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Post-epizootic microbiome associations across communities of neotropical amphibians

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Abstract

Microbiome–pathogen interactions are increasingly recognized as an important element of host immunity. While these host-level interactions will have consequences for community disease dynamics, the factors which influence host microbiomes at larger scales are poorly understood. We here describe landscape-scale pathogen–microbiome associations within the context of post-epizootic amphibian chytridiomycosis, a disease caused by the panzootic chytrid fungus *Batrachochytrium dendrobatidis*. We undertook a survey of Neotropical amphibians across altitudinal gradients in Ecuador ~30 years following the observed amphibian declines and collected skin swab-samples which were metabarcoded using both fungal (ITS-2) and bacterial (r16S) amplicons. The data revealed marked variation in patterns of both *B. dendrobatidis* infection and microbiome structure that are associated with host life history. Stream breeding amphibians were most likely to be infected with *B. dendrobatidis*. This increased probability of infection was further associated with increased abundance and diversity of non-*Batrachochytrium* chytrid fungi in the skin and environmental microbiome. We also show that increased alpha diversity and the relative abundance of fungi are lower in the skin microbiome of adult stream amphibians compared to adult pond-breeding amphibians, an association not seen for bacteria. Finally, stream tadpoles

exhibit lower proportions of predicted protective microbial taxa than pond tadpoles, suggesting reduced biotic resistance. Our analyses show that host breeding ecology strongly shapes pathogen–microbiome associations at a landscape scale, a trait that may influence resilience in the face of emerging infectious diseases.

KEYWORDS
B. dendrobatidis, *Batrachochytrium*, chytridiomycosis, Darwin's naturalisation conundrum, endemic, enzootic, invasive pathogen

1 | INTRODUCTION

The spread of invasive pathogens linked to increasing global connectivity is a major driver of biodiversity losses worldwide and the attendant attrition of ecosystem functions (Crowl et al., 2008; Thakur et al., 2019). The process of ecological invasion has four distinct stages—transport, introduction, establishment and spread (Blackburn et al., 2011)—that precede potential naturalization of the invader. To progress through these stages, an invading pathogen must successfully navigate a variety of biotic and abiotic filters which are scale-dependent (Cohen et al., 2016). At larger spatial scales, more diverse native communities have a greater intrinsic capability for supporting invasive species through increased resource availability (Ma et al., 2016). However, at finer spatial scales, invasion is governed by increased competition and interactions with native microbial species, which can decrease the probability of a pathogen successfully establishing (Ma et al., 2016).

The spread and establishment of a pathogen during an initial epizootic outbreak phase does not guarantee naturalization within a host system (Anderson & May, 1986) and, following an epizootic, the long-term persistence of a pathogen is governed by a combination of biotic and abiotic factors (Hawley & Altizer, 2011). Therefore, understanding these factors is a critical step for informing disease mitigation strategies as well as assessing the risk of invasion to naïve communities (Beukema et al., 2018; Canessa et al., 2020). To this end, the neotropical Andes present an ideal system to study the biotic and abiotic factors associated with multihost wildlife pathogens due to the occurrence of megadiverse host communities (Cadena et al., 2012) that are embedded in large elevation gradients.

The fungal pathogen *Batrachochytrium dendrobatidis* is a key driver of amphibian biodiversity loss on all continents with amphibians, except for Asia—the putative region of origin for the species (O'Hanlon et al., 2018). Spread globally through the amphibian trade (Fisher & Garner, 2007), *B. dendrobatidis* has been reported as a factor in the decline of over 500 species of amphibian worldwide and the extinction of over 90 (Scheele et al., 2019; but see Lambert et al., 2020; Scheele et al., 2020). Introduced to the Neotropics in the 1970 s (Cheng et al., 2011), the pathogen is now widely established across South and Central America. While Neotropical communities have been disproportionally affected by chytridiomycosis-driven declines, not all amphibian species have been affected equally by this disease. Observed heterogeneity in disease susceptibility has been attributed to a range of biotic and abiotic factors that includes the intrinsic variation in resilience of hosts to *B. dendrobatidis* that is lent by immune-mechanisms (Cohen et al., 2017; Woodhams et al., 2006, 2014). This local web of interactions comprises the realized niche of *B. dendrobatidis* and ultimately determines whether the pathogen thrives or dies (Hawley & Altizer, 2011).

The host's skin innate immune defences are the first-line defence against *B. dendrobatidis* infection and are primarily composed of antimicrobial compounds in granular gland secretions (Conlon et al., 2009). However, also of importance is an extended "immune phenotype" that is due to the amphibian skin microbiome; this has been demonstrated as being an important factor in resisting *B. dendrobatidis* infection (Rollins-Smith et al., 2011). The skin microbiome of amphibians is composed of a mixed commensal microbial assemblage, including taxa from both the bacterial and the archaeal domains alongside microbial eukaryotes, which primarily reside within the fungal kingdom. This complex skin microbiome consists of a wide phylogenetic breadth of microorganisms, some of which have probably evolved to survive on amphibian skin whilst others are more transient taxa obtained from the environment (Muletz et al., 2012). Notably, the structure of the amphibian skin microbiome and its inferred resistance to infection by *B. dendrobatidis* has been shown to correlate with the environment at a landscape (Bates et al., 2018; Kueneman et al., 2019) and microhabitat scale (Garcia-Recinos et al., 2019; Harrison et al., 2019). Consequently, the probability of successful long-term persistence of *B. dendrobatidis* within a community of amphibian hosts will be modified by its antagonistic or mutualistic interactions with native microbial species (Gallien & Carboni, 2017).

The outcomes of interactions between *B. dendrobatidis* and resident microbes are complex. On the one hand, native species which are phylogenetically similar to the invasive species, such as non-*Batrachochytrium* chytrid fungi, can potentially provide intense competition with the invader, resulting in "biotic resistance" (Ma et al., 2016). On the other hand, and provided there are no significant antagonistic interactions, these native species may also be a good predictor of invasion success, and hence of long-term persistence (Park et al., 2020). These contradictory arguments are known as "Darwin's naturalization conundrum" (Darwin, 1859).

While several studies have shown the amphibian skin bacteria to be an important factor in mitigating the impacts of *B. dendrobatidis* infection at an individual and population scale (Bates et al., 2018; Ellison et al., 2019), the role of the mycobiome (the fungal community) has been largely ignored (Kearns et al., 2017), especially when considering taxa that are phylogenetically similar to *B. dendrobatidis*. Antagonistic biotic interactions (biotic resistance) with the host skin microbiome could potentially exclude *B. dendrobatidis* from some host microbial communities, while opportunistic pathogens could take advantage of immunocompromised *B. dendrobatidis*-infected hosts (Claytor et al., 2019; Rollins-Smith et al., 2015) leading to secondary infections (Perpiñán et al., 2010). Hence, it has been argued that there will be a suite of microbial taxa that are positively associated (opportunistic taxa) and negatively associated (antagonistic taxa) with the presence of *B. dendrobatidis* and, perhaps, disease.

While the exact date of invasion by *B. dendrobatidis* in Ecuador is unknown, the majority of declines took place during the late 1980 s (Merino-Viteri et al., 2005) as part of a series of epizootic waves across the Neotropics (Lips *et al*. 2008) and before the discovery of *B. dendrobatidis* (Berger et al., 1998). Invasion of *B. dendrobatidis* in Ecuadorian amphibian communities was originally thought to be largely dependent upon abiotic conditions (Ron, 2005) and previous studies have highlighted the fact that 89% of the country's critically endangered species of amphibian live in locations which are predicted to be "suitable" for the proliferation of the pathogen (Menéndez-Guerrero & Graham, 2013). Host life history is associated with the probability of chytridiomycosis (Bielby et al., 2008), and anecdotal reports suggest greater impacts by chytridiomycosis on high-elevation, stream-breeding amphibian communities. Associations between breeding habitat and the prevalence of *B. dendrobatidis* has been observed for amphibians in Australia (Kriger & Hero, 2007), where amphibians associated with permanent water bodies, particularly streams, showed higher prevalence. In Ecuador, evidence of the scale of the declines in individual species has been difficult to quantify, and there have been few systematic *B. dendrobatidis* surveys at a landscape scale (Bresciano et al., 2015). However, published field surveys have showed that once-common species such as the Quito stubfoot toad (*Atelopus ignescens*) experienced total extirpation across known populations (Ron et al., 2003) while recent efforts revealed that some species have been persisting in isolated relict populations, including the Quito stubfoot toad and other *Atelopus* species (Jervis et al., 2020; Tapia et al., 2017).

Here we assess pathogen–microbiome associations of *B. dendrobatidis* in this megadiverse Neotropical amphibian community through a landscape scale sampling design across a climatic gradient in Ecuador. Specifically, we (i) assess the relative importance of abiotic (elevation) and biotic (host breeding mode and life stage) as factors in determining the presence of *B. dendrobatidis*; (ii) correlate domain-level host microbiome composition with factors predicting *B. dendrobatidis* presence; and (iii) determine the presence and diversity of non-*Batrachochytrium* chytrid fungi in relation to factors associated with *B. dendrobatidis* presence.

2 | METHODS

2.1 | Sample collection

Field sampling took place at 17 sites across Ecuador between February 28 and April 28, 2017. We conducted sample collection within a constrained time frame to limit the impact of seasonality upon disease and microbiome data. Sampling was conducted across an elevational range of 4,000 m (200–4,200 m a.s.l.) which encompasses the full range of habitats inhabited by Ecuadorian amphibians (Figure 1).

Animals were located by listening for calling males and visual encounter surveys around suitable breeding habitats. Premetamorphic animals had their mouthparts swabbed with an MW100 cotton-tipped swab and post-metamorphic animals were swabbed according to a standard protocol (Hyatt et al., 2007; Retallick et al., 2006); each specimen was swabbed by taking five strokes down the centre of the underside, on each flank, the inside of the hind legs and bottom of the feet. Swabs were stored below 4°C until DNA extraction. Each animal was contained in a new plastic bag and handled with a new pair of nitrile gloves to prevent cross-infection of individuals (Phillott et al., 2010). The coordinates and elevation were taken using a Garmin etrex GPS.

To characterize the microbial community of the aquatic breeding sites, we collected sediment samples by passing 1 L of water through a 0.45-μm nitrocellulose filter (Walker et al., 2007). In the event that the filter blocked due to large amounts of sediment, or the breeding site contained less than 1 L of water, the filtered volume was recorded. Filter papers were folded into 2-ml Eppendorf tubes, dried in a Laminar-flow cabinet and stored at 4°C until analysis. All equipment was sterilized in 5% chlorhexidine solution between sites.

2.2 | DNA extraction and B. dendrobatidis diagnostics

DNA from all samples was extracted in a PrepMan Ultra (Applied Biosystems; Hyatt et al., 2007). To assign the infection status and to quantify the disease burden of each individual, we performed *Batrachochytrium dendrobatidis*-ITS (internal transcribed spacer) qPCR (quantitative polymerase chain reaction) according to Boyle et al. (2004). Extractions were diluted 1:10 before qPCR amplification, run in duplicate, and considered to be positive if both wells amplified and were over 0.1 genomic equivalents (GE; where 1 GE is the quantity of genetic material present in a single zoospore). All samples were run against positive standards of 0.1, 1, 10 and 100 GE of *B. dendrobatidis* GPL (isolate IA042), and negative control standards. Plates with a standard curve of $R^2 < .95$ were repeated. All DNA

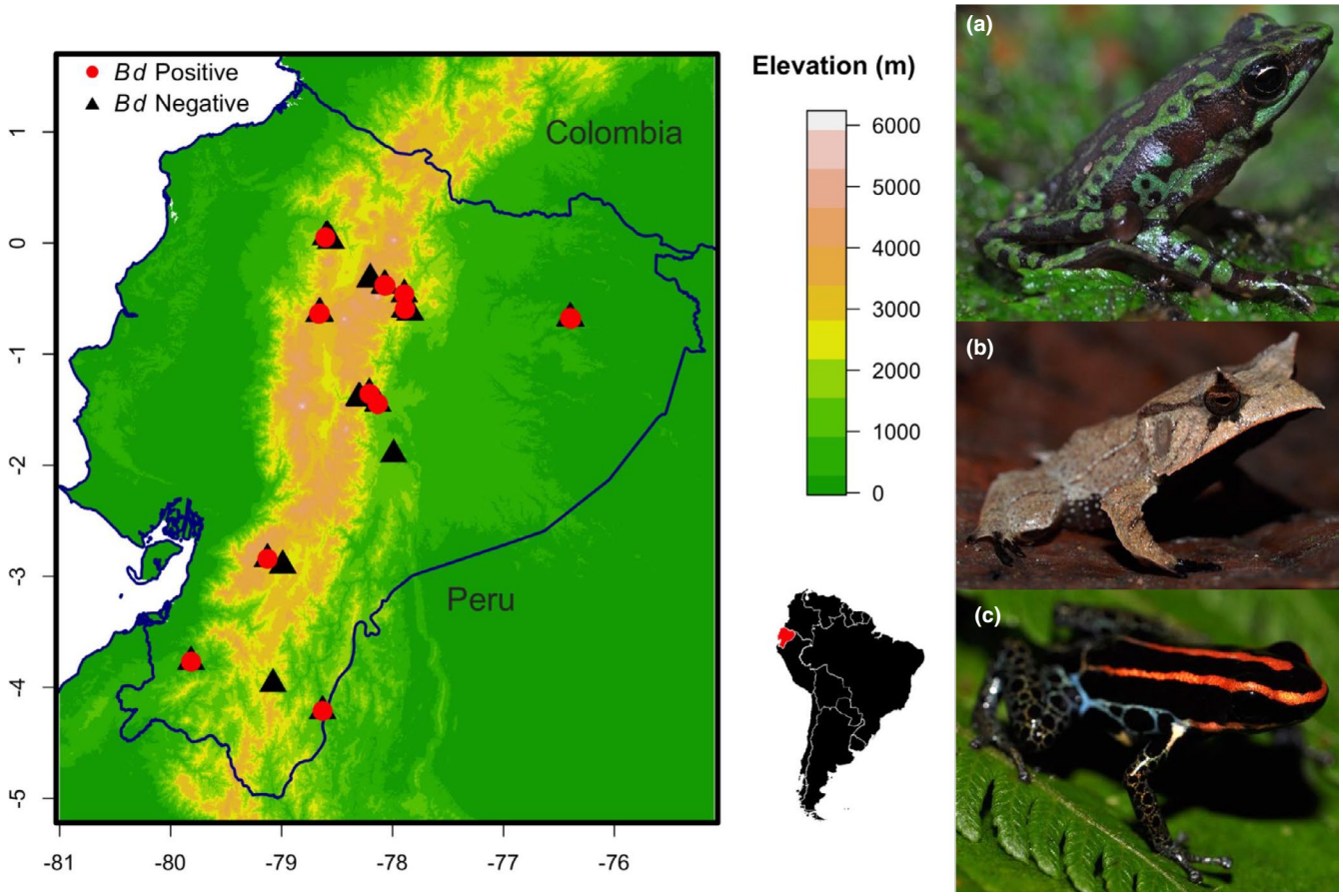


FIGURE 1 Map showing sampling locations and site infection status. Breeding modes were assigned as: "Stream breeding" if the amphibian species has an aquatic larval stage in flowing water (a, *Atelopus palmatus*); "Direct development" if the species has no aquatic larval stage (b, *Hemiphractus scutatus*); and "Pond breeding" if the species has an aquatic larval stage in standing water (c, *Ranitomeya ventrimaculata*) [Colour figure can be viewed at wileyonlinelibrary.com]

extractions were then used to amplify and assess both the bacterial (V4-16S rRNA) and fungal (ITS-2) communities.

2.3 | Mycobiome laboratory procedure

To identify which fungal samples contained sufficient genetic material for library preparation, a screening PCR using an *ITS3/4* primer set was performed and amplicons were visualized using 1.5% gel electrophoresis. Samples with a band had sufficient genetic material to undergo *ITS-2* amplification and were selected for metagenomic library preparation. *ITS-2* library preparation was performed using dual-indexed ITS-3F and ITS-4R primers (Kozich et al., 2013) and PCR protocols (Bates et al., 2019). Samples were run in duplicate, amplicons were visualized on a 1.5% agarose gel and samples that produced a band were pooled to yield a final per-sample volume of 50 µl. Pooled amplicon DNA was purified using AMPure XP bead clean-up (Beckman Coulter). Equimolar pooling of samples was performed according to band intensity of cleaned-up product following visualization on a 1.5% agarose gel. The samples then underwent 300-bp paired-end sequencing using V3 chemistry on an Illumina MiSeq platform (Illumina).

2.4 | Microbiome laboratory procedure

Amplification and sequencing of bacteria were performed using methods previously described by Harrison et al. (2019). Briefly, library preparation was performed with modified 515F and 806R primers designed to amplify the V4 region of the 16S rRNA gene using a dual indexing protocol (Kozich et al., 2013). Library cleanup and pooling were conducted following the method described by Harrison et al. (2019), with a final sequencing run performed on a MiSeq system using a 500 cycle V2 chemistry kit (Illumina).

2.5 | Mycobiome sequence processing

Raw sequence data were processed using DADA2 (Callahan, McMurdie, et al., 2016) running a pipeline recommended by Pauvert et al. (2019) with minor modifications. Fungal sequences with uncalled bases were removed before primers were identified and removed using CUTADAPT (Martin, 2011) and the reverse reads were discarded. *ITS* is a variable-length region and use of a merging step leads to a bias for fungi with short *ITS* regions. The forward reads were trimmed to 200 bases, processed using the DADA2 denoising

algorithm and dereplicated. Taxonomic assignment of amplicon sequence variants (ASVs) was performed using the fungal *ITS-2* UNITE database version 8 with modifications to include the 26 *B. dendrobatidis* sequences from UNITE version 7 which were not included in the most up-to-data FASTA release at the time of writing (Abarenkov et al., 2010). Suspected contaminants were removed using the R-package DECONTAM (Davis et al., 2018) with the threshold set to 0.5. Reads which were not assigned to the fungal kingdom were excluded to remove the influence of nontarget taxa.

Following the ITS3/4 screening PCR, amplicons from all sequenced samples (see Table S1: total, n = 181; amphibian swabs, n = 168; water filters, n = 13), after data trimming and quality filtering, produced a total of 5,582,691 sequences, with amphibian swabs contributing 96.34% of the total sequences. Each amphibian swab (n = 160) produced an average of 31,636 sequences (SD = 53,472.42, range 0–313,580). A total of 12 phyla, 39 classes, 129 orders, 301 families and 642 genera were detected from the amphibian swabs (Figure S1). The most abundant phyla were Ascomycota (average 82% of total sequences) and Basidiomycota (17%). From the 642 genera detected, 16 were dominant with a mean relative abundance >1% of the total sequences: *Debaryomyces* (35%), *Cladiosporum* (20%), *Vishniacozyma* (5%), *Gibberella* (4%), *Rhodotorula* (4%), *Cephalotrichum* (2%), *Aspergillus* (2%), *Plectosphaerella* (2%), *Didymella* (2%), *Cryptococcus* (1%), *Pyrenochaetopsis* (2%), *Acremonium* (2%), *Fusarium* (2%), *Myrothecium* (1%), *Sarocladium* (1%) and *Penicillium* (1%). A phylogenetic tree of ASVs was produced using the GTR model in FASTTREE version 2.1.9. Downstream analysis of fungal ASVs was conducted using the R package PHYLOSEQ (McMurdie & Holmes, 2013) in R version 3.6.3 (R Core Team, 2020). A phylogenetic tree of fungal ASVs was produced using the GTR model in FASTTREE version 2.1.9.

2.6 | Microbiome sequence processing

Raw sequences were processed using DADA2 (Callahan, McMurdie, et al., 2016) using the default pipeline for bacterial sequences, forward and reverse reads were trimmed to 240 and 160 bp respectively, while other filtering parameters were set to default. Filtered reads were processed using the DADA2 denoising algorithm, dereplicated and went through chimera removal. Taxonomic assignment of ASVs was performed using the bacterial 16S rRNA SILVA (version 132) training set (Yilmaz et al., 2013). Suspected contaminants were removed using the R-package DECONTAM (Davis et al., 2018) with the threshold set to 0.5.

Amplicons from all sequenced samples (see Table S2: total, n = 367; amphibian swabs, n = 347; water filters, n = 20), after data trimming and quality filtering, produced a total of 14,570,591 sequences, with amphibian swabs contributing 97.46% of the total sequences. Each amphibian swab (n = 347) produced an average of 41,523 sequences (SD = 28,975, range 14,996–523,782). A total of 35 phyla, 65 classes, 145 orders, 236 families and 574 genera were detected from the amphibian swabs (Figure S1). The most abundant phyla were Proteobacteria (average 74% of the total sequences), Bacteroidetes (11%), Firmicutes (8%) and Actinobacteria (5%). From the 574 genera detected, 16 were dominant with a mean relative abundance >1% of the total sequences: *Pseudomonas* (32%), *Acinetobacter* (8%), *Stenotrophomonas* (8%), *Staphylococcus* (7%), *Klebsiella* (6%), *Verticia* (4%), *Chryseobacterium* (3%), *Sanguibacter* (2%), *Sphingobacterium* (2%), *Citrobacter* (1%), *Raoultella* (1%), *Pantoea* (1%), *Yersinia* (1%), *Glutamicibacter* (1%), *Empedobacter* (1%) and *Flavobacterium* (1%). A phylogenetic tree of ASVs was produced using the GTR model in FASTTREE version 2.1.9. Downstream analysis of bacterial ASVs was conducted using the R package PHYLOSEQ (McMurdie & Holmes, 2013).

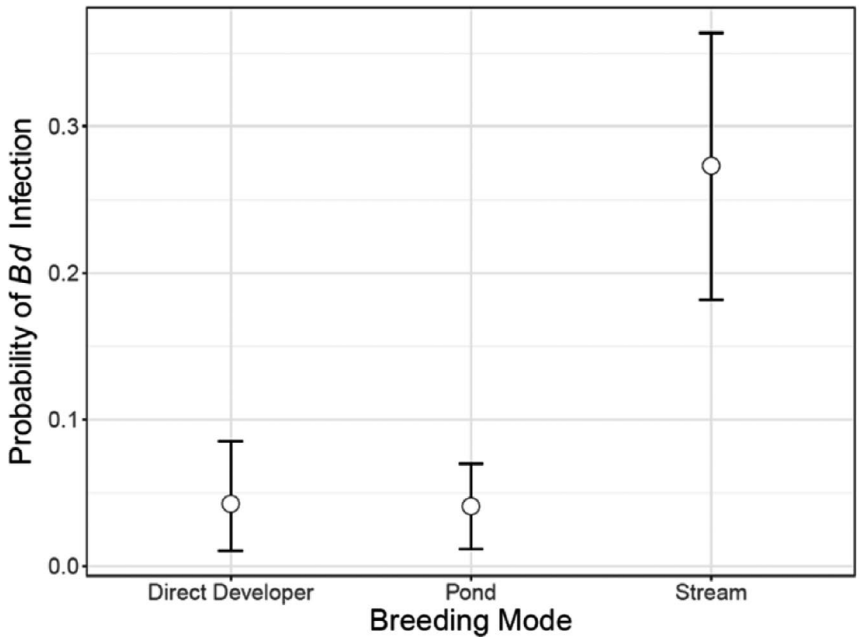
2.7 | Rhizophydiales phylogeny

Sequences were subset to the order Rhizophydiales using the R package PHYLOSEQ (McMurdie & Holmes, 2013) before following the methods described for the BIOCONDUCTOR workflow (Callahan et al., 2016). Briefly, sequences were aligned using the R package DECIPHER (Wright, 2016), before generating a phylogenetic tree using the R package PHANGORN (Schliep, 2011). We fitted a neighbour-joining tree which was optimized using a GTR model to generate a maximum-likelihood tree before bootstrapping to provide confidence estimates.

2.8 | Statistical analyses

Statistical analyses were performed within R version 3.6.3 (R Core Team, 2020). To determine the factors driving the presence–absence of *B. dendrobatidis*, we constructed a generalized linear mixed effects model using the R package LME4 (Bates et al., 2014). The *B. dendrobatidis* infection status of each amphibian was set as the binary response variable, with breeding mode (three-level factor), life stage (two-level factor) and elevation fitted as explanatory variables, and site was set as a random factor within a binomial error structure. To reduce problems associated with predictors being on different scales, the climate and elevation variables were centred and standardized to SD = 1. The significance of each of these variables was calculated using a stepwise simplification procedure with p-value filtering (Figure 2).

The 16S rRNA data were rarefied to the minimum library size of 14,996 reads; ITS-2 data were not rarefied owing to the lower read-depth and diversity of these sequences (Pauvert et al., 2019). The combined microbial analysis was performed through the concatenation of these data. To generate heatmaps and relative abundance plots, taxa were aggregated at the relevant taxonomic level, filtered by percentage contribution to overall reads (>3.5%) and then plotted as a heatmap using the R packages GPLOTS (Warnes et al., 2015) and GGPLOT2 (Wickham, 2016) respectively. Hierarchical clustering using Bray–Curtis distances was performed on x and y axes using R function "Hclust" (Müllner, 2013). Alpha diversity (Shannon) for the fungal and bacterial communities was calculated using the



Term Removed	df	χ2	p value
Life stage	1	0.04	0.84
Elevation	1	1.68	0.19
Breeding mode	2	17.07	<0.001

(b) Top Model Results

Random Effects	Variance	Std. Dev.
Site	0.308	0.56

Fixed Effects

Parameter	Std. Error	95% CI
Direct developer	0.55	<0.001
Pond breeder	0.68	0.88
Stream breeder	0.63	0.001

r^2	
Marginal	0.24
Conditional	0.27

FIGURE 2 Mixed model results show that host breeding mode, not elevation or life stage, is the only significant factor in predicting the presence of *Batrachochytrium dendrobatidis*. A summary of life history groups is given in Table S4

function "estimate_richness" in the R package PHYLOSEQ (McMurdie & Holmes, 2013). Differences in alpha diversity between breeding mode and life stage groups were determined through the use of an ANOVA with post-hoc Tukey tests in base R (R Core Team, 2020). Beta diversity analysis was performed using principal components analysis (PCA) ordination on Aitchison distances of centred-log-ratio (clr)-transformed data (Gloor et al., 2017). We used PERMANOVA to quantify differences in microbial community structure among samples, using the "adonis" function in the R package VEGAN (Oksanen et al., 2013).

To find microbial taxa with positive and negative associations with *B. dendrobatidis*, we used the R package COOCCUR (Griffith et al., 2016) independently on both life stage (pre- and post-metamorphic hosts) and on each of the 16S rRNA and ITS-2 data sets with a co-occurrence threshold set at 0.1.

3 | RESULTS

3.1 | Field sampling and predictors of B. dendrobatidis infection

A total of 377 samples were collected from 43 species of amphibians and their immediate environment. These consisted of 22 water sediment filters, 250 adult amphibian swabs and 105 tadpole swabs. Direct-developers (96 adults) were present at 191–4,233 m, pond breeders (69 tadpoles, 102 adults) were present at 207–3,529 m and stream breeders (36 tadpoles, 52 adults) were present at 260–2,722 m. A summary of breeding mode categories is provided in Table S4. Fewer stream breeders were sampled due to their restricted elevational range and low encounter rate. A summary of species and sites samples is presented in Table S3.

In total, 35 out of 377 animals (9.2%, Clopper-–Pearson confidence interval [CI] 7%–13%) sampled were found to be *Batrachochytrium dendrobatidis* positive through *B. dendrobatidis*-ITS qPCR, with an average infection intensity of 17.1 GE (*SD* = 36.0, range 0.1–194 GE). This "low-prevalence, low-intensity" pattern is characteristic of an enzootic disease system following invasion by *B. dendrobatidis*. We hypothesized that stream-breeding amphibians at high elevations would be associated with *B. dendrobatidis* presence. In the global model, *B. dendrobatidis* status was run with elevation, life stage (adult or tadpole) and breeding mode (direct developer, stream breeder or pond breeder) as predictors, and site as a random factor (Figure 2). Stream breeding was determined to be the only significant factor in the presence of *B. dendrobatidis* (Figure 2).

3.2 | Bacterial and fungal community analysis

To compare the bacterial (16S rRNA) and fungal (ITS-2) communities of both the environmental samples and the amphibian swabs, we split the samples by breeding habitat (environmental and skin swabs) and life stage (skin swabs). Despite life stage being a non-significant predictor of *B. dendrobatidis* infection (Figure 2), we analysed amphibians by groups defined by life stage and breeding mode, as the microbial communities are known to be heavily influenced by host life stage (Bates et al., 2018). Hierarchical

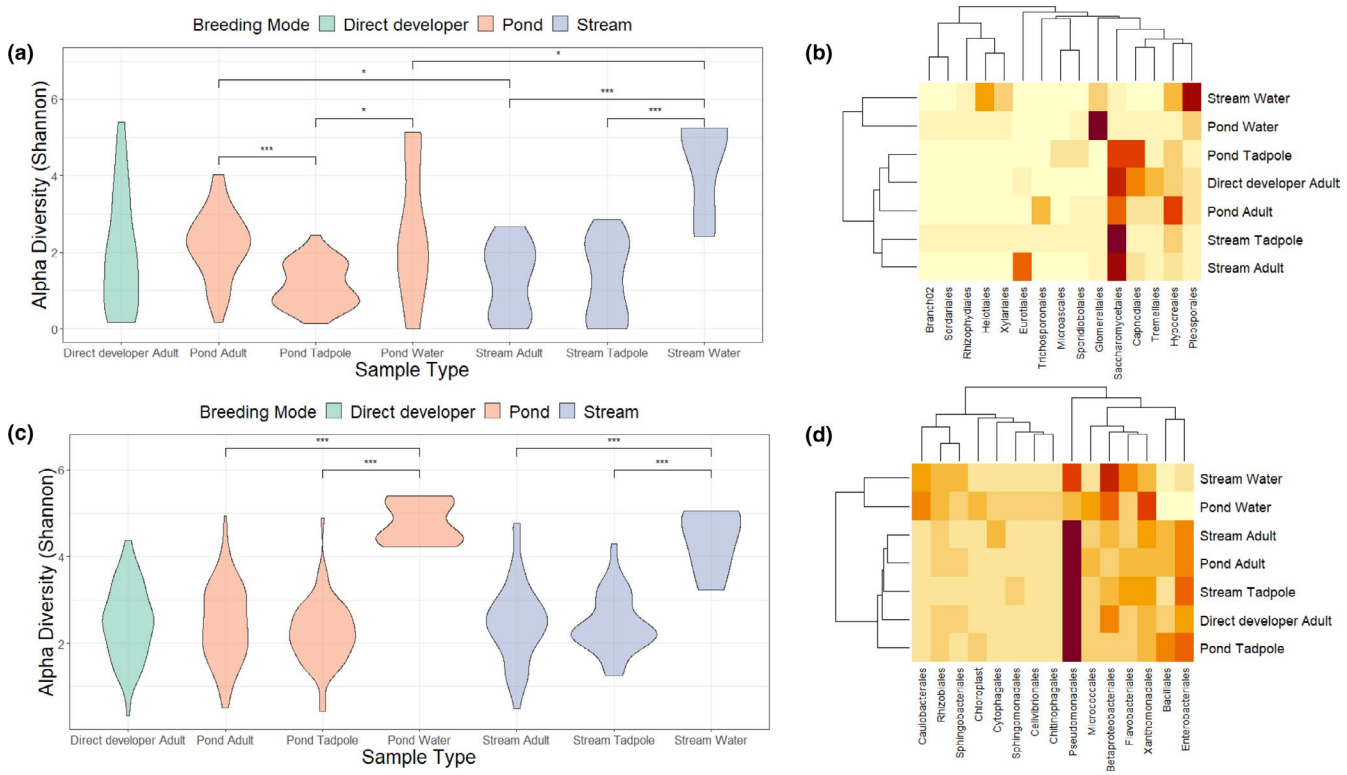


FIGURE 3 Fungal and bacterial community structure shown across environmental and life history groups. (a) Fungal (ITS2) alpha diversity; (b) fungal (ITS2) heatmap showing common (>3.5% of total reads) orders with hierarchical Bray–Curtis clustering of axes; (c) bacterial (16S) alpha diversity; and (d) bacterial (16S) heatmap showing common (>3.5% of total reads) orders with hierarchical Bray–Curtis clustering of axes. Sample sizes are given in Table S4. Significance on alpha diversity plots was determined by ANOVA with post-hoc Tukey test and significance is denoted by $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ [Colour figure can be viewed at wileyonlinelibrary.com]

Bray–Curtis clustering grouped environmental samples and amphibian swabs separately in both the bacterial (Figure 3b) and the fungal (Figure 3d) data sets.

Following the mixed model results (Figure 2), we expected that stream-breeding amphibians would present reduced biotic resistance to *B. dendrobatidis* through a reduced diversity or differences in their microbiome community structure. We investigated these differences both as independent domains and as a mixed microbial assemblage. Alpha diversity, as measured by the Shannon diversity index, of the fungal community showed significant differences between environments, breeding modes and life stages (Figure 3a). The environmental fungal community was significantly richer in streams than ponds and we found that pond-breeding adults had significantly greater fungal alpha diversity than pond-breeding tadpoles and stream-breeding adults (Figure 3). Moreover, we did not find significant differences in the fungal diversity between larval amphibian groups. These results show significantly greater fungal diversity in streams relative to ponds, a trend which does not correspond to the fungal diversity patterns in the amphibian mycobiomes that are associated with these habitats. In marked contrast, different environments, life stages and breeding modes were not good predictors of bacterial alpha diversity (Figure 3c), although all amphibian groups had a significantly lower bacterial diversity than their environment.

3.3 | Combined bacterial and fungal community analysis

To examine the combined microbial (bacteria and fungi) community structure, we combined the ITS2 and 16S rRNA data for each sample. The alpha diversity, as measured with Shannon diversity index, of the microbial community (Figure 4c) showed significant differences between the stream environment and both stream-breeding life stages. Following the same trend as the individual domain analyses (Figure S2), groupings of amphibians with differing life histories (Figure 4a) were a significant factor in the community structure (PERMANOVA $F = 4.3$, $R^2 = .07$, $p = .001$), but far more of the variance was explained by site (PERMANOVA $F = 3.5$, $R^2 = .13$, $p = .001$) and host genus (PERMANOVA $F = 3.4$, $R^2 = .18$, $p = .001$).

Co-occurrence analysis found that 56 taxa across the fungal and bacterial communities are positively and negatively associated with the presence of *B. dendrobatidis* (Table S5). Following the mixed model results, we expected stream-breeding amphibians to have a reduced association with potentially protective microbial taxa. Due to differences between the microbial taxa of adults and tadpoles (Figure 3), co-occurrence analysis was run separately on each life stage. Pond tadpoles were found to be enriched for potentially protective taxa compared to stream tadpoles (Figure 5),

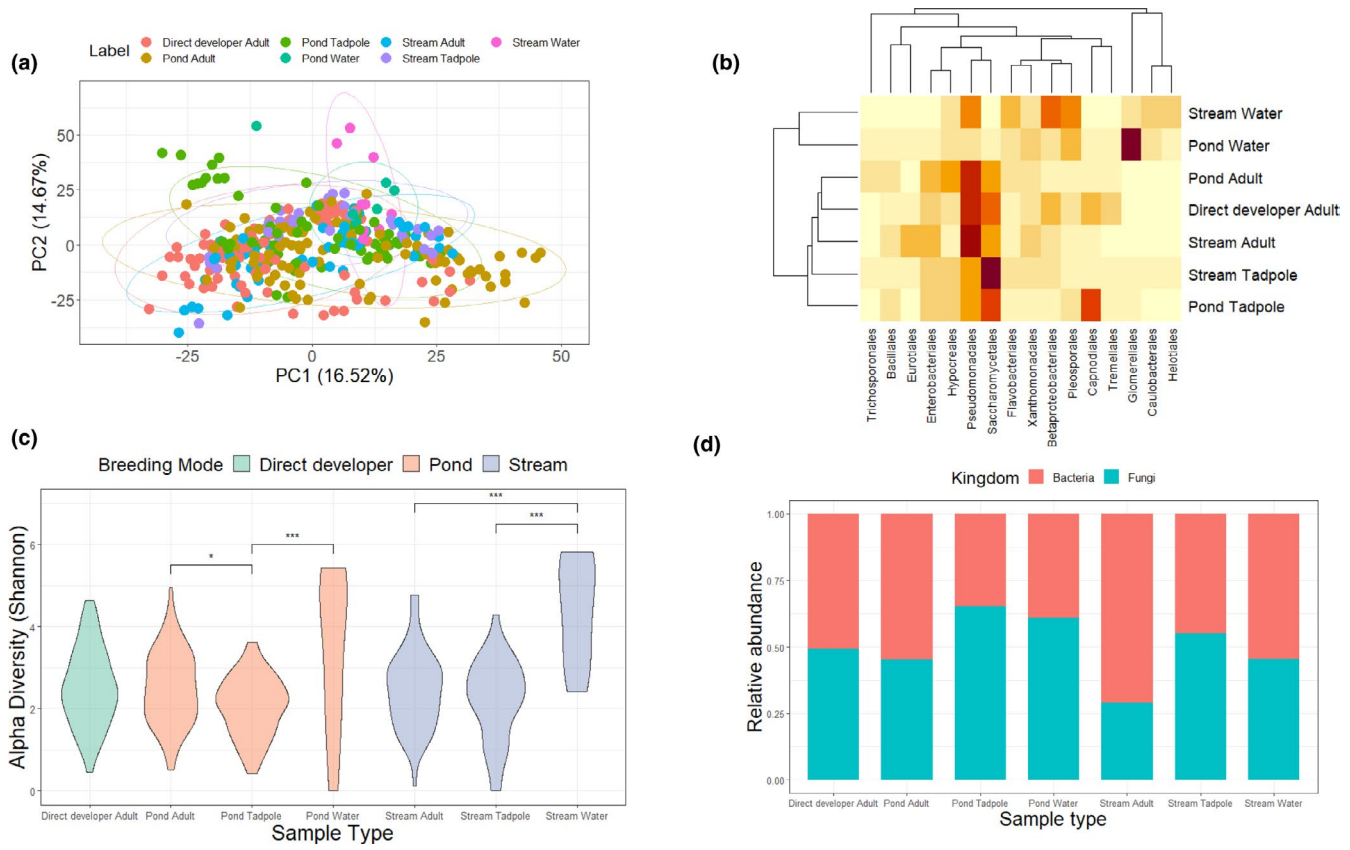
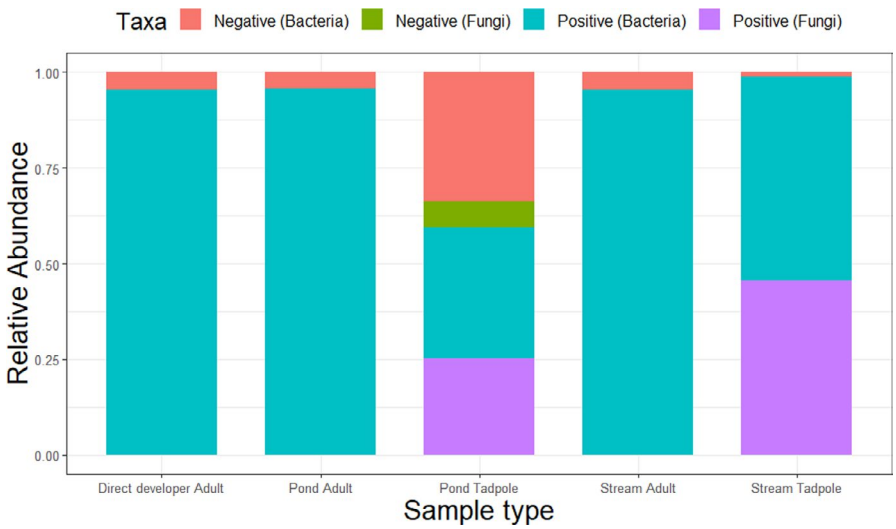


FIGURE 4 Combined fungal and bacterial community structures across environmental and life history groups. (a) Microbial PCA plot of clr-transformed Aitchison distance; (b) microbial heatmap with hierarchical Bray–Curtis clustering of axes; (c) violin plot of microbial Shannon diversity; (d) microbial relative abundance at Kingdom level. Sample sizes shown in Table S4. Significance was determined by ANOVA with post-hoc Tukey test and significance is denoted by $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 5 Relative abundance of taxa found to have a negative association with *Batrachochytrium dendrobatidis* (predicted antagonistic taxa) or a positive association with *B. dendrobatidis* (predicted opportunistic pathogens or mutualistic taxa) [Colour figure can be viewed at wileyonlinelibrary.com]



which may explain the absence of *B. dendrobatidis* detected within this group. Adult amphibians had a consistently low abundance of potentially protective taxa regardless of breeding habitat association.

The ITS2 chytrid sequences that we recovered span across 96 ASVs with representatives from eight different families (Figure 6). Additionally, we found clades of unknown chytrids, some of which were enriched on amphibians. These chytrid taxa were concentrated in stream-breeding environments with 59% of these 120 chytrid detections being in either stream-breeding amphibians or their breeding sites. Notably, none of these batrachochytrid taxa showed significant relationships with *B. dendrobatidis*.

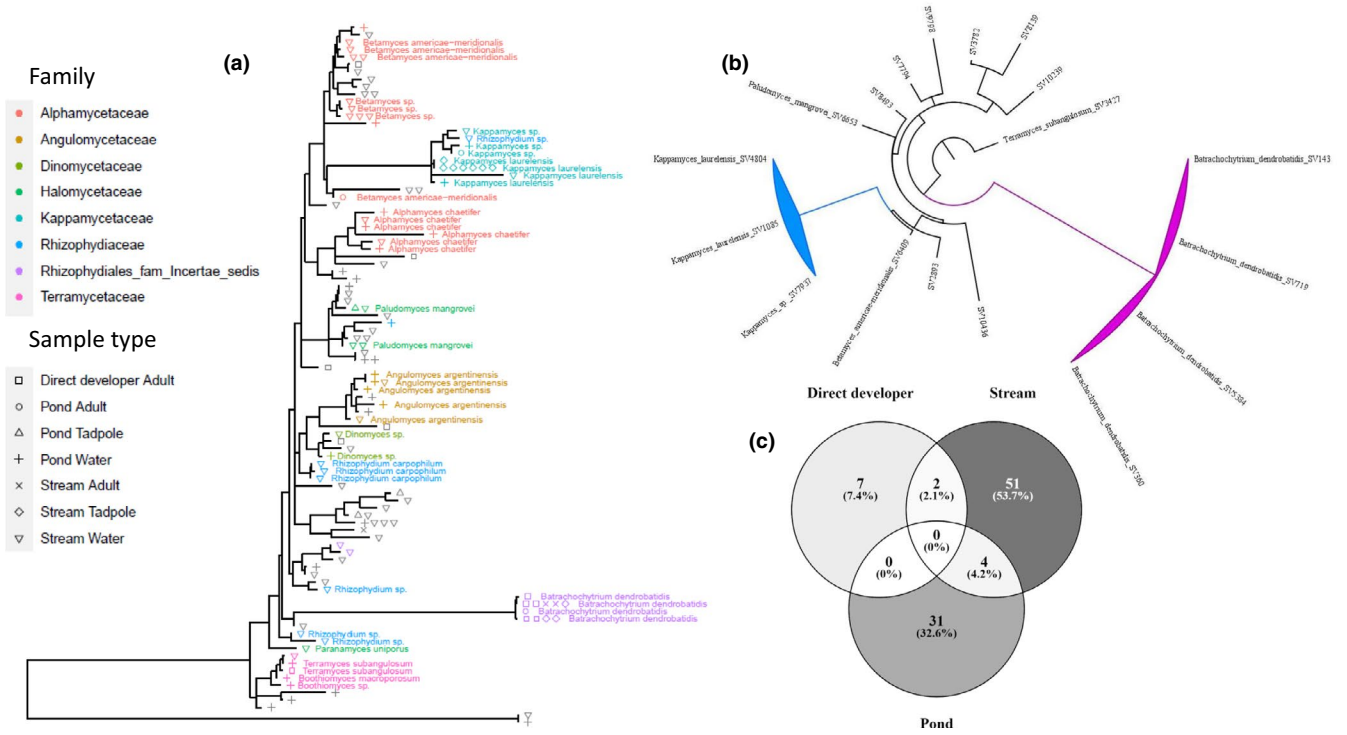


FIGURE 6 Chytrid taxa (ITS2-Order: Rhizophydiales). A total of 120 chytrid sequences were recovered in the mycobiome of both amphibians and their breeding environments. (a) Maximum-likelihood tree showing all chytrid taxa detected in both skin swabs and environmental samples. (b) Maximum-likelihood tree showing taxa detected from amphibian skin swabs. (c) Venn diagram showing the distribution of chytrid taxa between environmental samples (Pond, Stream and Terrestrial) and amphibian skin swabs (Pond, Stream and Direct developer) split by breeding mode [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

Over 30 years following epizootic declines caused by chytridiomycosis in Neotropical amphibian populations we find that the presence of *B. dendrobatidis*, as well as novel undescribed native chytrids, are strongly associated with stream-breeding amphibian communities. Our data show that amphibian life history traits modify both host bacterial and fungal microbiomes, and susceptibility to infection with *Batrachochytrium dendrobatidis*. We observed marked variation among host life stages and breeding modes in the frequency and direction of putative interactions between microbiome and *B. dendrobatidis*, indicating that functionally relevant interactions occur. Although we detected a high diversity of native chytrids, there did not appear to be strong competition with *B. dendrobatidis*, as evidenced by a lack of negative association with the pathogen. Critically, these empirical data expand the pathogens distribution below the lower limit of elevation of 1,000 m predicted by envelope modelling of abiotic variables alone (Ron, 2005). Our work highlights the importance of understanding both abiotic and biotic interactions when attempting to quantify heterogeneity amongst host species in susceptibility to a lethal pathogen post-invasion. Our work also underlines the complex suite of factors that have combined to govern this pathogen's transition to endemism across local and landscape scales, and which ultimately define the "realized niche" of *B. dendrobatidis* in this region of the neotropics.

We detected *B. dendrobatidis* from 200 to 3,500 m in elevation, which extends the elevational distribution of the pathogen beyond the observed chytridiomycosis-driven declines at high elevation. By confirming the presence of *B. dendrobatidis* within the Ecuadorian Amazon basin, our data agree with other contemporary studies in the region (McCracken et al., 2009; Russell et al., 2019). The prevalence and mean infection intensity (9.2%, 17.1 GE) of *B. dendrobatidis* that we report is higher than is observed within the native range of *B. dendrobatidis* GPL (Swei et al., 2011; Tapley et al., 2020) but is comparable to or lower than other *B. dendrobatidis* disease systems in the Neotropics that have probably transitioned to endemism (Bresciano et al., 2015; Flechas et al., 2017; Jervis et al., 2020; Kilburn et al., 2010; Puschendorf et al., 2006). The observed association of elevation with historical amphibian declines is probably due to differences in disease progression as the presence of *B. dendrobatidis* does not necessarily cause chytridiomycosis within wild amphibian populations (Lips, 2016). Here, we found that life stage and elevation do not appear to be significant predictors of the presence of *B. dendrobatidis*. This suggests that observations of heavier declines in high-elevation sites is due to differences in individual disease progression rather than pathogen distribution per se.

Both life stages of stream-breeding amphibians were more likely to be infected with *B. dendrobatidis*, a finding that is consistent with these taxa being more at risk from chytridiomycosis during epizootics (Kriger & Hero, 2007). However, we found that microbiomes

structures associated with stream breeders differed between larval and adult life stages. Adult stream-breeding amphibians showed significantly lower fungal alpha diversity (Figure 3a) and a lower relative abundance of fungi (Figure 4d) than pond-breeding adults. We speculate that adult pond-breeding amphibians present a more diverse, varied fungal community to *B. dendrobatidis* which could hamper long-term persistence of the pathogen on the host through intensified competition. This competition would lower the infection burden of an individual through biotic resistance (Ma et al., 2016), and hence reduce transmission which will have consequences at a population level (Woodhams et al., 2011).

We find 56 microbial taxa that are positively or negatively associated with the presence of *B. dendrobatidis* and show that stream tadpoles house a lower proportion of potentially protective microbial taxa than pond-breeding tadpoles. There is evidence that there are trade-offs in host innate immunity and protective microbiome (Metcalf & Koskella, 2019). Given that tadpoles have a lower immune competence than adults (Grogan et al., 2018), it is possible that they rely more heavily upon a protective microbiome, a hypothesis which may explain the lack of observed variation in the proportion of protective taxa across the adult amphibian groups. Although these associations are correlative, our results are consistent with the argument that there are antagonistic interactions between *B. dendrobatidis* and taxa in the amphibian skin microbiome. In support of this argument, we found negative associations with *Trichosporon asahii* (El-Tarabily, 2004) and *Raoultella* sp. (Fiołka et al., 2012) which are known to produce compounds that inhibit the growth of fungal competitors, and potentially *B. dendrobatidis*. Microbial taxa positively associated with *B. dendrobatidis* may represent secondary infections of hosts that are immunocompromised by *B. dendrobatidis* infection (Rollins-Smith et al., 2015) or mutualistic interactions amongst the host microbiota (Partida-Martinez & Hertweck, 2005). Although these results are interesting, further experimental work is required to dissect the directionality of these associations and provide a mechanistic basis for these microbiota–pathogen interactions.

Across our fungal ITS2 data set, we found strong signal that stream environments and stream-breeding amphibians had the highest number of presumed native chytrids in their microbiome when compared to pond environments and pond-breeding amphibians, or direct developing amphibians (Figure 6). The absence of chytrid that which have significant positive or negative associations with *B. dendrobatidis* implies that there may not be significant competition between the pathogen and the native chytrid taxa across the scales that we investigated. While Darwin's naturalization conundrum presents contrasting arguments on the impact of phylogenetic similarity of native communities in relation to the successful establishment of invasive species (Darwin, 1859), evidence suggests that phylogenetic similarity of the native species can be a good predictor of the success of an invasive species (Tan et al., 2015), provided there is little antagonistic interaction between them. This argument suggests that the association between *B. dendrobatidis* and stream-breeding amphibians may be partly due to streams and stream-breeding amphibians being suitable habitats for chytrids and a lack of competition between *B. dendrobatidis* and these native chytrids may explain the proliferation of the pathogen in these environments. This may represent an example of the "Enemy release hypothesis" (Colautti et al., 2004), which predicts that an invasive species will be successful in new environments which lack competition compared the native range of the invader. In our study, enrichment of chytrids in the stream-breeders therefore appears to be a reliable proxy for the establishment of the non-native *B. dendrobatidis* and is perhaps a predictor of ecosystem impact, if it predicts susceptibility of native amphibians to chytridiomycosis. This is in contrast to observed patterns in Asia, where the probable coevolution of amphibians and *B. dendrobatidis* within their native range means that chytridiomycosis is not observed (Swei et al., 2011). Further studies would be required within the native range of *B. dendrobatidis* to assess the level of competition between *B. dendrobatidis* and other Asian chytrids, and these studies may give insight explaining *B. dendrobatidis's* success once introduced to naïve systems. Conversely, it could be reasoned that the Ecuadorean non-*Batrachochytrium* chytrid taxa that we describe may pose a biosecurity concern if they are pathogenic to naïve amphibian communities outside of their native range.

Caution needs to be exercised when invoking contemporary patterns of microbial communities in order to describe historical observations of amphibian declines, such as those observed in the Ecuadorian highlands. It can be argued that the host and its microbiome—the combined "hologenome"—can be considered as a single unit under selection (Wilson, 1997). In this case, the data presented in this study will only be representative of the current post-epizootic microbial community. This may differ from the predisease amphibian community due to an under-representation of susceptible hosts and maladaptive microbiota following their extirpation (Roughgarden et al., 2018).

Overall, our study presents evidence that post-epizootic realized ecological niche of *B. dendrobatidis* is shaped more by the biotic factors of host ecology and microbiome than it is by landscape-scale abiotic factors. In particular, we show that stream-breeding amphibians are associated with *B. dendrobatidis*, as well as other previously unrecorded species of chytrids, and that these are home to a less diverse microbial assemblage that includes a lower abundance of taxa predicted to inhibit the proliferation. While functional dissection of these associations is required, that the enzootic persistence of *B. dendrobatidis* across neotropical landscape scales appears shaped by biotic factors lends hope that microbial ecology approaches such as we detail here will lend new insight into the longer term consequences of microbial invasions. More broadly, we have shown that host breeding ecology strongly shapes pathogen–microbiome associations at a landscape scale in megadiverse communities, a trait that may influence resilience in the face of emerging infectious diseases.

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AUTHOR CONTRIBUTIONS

P.J., P.P., D.A.R., S.R., A.M.V. and M.C.F. designed the study; P.J., P.P., D.A.R. and A.M.V. collected field data; P.J., K.H. and C.W. performed the laboratory analysis; P.J., J.M.G.S., E.S., G.M.R., X.H. and M.C.F. contributed to the bioinformatics and statistical analysis; P.J. wrote the first draft of the manuscript and all authors contributed substantially to revisions.

DATA AVAILABILITY STATEMENT

Data and R scripts are available at Data Dryad: https://doi.org/10.5061/dryad.pg4f4qrnb

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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