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INFECTION DYNAMICS OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN TWO FROG SPECIES INHABITING QUITO'S METROPOLITAN GUANGÜILTAGUA PARK, ECUADOR

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ABSTRACT: *Batrachochytrium dendrobatidis* (*Bd*) infection is one of the principal causes of amphibian declines worldwide. The presence of *Bd* has been determined in *Gastrotheca riobambae* tadpoles that inhabit ponds in Quito's Metropolitan Guangüiltagua Park, Ecuador. This study sought to determine whether these tadpoles are infected and to determine the presence of chytridiomycosis in another frog species, *Pristimantis unistrigatus*, which also inhabits the park and has different reproductive biology and distinct behavioral habits. We used end-point and real-time PCR techniques to detect and quantify *Bd* infection. At 1 yr, samples were taken from the skin of *P. unistrigatus* using swabs and were also taken from the mouthparts of *G. riobambae* tadpoles. It was found that the two species were infected with a *Bd* prevalence of 39% (53/135) in *G. riobambae* tadpoles and 15% (57/382) in *P. unistrigatus* frogs. The two types of samples (tissue and swabs) from mouthparts showed differences in the zoospores per microliter loads ($\bar{x}$=1,376.7±3,450.2 vs. $\bar{x}$=285.0±652.3). Moreover, a correlation (r^2=0.621) was discovered between the monthly mean maximum temperature of the pond with disease prevalence in *G. riobambae* tadpoles. Infection levels in the *P. unistrigatus* population varied significantly over time, and distance to the pond was a determinant factor for infection intensity.

Key words: Amphibians declines, chytridiomycosis infection dynamics, *Gastrotheca riobambae*, *Pristimantis unistrigatus*, urban parks.

INTRODUCTION

The appearance of emerging diseases, such as chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), has become one of the most serious threats to amphibian populations worldwide. This chytrid fungus has been widely researched during the past 20 yr (e.g., Berger et al. 1998; Longcore et al. 1999; Daszak et al. 2003; Lips et al. 2003; Fisher et al. 2009a, b; Rosenblum et al. 2013; Rodriguez et al. 2014; Talley et al. 2015; De León et al. 2017; O'Hanlon et al. 2018; Scheele et al. 2019). This pathogen is present in the keratinized structures of infected postmetamorphic animals (skin) and tadpoles (mouthparts; Fellers et al. 2001; Rachowicz and Vredenburg 2004), infecting cells in the stratum granulosum and the stratum corneum of the superficial epidermis (Berger et al. 2005), altering the skin's osmoregulatory function and producing an electrolyte imbalance, which ends with death (Voyles et al. 2009).

Previously, detection of chytridiomycosis was performed using invasive techniques. Currently, molecular techniques (end-point PCR and real-time or quantitative [q]PCR)

for the amplification of *Bd*'s DNA have become the most reliable noninvasive techniques for detecting the presence of this pathogenic fungus (Kriger et al. 2006b). These techniques allow determination of the prevalence of the fungus and of infection levels.

The distribution of *Bd* and chytridiomycosis is significantly associated with environmental variables (Fisher et al. 2009b). An optimal growth temperature of *Bd* has been hypothesized, suggesting that both daytime cooling (local or microscale) and nighttime warming accelerate disease development (Pounds et al. 2006). In this regard, the decline of amphibians is related to global warming and temperature variability (Pounds and Crump 1994; Bosch et al. 2007; Cohen et al. 2019).

The Neotropics are among the most affected regions. Since the 1980s, sudden losses of frog species and a high prevalence of chytridiomycosis have been reported in Central and South America (e.g., Bonaccorso et al. 2003; Ron et al. 2003; Burrowes et al. 2004; Bustamante et al. 2005; Eterovick et al. 2005; Carnaval et al. 2006; Lips et al. 2006; Longo et al. 2010; Lescano et al. 2013; Guayasamin et al. 2014; Cusi et al. 2015; Flechas et al. 2017).

Ecuador is among the Neotropical countries most affected with population declines and amphibian extinctions. It ranks third worldwide in terms of number of threatened species (Ron et al. 2019). A decline of amphibian populations in Ecuador has been reported since the early 1990s (Coloma 1995), and the earliest record of chytridiomycosis dates back to 1980 (Ron and Merino-Viteri 2000), indicating that *Bd* was present in wild populations before the decline problem was noted. Several studies have aimed to understand the biology of *Bd*, along with its distribution, genetic characterization, and how it has affected (and is still affecting) the decline in frog populations in Ecuador (Ron 2005; Ron et al. 2011; Menéndez-Guerrero and Graham 2013; Vizcaíno et al. 2013; Burgos et al. 2019). More studies are needed to understand the dynamics of the disease and how it is affected by environmental changes.

Our study was conducted at Quito's Metropolitan Guangüiltagua park, the second-largest park in the Metropolitan District of Quito, Ecuador, with an area of approximately 571 ha. It is located at 2,988 m above sea level and has an average temperature range of 12 to 18 C (Espinoza-Gracia 2009). The park houses several species of birds, some marsupials, and two species of frogs: the striped robber frog (*Pristimantis unistrigatus*) and the Riobamba marsupial frog (*Gastrotheca riobambae*). *Pristimantis unistrigatus* is a nocturnal, direct-developing frog that is a common species in gardens within Quito (Ron et al. 2018). *Gastrotheca riobambae* is an arboreal nocturnal frog that has larval stages, and is usually found near water sources such as irrigation canals, ponds, streams, gaps, marshes, among other such sites (Chasiluisa et al. 2020).

It is known that *G. riobambae* tadpoles are infected with *Bd* (Manzano-Pasquel 2010). Because *P. unistrigatus* frogs coexist with *G. riobambae* and are exposed to a habitat containing infected *G. riobambae* tadpoles, juvenile and adult *P. unistrigatus* could also be infected. Our study aimed to evaluate the dynamics of infection over time in both species, by correlating the number of zoospores (maxima and minima) with environmental factors.

MATERIALS AND METHODS

Sampling site and study species

The study was conducted on a permanent pond located in Quito's Metropolitan Guangüiltagua Park (00°11′01″S, 078°28′08″W). From September 2012 to August 2013, we carried out 19 sampling periods collecting *G. riobambae* tadpoles and 21 sampling periods collecting *P. unistrigatus* adults and juveniles. Using a mesh network across the pond, 154 *G. riobambae* tadpoles were collected during daytime hours. The net was moved slowly along the stream substrate, especially near the banks. In each sampling period, between 5 and 10 tadpoles were collected, with larval stages of 28–46 based on the Gosner (1960) advanced development stages. Each captured tadpole was placed in a separate container to avoid cross-contamination and was moved to the Museum of Zoology at the Pontificia Universidad Católica del Ecuador (QCAZ). At the laboratory, mouthparts were swabbed from all tadpoles through sterile cotton swabs (MW100,

TABLE 1. Monthly precipitation (mm) and temperature (C) values from a permanent pond located in the Metropolitan Guangüiltagua Park in Quito, Ecuador, from September 2012 to August 2013.

Months	Precipitation[a]	Air maximum[b]	Air median[b]	Air minimum[b]	Pond maximum[b]	Pond median[b]	Pond minimum[b]
September 2012	12.50	23.97	14.62	9.30	14.70	13.65	12.60
October 2012	133.50	19.16	12.95	8.99	14.09	13.57	13.16
November 2012	145.90	17.45	12.75	9.41	14.93	13.96	13.08
December 2012	60.80	18.35	12.81	9.00	14.52	13.81	13.13
January 2013	42.70	20.38	14.03	9.62	14.36	14.04	13.67
February 2013	195.10	16.93	12.73	9.94	13.77	13.51	13.28
March 2013	82.70	20.06	13.92	10.13	14.29	13.90	13.70
April 2013	110.90	21.61	13.75	9.75	14.08	13.68	13.29
May 2013	115.70	21.71	13.30	10.11	13.36	13.02	12.85
June 2013	0.30	28.33	14.53	8.99	13.51	13.14	12.88
July 2013	0.60	27.88	14.36	9.23	13.49	12.92	12.51
August 2013	18.40	23.89	13.91	9.18	13.76	13.23	12.72

[a] Precipitation values based on the monthly meteorological bulletins of the National Institute of Meteorology and Hydrology belonging to the city of Quito, Ecuador.

[b] Temperature values (maximum. median and minimum) of the environment and the pond obtained through automatic temperature recorders (iButton DS1922L temperature loggers).

Medical Wire and Equipment Co, Durham, North Carolina, USA) by gently introducing the swab into the oral mouthparts and twirling it about five to seven times (Hyatt et al. 2007). Each swab was placed in a tube with an individual code and stored in a refrigerator (2–4 C) for 15 d before DNA extraction. The snout-vent length (SVL, in mm) of each tadpole was measured, and they were euthanized with an overdose of MS-222 (Sigma-Aldrich, St. Louis, Missouri, USA; Julian et al. 2016) to obtain their mouth tissue (Hyatt et al. 2007). The mouthpart pieces were placed into 1.5 mL Eppendorf tubes with absolute ethanol for later molecular analysis.

We captured 382 *P. unistrigatus* adults by hand using a plastic bag at night (hand covered by the bag, then the bag turned inside out to collect the individual without touching it with the hand). In each sampling period, 15–20 frogs were collected and placed in individual plastic bags. We obtained skin samples via cotton swabs (MW100, Medical Wire and Equipment Co, Durham, North Carolina, USA) that were passed 10–12 times through the pelvic patch and 3–5 times between the toe digits (Boyle et al. 2004). Each swab was placed in a tube with an individual identification code and refrigerated (2–4 C). The place where each frog was captured was registered (inside or outside the pond), plus the distance from the pond at which it was found. The SVL was measured with the aid of a digital caliper. Eight metamorphs of *G. riobambae* were found, from which samples were taken using the same technique as with *P. unistrigatus*.

Collection of environmental data

Two automatic temperature recorders (iButton® DS1922L temperature loggers; Maxim Integrated, San Jose, California, USA) were positioned, one inside and one outside the pond (3 m from the level of the pond). Data were recorded at 1-hr intervals during all months of the year (Table 1). Precipitation values were obtained through monthly meteorological bulletins of the National Institute of Meteorology and Hydrology for the period 2012–13 (https://www.inamhi.gob.ec/boletines-meteorologicos/; Table 1).

DNA extraction and Bd detection by PCR

We extracted DNA from all samples (536 swabs and 135 tissues) using a PrepMan Ultra buffer (Applied Biosystems, Carlsbad, California, USA). Briefly, both tissues and swabs were placed in 1.5 mL Eppendorf tubes with 0.02 to 0.03 g of Zirconia beads (0.5 mm diameter, Biospec Products, Bartlesville, Oklahoma, USA) and were then mixed well with the buffer (Boyle et al. 2004; Hyatt et al. 2007).

Two PCR molecular techniques were used to diagnose *Bd* infection: PCR and qPCR, as described by Boyle et al. (2004) with some modifications. End-point PCR amplifications were performed using a SureCycler 8800 Thermal Cycler (Agilent Technologies©, Santa Clara, California, USA), as follows: 10 min at 93 C, plus 35 cycles (45 s at 93 C, 40 s at 64 C, and 45 s at 72 C), and a final extension step at 72 C for 10 min.

The PCR reaction mixture was performed with the GoTaq® DNA polymerase kit (Promega, Madison, Wisconsin, USA) in a final volume of 25 μL, with 0.2 μM of each primer and 450 ng of genomic DNA. Amplified products were tested by electrophoresis in a 2% agarose gel.

For real-time PCR and quantification of *Bd* zoospores, labeled probes with fluorochromes (TaqMan probes, Chytr MGB2) were used. The qPCR reaction mixture was performed with the GoTaq Probe qPCR Master Mix (Promega) in a final volume of 20 μL, with 0.2 μM of each primer, 0.25 μM of probe and 450 ng of genomic DNA. Amplifications were performed using Stratagene Mx3000P® (Agilent), as follows: 2 min at 95 C and 40 cycles of 15 s at 95 C and 1 min at 64 C. A standard curve was generated through an absolute quantification (R^2=0.999) of seven serial dilutions of *Bd* control target sequence into plasmids (Pisces Molecular, Boulder, Colorado, USA; 2.1 copies × 10^6/μL to 10^0/μL). Negative, nontemplate controls (nuclease-free water) were also used.

To confirm *Bd* identification, 20 positive samples from tadpole mouthpart tissues were sequenced via the Sanger technique, using the internal transcribed spacer and 5.8S primers (Boyle et al. 2004).

Statistical analysis

All the statistical tests were performed using IBM SPSS® Statistics software, version 22 (SPSS, Chicago, Illinois, USA). The prevalence of the disease (percentage of positive animals for *Bd*) was used to determine the differences between the two molecular techniques employed and between the two types of samples analyzed. The *Bd* infection levels (zoospores per millimeter) were obtained by dividing the number of DNA copies (zoospores per microliter) for the size of each animal (SVL) to adjust to a more precise value based on the size of each species. However, to determine whether there are differences between the infection levels and larval stages, the number of zoospores per microliter was used instead of the number of zoospores per millimeter.

The Kruskal-Wallis nonparametric test was used to determine the differences between the two variables (infection levels and disease prevalence) in the two species over time; in which, the months were grouped by periods of 3 mo. The same procedure was used to evaluate the infection levels between larval stages; in which, they were grouped by three-stage ranks per group. Correlations and regressions were used to evaluate whether the distance from the pond was a determining factor in the infection level of *P. unistrigatus*; to determine the existence of any links between the two variables, both within the population of each species and between the two species; and, finally, determine whether there is any link between the two variables with environmental factors, such as temperature and precipitation.

RESULTS

Mouthpart tissues and swab samples

A total of 154 *G. riobambae* tadpoles were collected and 154 swabs were obtained but only 135 mouthpart tissues were analyzed, due to some mouthparts being unviable. We found that 42 of the 135 oral tissue samples (31%) were positive for *Bd* through PCR, and 53 (39%) of the 135 tissue samples were positive for *Bd* through qPCR (Table 2). The tadpole with the greatest number of zoospores had 21,753 zoospores/μL ($\bar{x}$=1,376.7±3,450.2) with an infection level of 345 zoospores/mm ($\bar{x}$=24.8±54.7).

For the 154 oral swabs from *G. riobambae* tadpoles, we found that only six (4%) were positively diagnosed for *Bd* through PCR, whereas 22 (14%) were positive for *Bd* through qPCR (Table 2). The swab with the greatest quantity of zoospores had 3,092 zoospores/μL ($\bar{x}$=285.0±652.3) with an infection level of 43 zoospores/mm ($\bar{x}$=4.9±9.1).

From the 382 skin samples of *P. unistrigatus,* we collected, only two animals (0.5%) were positive for *Bd* through PCR (Table 2), whereas 57 individuals (15%) were positive for *Bd* through qPCR. The swab with the greatest quantity of zoospores had 7,556 zoospores/μL ($\bar{x}$=556.04±1,426.1) and had an infection level of 459 zoospores/mm ($\bar{x}$=29.27±78.77).

Finally, the 20 oral tissue samples from the *G. riobambae* tadpoles sequenced showed a high match (>95%) to *Bd* sequences in GenBank (accession no. MG601126.1), confirming the detection of this chytrid fungus.

Comparison of the two molecular techniques

The two molecular techniques (PCR and qPCR) used with tissue samples and swabs from tadpoles' mouthparts presented differences (P=0.049 and P=0.018) in the detection

TABLE 2. Prevalence (%) of detection of *Batrachochytrium dendrobatidis* with the two molecular techniques and the type of sample analyzed from mouthpart tissues of the Riobamba marsupial frog (*Gastrotheca riobambae*) tadpoles and the skin of adults and juveniles of the striped robber frog (*Pristimantis unistrigatus*) inhabiting the Metropolitan Guangüiltagua Park in Quito, Ecuador, from September 2012 to August 2013.

Sample	End-point PCR					Quantitative PCR				
	Positives	Prevalence	Negatives	Prevalence	Total	Positives	Prevalence	Negatives	Prevalence	Total
Mouthpart tissues, *G. riobambae*	42	31	93	69	135	53	39	82	61	135
Mouthpart swabs, *G. riobambae*	6	4	148	96	154	22	14	132	86	154
Skin swabs, *P. unistrigatus*	2	0.5	380	99.5	382	57	15	325	85	382
Total					671					671

of *Bd* (tissue: 31%, 42/135, and 39%, 53/135; swabs: 4%, 6/154, and 14%, 22/154, respectively). In addition, the prevalence detected from the two sampling methods (tissue: 39% and swabs: 14%) with qPCR showed highly significant differences ($P<0.01$; Table 2).

Infection levels and prevalence of *Batrachochytrium dendrobatidis*

Levels of infection with *Bd* varied over time in the *P. unistrigatus* population ($P=0.009$), where the highest average of infection level was during the period between June and August (see Fig. 1). Despite not being able to find any links between the infection average and the prevalence of *Bd* over time in the populations of the two species studied ($P=0.208$; $P=0.349$; $P=0.009$; $P=0.274$, respectively; Tables 3, 4), a significant correlation ($P=0.015$) was found between the distance from the pond and the infection levels in the *P. unistrigatus* population. Specifically, the further away the individuals were found from the pond, the lower the infection level (see Table 5).

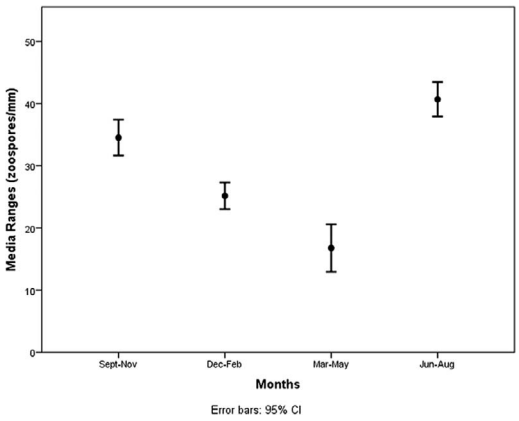

FIGURE 1. Comparison of the averages of *Batrachochytrium dendrobatidis* infection levels in the striped robber frog (*Pristimantis unistrigatus*; $N=57$) inhabiting the Metropolitan Guangüiltagua Park in Quito, Ecuador, from September 2012 to August 2013. CI=confidence interval.

Infection average and prevalence vs. environmental factors

In spite of there being no significant differences between the infection levels over time in *G. riobambae* tadpoles or in their larval stages ($P=0.226$ and $P=0.260$, respectively), this study found that the larval stages of 34 to 42 had a greater average level of infection than the other stages had (Table 6).

The temperature of the pond was a determinant factor in *Bd* infection, because both variables (infection level and prevalence) showed a significant correlation in *G. riobambae* tadpoles ($r^2=0.582$, $P=0.046$; $r^2=0.621$, $P=0.007$). Nevertheless, the two variables behaved differently. As the temperature in the pond increased, the infection average in the tadpole population decreased (see Fig. 2). In contrast, when the temperature in the pond increased, the prevalence of the disease also increased (see Fig. 3).

TABLE 3. Monthly prevalence (%) and infection average (zoospores per millimeter) of *Batrachochytrium dendrobatidis* in mouthpart tissues of the Riobamba marsupial frog (*Gastrotheca riobambae*) tadpoles inhabiting a permanent pond located in the Metropolitan Guangüiltagua Park in Quito, Ecuador, from September 2012 to August 2013.

Months[a]	Positives	Prevalence	Negatives	Prevalence	Infection average	Total
September	10	71	4	29	39.19	14
October	8	73	3	27	21.41	11
November	5	83	1	17	9.18	6
December	1	—	0	—	—	1
January	15	56	12	44	11.82	27
February	3	19	13	81	15.28	16
March	1	10	9	90	—	10
April	7	37	12	63	39.43	19
May	2	12	15	88	55.48	17
June	0	0	10	100	—	10
July	1	25	3	75	—	4
Total	53		82			135

[a] Months in bold were not considered as they did not have enough individuals to analyze (a minimum of two) and were not able to yield *Bd* infection averages.

TABLE 4. Monthly prevalence (%) and infection average (zoospores per millimeter) of *Batrachochytrium dendrobatidis* in skin swabs of adults and juveniles of the striped robber frog (*Pristimantis unistrigatus*) inhabiting the Metropolitan Guangüiltagua Park in Quito, Ecuador, from September 2012 to August 2013.

Months[a]	Positives	Prevalence	Negatives	Prevalence	Infection average	Total
September	6	22	21	78	22.55	27
October	1	—	1	—	—	2
November	6	12	45	88	21.45	51
December	14	26	39	74	7.68	53
January	4	10	36	90	1.61	40
February	9	23	31	78	11.88	40
March	0	0	40	100	—	40
April	6	19	26	81	50.04	32
May	1	5	20	95	—	21
June	5	23	17	77	12.78	22
July	0	0	20	100	—	20
August	5	15	29	85	163.49	34
Total	57		325			382

[a] Months in bold were not considered because they did not have sufficient numbers of individuals to analyze (a minimum two) and, therefore, were not able to yield *Bd* infection averages.

TABLE 5. Zoospore loads and infection levels (mean±SD) in the striped robber frog (*Pristimantis unistrigatus*) in relation to the distance from a permanent pond located in the Metropolitan Guangüiltagua Park in Quito, Ecuador, from September 2012 to August 2013.

Individual	*N*	Load range (zoospores/μL)	Infection level (zoospores/mm)	Average distance (m)
Positives	57	10–7,556 (556.04±1,426.14)	0.45–458.77 (29.27±78.77)	25.56
Negatives	325	0–9 (1.89±2.26)	0–0.53 (0.09±0.11)	34.78

TABLE 6. Zoospore loads and infection levels (mean±SD) in the different groups of larval stages of the Riobamba marsupial frog (*Gastrotheca riobambae*) tadpoles inhabiting a permanent pond located in the Metropolitan Guangüiltagua Park in Quito, Ecuador, from September 2012 to August 2013.

Larval stages[a]	*N*	Load range (zoospores/μL)	Infection level (zoospores/mm)
28–30	10	20–821 (259.80±307.10)	0.80–31.24 (10.02±10.88)
31–33	9	31–1,535 (354.89±509.73)	0.78–56.48 (13.90±19.13)
34–36	13	20–7,960 (2,277.62±2,822.36)	0.29–128.22 (39.72±46.87)
37–39	10	11–21,753 (2,393.20±6,806.98)	0.17–344.74 (37.83±107.91)
40–42	11	31–8,449 (1,239.36±2,459.89)	0.46–118.45 (17.62±34.48)

[a] Tadpole larval stages grouped by three-stage ranks per group based on the Gosner (1960) advanced development stages to evaluate the infection levels.

DISCUSSION

The qPCR technique was much more sensitive than the PCR was in both samples analyzed (tissue and swabs samples). Similar results were obtained in the detection of *Bd* with both techniques using swabs, following Guayasamin et al. (2014; 42% with qPCR and 22% with PCR). Moreover, the prevalence determined with qPCR in both types of samples was similar to those reported by Retallick et al. (2006; prevalence: tissue, 41.1%; average prevalence: swabs, 21.4%) and Hyatt et al. (2007; prevalence: swabs, 15–22%). The technique allows detection of a greater frequency of zoospores in the early stages of infection in samples from tissue than those from swabs (Longo et al. 2010), causing the prevalence to increase and is related to the life cycle of *Bd* (Hyatt et al. 2007). Our study has confirmed that, as established previously, the best PCR technique for detecting *Bd* is qPCR (Hyatt et al. 2007). We found that the sampling methodology with the greatest sensitivity of *Bd* detection in tadpoles was the extraction of mouthparts. However, the mouthparts of tadpoles can also be swabbed and analyzed by qPCR, providing an estimated prevalence with an acceptable confidence level, using the formula cited by Retallick et al. (2006). Therefore, mouthpart swabbing of tadpoles is a noninvasive and valid technique for the diagnosis of chytridiomycosis, with a

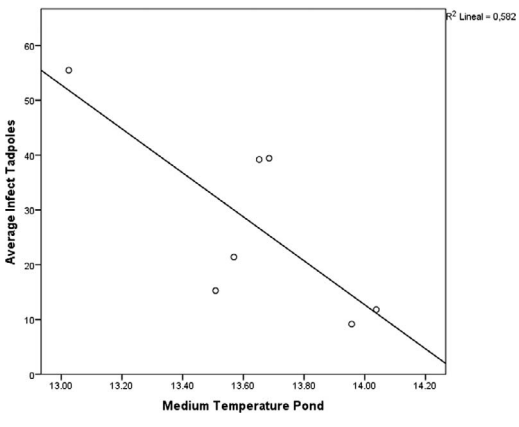

FIGURE 2. Regression between the infection average of the Riobamba marsupial frog (*Gastrotheca riobambae*) tadpoles (*N*=7 mo) and the median temperature of a permanent pond located in the Metropolitan Guangüiltagua Park in Quito, Ecuador, from September 2012 to August 2013.

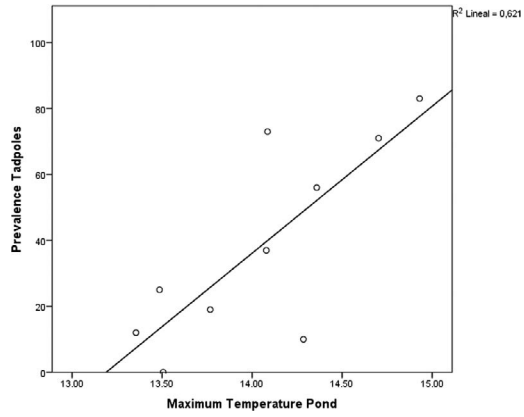

FIGURE 3. Regression between the prevalence of the disease of the Riobamba marsupial frog (*Gastrotheca riobambae*) tadpoles (*N*=10 mo) and the maximum temperature of a permanent pond located in the Metropolitan Guangüiltagua Park in Quito, Ecuador, from September 2012 to August 2013. The correlation was significant (r^2=0.621, *P*=0.007).

significant cost reduction (Kriger et al. 2006a, b). In all comparisons between the techniques, 11–14% of "false-negative" samples were obtained because, in the PCR, the proportions of amplified fragments did not reflect the real abundance of sequences present in the medium; detection based on fluorescence provided greater sensitivity and allowed for discrimination of the numbers of genes across a wider dynamic range (Smith and Osborn 2009).

Dynamics of *Batrachochytrium dendrobatidis* over time

The *Bd* infection levels behaved in different ways between both species. We found that the infection levels varied substantively over time in the *P. unistrigatus* population, but not in the *G. riobambae* tadpoles. Infection levels fluctuated, first decreasing, then increasing once more. These differences detected during the study period are probably due to the following three factors: 1) *Pristimantis unistrigatus* frogs have nocturnal habits, and temperatures differ from diurnal and nocturnal activity periods, which can affect immune responses to infection or affect the host's ability to reduce infection loads (Ruggeri et al. 2015); 2) Quito's seasonality, with the dry season from June to September and the rainy season from October to April, which is similar to the Longo et al. (2010) results, who found that the levels of *Bd* infection in two species of Puerto Rican frogs with direct development varied significantly between the country's two climatic seasons; and 3) the reproductive behavior of *P. unistrigatus* because they do not congregate in ponds or streams to reproduce, which is closely related to Quito's seasonality. This could appear to be an advantage to avoid exposure to the aquatic infectious phase of *Bd* (Olson et al. 2013). Despite that, some studies have reported a higher prevalence of infection in direct-developing species (Guayasamin et al. 2014; Flechas et al. 2017). Further long-term studies are needed to consider those possibilities.

Environmental factors could influence the infection levels in the *P. unistrigatus* population. They might exert stress on individuals, since this species of frogs is sensitive to subtle climatic changes. Precipitation is a very important factor for amphibians, and rehydration depends on the humidity of the vegetation, so extensions of the dry season can greatly affect their reproductive activity and the recruitment of individuals (Burrowes et al. 2004; Ruggeri et al. 2018). These stressful conditions can affect an individual's immune system (Whitfield et al. 2012), favoring *Bd* development and promoting the evolution of the chytrid (faster growth or higher pathogenicity; Flechas et al. 2017). Moreover, similar to the results of Retallick et al. (2006) and Julian et al. (2016), our study did not find differences in the infection levels in *G. riobambae* tadpole larval stages. In all likelihood, as Smith et al. (2007) pointed out, the infection level could be more related to the time of exposure than to the surface area of mouthparts, per se.

No differences were found in the prevalence of the disease over time in the *P. unistrigatus* population or in the *G. riobambae* tadpoles. Nonetheless, a higher prevalence was found in tadpoles (39%, 53/135) than results previously reported (33.9%; Manzano-Pasquel 2010), given that low levels of *Bd* infection were detected. However, the prevalence (31%, 42/135) was similar when comparing with the same technique (PCR). Based on that, it appears likely that the prevalence has been maintained over time. This is probably due to the fungus not usually being able to kill tadpoles and to the ability of the frogs to live and grow with damaged mouthparts to become a *Bd* reservoir (Sapsford et al. 2018; Ruggeri et al. 2018) or because of the presence of microbiota defenses that allow the species to tolerate infections so that the population can persist with a high prevalence of *Bd* infection (Bresciano et al. 2015).

The dynamic of *Bd* was different between the two species over time. This suggests that each species reacts differently to the pathogen because certain species are more prone to infections than other species are (Kriger and Hero 2007; Ruggeri et al. 2018). Prevalence

describes the infection status in a population at a given time, whereas infection levels provide information on the response of an individual to the fungus (Longo et al. 2010). We observed that, in some monthly groups, although infection levels decreased, prevalence increased and vice versa: prevalence decreased, and infection levels increased. Thus, when chytridiomycosis becomes an enzootic disease, and environmental conditions are not stressful for amphibians or act in favor of the physiology of the fungus, the prevalence of the disease may be high, whereas the level of infection is low. In contrast, when environmental conditions are stressful for amphibians (low rainfall), the prevalence does not change significantly. but the contaminated individuals have high infection levels (Longo et al. 2010; Ruggeri et al. 2018).

Additionally, the two frog species have different habitats: one is terrestrial (*P. unistrigatus*), whereas the other is aquatic (tadpoles), so the ecology of the chytrid will vary (Kriger and Hero 2007; Ruggeri et al. 2018). Because our study did not find any relationship between the two variables and the two species, it can be inferred that the presence of *Bd* in *P. unistrigatus* is not a consequence of the prevalence of the disease in tadpoles and that the frogs are acquiring the infection from other sources, such as 1) water from the pond (Kirshtein et al. 2007), 2) rainfall (Kolby et al. 2015), or 3) potential contact with infected skin (Kriger and Hero 2007; Ruggeri et al. 2018) of *G. riobambae* metamorphs or adults by sharing wet shelters (microhabitats) with *P. unistrigatus*, where there may be encysted *Bd* zoospores (Woodhams et al. 2008; Daversa et al. 2018; Sapsford et al. 2018), when environmental conditions are stressful. Out of the eight metamorphs found, five were positive for *Bd*. Finally, it was found that the distance from the pond is a determinant factor for infection levels in *P. unistrigatus* (infection level until ~50 m), given that the animals tagged as positive for *Bd* were found closer to the pond than those who with negative results ($\bar{x}$=25.56 m vs. $\bar{x}$=34.78 m, P=0.042).

Dynamics of *Batrachochytrium dendrobatidis* with environmental factors

Both the infection average and prevalence of the disease in *G. riobambae* tadpoles were strongly related to the pond's temperature but with opposite correlations. As the pond's median temperature increased, the infection average levels decreased (r^2=0.582, P=0.046). However, a minimum of eight or 10 observations are necessary to statistically validate the correlation as a significant result. Despite not obtaining the minimum observations required to make our results more robust, a negative regression can be observed. More studies would be needed to corroborate this relationship by increasing the population size studied. Even though the optimum temperature of the fungus is 17–25 C (Piotrowski et al. 2004), some strains have a wider range of thermal tolerance, supporting freezes (–12 C) or heat shocks (28 C; Sapsford et al. 2018), thereby enabling them to persist and slowly grow at low temperatures. In contrast, the prevalence of the disease was directly related to the pond's temperature, that is, as the pond's maximum temperature increased, the prevalence also increased (r^2=0.621, P=0.007). These results are similar to those that have been previously reported (Manzano-Pasquel 2010), and it seems that temperature is a key factor in more tadpoles acquiring the infection (Alford et al. 2007; Cohen et al. 2019).

Finally, this study confirmed that *G. riobambae* tadpoles are still infected with the chytrid fungus, and the situation has remained stable over time. The pond's temperature represented an important increasing factor in the infection levels and in the prevalence of the disease in this population. Furthermore, this study reports for the first time the presence of chytridiomycosis in a population of *P. unistrigatus* living near a pond in a metropolitan park in Quito. In the case of that species, the infection levels varied over time, and a strong relationship was found between infected individuals and their proximity to the pond. More studies are needed that focus on understanding the evolutionary potential of thermal tolerance in different endemic *Bd*

strains and to evaluate the long-term link between the hosts and the pathogen at different elevations.

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