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Detection of *Batrachochytrium dendrobatidis* in Amphibians from the Forest Floor to the Upper Canopy of an Ecuadorian Amazon Lowland Rainforest

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During the last two decades significant declines and extinctions of amphibians have been observed worldwide (Blaustein and Wake 1995; Houlahan et al. 2000; Stuart et al. 2004). The Neotropics are among the most impacted areas, with sudden losses of anuran species noticed during the 1980s in Costa Rica, Ecuador and Venezuela (Pounds and Crump 1994; Pounds et al. 1997; Young et al. 2001; Ron et al. 2003). Population declines and losses have been more significant at high elevation (above 1000 m) with one study showing all species of *Atelopus* with sufficient data (N = 28) declining and 75% disappearing (La Marca et al. 2005). A serious contributor to amphibian declines worldwide is the fungus *Batrachochytrium dendrobatidis* (*Bd*), which was present in the Neotropics before declines were noticed in the 1980's (Berger et al. 1999; Speare and Berger 2000; Bosch et al. 2001; Bradley et al. 2002; Lips et al. 2005; Ron and Merino 2000). *Bd* is now implicated as one of the causes of the extinction of several species of anurans from these regions (Ron et al. 2003; La Marca et al. 2005; Merino-Viteri et al. 2005). While *Bd* has been detected in amphibians from many high elevation sites in the Neotropics (e.g., Ron and Merino 2000; Young et al. 2001; Lips et al. 2003),

low elevation sites in the Neotropics have received comparatively little attention and thus reports on the detection of *Bd* at these sites are less common (Puschendorf et al. 2006; Oliveira de Queiroz Carnaval et al. 2006).

The aim of this study was therefore to examine the occurrence of *Bd* in amphibian species of a Neotropical lowland rainforest, i.e., at sites in the Upper Amazon Basin of eastern Ecuador at low elevation (about 200 m). This study included analyses of amphibians occupying ecological niches above the forest floor and shrub layers, specifically the mid- to upper canopy, because the upper strata of Amazonian rainforests plays an integral role in ecosystem function and data are missing for the interaction of *Bd* with amphibians in this important microhabitat.

Methods.—Samples were collected from sites surrounding Tiputini Biodiversity Station – Universidad San Francisco de Quito (0.6384694°S, -76.1490806°W, 217 m elev.), at the border of Yasuni National Park in the eastern lowlands of Ecuador (Fig. 1) during May–August of 2004 and 2006. The vegetation of the region has been defined as Amazonian Evergreen Lowland Forest, where annual rainfall averages 2425–3145 mm, temperature averages 25°C (range is 15°–38°C), and average humidity is 88% (Balslev et al. 1987; Blandin Landívar 1976; Duellman 1978). This area is part of the Napo refugium, a subset of the Amazonian system, and it is likely to represent some of the oldest undisturbed forests and greatest biodiversity in the system (Prance 1982). Amphibians from the forest floor and shrub layer were collected near the banks of the Rio Tiputini at elevations between 190–250 m during visual encounter surveys. Amphibians occupying higher strata were collected during bromeliad patch sampling following the procedure outlined by McCracken and Forstner (2008).

FIG. 1. Schematic presentation of the sampling site within the Yasuni National Park near Tiputini Biodiversity Station (TBS) – Universidad San Francisco de Quito, in the Upper Amazon Basin of eastern Ecuador used for collection of amphibians to be tested for infection by *Batrachochytrium dendrobatidis*.

Toe clips and skin from thigh muscle samples were used to test for the presence of *Bd* (Table 1). Samples were grouped into three categories (fossorial/forest floor, shrub/sub-canopy, and upper canopy) along the vertical axis based on historical and current collection locations for each species tested. Species placed in the fossorial/forest floor category are those which utilize subterranean habitat such as burrows and holes or typically occur amongst the forest floor leaf litter. Thirty-six individuals of 15 species in the order Anura were tested from the fossorial/forest floor category. The second category contains species which occupy the forest understory (shrub/sub-canopy). Whereas some of these amphibians may occasionally be found on the forest floor, they are most commonly found on low vegetation (< 2 m); a few animals which were tested and placed in this category have been found up to 4 m. Twenty-nine individuals of 12 species in the orders Anura and Caudata were tested from the shrub/sub-canopy category. The final category contains species found in the upper canopy occupying tank bromeliad habitat above 4 m (4–38 m). Twenty-one individuals of 4 species in the order Anura were tested from the upper canopy layer.

Water temperature was collected in three tank bromeliads of the species *Aechmea zebrina* located in the upper canopy of an emergent tree, *Parkia multijuga*, at 32, 34, and 35.5 m vertical height and within 2 m horizontal distance from the tree bole. One Thermochon iButton (model DS1922L) temperature logger was placed in an outer leaf bract of each bromeliad a minimum of 8 cm below the water level. Data loggers were set to collect water temperature every 30 minutes (35.5 m) and every 60 minutes (32 m, 34 m) at a resolution of 0.5°C with an accuracy of ±1°C from -30°C to +70°C. Duration of total data collection varied from 43 to 142 days from 31 March 2008 to 24 August 2008. Water pH was taken during bromeliad patch sampling according to McCracken and Forstner (2008) for five *A. zebrina* in each of 18 trees for a total of 90 bromeliads during field seasons in 2006 and 2008. An Oakton pHTestr 30 was used to collect water pH at a resolution of 0.01 pH with an accuracy of ±0.01 pH; a three-point calibration was performed before each tree sampled.

A nested PCR approach that has shown increased sensitivity of detection in samples with low template numbers and is outlined in detail in Gaertner et al. (*in press*) was used for detection of *Bd*. This approach used primers ITS1f (5'CTT GGT CAT TTA GAGC GAA GTA-3') and ITS4 (5'TCC TCC GCT TAT TGA TAT GC-3') targeting conserved regions of the 28S and 18S rRNA to amplify the 5.8S rRNA gene and the flanking internal transcribed spacer (ITS) of all fungi (White et al. 1990). PCR products from this reaction were purified and then used as a template for the subsequent PCR reaction using the *Bd*-specific primer set Bd1a (5'CAG TGT GCC ATA TGT CAC G-3') and Bd2a (5'CAT GGT TCA TAT CTG TCC AG-3') (Annis et al. 2004). The product from this reaction was examined by gel electrophoresis (2% agarose in TAE buffer) (Sambrook et al. 1989) for a fragment of approximately 300 bp (Annis et al. 2004). DNA from a sample positive for *Bd* from a previous study (Gaertner et al., *in press*) and sterilized distilled water were used as positive and negative controls, respectively. PCR products from samples showing the 300 bp amplicons were then sequenced using the CEQ 8800 Quickstart Kit with the addition of 5% DMSO to the reaction mix on a CEQ 8800 sequencer (Beckman Coulter, Fullerton, California). The sequences were

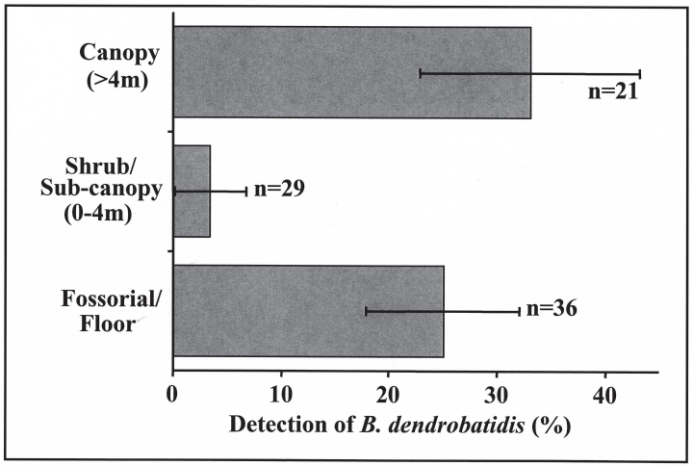

FIG. 2. Detection of *Batrachochytrium dendrobatidis* (% ± 95% confidence intervals, calculated in Microsoft Office Excel) in samples from amphibians collected within the Yasuni National Park, Ecuador, at three different vertical strata groups (fossorial/floor; shrub/sub-canopy, 0–4 m; and canopy, > 4 m).

validated against the GenBank/EMBL databases using BLASTn (Pearson and Lipman 1988).

Results.—Eighty-six amphibians of two orders (Anura N = 84, and Caudata N = 2) belonging to 7 families and 31 species were screened (Table 1) using nested PCR analysis (Gaertner et al., *in press*). Of those tested, 17 individuals (20%) representing eight species were positive for *Bd*. *Bd* was detected from within each of the strata, with the fossorial/floor group consisting of nine positive from 36 individuals tested (25%), shrub/sub-canopy group consisting of one positive from 29 tested (3%), and the canopy group consisting of seven positive of 21 tested (33%) (Fig. 2). Comparative sequence analysis of all 17 amplicons retrieved from PCR amplification with existing sequences of *Bd* available in GenBank/EMBL databases confirmed the detection of *Bd*. All sequences exhibited more than 99% similarity, with all sequences of the 5.8S rRNA gene being identical to the published sequence of *Bd* (AY997031), and those of seven amplicons displaying small differences in the ITS regions (Table 2).

Water temperatures recorded inside *A. zebrina* bromeliads at 32 m were 20.5–30.5°C (23.8 ± 1.9 [$\overline{x}$ ± SD], N = 1369), at 34 m they were 20.0–31.0°C (24.1 ± 1.9, N = 3422), and at 35.5 m were 20.5–32.5°C (24.5 ± 2.5, N = 2017). The mean pH recorded was 4.48 ± 0.67 (N = 90).

Discussion.—The presence of *Bd* was demonstrated in amphibians of the Upper Amazon Basin of eastern Ecuador. *Bd* has been detected from several high altitude sites in the Neotropics (Lips et al. 2003; Ron and Merino 2000; Young et al. 2001), however, detections at low elevation sites are less common (Oliveira de Queiroz Carnaval et al. 2006). Our results have shown that *Bd* is present in Amazonian lowland rainforests at elevations less than 300 m. This finding is significant because even though amphibian declines are not as prevalent at low elevations (Oliveira de Queiroz Carnaval et al. 2006; Puschendorf et al. 2006), *Bd* is present and could be contributing to declines. Amphibians may also be serving as a reservoir for *Bd* which could then move to and infect amphibians at higher elevation sites.

Another important finding of this study is the demonstration of clinical signs of chytridiomycosis and a positive detection for *Bd* by one individual (*Leptodactylus pentadactylus*) included in the study. In laboratory experiments, *Bd* grew best and was most lethal under cool (22ºC), moist conditions (Piotrowski et al. 2004; Woodhams et al. 2003) suggesting that montane species should be the most likely candidates for declines. This scenario is certainly supported by studies in the area with one study failing to find even a single apparently healthy population of *Atelopus* at elevations above 1000 m (La Marca et al. 2005). Despite the apparent virulence of *Bd* at high elevation sites, there are very few accounts of population declines caused by *Bd* infection at low elevations. The discovery of *L. pentadactylus* at this elevation showing clinical signs suggests that *Bd* might play a more important role in lowland declines than previously thought.

TABLE 1. Amphibian species collected from three vegetation strata (canopy, shrub/sub-canopy, fossorial/floor) in Yasuni National Park, Ecuador, and analyzed for *Batrachochytrium dendrobatidis* (*Bd*).

Amphibian species	Number of samples analyzed	Number of *Bd*-positive samples
Canopy		
Hypsiboas boans	1	0
Osteocephalus taurinus	4	0
Pristimantis aureolineatus	7	4
Pristimantis waoranii	9	3
Total	21	7
Shrub/sub-canopy		
Dendropsophus parviceps	2	0
Hypsiboas cinerascens	1	0
Hypsiboas geographicus	1	0
Hypsiboas lanciformis	2	0
Osteocephalus planiceps	2	0
Scinax cruentommus	2	0
Scinax ruber	1	0
Pristimantis acuminatus	3	0
Pristimantis altamazonicus	6	0
Pristimantis ockendeni	6	1
Pristimantis peruvianus	1	0
Bolitoglossa equatoriana	2	0
Total	29	1
Fossorial/floor		
Rhinella margaritifera	2	0
Rhinella marina	2	0
Engystomops petersi	6	1
Leptodactylus andrea	1	0
Leptodactylus discodactylus	5	2
Leptodactylus hylaedactyla	1	0
Leptodactylus pentadactylus	5	4
Leptodactylus rhodomystax	1	1
Leptodactylus wagneri	1	0
Chiasmocleis bassleri	3	0
Oreobates quixensis	2	0
Pristimantis lanthanites	1	1
Pristimantis malkini	2	0
Strabomantis sulcatus	2	0
Total	36	9

TABLE 2. Base pair differences of sequences generated from nested PCR reactions of samples collected from Yasuni National Park, Ecuador, using existing GenBank entry AY997031 for reference positions.

Accession Number	Difference
FJ232005	1 bp insertion at base 68 (A)
FJ232006	1 bp insertion at base 68 (A), deletion of positions 80–86
FJ232007	1 bp insertion at base 68 (A)
FJ232009	SNP at position 47 (G to T)
FJ232019	deletion of positions 13–20, SNP at position 55 (T to A)
FJ232020	deletion of positions 36–39, SNP at position 51 (A to G)
FJ232021	deletion of positions 31–33

All previous studies on *Bd* have used amphibians caught at or near ground level. This allows for testing of only the portion of amphibians contained in the lower strata of a structurally complex system. We detected *Bd* on amphibians inhabiting all strata of the forest which demonstrates that searches not including vertical strata may be lacking important data on the system. Furthermore, we found evidence that the occurrence of *Bd* infection on amphibians along a vertical axis is non-random in this system. Infection by *Bd* was found to be significantly higher in the fossorial/floor and canopy groups, showing 25% and 33% of individuals infected, respectively, than in the shrub/sub-canopy group with only one individual (3%) infected. Each of the strata of the rainforest has unique microclimate characteristics that could potentially affect *Bd* infections. In this case the availability of water may play a role in the prevalence of infection in each of the groups. Amphibians of the fossorial/floor group have water available in the form of streams and standing pools and the canopy group has access to water captured in phytotelmata, primarily tank bromeliads. Because *Bd* is transmitted via aquatic zoospores (Longcore et al. 1999), the absence of an abundance of standing water available to amphibians of the shrub/sub-canopy group may be reducing their exposure to the fungus. Previous studies have demonstrated that environmental conditions at the landscape level can have strong effects on host-pathogen dynamics (Woodhams et al. 2006), and future studies should include the investigation of interactions of *Bd* with environmental conditions, including water availability, on both the landscape and microhabitat level.

Environmental parameters for *Bd* have been found to have a significant influence on its pathogenicity (Andre et al. 2008; Piotrowski et al. 2004). Piotrowski et al. (2004) found isolates of *Bd* to grow and reproduce (in culture) between 4–25ºC and pH 4–8. Optimal growth of *Bd* zoospores occurred at temperatures of 17–25ºC and pH 6–7 (Piotrowski et al. 2004). Mortality rates in studies of *Bd*-infected frogs exposed to ambient temperatures of 17–25ºC have been >50%, although several studies show increased survival and decreased infection rates with temperatures >22ºC (Andre et al. 2008; Berger et al. 2004; Carey et al. 2006; Kriger and Hero 2007; Woodhams et al. 2003). Exposure to temperatures >25ºC has been shown to kill *Bd* zoospores and cure infected frogs (Berger et al. 2004; Kriger and Hero 2006; Piotrowski et al. 2004; Woodhams et al. 2003). While *Bd* prefers a near neutral pH, swimming zoospores were found in cultures at pH 4 and a temperature of 23ºC for 14 days (Piotrowski et al. 2004).

In our study, basic environmental water parameters collected from *A. zebrina* tank bromeliads indicate optimal temperature conditions for the persistence of *Bd* in the canopy. While pH levels were lower than previously reported as optimal conditions in fungal culture experiments, it remains unclear what effect this has *in situ*. *Pristimantis aureolineatus* and *P. waoranii* are known permanent inhabitants of *A. zebrina* bromeliads and with 44% of those individuals screened in this study testing positive it seems evident that *Bd* is present in canopy phytotelmata. However, definite proof of this assumption requires detection of *Bd* directly in water of the bromeliads. Many other species of anurans utilize bromeliad habitat for egg and tadpole deposition sites and may act as a reservoir for transferring the pathogen between sites. Several species of Dendrobatidae are of particular concern since they transfer tadpoles from terrestrial deposition sites to bromeliads in the canopy for final development. This broad traverse of habitats may facilitate the movement of *Bd* between terrestrial and canopy water sources, additional studies are needed to track potential movement of *Bd* along the vertical axis.

Four ecological traits are commonly associated with amphibian populations in decline including: aquatic mode of life, occurrence at mid to high altitudes, low fecundity and endemic distribution (Laurance et al. 1996; Lips 1998; Lips et al. 2003; Williams and Hero 1998). The observation of clinical signs of chytridiomycosis at a low elevation site and the characteristics of microhabitat utilized by permanent canopy anuran inhabitants infected by *Bd* demonstrate that the threat of chytridiomycosis may still be significant at sites even in the absence of those traits.

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