



High prevalence of *Batrachochytrium dendrobatidis* in an Andean frog community (Reserva Las Galarias, Ecuador)

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Abstract.—We report patterns of infection of *Batrachochytrium dendrobatidis* (*Bd*) in a cloud forest amphibian community in the Andean Western Cordillera of Ecuador (Reserva Las Galarias). Data were obtained during the rainy seasons of two consecutive years, using qPCR (year 2012) and end-point PCR (year 2013). We show that average *Bd* prevalence in this amphibian community is high (2012: 35–49%; 2013: 14–32%), but found no evidence of population declines or that *Bd* is negatively affecting host populations. We found a significant correlation between *Bd* prevalence and taxonomy, reproductive mode, and habitat, but no correlation between *Bd* infection intensity and the same three variables. Contrary to our expectations, frog species with aquatic reproductive modes (glassfrogs, treefrogs) showed lower *Bd* prevalence than direct-developing frogs (*Pristimantis* spp.). Although further monitoring is needed to determine long-term population trends, our two-year dataset on disease and population size support the hypothesis that frogs are tolerant to infection, a condition that could potentially have resulted from exposure to previous *Bd* epidemic outbreaks.

Resumen.—En este estudio reportamos datos sobre los patrones de infección de *Batrachochytrium dendrobatidis* (*Bd*) en una comunidad de anfibios en la Cordillera Occidental de los Andes del Ecuador (Reserva Las Galarias). Los datos fueron obtenidos durante la estación lluviosa en dos años consecutivos, utilizando qPCR (año 2012) y PCR de punto final (año 2013). Los resultados muestran una alta prevalencia de *Bd* en la comunidad (2012: 35–49%; 2013: 14–32%); sin embargo, no se encontró evidencia de disminuciones poblacionales o de que *Bd* esté afectando negativamente a las especies de anfibios. Existe una relación significativa entre la prevalencia de *Bd* y la taxonomía, modo reproductivo y hábitat de los anfibios, pero no hubo correlación entre la intensidad de infección de *Bd* y las mismas tres variables. Contrario a nuestras predicciones, las especies de anuros con larvas acuáticas (ranas de cristal, ranas arbóreas) presentaron prevalencias de *Bd* más bajas que los anuros de desarrollo directo (*Pristimantis* spp.). A pesar de que se requiere de un monitoreo continuo para determinar las dinámicas poblacionales a largo plazo, los datos obtenidos hasta el momento apoyan un escenario donde las especies de ranas de la Reserva Las Galarias parecen tolerar la infección de *Bd*, una condición posiblemente adquirida mediante la exposición a brotes epidémicos previos.

Key words. Chytridiomycosis, emerging disease, amphibian declines, Andes, conservation

Palabras claves. Quitridiomycosis, enfermedad emergente, declinación de anfibios, Andes, conservación

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Introduction

A third of global amphibian species are threatened with extinction (Stuart et al. 2004; Wake and Vredenburg 2008) and, most concerning, numerous local population declines and extinctions have occurred in relatively pristine areas, where anthropogenic habitat destruction is low (Lips 1998, 1999; Drost and Fellers 1996; La Marca et al. 2005). In the last two decades, several studies have attributed anuran mass mortality events to the emergence of the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*), a pathogen with widespread geographic and ecological distribution (Berger et al. 1998; Daszak et al. 1999, 2003; Lips et al. 2006; Becker and Zamudio 2011; Rodriguez et al. 2014). Alternative explanations to amphibian declines add a role to global warming and temperature variability (Pounds et al. 2006; Rohr and Raffel 2010; Menéndez-Guerrero and Graham 2013).

Batrachochytrium dendrobatidis infects the keratinized skin of amphibians and disrupts the regulatory functioning of the integument (Berger et al. 1998; Voyles et al. 2009). Infection inhibits host immune responses in some species (Fites et al. 2013) and in severe cases of infection, electrolyte depletion and osmotic imbalance may lead to mortality (Voyles et al. 2007, 2009). However, not all amphibian species are equally susceptible to the pathogen. For example, at Santa Fé, Panama, *Bd* has caused declines or local extinctions of most anurans in the original community, but six species of frogs and toads remain abundant, despite being infected by the fungus (Lips et al. 2006). In laboratory challenge experiments, amphibian mortality rates range from 0% to 100%, depending on the species, host age, pathogen genotype, and dosage (Berger et al. 2005a; Daszak et al. 2004; Longo et al. 2014). The reasons for host differences in susceptibility include immunogenic variation (Ellison et al. 2014; Savage et al. 2014), microhabitat use (Kriger and Hero 2007; Gründler et al. 2012), association with water as embryos, tadpoles, or adults (Lips et al. 2003), and host thermoregulatory behavior (Richards-Zawacki 2010). Because *Bd* transmission may happen through frog-frog contact, or through motile zoospore movement from one host to another, frogs and toads that spend more time in water are expected to have higher exposure and susceptibility to infection than species that are primarily terrestrial (i.e., direct developers; Lips et al. 2003; Kriger and Hero 2007).

In this study, we report data on infection patterns of *Bd* obtained during the rainy seasons of two consecutive years in the amphibian community of Reserva Las Galarías, a cloud forest site in the Andean Western Cordillera of Ecuador. We found that *Bd* prevalence in all amphibian species is high, but found no evidence that *Bd* is negatively affecting amphibians (i.e., no apparent population declines, or records of clinical signs of chytridiomycosis). We report on infection prevalence and intensity dynamics for the two-year period, and examine

patterns of *Bd* infection in species that vary in their taxonomy, reproductive mode, and habitat.

Materials and Methods

Study site: The study was conducted at Reserva Las Galarías (0°01' S, 78°44' W; 1822–2400 m), a private reserve covering an area of 1,063 acres (425 ha) located on the Pacific slopes of the Andes, Pichincha Province, Mindo Parish, Ecuador. The study site has an elevational range of 1,825–2,400 m and includes primary and secondary forest, regenerating pasture, and numerous ephemeral and permanent streams and creeks (Hutter and Guayasamin 2012).

Amphibian taxonomy: For generic and suprageneric classification, we follow the taxonomic proposals of Hedges et al. (2008), Guayasamin et al. (2009), and Faivovich et al. (2005), Pyron and Wiens (2011), as summarized in Frost (2014).

Amphibian richness and abundance: During the rainy seasons of 2012 (23 January–29 March) and 2013 (14 March–22 April), we sampled trails of Reserva Las Galarías during the night, including most of its habitat heterogeneity and elevational gradient, to record the species richness of the reserve. We placed eight transects, each with an area of 500 × 4 m (Appendix 1), to maximize species detection and to obtain a baseline dataset on population size and *Bd* prevalence. Each transect was sampled by two people for 3–4 hours during the night (generally starting at 8 pm); temperatures during sampling varied between 11–15 °C. All detected amphibians were, when possible, photographed. Calling males were also reported and identified with the aid of photographic and acoustic guides (Arteaga et al. 2013; Centro Jambatu 2011–2014). We used a Student's *t*-test to quantify differences in population sizes in transects that were sampled multiple times during the rainy season of 2012 and 2013 (Lucy's Creek and Kathy's Creek); the normality of species abundance was assessed using a Shapiro-Wilk Test.

Diagnosis of *Batrachochytrium dendrobatidis*: We swabbed the ventral regions of all amphibians captured in our survey, following the standard procedures in Hyatt et al. (2007; Fig. 1); dry swabs were stored in -4 °C until analysis. Testing for *Bd* was carried out using Real-Time PCR (q-PCR) for samples obtained during 2012 and endpoint Polymerase Chain Reaction (PCR) for samples obtained in 2013; the use of these two methods was contingent on access to q-PCR (available during 2012). In both cases, DNA extractions were carried out using guanidinium thiocyanate. For samples obtained during 2012, we used a 1:10 dilution of the extract as template in Taqman q-PCR assays for the detection of *Bd* (Boyle et al. 2004). This assay uses *Bd*-specific primers ITS1-3 Chytr and 5.8S Chytr, in addition to the fluorescently-labeled probe Chytr MGB2, and amplifies the ITS-1 fragment of the



Fig. 1. Swab sample obtained from *Centrolene heloderma* at Reserva Las Gralarias, Ecuador.

Bd genome at the junction of the ITS-1 and 5.8S regions. We used a standard curve that included 1000, 100, 10, 1, and 0.1 zoospore genome equivalents, and followed qPCR conditions described in Boyle et al. (2004). For samples obtained during 2013, *Bd* presence was tested using the internal transcribed spacer regions (ITS-1, ITS-2) primers Bd1a (5'-CAGTGTGCCATATGTCACG-3') and Bd2a (5'-CATGGTTCATATCTGTCCAG-3') developed by Annis et al. (2004); the presence/absence of *Bd* was determined via the visualization of the amplified band in agarose gel electrophoresis. The two methods to detect *Bd* have different sensitivities; therefore, direct comparisons of *Bd* prevalence between years should be considered with caution. However, family and habitat correlates with infection status should not be biased by detection method, and qPCR offers the additional advantage of quantifying infection intensity (load).

Prevalence and correlates of *Batrachochytrium dendrobatidis* in amphibians: We estimated prevalence of

Bd within each anuran species as the number of frogs that tested positive for *Bd*, divided by the total number of sampled frogs for that particular species in a given year. We estimated the 95% confidence interval for prevalence in each species, (Wilson 1927; Newcomb 1998). We modeled *Bd* presence or absence in each individual by using a logistic regression. We tested for possible associations of *Bd* prevalence with the following variables: habitat (terrestrial, riparian, lentic), reproductive mode (aquatic, terrestrial), and taxonomy (family). Statistical significance of results was assessed with a chi-square test.

Infection intensity of *Batrachochytrium dendrobatidis* and correlates in amphibians: We tested for possible associations of *Bd* intensity (measured as zoospore genomic equivalents) with the following variables: habitat (terrestrial, riparian, lentic), reproductive mode (aquatic, terrestrial), and taxonomy (family, genus, species; Appendix 2). Given the strong right skew of infection load, we used the non-parametric Kruskal-Wallis test. All statistical analyses were performed using R v. 2.15.3 (R CoreTeam 2012).

Results

Species richness and abundance: During the two sampling periods, we recorded a total of 2,450 individuals of 28 species (Appendix 2). The abundance of species at Lucy's Creek and Kathy's Creek is summarized in Tables 1 and 2. Because most taxa were scarce, we restricted the comparisons between years to relatively abundant species (glassfrogs). Abundances of glassfrogs at Lucy's Creek and Kathy's Creek were not significantly different between years (Tables 1, 2).

Prevalence of *Batrachochytrium dendrobatidis* in amphibians: Swabs of 320 frogs were tested for *Bd*, and

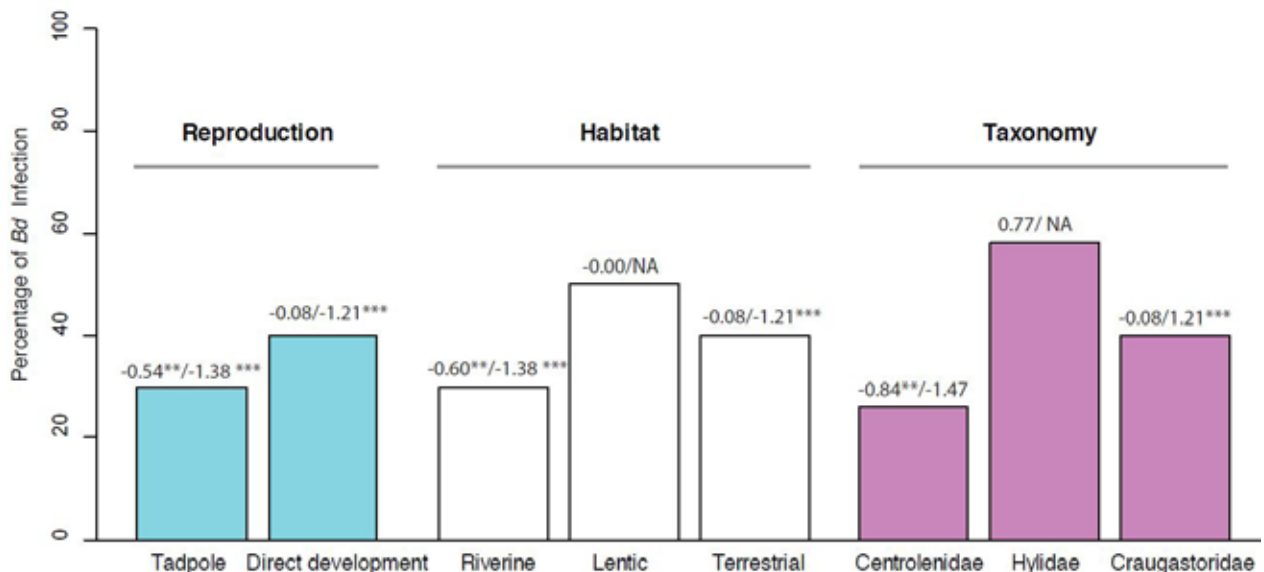


Fig. 2. Significant *Bd* infection differences in amphibians according to reproductive modes, habitat use, and taxonomy. *P* values are reported for 2012 and 2013; significance is noted by ** (*p* < 0.01) and *** (*p* < 0.001).

Table 1. Abundance of amphibian species at Lucy's Creek, Reserva Las Gralarias. Abundances are presented as minimum–maximum, followed, in parenthesis, by mean \pm standard error. The Student's *t*-test was performed only in species with normally distributed abundances.

Year	Lucy's Creek		t-test (<i>p</i>)	Population trend
	2012	2013		
Number of nights sampled	10	6		
Family/Species				
Centrolenidae				
<i>Centrolene lynchi</i>	5–20 (13.1 \pm 5.13)	2–18 (9.0 \pm 6.54)	0.184	No difference
<i>Centrolene peristictum</i>	4–35 (20.7 \pm 11.68)	6–26 (15.2 \pm 7.00)	0.314	No difference
<i>Nymphargus grandisonae</i>	1–10 (6.2 \pm 3.12)	0–8 (3.8 \pm 2.93)	0.155	No difference
Hylidae				
<i>Hyloscirtus alytolylax</i>	3–7 (4.3 \pm 1.42)	0–6 (3.3 \pm 2.16)	0.295	No difference
Craugastoridae				
<i>Pristimantis achatinus</i>	0–2 (0.3 \pm 0.67)	0–1 (0.2 \pm 0.41)	—	—
<i>Pristimantis appendiculatus</i>	0–2 (0.8 \pm 0.92)	0–1 (0.5 \pm 0.55)	—	—
<i>Pristimantis calcarulatus</i>	0–2 (0.2 \pm 0.63)	0–1 (0.2 \pm 0.41)	—	—
<i>Pristimantis eremitus</i>	0	0–2 (0.5 \pm 0.84)	—	—
<i>Pristimantis eugeniae</i>	0	0–2 (0.3 \pm 0.82)	—	—
<i>Pristimantis illotus</i>	0–1 (0.1 \pm 0.32)	0	—	—
<i>Pristimantis parvillus</i>	0–1 (0.1 \pm 0.32)	0	—	—
<i>Pristimantis sobetes</i>	0	0–2 (0.3 \pm 0.82)	—	—
<i>Pristimantis w-nigrum</i>	0–2 (0.6 \pm 0.84)	0–2 (0.7 \pm 0.82)	—	—

approximately a third of those were positive. In samples from 2012, prevalence of *Bd* was relatively high, with 42% of all frogs testing positive for *Bd* infection. During 2013, *Bd* prevalence was 22%. Differences in prevalence between the two years are likely caused by detection method. Most species infected in 2012 carried low *Bd* loads as determined by qPCR; the highest *Bd* load obtained was in *Centrolene ballux* with 22.5 genomic equivalents. Prevalence per species per year is summarized in Table 3.

The logistic regression shows a significant relationship ($p < 0.001$) of *Bd* infection with species reproductive mode, habitat, and taxonomy (Fig. 2). Frogs with a terrestrial reproductive mode (direct developers; i.e., genus *Pristimantis*; see Duellman and Trueb 1986) have a higher *Bd* prevalence than amphibians with aquatic reproduction (i.e., glassfrogs and treefrogs). Frog species that are dependent on riverine habitats for reproduction show significantly less infection than anurans that use terrestrial or lentic habitats for reproduction ($p < 0.001$). Also, species in the Centrolenidae family (glassfrogs) show a lower *Bd* prevalence than species in Craugastoridae and Hylidae (Table 3). Although, *Bd* prevalence during 2012 was significantly higher than in 2013 (probably as a result of higher sensitivity of qPCR), we found no significant interaction among sampling year and reproductive mode, habitat, or taxonomy.

Infection intensity of *Batrachochytrium dendrobatidis* and correlates in amphibians: We found no relation-

ship between *Bd* infection intensity (*Bd* load, year 2012) and taxonomy, reproductive mode, or habitat.

Discussion

Our results show a relatively high mean prevalence of *Bd* (36%) across both years in the Andean frog community of Reserva Las Gralarias (see Hossack et al. 2010 for comparison). From a total of 20 species analyzed, only three (*Nymphargus griffithsi*, *Pristimantis illotus*, and *P. pteridophilus*) tested negative for *Bd*; however, sample sizes for non-infected species were low (5, 1, and 3 individuals, respectively). Infected species included frogs with very different reproductive modes, including taxa with terrestrial direct development (*Pristimantis* spp.), species that deposit eggs in ponds (*Dendropsophus carnifex*), and others that place their eggs on vegetation from where hatching tadpoles drop into streams (*Centrolene* spp., *Nymphargus* spp., *Hyloscirtus* spp.).

Because *Bd* is an aquatic pathogen (Berger et al. 2005b) we expected amphibian species with aquatic reproductive modes to show higher infection prevalence (Lips et al. 2005; Brem and Lips 2008). In fact, the most dramatic amphibian declines and extinctions in the Andes have occurred in species with aquatic larvae (La Marca et al. 2005; Bustamante et al. 2005; Merino-Viteri et al. 2005; Coloma et al. 2010). Our results indicate, surprisingly, a higher *Bd* prevalence in frogs with a terrestrial reproductive mode (*Pristimantis* spp.) than in those that

Table 2. Abundance of amphibian species at Kathy’s Creek, Reserva Las Galarías. Abundances are presented as minimum–maximum, followed, in parenthesis, by mean ± standard error. The Student’s *t*-test was performed only in species with normally distributed abundances.

Year	Kathy’s Creek		t-test	Population trend
	2012	2013		
Number of nights sampled	10	5		
Family/Species				
Centrolenidae				
<i>Centrolene ballux</i>	5–37 (22.7 ± 11.6)	3–25 (11.4 ± 8.67)	0.078	No difference
<i>Centrolene peristictum</i>	0–5 (2.1 ± 1.66)	0–5 (2.0 ± 1.87)	0.918	No difference
<i>Nymphargus grandisonae</i>	0–7 (3.7 ± 2.21)	0–6 (2.2 ± 2.28)	0.242	No difference
<i>Nymphargus griffithsi</i>	0–8 (2.3 ± 2.26)	0–3 (1.4 ± 1.34)	—	—
<i>Nymphargus lasgalarías</i>	3–28 (19.4 ± 8.54)	7–28 (15.0 ± 8.69)	0.366	No difference
Hylidae				
<i>Hyloscirtus alytolylax</i>	0–1 (0.1 ± 0.32)	0–1 (0.4 ± 0.59)	—	—
Craugastoridae				
<i>Pristimantis achatinus</i>	0–3 (0.3 ± 0.95)	0–1 (0.2 ± 0.45)	—	—
<i>Pristimantis appendiculatus</i>	0–7 (1.2 ± 2.10)	0–1 (0.4 ± 0.59)	—	—
<i>Pristimantis calcarulatus</i>	0–3 (1.1 ± 0.74)	0–3 (1.0 ± 1.23)	—	—
<i>Pristimantis eremitus</i>	0–1 (0.1 ± 0.32)	0–1 (0.2 ± 0.45)	—	—
<i>Pristimantis eugeniae</i>	0–1 (0.1 ± 0.32)	1–2 (0.8 ± 0.84)	—	—
<i>Pristimantis sobetes</i>	0–1 (0.2 ± 0.42)	0–2 (0.4 ± 0.89)	—	—
<i>Pristimantis w-nigrum</i>	0–1 (0.1 ± 0.32)	0–1 (0.4 ± 0.59)	—	—

reproduce in water (mainly glassfrogs; *Centrolene* spp., *Nymphargus* spp.). This finding supports the idea that even terrestrial breeders may serve as reservoirs for the pathogen in diverse amphibian communities (Longo et al. 2013). Higher prevalence in terrestrial frogs requires that *Bd* zoospores survive in terrestrial habitats. Johnson and Speare (2003) indicated that *Bd* can survive in moist soil for up to three months. Cloud forests in western Ecuador typically have near constant rain and high levels of humidity during the rainy season (Hutter and Guayasamin 2012; Arteaga et al. 2013), and this may extend zoospore survival in terrestrial environments at Las Galarías. Higher *Bd* prevalence in terrestrial frogs compared to that in frogs with aquatic reproduction might also be related to intrinsic differences in, for example, the efficacy of immune responses (Rosenblum et al. 2009; Woodhams et al. 2007) or differences in anuran skin microbiota (Flechas et al. 2012).

Our results also show that *Bd* prevalence is significantly associated with taxonomy (i.e., family). Thus, glassfrogs (family Centrolenidae) might have immune responses or skin microbiota that work as better barriers to the pathogen than those in terrestrial (i.e., *Pristimantis*) frogs. The strong correlation of prevalence with taxonomy, habitat, and reproductive mode (Appendix 2) indicates that further studies need to focus on the specific effects of each of these factors; in other words, phylogeny (and taxonomy) correlates with reproductive mode and habitat use.

A second surprising finding of our study is that, although prevalence of *Bd* is high in most anuran species,

we did not observe any sign of population declines or abrupt crashes, nor have we found dead or sick frogs during four years of intensive fieldwork (2010–2014; JMG pers. obs.). Thus, this frog community persists with an endemic pathogen and with relatively low loads (less than 10 zoospores, Table 3). The apparent increased resistance or tolerance of amphibians from Reserva Las Galarías to *Bd* infection may be explained by one or several of the following mechanisms: (i) amphibian innate and/or acquired defense mechanisms (Savage and Zamudio 2012; Woodhams et al. 2007); (ii) skin bacterial commensals with anti-fungal properties (Harris et al. 2006); (iii) behavioral and ecological factors that reduce the likelihood of infection and disease (e.g., microhabitat selection, reproductive mode; Lips et al. 2003; Rowley and Alford, 2007), and/or (iv) variation in *Bd* virulence (Berger et al. 2005a; Fisher et al. 2009). Our amphibian monitoring took place during the rainy season when most species are active. Therefore, future studies should determine whether or not this apparent tolerance to *Bd* is stable through longer periods of time or if it fluctuates depending on environmental variables influencing host immunity, behavior, microbiota, or pathogenicity.

After the emergence of an infectious disease, surviving hosts can evolve tolerance or resistance (Retallick et al. 2004; Savage and Zamudio 2011). The earliest known record of *Bd* in Ecuador is in 1980, in the Harlequin frog *Atelopus bomolochos* (Ron and Merino-Viteri 2000), a species that is now probably extinct (Coloma et al. 2014). If *Bd* reached and spread in Ecuador during the early 1980s (Ron et al. 2003; Lips et al. 2008), we hy-

Table 3. Prevalence of *Batrachochytrium dendrobatidis* (*Bd*) in amphibians at Reserva Las Galarías, Ecuador, during the rainy seasons of 2012 and 2013. *Bd* prevalence for each species is followed, in parenthesis, by a 95% confidence interval. *Bd* load summary data includes only samples that tested positive for *Bd*.

	2012				2013		
	N	Positive <i>Bd</i>	Prevalence	<i>Bd</i> Load (mean ± sd)	N	Positive <i>Bd</i>	Prevalence
Family: Centrolenidae							
<i>Centrolene ballux</i>	17	8	47% (24–71%)	6.5 ± 10.7	9	2	22% (39–59%)
<i>Centrolene heloderma</i>	6	1	17% (1–63%)	0.6	1	0	0% (0–95%)
<i>Centrolene lynchi</i>	6	1	17% (1–63%)	–	5	1	20% (1–70%)
<i>Centrolene peristictum</i>	21	6	29% (12–52%)	2.1 ± 1.5	16	3	19% (5–46%)
<i>Nymphargus grandisonae</i>	21	5	24% (9–48%)	4.7	–	–	–
<i>Nymphargus griffithsi</i>	3	0	0% (1–69%)	–	2	0	0% (0–80%)
<i>Nymphargus lasgalarías</i>	16	6	38% (16–64%)	6.5 ± 1.5	10	2	20% (4–56%)
Family: Hylidae							
<i>Dendropsophus carnifex</i>	10	5	50% (20–80%)	–	–	–	–
<i>Hyloscirtus alytolylax</i>	9	8	89% (51–99%)	2.4 ± 2.4	7	2	29% (5–70%)
Family: Craugastoridae							
<i>Pristimantis achatinus</i>	7	4	57% (20–88%)	–	–	–	–
<i>Pristimantis appendiculatus</i>	23	10	44% (24–65%)	1.9 ± 2.4	–	–	–
<i>Pristimantis calcarulatus</i>	15	2	13% (2–42%)	1.1	15	1	7% (4–34%)
<i>Pristimantis eremitus</i>	4	4	100% (40–100%)	0.9 ± 0.2	4	2	50% (9–91%)
<i>Pristimantis eugeniae</i>	18	12	66% (41–86%)	2.5	2	1	50% (3–97%)
<i>Pristimantis hectus</i>	8	2	25% (4–64%)	–	14	4	29% (10–58%)
<i>Pristimantis illotus</i>	–	–	–	–	1	0	0% (0–95%)
<i>Pristimantis parvillus</i>	9	4	44% (15–77%)	–	–	–	–
<i>Pristimantis sobetes</i>	8	3	38% (10–74%)	–	9	3	33% (9–69%)
<i>Pristimantis pteridophilus</i>	–	–	–	–	3	0	0% (0–69%)
<i>Pristimantis w-nigrum</i>	21	13	62% (39–81%)	1.5 ± 0.7	–	–	–
TOTAL	222	94	42% (35–49%)		88	19	22% (14–32%)

pothesize that many of the population declines observed in the country at that time (e.g., Coloma 1995, 2002; Coloma et al. 2000; Ron et al. 2003; Bustamante et al. 2005; La Marca et al. 2005; Lips et al. 2008; Coloma et al. 2010) could be attributable to chytridiomycosis. Thus, it is probable that most Andean amphibian communities

have been exposed to *Bd* for more than three decades and that current sampling finds remnant species that are tolerant to *Bd* while the susceptible species are already extinct. Under this scenario, selection should have favored the persistence of amphibian species or specific populations that have developed defenses against *Bd*; therefore,

changing host composition of these communities right after pathogen emergence. We acknowledge, however, that this is a working hypothesis which assumptions depend on our knowledge of the historical distribution of the chytrid. For example, if new data shows that *Bd* was present in the Ecuadorian Andes before amphibian declines were noticed, such piece of information would support the endemic pathogen hypothesis, which states that environmental changes triggered *Bd* outbreaks (Rachowicz et al. 2005).

Reserva Las Gralarias is one of the most studied and species-rich area in the cloud forest of the tropical Andes, containing numerous species considered endangered by the IUCN (2014; see Appendix 2). However, the community (and surrounding areas) lacks at least three groups of species that were conspicuous in Ecuadorian cloud forests: marsupial frogs (*Gastrotheca plumbea*, *G. guentheri*), harlequin frogs (*Atelopus longirostris*, *A. mindoensis*), and dendrobatid frogs (*Hyloxalus lehmani*, *H. maquipucuna*) (Coloma et al. 2011–2014; Arteaga et al. 2013). Marsupial and harlequin frogs are particularly susceptible to *Bd* (Lips et al. 2003; Flechas et al. 2012; Ellison et al. 2014; DiRenzo et al. 2014) and are the primary species that suffered population declines and extinctions in Ecuador (Lips et al. 2002; La Marca 2005) even in pristine areas. The absence of these lineages at Reserva Las Gralarias supports to the hypothesis that this is a post-decline amphibian community. Understanding the long-term effects of pathogens (eg., chytrid) and temperature variability in such a community is essential for the continued effective management of endangered species in the Andean cloud forests.

Considering Ecuador's high diversity of amphibian species, life history modes, and evolutionary history, our study provides a baseline to study the evolution of defense strategies against *Bd*. We recommend further research to determine the mechanisms driving the observed differences in pathogen exposure among hosts differing in reproductive modes, habitat, and taxonomy.

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Appendix 1. Transects sampled at Reserva Las Gralarias. Each transect has an area of 500 × 4 m. Latitude and longitude are in decimal degrees.

Transect	Elevation (m)	Latitude	Longitude	Habitat
Lucy’s creek	1822–1858	At start: -0.00492 At end: -0.00342	At start: -78.73344 At end: -78.74051	Riverine vegetation along creek
Kathy’s creek	2041–2066	At start: -0.01696 At end: -0.0156	At start: -78.7314 At end: -78.73386	Riverine vegetation along creek
Santa Rosa river	1884–1882	At start: -0.0133 At end: -0.01054	At start: -78.72368 At end: -78.7211	Riverine vegetation along river
Waterfall trail	1897–2107	At start: -0.0135 At end: -0.01379	At start: -78.72461 At end: -78.7269	Primary and secondary terra firme forest
Five-Frog creek	2141–2156	At start: -0.03166 At end: -0.03098	At start: -78.70421 At end: -78.70853	Riverine vegetation along creek
Osoverde & Guarumo trail	2141–2156	At start: -0.03166 At end: -0.03098	At start: -78.70421 At end: -78.70853	Primary and secondary terra firme forest
Puma trail	1923–2031	At start: -0.00954 At end: -0.00708	At start: -78.7346 At end: -78.73662	Primary and secondary terra firme forest
TKA trail	2192–2216	At start: -0.0275 At end: -0.02516	At start: -78.70477 At end: -78.70353	Primary and secondary terra firme forest
Peccary trail	1803–1896	At start: -0.00750 At end: -0.0076	At start: -78.72635 At end: -78.72862	Primary and secondary terra firme forest

Appendix 2. Amphibians at Reserva Las Gralarias, with corresponding IUCN (2014) conservation status. The list includes three potential new species (*Pristimantis* sp. 1, *Pristimantis* sp. 2, and *Pristimantis* sp. 3). Reproductive modes are sensu Haddad and Prado (2005).

Species	Reproductive mode	Habitat for reproduction	Conservation status
Family: Centrolenidae (7 spp.)	Mode 25: Eggs hatching into exotrophic tadpoles that drop in lotic water		
<i>Centrolene ballux</i>	Mode 25	Vegetation along fast-flowing streams	Critically Endangered
<i>Centrolene heloderma</i>	Mode 25	Vegetation along fast-flowing streams	Critically Endangered
<i>Centrolene lynchi</i>	Mode 25	Vegetation along fast-flowing streams	Endangered
<i>Centrolene peristictum</i>	Mode 25	Vegetation along fast-flowing streams	Vulnerable
<i>Nymphargus griffithsi</i>	Mode 25	Vegetation along fast-flowing streams	Vulnerable
<i>Nymphargus grandisonae</i>	Mode 25	Vegetation along fast-flowing streams	Least Concern
<i>Nymphargus lasgralarias</i>	Mode 25	Vegetation along fast-flowing streams	Data Deficient

Batrachochytrium dendrobatidis in an Andean frog community

Appendix 2 (continued). Amphibians at Reserva Las Gralarias, with corresponding IUCN (2014) conservation status. The list includes three potential new species (*Pristimantis* sp. 1, *Pristimantis* sp. 2, and *Pristimantis* sp. 3). Reproductive modes are sensu Haddad and Prado (2005).

Species	Reproductive mode	Habitat for reproduction	Conservation status
Family: Craugastoridae (16 spp.)	Mode 23: Direct development of terrestrial eggs		
<i>Pristimantis achatinus</i>	Mode 23	Terrestrial, mainly in pastures and modified environments	Least Concern
<i>Pristimantis appendiculatus</i>	Mode 23	Terrestrial, mainly primary and secondary forests	Least Concern
<i>Pristimantis calcarulatus</i>	Mode 23	Terrestrial, mainly primary and secondary forests	Vulnerable
<i>Pristimantis crenunguis</i>	Mode 23	Terrestrial, mainly primary and secondary forests	Endangered
<i>Pristimantis eremitus</i>	Mode 23	Terrestrial, mainly primary and secondary forests	Vulnerable
<i>Pristimantis eugeniae</i>	Mode 23	Terrestrial, mainly primary and secondary forests	Endangered
<i>Pristimantis hectus</i>	Mode 23	Terrestrial, mainly primary and secondary forests	Data deficient
<i>Pristimantis illotus</i>	Mode 23	Terrestrial, mainly primary and secondary forests	Near Threatened
<i>Pristimantis parvillus</i>	Mode 23	Terrestrial, mainly primary and secondary forests	Least Concern
<i>Pristimantis peridophilus</i>	Mode 23	Terrestrial, mainly primary and secondary forests	Endangered
<i>Pristimantis sobetes</i>	Mode 23	Terrestrial, mainly primary and secondary forests	Endangered
<i>Pristimantis verecundus</i>	Mode 23	Terrestrial, mainly primary and secondary forests	Vulnerable
<i>Pristimantis w-nigrum</i>	Mode 23	Terrestrial, mainly primary and secondary forests	Least Concern
<i>Pristimantis</i> sp. 1	Mode 23	Terrestrial, mainly primary and secondary forests	Not evaluated
<i>Pristimantis</i> sp. 2	Mode 23	Terrestrial, mainly primary and secondary forests	Not evaluated
<i>Pristimantis</i> sp. 3	Mode 23	Terrestrial, mainly primary and secondary forests	Not evaluated
Family: Hylidae (3 spp)			
<i>Dendropsophus carnifex</i>	Mode 1: Eggs and exotrophic tadpoles in lentic water	Ponds	Least Concern
<i>Hyloscirtus alytolylax</i>	Mode 25	Vegetation along fast-flowing streams	Near Threatened
<i>Hyloscirtus criptico</i>	Mode 25	Vegetation along fast-flowing streams	Not evaluated
Family: Caeciliidae (1 sp.)			
<i>Caecilia buckleyi</i>	Unknown	Unknown	Not evaluated
Family: Rhinatrematidae (1 sp.)			
<i>Epicrionops bicolor</i>	Unknown	Unknown	Least Concern



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