EcoHealth
https://doi.org/10.1007/s10393-021-01570-8

ECOHEALTH

Original Contribution

Three Pathogens Impact Terrestrial Frogs from a High-Elevation Tropical Hotspot

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Abstract: Three infectious pathogens *Batrachochytrium dendrobatidis* (*Bd*), *Ranavirus* (*Rv*) and Perkinsea (Pr) are associated with widespread and ongoing amphibian population declines. Although their geographic and host ranges vary widely, recent studies have suggested that the occurrence of these pathogens could be more common than previously thought, even in direct-developing terrestrial species traditionally considered less likely to harbor these largely aquatic pathogens. Here, we characterize *Bd*, *Rv*, and Pr infections in direct-developing terrestrial amphibians of the *Pristimantis* genus from the highland Ecuadorean Andes. We confirm the first detection of Pr in terrestrial-breeding amphibians and in the Andean region, present the first report of *Rv* in Ecuador, and we add to the handful of studies finding *Bd* infecting *Pristimantis*. Infection prevalence did not differ significantly among pathogens, but infection intensity was significantly higher for *Bd* compared to Pr. Neither prevalence nor intensity differed significantly across locality and elevation for *Bd* and *Rv*, although low prevalence in our dataset and lack of seasonal sampling could have prevented important epidemiological patterns from emerging. Our study highlights the importance of incorporating pathogen surveillance in biodiversity monitoring in the Andean region and serves as starting point to understand pathogen dynamics, transmission, and impacts in terrestrial-breeding frogs.

Keywords: *Batrachochytrium dendrobatidis*, ranavirus, perkinsea, andes, tropics, *Pristimantis*

Introduction

Infectious diseases pose threats to amphibian persistence due to their potential to spread into new areas, infect naïve hosts, and thrive in novel environments (Daszak et al., 2000; Crowl et al., 2008). Understanding pathogen geographic distributions and host ranges is widely recognized as a priority in amphibian conservation strategies (DiRenzo et al., 2019). Currently, the three pathogens *Batrachochytrium dendrobatidis (Bd)*, *Ranavirus* (*Rv*) and a yet undescribed Perkinsea protist (Pr) are the cause for the most common infectious diseases affecting amphibians worldwide (Isidoro-Ayala et al., 2017) and have been associated with multiple population declines across a wide diversity of anuran clades. Given the apparent absence of

Supplementary Information: The online version contains supplementary material available at https://doi.org/10.1007/s10393-021-01570-8.

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Published online: 11 December 2021

host specificity and the potential severity and amplitude of these infectious pathogens, analyzing their presence, especially in amphibian-diverse regions, remains critical to inform local conservation plans and establish baselines for future monitoring.

Studies of amphibian pathogens have overwhelmingly focused on *Bd* because it is the most widespread and devastating pathogen (see Scheele et al., 2019) affecting thousands of amphibian species (Lips, 2016). *Bd* is a waterborne, single-celled chytrid fungus that infects keratinized tissues of amphibians, including skin of metamorphosed frogs and mouthparts of tadpoles (Moss et al., 2008). *Bd* is particularly linked to amphibian population declines in high-elevation tropical regions (Catenazzi et al., 2013; La Marca et al., 2005; Longo et al., 2010). Most notably, dramatic effects of *Bd* have been recorded in the highland Andes of South America (e.g., Lips et al., 2008; Catenazzi et al., 2011), Central America (Campos-Cerqueira and Aide, 2017) and lowland Amazon (von May et al., 2018). However, even within locations, community *Bd* dynamics can vary widely across host species (including closely related congeners; Savage et al., 2018), life history traits (Gervasi et al., 2013), and environmental gradients (Karvemo et al., 2018). For instance, higher *Bd* prevalence is generally associated with higher elevation ecosystems in tropical regions because these areas present moist and cool climates ideal for *Bd* growth (Piotrowski et al., 2004). However, in mountain regions such as in the Andes where peaks can reach over 4000 m, elevations and associated climatic conditions shift rapidly, thus *Bd* prevalence may also vary within small spatial scales (Zimkus et al., 2020). In addition, distinct degrees of susceptibility and infection risk of *Bd* have been associated with host life history, with a general trend of water-associated species having increased infection risk compared with terrestrial breeding species (Krieger and Hero, 2007). Due to this increased infection risk, most studies have focused on understanding the prevalence and community dynamics of *Bd* in clades linked to water bodies (Ruggeri et al., 2015; Valencia-Aguilar et al., 2016), However, recent community-wide studies demonstrate that, although the prevalence of *Bd* is comparatively low in terrestrial breeding amphibians, it could still impact population persistence over time (Becker et al., 2019). Interestingly, the prevalence of *Bd* in terrestrial amphibians likely results from spillover infections originating in sympatric aquatic and semi-aquatic infected hosts (Longo et al., 2010; Becker et al., 2019), thus, highlighting the importance of assessing the prevalence of *Bd* on terrestrial breeding frogs, especially in regions where *Bd* infections have been historically documented.

Although far less is known about disease dynamics of Pr and *Rv* compared to *Bd*, some general patterns have been established. Pr mostly infects amphibian embryos and larvae, although some studies have reported this pathogen in adult individuals from aquatic and semiaquatic species only (Cook et al., 2008; Karwacki et al., 2018, 2021; Galt et al. 2021). The geographic distribution of Pr is only known from a few localities throughout the world, including Panama (Smilansky et al. 2021) and a single record in South America from French Guiana (Chambouvet et al., 2015). *Rv* is known to infect over a hundred amphibian species; however its hosts also include a variety of fish and reptile clades (Schock et al., 2008; Price et al., 2017). *Rv* has been associated with multiple population declines that mainly occur at higher latitudes in North America and Europe (Price et al., 2014), although more recent reports have also confirmed its presence in South and Central America (Warne et al., 2016). While *Rv* can infect all life stages that use aquatic environments, correlating with known waterborne transmission, the presence of *Rv* in frogs without an aquatic phase has been documented in high-elevation ecosystems in the tropical Andes of Peru (Warne et al., 2016). However, due to the scarcity of *Rv* studies, overall presence and prevalence in the tropics and across host species, including within other Andean regions, is still unknown. While the spatial range of Pr, *Rv,* and *Bd* has increased significantly, recent evidence points towards long-term prevalence and coinfection patterns of these three pathogens across native populations (Talley et al., 2015; Karwacki et al., 2021). These recent findings suggest that the co-occurrence of Pr, *Rv*, and *Bd* across regions could be more common than previously thought.

One region where amphibians have been severely affected by pathogenic infections is the highland Andes of Ecuador. Most notably, dramatic population declines and extinctions of species in the highly endangered genera *Atelopus* and *Telmatobius* have been attributed to *Bd* (Coloma et al., 2000; Ron and Merino-Viteri, 2000; Ron et al. 2019). Most studies in this area have consequently been focused on documenting the presence of *Bd* in aquatic-breeding groups such as *Atelopus* and *Gastrotheca* (Ron and Merino-Viteri, 2000; Bresciano et al., 2015). However, the majority of amphibian species in this region are terrestrial-breeding frogs from the *Pristimantis* genus (Ron et al., 2019). To date, only a handful of studies have investigated *Bd* in *Pristimantis* from high-elevation Andean ecosystems

(Catenazzi et al., 2011; Guayasamin et al., 2014; Caceres-Andrade, 2014), and *Rv* and Pr have never been document infecting amphibians in Ecuador. To address this knowledge gap, we used non-invasive swabbing coupled with molecular diagnostics to analyze the prevalence and intensity of *Bd*, *Rv* and Pr in *Pristimantis* frogs from the high-elevation ecosystems in the southern Ecuadorian Andes. In addition, we tested for differences in prevalence and intensity of each pathogen across elevation, localities and species groups.

Methods

Study Area

We collected data across 17 localities in the provinces of Azuay and Cañar in the southern Ecuadorian Andes (Fig. 1). All localities are distributed in an elevational range between 2800 and 4000 m, with an annual average temperature between 2 and 14 °C and an annual average precipitation between 500 and 1500 mm (SIGAGRO, 2002). The study localities are distributed across two ecosystem types: Paramo grassland (12 localities), and Evergreen high montane forest (5 localities) (Ministerio de Ambiente del Ecuador, 2012).

Data Collection

Samples were collected opportunistically between May and July 2018 with authorization from the Ecuadorian Environmental Ministry MAE-DPC-AIC-B-2018–003. We collected specimens of the *Pristimantis* genus between 19:00 and 23:00 h. Before and after visiting each locality, we followed a disinfection protocol according to Aguirre and Lampo (2006) to prevent the potential dispersion of pa-

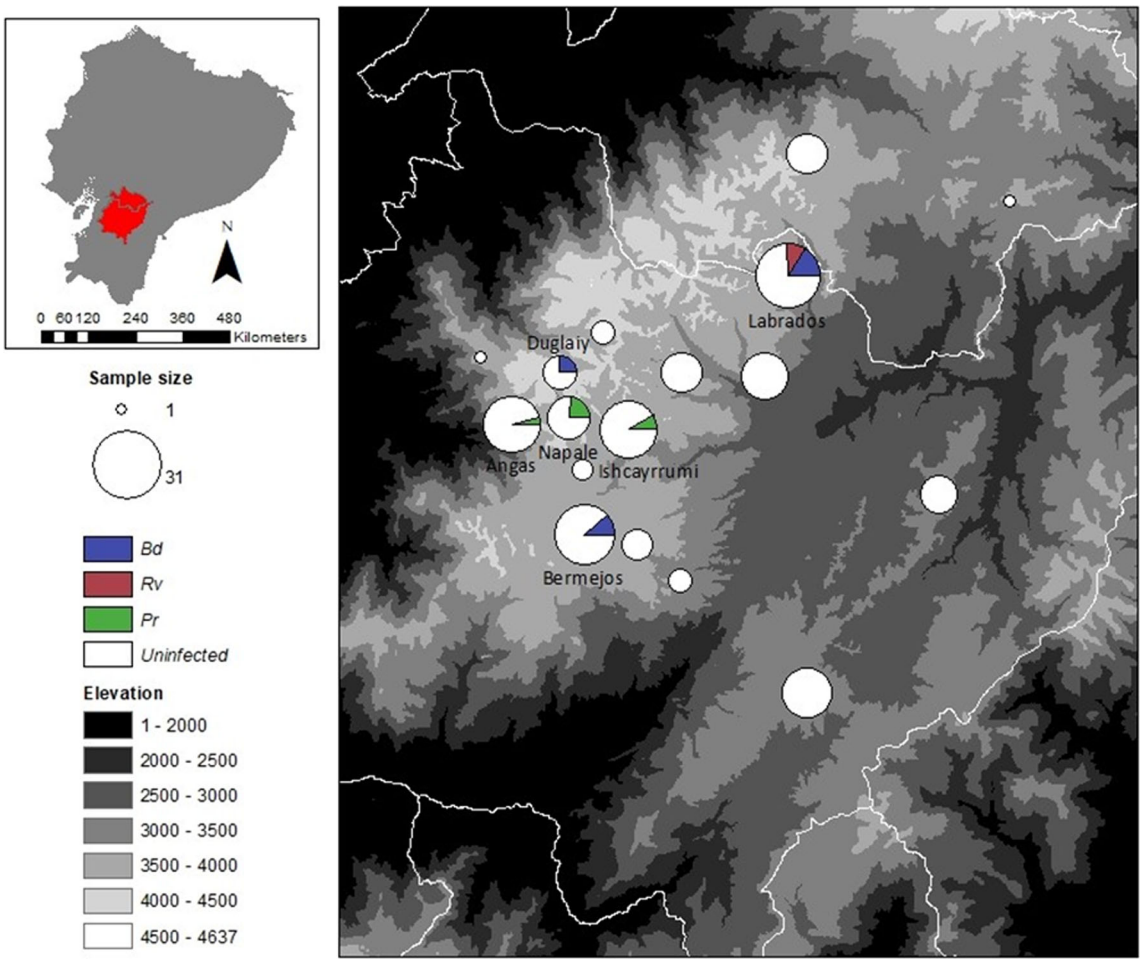

Figure 1. *Bd*, *Rv* and Pr prevalence across sampling localities in the southern Ecuadorean Andes collected between May and July 2018. The colors represent the proportion of infected individuals by each pathogen. The red color highlights the provinces where the sampling localities are located in relation to Ecuador.

thogens among sampling sites. Each individual was collected with disposable gloves (one pair per individual) and deposited inside a transparent plastic bag with dampened pads to maintain humidity. Next, we swabbed each individual using sterile swabs (MW113, MWE, United Kingdom). To standardize our sampling protocol across individuals, we swabbed as follows: five strokes in venter (taken longitudinally from the chest to the lower abdomen), five strokes in dorsum (taken longitudinally from the occipital region to the cloaca), five longitudinal strokes in each thigh, five strokes in each hand and five strokes in each foot (Hyatt et al., 2007). After collecting the 40 strokes, swabs were deposited in sterile 2 mL screw-cap collection tubes pre-filled with 96% ethanol, and were stored at −20 °C until DNA extraction. All protocols used in this study were approved by the University of Central Florida's IACUC protocol #18-16 W.

Species Group Identification

Pristimantis is a genus with notoriously complex taxonomy, and considerable species diversity remains undescribed, especially in the high-elevation ecosystems of our sampling region (Urgiles et al., 2019). Thus, to avoid misleading identification at the species level, we categorized our individuals more conservatively into previously defined species groups. We achieved this by reviewing morphological characters of our specimens and geographical distributions of a subsample of museum collections available from our sampling localities. We referred to the works of Hedges et al., (2008), Paez and Ron (2019), Urgiles et al., (2019) and Sanchez-Nivicela et al., *in prep* to generate morphological comparisons with our individuals. The individuals analyzed were grouped into five species groups: *P. cryophilius*, *P. lacrimosus*, *P. orestes*, *P. phoxocephalus*, and *P. unistrigatus*.

Pathogen Quantification and Validation

DNA was extracted from each swab using Qiagen blood and tissue extraction kits (Qiagen, Valencia, CA, USA) following the manufacturer instructions. The DNA concentration (ng/µl) in each extraction was quantified using NanoDrop One (Thermo Scientific). Each sample was analyzed via qPCR to determine the presence/absence of *Bd*, *Rv*, and Pr and to quantify the pathogen infection intensity (the number of genome equivalents [GE]). We used the primers and the protocols for *Bd*, *Rv*, and Pr from Boyle et al., (2004), Allender et al., (2013), and Karwacki et al., (2018), respectively. The total reaction of 25 µl included 8ul of Supermix (Bio-Rad), 2 µl of each 10 µM primer (forward and reverse), 5 µl of 1 µM probe, and 5 µl of DNA. Following Karwacki et al (2018) all reactions were performed in an ultraclean room in an AC600 PCR Workstation with HEPA filter and UV irradiation (Air-Clean Systems, Creedmoor, NC). We performed the analysis in a Bio-Rad thermocycler CFX96 Real-Time System and analyze the results using the Bio-Rad CFX Manager software. We used gBlock gene fragments designed for each pathogen, following Karwacki et al., (2021), as qPCR standards for absolute pathogen quantification. The gBlock standard dilutions for each pathogen were run in triplicate on each plate. The highest dilution for *Bd* was 2 × 10^7 Genome Equivalents (GE)/reaction and the lowest dilution was 2 × 10^0 GE; for Pr the highest dilution was 2 × 10^9 GE and the lowest dilution was 2 × 10^2 GE; finally for *Rv* the highest dilution was 2 × 10^8 GE and the lowest was 2 × 10^0 GE. We included two negative controls consisting of molecular-grade water on each qPCR plate. qPCR conditions were 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C (*Bd* and *Rv*) or 59 °C (*Pr*) for 1 min.

Initially, we performed analysis of presence/absence of each pathogen by pooling together samples from each locality. We used a mix of 5 µl of DNA per individual per locality and repeated the procedure three independent times for each pathogen. When we found positive results in at least one of three runs, we repeated the analysis but this time using each sample individually. For the individual qPCRs, we ran each sample three independent times and used the mean GE as the infection intensity value in all downstream analyses. We only considered samples that amplified before 38 cycles in at least two of the three independent runs to be true positives.

To confirm that qPCR primers and probes were truly pathogen-specific, we randomly amplified five positive samples via PCR, using the primers and protocols detailed in Karwacki et al., (2018) and Karwacki et al., (2021). We ran all PCRs in 20 µl reactions consisting of: 5.6 µL of 1X concentration OneTaq Standard Buffer with MgCl2 (New England BioLabs), 1.6 µL of 10 mM dNTP, 0.4 µL of 10 mM DMSO, 0.4 µL of each primer (10 µM), 0.25 µL of 1U OneTaq Polymerase (Applied Biosystems), 2 µL of template DNA, and 9.35 µL of molecular grade water. The PCR products were visualized in a 2% agarose gel and Sanger sequenced in Eurofins Genomics (Louisville, Ken-

tucky, USA). We cleaned each sequence by eye and aligned the forward and reverse fragments in Geneious 10 (BioMatters); then we used Blastn to compare the newly generated sequences (accession numbers: AES2268 OL770099, AES2583 OL770100, AES2212 OL672750, AES2215 OL672751, AES2246 OL672752) with similar sequences available in GenBank.

Data Analysis

All statistical analyses were performed using R statistical software (R Core Team, 2019). First, we compared the overall prevalence and intensity of each of the three pathogens using a Chi-square test and a Kruskal–Wallis test, respectively. When the Kruskal–Wallis test resulted in significant differences, we performed a post hoc test to generate pairwise comparison across the three pathogens using a Dunn test with the *dunnTest* function from the FSA package. To compare *Bd*, *Rv* and Pr prevalence (the proportion of positive samples out of the total number of samples), we calculated 95% Clopper–Pearson binomial confidence intervals. To compare prevalence of each pathogen across elevations, localities, and species groups, we used Chi-square tests. For elevation comparisons, we separated sampling locations into two discrete categories: mid-elevation (2830 to < 3400 m) and high-elevation (> 3700 m to 3950). We did not compare *Rv* prevalence across localities because we only found positive samples in one study site. When overall Chi-square tests resulted in significant differences, we generated post hoc pairwise comparisons between factors using an adjusted p-value for multiple comparisons to decrease the chance of type I error. The Chi-square and post hoc tests were conducted in base R using the *chisq.test* and *pairwise.prop.test* functions.

To test the effects of localities, species groups, and environmental variables (elevation and precipitation) on the prevalence of each pathogen, we used generalized linear models (glm) with a binomial distribution. The precipitation data was obtained from WorldClim v2 with a 1km^2 scale (Fick et al., 2017) and represents the average precipitation from May to July. We also obtained temperature data from WorldClim, however a subsequent analysis showed a strong correlation of temperature with elevation (Pearson correlation = 0.85), and thus we conducted our analysis using elevation as a continuous variable and not including temperature. We created candidate models considering pathogen prevalence as a function of each individual variable and then as a function of all the additive combinations of variables. In addition, because the effects of precipitation on pathogen prevalence could vary at different elevations, we built a model with an interaction between elevation and precipitation. Moreover, because amphibian taxa are likely not equally distributed among sampling localities, we built a model with an interaction between species groups and localities. Furthermore, we tested the effects of each variable on pathogen prevalence and intensity with linear models (lm) using the same variables, additive combinations, and interactions. However, the models that best explained infection intensity (average GE detected among infected individuals) did not converge for any of the three pathogens due to the small sample sizes of infected individuals. We therefore only report models evaluating pathogen prevalence. We ranked all our models (including a null model) based on the AICc value, model weights and delta value (dAICc) using the AICctab function (*bbmle,* Bolker, 2020).

Results

We analyzed 213 *Pristimantis* individuals, of which we confidently assigned 76% to the *P. orestes* species group, 14% to the *P. phoxocephalus* species group, 8% to the *P. cryophilus* species group, 1% to the *P. unistrigatus* species group, and 1% to the *P. lacrimosus* species group (Table 1). All individuals were actively moving within small patches of vegetation during sampling, and several males were observed vocalizing. Overall, 10 individuals (5%) were infected with *Bd*, three individuals (1%) were infected with *Rv*, and six individuals (3%) were infected with *Pr*. The average infection intensity among infected individuals was 2,570,008 GE for *Bd*, 31,815 GE for *Rv*, and 106 GE for *Pr*. Intensity was significantly different among pathogens ($\chi^2_{(df = 2)} = 11.8$, p-value = 0.002; Fig. 2b): *Bd* intensity was significantly higher than Pr (p-value = 0.002), but not *Rv* (adjusted p-value = 0.75), and although *Rv* intensity was notably higher than Pr, this difference was not statistically significant (adjusted p-value = 0.06). Likewise, *Bd* prevalence was higher than *Rv* or Pr prevalence; however we did not recover significant prevalence differences among the three pathogens ($\chi^2_{(df = 2)} = 4.01$, p-value = 0.13). We did not detect any coinfected individuals harboring more than one pathogen.

We detected *Bd* in three of the 17 localities (Table 2, Fig. 1), and the average intensity per positive site was 2,164,349 GE (Table 2, Fig. 4c). *Bd* prevalence did not

Table 1. Average and 95% confidence intervals of *Bd*, *Rv* and Pr prevalence based on qPCR analysis across species groups. Prevalence represents the number of infected individuals divided by the total number of individuals in each species group.

Species groups	N	*Bd*	*Rv*	Pr
P. cryophilius	16	0	0	0
P. lacrimosus	2	0	0	0
P. orestes	162	0.028 CI[0.021 – 0.094]	0.009 CI[0.0015 – 0.043]	0.18 CI[0.013 – 0.078]
P. phoxocephalus	30	0.019 CI[0.0008 – 0.17]	0.005 CI[0.0008 – 0.17]	0
P. unistrigatus	3	0	0	0

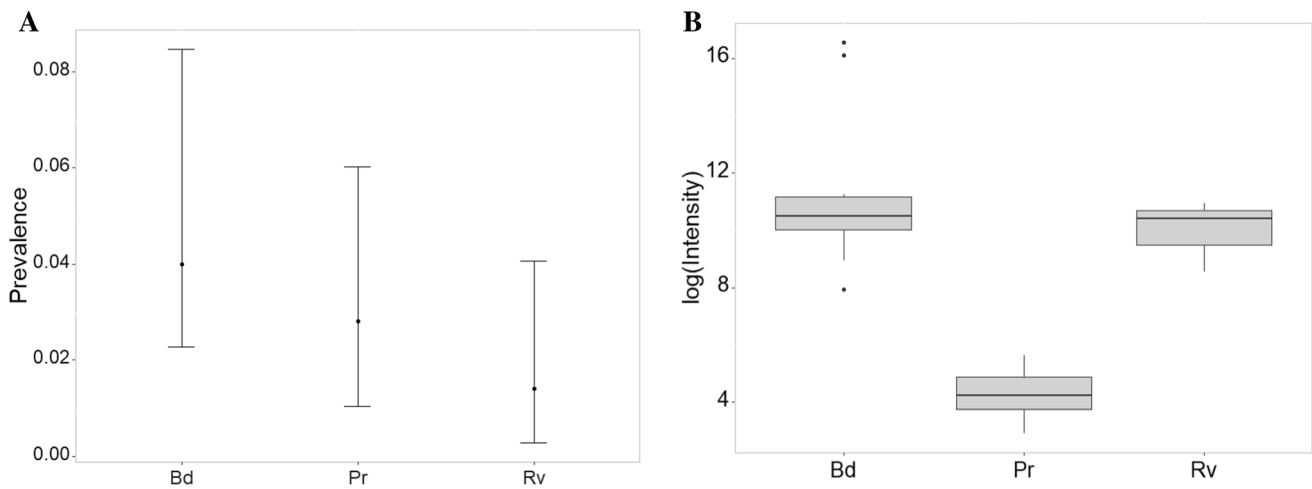

Figure 2. Overall *Bd*, *Rv* and Pr **a** prevalence and **b** log-transformed average infection intensity based on analysis of 213 *Pristimantis* individuals from the southern Andes of Ecuador. Prevalence error bars represent Clopper–Pearson 95% binomial confidence intervals.

differ across species groups ($\chi^2_{(df\ =\ 4)} = 6.3$, *p*-value = 0.17) or elevations ($\chi^2_{(df\ =\ 1)} = 0.09$, *p*-value = 0.70) (Fig. 3). *Bd* prevalence differed significantly across localities ($\chi^2_{(df\ =\ 16)} = 26.4$, *p*-value = 0.04), but posterior comparisons did not show any significant pairwise differences (Table S1). The top two models that predicted *Bd* prevalence were a model that included only elevation as a variable (AICc = 82.3, $w_i = 0.41$) and the null model (AICc = 82.7, $w_i = 0.33$). However, the difference in the dAICc between these two models was 0.4; therefore we could not distinguish a best model to predict *Bd* prevalence (Table S2). We recorded *Rv* in only one of the 17 localities (Table 1, Fig. 1) with an average infection intensity of 31,815 GE (Fig. 4c). *Rv* prevalence did not differ among species groups ($\chi^2_{(df\ =\ 4)} = 1.14$, *p*-value = 0.88), localities ($\chi^2_{(df\ =\ 16)} = 17.865$, p-value = 0.33), or elevations ($\chi^2_{(df\ =1)} = 0.70$, *p*-value = 0.39) (Fig. 3). In addition, the model that best explained *Rv* prevalence was the null model (AICc = 33.6, $w_i = 0.7$) (Table S2). Finally, we recorded Pr in three of the 17 localities (Table 2, Fig. 1) and the average intensity per positive site was 60.3 GE (Table 2, Fig. 4). Pr prevalence did not differ by species groups ($\chi^2_{(df\ =4)} = 2.3$, *p*-value = 0.68), or elevations ($\chi^2_{(df\ =1)} = 3.07$, *p*-value = 0.07) (Table 1, Fig. 3). The model that best explained Pr prevalence included an interaction between elevation and species group (AICc = 50.0, $w_i = 0.62$), however we could not differentiate this model from a model that included elevation only (*p*-value = 0.04, AICc = 51.4, $w_i = 0.32$) because the AICc difference was only 1.3 (Table S2). All three pathogens were present in the *P. orestes* species group, *Bd* and Pr occurred in members of the *P. phoxocephalus* species group, and only *Bd* was present in members of the *P. cryophilus* group (Fig. 3a, Table 1).

We obtained clean *Bd* sequence fragments of 52 and 92 bp from positive samples MZUA2268 and MZUA2583, respectively, and clean Pr sequence fragments of 75, 129 and 130 bp from positive samples MZUA2212, MZUA2215, and MZUA2246. BLAST searches showed that

Table 2. Prevalence, 95% confidence intervals and mean infection intensity of *Bd*, *Rv* and Pr detected by qPCR analysis by locality and elevation (mid elevation < 3400 m and high elevation > 3700 m).

Locality	N	*Bd*	*Rv*	Pr
Bermejos	26	0.12 CI[0.024–0,3] (3,331,312 ± 5,696,318)	0	0
Chacayacu	1	0	0	0
Galgal	3	0	0	0
Ishcayrrumi	24	0	0	0.08 CI[0.01–0,26] (79 ± 71.3)
Laguna duglaiy	8	0.25 CI[0.03–0,65] (34,178 ± 100.8)	0	0
Laguna napale	13	0	0	0.23 CI[0.05–0,53] (54 ± 43.9)
Miguir	4	0	0	0
Quimsacocha	7	0	0	0
All high-elevation	*86*	0.023 CI[0.019–0,13] (2,012,459 ± 2,643,204)	0	0.023 CI[0.019–0,13] (117 ± 104.6)
Angas	23	0	0	0.04 CI[0.011–0,21] (48)
Cerro Cabogana	16	0	0	0
Duraznos	4	0	0	0
Jima	18	0	0	0
Labrados	31	0.16 CI[0.054–0,33] (3,127,557 ± 4,899,079)	0.1 CI[0.02–0,25] (31,815 ± 26,008.7)	0
Llavircay	12	0	0	0
Llaviucu	12	0	0	0
Merced	10	0	0	0
Pugiloma	1	0	0	0
All mid-elevation	*127*	0.023 CI[0.012–0,08] (3,127,557 ± 4,564,362.7)	0.014 CI[0.004–0,067] (31,815 ± 26,008.7)	0.005 CI[0.0001–0,04] (48)

Prevalence represents the proportion between the number of infected individuals by the total number of individuals in each locality and elevation. The infection intensity (shown in parenthesis) is the average of genomic equivalents (GE) detected in positives samples.

our *Bd* fragments were extremely similar to over 100 other *Bd* sequences (% identity > 97.0). However, we lack enough resolution in this short DNA fragment to assign our *Bd* sequences to any of the major *Bd* lineages. In contrast, our Pr fragments were 100% identical to other Pr sequences sampled from 33 amphibians across the USA, all of which belong to the Pathogenic Perkinsea Clade (PPC; Isidoro-Ayza et al. 2017). We did not recover any clean sequence data for *Rv*.

Discussion

Our study is the first to detect Pr, or *Rv* infecting direct-developing terrestrial amphibians from the Ecuadorian Andes. In general, we found low prevalence of all three pathogens across localities, elevations, and species groups. *Bd* was relatively more common than Pr and *Rv*, but we found no significant differences in the prevalence of any pathogen across species groups, localities, or elevations. In contrast, the infection intensity of Pr was significantly lower than *Bd*. We interpret these comparisons with caution, as our skin swab data only targets the focal tissue for *Bd* infections, and does not reflect the potential infection severity of Pr and *Rv* that may be detected from the internal organs that are the primary targets of those pathogens. However, our swab-based study provides a useful non-invasive method for general pathogen comparisons, and the fact that we can still detect Pr and *Rv* from these samples highlights the utility of non-invasive techniques for mon-

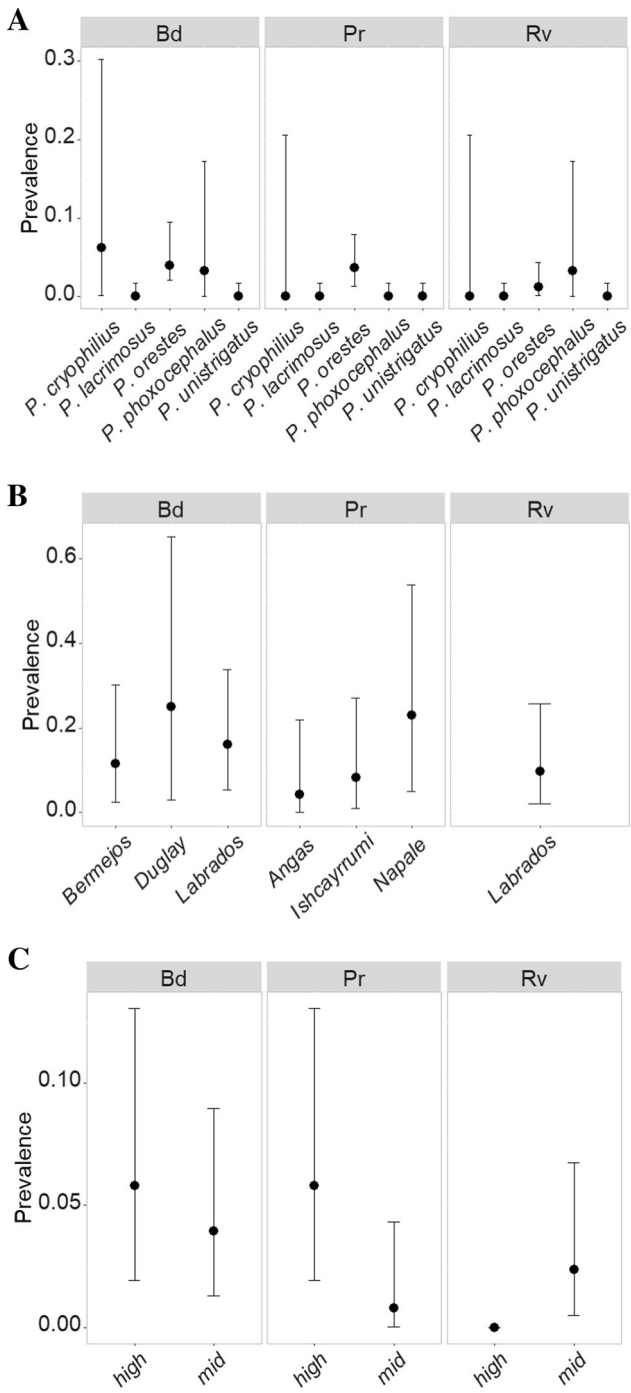

Figure 3. Prevalence of *Bd*, *Rv* and Pr across **a** species group, **b** locality, and **c** elevation. Mean infection prevalence is shown with 95% Clopper–Pearson binomial confidence intervals.

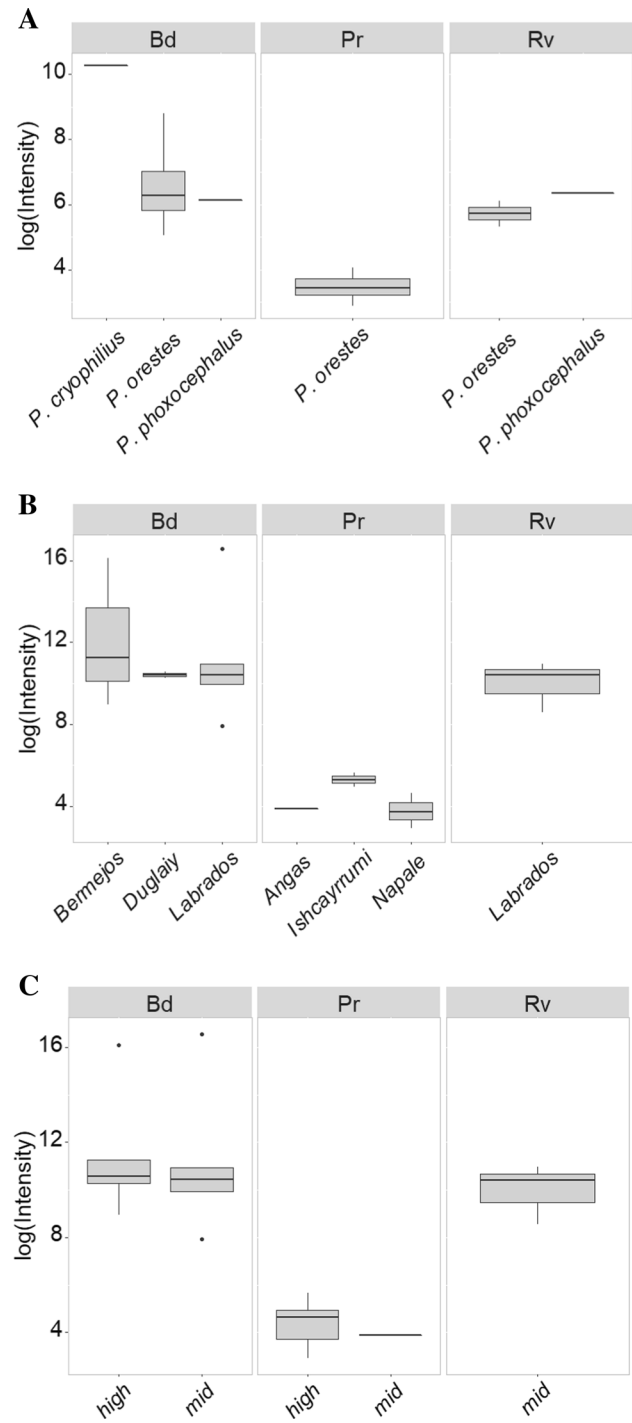

Figure 4. Log-transformed infection intensity of *Bd*, *Rv* and Pr across **a** species group, **b** locality, and **c** elevation.

itoring the occurrence of pathogens in specific regions and species. Overall, our results are consistent with the previously recorded presence of *Bd* in southern Ecuador and in *Pristimantis* (Ron and Merino-Viteri, 2000; Guayasamin et al., 2014; Caceres-Andrade, 2014) and suggest a wider distribution of *Rv* in the Andes of South America that previously documented (Warne et al., 2016). Importantly, this first recorded occurrence of Pr in direct-developing frogs raises interesting questions regarding the dynamics of infection in amphibians without a tadpole stage, as this poorly understood protistan pathogen has largely been

associated with infections and mortality in larval-stage anurans (Isidoro-Ayza et al. 2017; Karwacki et al., 2018).

Previous reports of *Bd* in *Pristimantis* (Guayasamin et al., 2014; Warne et al., 2016) as well as in other direct-developer groups, including *Austrochaperina* (Hauselberger and Alford, 2012), *Brachycephalus* (Mesquita et al., 2017), *Eleutherodactylus* (Longo et al., 2010; Mesquita et al., 2017), *Craugastor* (Kolby et al., 2010), and *Ischnocnema* (Mesquita et al., 2017), suggest not only that transmission in terrestrial habitats is frequent in wild populations, but also that multiple modes of direct and indirect transmission likely play a role. Aquatic-to-terrestrial host spillover has been experimentally demonstrated in laboratory settings (Becker et al., 2019) and suggested as a form of transmission in natural populations (Longo et al., 2010). In this scenario, even mild pathogenic loads in sympatric aquatic species can contribute to the transmission of *Bd* to terrestrial hosts (Becker et al., 2019). In addition to skin–skin contact among infected individuals, indirect transmission via contaminated water bodies or surfaces has been also suggested to facilitate *Bd* spread (Kolby et al., 2015). Recently, Burns et al., (2021) detected *Bd* in wetland and montane forest floor habitats using a novel environmental DNA sampling for terrestrial environments, and remarkably, *Bd* was detected even in sites with no water bodies nearby. In our study area, the presence of *Bd* was historically documented in tadpoles and adult individuals of *Atelopus* and *Gastrotheca,* associated to riverine habitats (Caceres-Andrade, 2014). Thus, the high *Bd* prevalence and intensity previously documented in these species might have contributed to indirect transmission of *Bd* to other species such as *Pristimantis.* In the paramo and montane forest ecosystems we sampled, the densities of *Pristimantis* species are particularly high, and a large number of individuals can typically be found within a very small area (V. Urgiles, personal observation). Thus, it is likely that in *Pristimantis* density-dependent transmission patterns occur due to increasing chance of contact with infected individuals and surfaces (Brannelly et al., 2012).

Bd has a relatively long history of amphibian infections in the high-elevation ecosystems of Azuay and Cañar, most notably with the report of *Bd* in the highly endangered Arlequin toad *Atelopus bomolochos* in 1980 (Ron and Merino-Viteri, 2000). Although the monitoring of pathogenic diseases in this region has been very limited, one technical report by Caceres-Andrade (2014) found *Bd* in 12 out of 14 sites located in Azuay, including two of our sampling localities Ishcayrrumi and Llaviucu. We did not find *Bd* in either of these localities, and overall, we found a low number of *Bd* positives across our study sites in Azuay. We also recoded a lower prevalence and infection intensity in *Pristimantis* compared with the results of Guayasamin et al. (2014) in the cloud forest of northern Ecuador, where overall *Bd* prevalence was 0.47. Furthermore, the previous studies by both Caceres-Andrade (2014) and Guayasamin et al., (2014) were conducted during different months (late June to early December) than our May–July sampling; therefore differences in *Bd* prevalence among these studies could reflect seasonal dynamics that would be interesting to evaluate in future long-term analyses. Seasonality strongly influences *Bd* dynamics, and overall infection intensity and prevalence both tend to be higher in winter months (Kinney et al., 2011; Ruggeri et al., 2015; Sonn et al., 2019). In the Andean region, winter (humid season) occurs between October and May, whereas our sampling was conducted mostly in the summer (dry season), which likely explains the higher *Bd* prevalence reported by Caceres-Andrade (2014) in these two localities and throughout this region of Ecuador.

Studies characterizing *Rv* in South American amphibians remain limited, but FV3-group *Rv* lineages have been documented in non-native bullfrogs (*Rana catesbianus*) in Brazil and Uruguay (Galli et al., 2006; Candido et al., 2019), non-native *Xenopus laevis* and *Calyptocephallela gayi* in Chile (Soto-Azat et al., 2016) and in captive-trade *Telmatobius marmoratus* in Cusco, Peru and wild *Hypsiboas gladiator* and multiple *Pristimantis* species in the Peruvian Andes (Warne et al., 2016). Additionally, probable *Rv* die-offs were documented in natural populations of *Atelognathus patagonicus* in Patagonia, Argentina (Fox et al., 2006). Thus, our detection of *Rv* in *Pristimantis* of the Ecuadorean Andes is an unsurprising but important contribution to the currently scarce knowledge of this pathogen in South American frog species. Ranaviruses can broadly infect fish, turtles and amphibians (Chinchar, 2002), and some iridoviruses appear to jump hosts directly from fish to amphibians (Mao et al., 1999; Jancovich et al., 2005). One potential source of *Rv* transmission to *Pristimantis* may therefore be one of the most abundant aquatic vertebrates in the ecosystems we sampled: non-native rainbow trout (*Oncorhynchus mykiss*) that have been widely introduced into high-elevation Andean lakes and rivers that are devoid of native fish (Mouillet et al., 2018). Experiments demonstrate that these Ecuadorean trout transmit pathogens to the native frog *Engystomops petersi* (Martín-Torrijos et al., 2016), and *O. mykiss* has been documented

with *Rv* infections in other locations (Marsh et al., 2002). While *Pristimantis* are direct-developing, terrestrial-dwelling frogs, anecdotal evidence (Yanez-Munoz et al., *in prep*) demonstrates that introduced Ecuadorean trout can consume *Pristimantis* in large quantities. These frogs are thus occupying areas close enough to lake edges to encounter and be consumed by fish, presenting a potential *Rv* transmission route.

Amphibian Pr infection dynamics remain poorly understood, despite recent evidence that this pathogenic protistan lineage infects amphibians worldwide (Chambouvet et al., 2015) and that, at least in North America, Pr may be the third leading cause of amphibian disease based on analysis of 247 mortality events over a 15-year period (Isidoro-Ayza et al., 2017). To date, the only study documenting Pr in South America found the pathogenic Pr lineage infecting four tadpoles sampled in French Guiana: one *Phyllomedusa tomopterna*, one *Hyla helenae*, and two *Hypsiboas geographicus* (Chambouvet et al., 2015). Thus, the extent and severity of infections, and the mortality threats imposed by Pr throughout South America and among different amphibian taxa, remains largely unknown due to the lack of monitoring programs in the region. Our detection of Pr in *Pristimantis* species from three localities in the Ecuadorean Andes therefore documents this pathogen in a new region of the world and a new taxonomic group of hosts. While the prevalence and infection intensities we measured were consistently low, in line with the findings of Chambouvet et al. (2015) in French Guiana (prevalence = 3%), suggesting only sub-clinical infections, the fitness impacts of these infections are unknown. Furthermore, Pr tends to cause mortality in larval frogs (Davis et al., 2007), and no data exist on how this may translate to direct-developing *Pristimantis* embryos, which are rarely observed, but could represent the life stage experiencing Pr mortality. Our results suggest an effect of elevation on *Pr* prevalence, with an increase in Pr positives at higher elevations. Expanded studies of Pr in *Pristimantis*, other terrestrial amphibians and across elevational gradients are clearly needed to assess the threat posed by this potentially widespread and damaging pathogen.

In the Andean region, studies based on historical reports suggest that *Bd* is an emergent infectious pathogen resulting from multiple independent introductions (Lips et al. 2008). In contrast, whether *Rv* and Pr infections result from invasive or endemic pathogenic lineages in South America requires further examination. So far, there are no reports of outbreaks or amphibian population declines linked with *Rv* in this region, and to our knowledge, no signs of apparent infection or symptoms associated with Severe Perkinsea Infections (SPI) have been reported. This conspicuous absence of mortality by *Rv* or Pr infections coupled with the low prevalence and infection intensity found in our study could potentially support the idea of native pathogenic strains in the Andes naturally infecting multiple hosts. Additional reports of FV3 *Rv* strains present throughout the American continent (Galli et al., 2006; Candido et al., 2019) could also support this hypothesis. However, without a comprehensive historical analysis of these pathogens in the region, we currently lack the data to support any assumption related to their origin and transmission.

Limited information regarding coinfection patterns in *Pristimantis* and other direct-developing Andean frogs is currently available. One previous study conducted in the cloud forest of Peru reported coinfection of *Bd* and *Rv* in six *Pristimantis* individuals (Warne et al., 2016). In our study, we did not recover any coinfections, and we only registered co-occurrence of *Rv* and *Bd* in one out of our 17 sampling localities. However, the extent to which these results reflect the reduced spatial and temporal sampling of both studies versus specific dynamics of coinfection in this clade needs to be further explored. One simple potential explanation for the low prevalence of coinfections is that the adult stages of *Pristimantis* species may have low susceptibility to pathogens (Warne et al., 2016). This pattern has been evident when analyzing pathogen co-occurrence in other montane amphibians (Bosch et al., 2020). Consequently, jointly monitoring these three common pathogens across *Pristimantis* species groups and life stages is important for future studies because their interaction can affect disease dynamics (Rosa et al. 2017) and potentially increase susceptibility to other infections (Ayres et al., 2020).

The use of skin swabs for pathogen screening has only been formally validated in *Bd* (Soto-Azat et al., 2009), but has also been shown to correspond with histologic severity of Pr infections (Karwacki et al., 2018). Because the skin is not the target affected organ in *Rv* or Pr infections, one potential caveat of infection estimates based on skin swabs is lower detection sensitivity and underestimated infection intensity. These results have been observed when comparing *Bd* detection efficacy (Retallick et al., 2006), but in contrast, when comparing Pr sensitivity, Karwacki et al., (2018) found similar detection efficacy across toe samples, skin swabs, and skin histology. Because limited studies have

been focused on large-scale comparisons across tissue and skin swabs for the detection of Pr and *Rv*, it is not possible to determine yet the extent to which swabs contribute to underestimations in prevalence and infection intensity. Regardless of potential differences in sensitivity, non-invasive swabbing techniques have been used in other studies to determine historic patterns of *Rv*, Pr and *Bd* from museum specimens (Karwacki et al., 2021), to explore the contribution of invasive species as pathogen reservoirs (Rivera et al., 2019; Galt et al., 2021), and to disentangle seasonal infection dynamics in subtropical communities (Karwacki et al., 2018). Thus, in the Andean region, this non-invasive technique has the potential to be a cost-effective method to determine presence and prevalence of Pr, *Rv* and *Bd* across multiple, often endemic or threatened taxonomic groups and ecosystems.

Pristimantis is a group representing the most specious genus of terrestrial vertebrates on the planet occupying some of the most threatened habitats in the world. A recent update of the Red list of Amphibians from Ecuador indicates that 67% of *Pristimantis* species are threatened (Ortega et al., 2021). Our study therefore underscores the need to drastically expand pathogen studies in Andean *Pristimantis*, which can readily be implemented as a complement to the high rate of ongoing species descriptions within this group (e.g. Paez and Ron, 2019) by adding straightforward swabbing and PCR techniques to current field surveys. We suggest that researchers conducting studies with amphibians in the region pay special attention to disinfection protocols to prevent the potential spread of pathogenic diseases and to guarantee the integrity of swabbing collection protocols. Certainly, pathogen surveys in *Pristimantis* have the potential to inform and support current and future conservation efforts in the highland Andes but also open an exciting opportunity to explore evolutionary patterns of pathogen susceptibility and immunity in a taxonomic and ecological diverse group of vertebrates with singular developmental characteristics.

Acknowledgements

Our field expeditions in Ecuador were supported by Juan C. Sanchez-Nivicela, Paul Szekely, Diana Szekely, Amanda Quezada, Bruno Timbe and Valentina Posse. Boris Tinoco and Rodrigo Caroca provided suggestions for sampling and data management in Ecuador. Yanez Munoz from Instituto Nacional de Biodiversidad del Ecuador facilitated sample exportation to UCF. Emily Karwacki and Matthew Atkinson helped define protocols for laboratory analysis; Katherine Martin provided suggestions for data analysis. We are grateful to Molly C. Womack and Jackson Phillips for providing edits and suggestions in an early version of this manuscript. This study was funded by grants from the National Geographic Society and the Explorers Club awarded to VLU.

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