aquatic breeding species and are not exposed to *Bd* during developmental larval stages. As a result, it appears they may experience weaker selection for *Bd* resistance than aquatic breeding species (Mesquita et al., 2017). For instance, Becker et al. (2019) found that even low loads of *Bd* spillover from aquatic breeding species resulted in significantly higher *Bd* infection loads and increased mortality of a direct-developing species. Thus, in enzootic conditions direct-developing frogs may still be susceptible, and low load pathogen spillover in the environment and direct heterospecific transmission can result in chytridiomycosis and die-offs, even in relatively stable environments (Catenazzi et al., 2017; Becker et al., 2019). These dynamics may further be exacerbated by seasonal variation and changing environmental stressors. For instance, although our temporal sampling is limited,

we found differences in both *Bd* prevalence and infection load between our 2011 (August–September; beginning of long wet season) and 2019 (April; short wet season) sampling periods, suggesting that both prevalence and infection load may vary seasonally and interannually in these communities. Drought conditions and even seasonal dry periods cause host behavioural changes in some direct-developing species that result in increased infection loads and mortality (Longo & Burrowes, 2010; Longo et al., 2010; Ruggeri et al., 2018; Moura-Campos et al., 2021). It is unclear whether similar dynamics are at play in afrotropical communities but the strong patterns we detected, particularly on Bioko Island, present an ideal setting for investigating spillover, seasonal variation, and disease risk in afrotropical direct-developers.

Currently, it is unclear whether *Bd*-related declines are occurring in afrotropical amphibian communities. Populations of high elevation spray toads in Tanzania began experiencing sharp declines in 2012, eventually becoming extinct in the wild. However, altered environmental conditions, as well as *Bd*, could have been contributing factors (Makange et al., 2014; Weldon et al., 2020). Studies in Cameroon originally failed to detect *Bd* in highland amphibian populations, but more recent studies have confirmed the presence of *Bd* and suggest a trend of *Bd*-related declines correlated with higher elevation (Baláž et al., 2012; Doherty-Bone et al., 2013; Hirschfeld et al., 2016). It is also unclear whether the presence of *Bd* in Cameroon, Equatorial Guinea, and other afrotropical communities is endemic versus novel or if these amphibian populations are naïve to *Bd*. Additionally, our understanding of *Bd* strains in central Africa is still quite limited, including information on genotypic diversity, current ranges, virulence, whether these strains are native or introduced, and whether co-infection and hybridisation occur (Zimkus et al., 2020). We have not observed die-off events or clear declines in Equatorial Guinea, but our results highlight the potential susceptibility of direct-developing species and high elevation communities. By focusing monitoring efforts on these high-risk amphibian communities, we will begin to better understand the role of host ecology, pathogen ecology, climate change and variability, and afrotropical environmental conditions on Central African *Bd* dynamics.

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Authors' Contributions

PJM and RCB conceived of the project; PJM, JEM, LJB, LAS, CI and RCB performed the fieldwork; VMM, PJM and RCB performed the lab work; VMM and CGB analysed the data; VMM and RCB produced the figures; VMM and PJM wrote the first draft of the paper; all authors contributed to reviewing and revising the paper.

Data Accessibility Statement

The complete dataset of museum specimen vouchers and corresponding *Bd* loads is available in Table S1.

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