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Infection Prevalence in Three Lowland Species of Harlequin Toads from the Threatened Genus *Atelopus*

Atelopus, a species-rich Neotropical genus within the family Bufonidae (Lötters 1996), is distributed from Costa Rica south to Bolivia and eastward into the Guyanas (La Marca et al. 2005). Despite declines and disappearances of *Atelopus* species occurring before *Batrachochytrium dendrobatidis* (hereafter, *Bd*) was described (Longcore et al. 1999), *Bd* has been implicated in losses (La Marca et al. 2005; Lampo et al. 2006). The majority of these species occur in the Andes Range above 1500 m, and are considered one of the most threatened genera of amphibians worldwide. At least 71% (69 of 97) of *Atelopus* species are listed as Critically Endangered or Extinct (IUCN 2015). Still, many species have uncertain conservation status because they remain undescribed (Coloma et al. 2010) or because they require systematic revision (Lötters et al. 2011). The most affected species within the genus occur along mid-to-high elevation streams with nearly 75% of those species facing serious declines (La Marca et al. 2005). Colombia harbors the highest number of *Atelopus* species (Lötters 1996) and, similar to other parts of its distribution, nearly all species that occur in the mid-to-high elevations have declined sharply (Rueda-Almonacid et al. 2005). In the Colombian lowlands, however, species still persist at altitudes ranging from 0–600 m and there are no reported declines in this group (Rueda-Almonacid et al. 2005). This difference in survival is usually attributed to *Bd* not being able to survive well at lower elevations, which in the tropics usually means higher and less variable environmental temperatures. In order to evaluate whether *Bd* can be considered as a potential threat for *Atelopus* species, we assessed *Bd* prevalence and infection intensity on three lowland species that occurring between 5–115 m elevation, examining live animals during contemporary field surveys in 2007 to 2012 and archived museum specimens collected between 1968 and 2000.

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We studied three *Atelopus* species occurring in three different localities in the coastal forests of Colombia (Fig. 1). We visited each site 3–5 times between 2007 and 2012 (Table 1). *Atelopus* aff. *elegans*, from Gorgona Island (2.966667°N, 78.166667°W; 6–115 m elev.) was sampled three times (July 2007, July 2010, and June 2012). *Atelopus spurrelli*, in the municipality of Arusí (5.566667°N, 77.5°W; 90 m elev.) on the Pacific coast was sampled five times (May and November 2009, June and September 2011, and June 2012). *Atelopus* aff. *limosus*, an undescribed species occurring near the municipality of Capurganá (8.6°N, 77.3333°W; 150 m), close to the border between Panama and Colombia, was sampled three times (September 2008, October 2009, and July 2012).

Survey teams composed of 2–5 people conducted field trips to each locality for 2–8 days per trip. To maximize capture success, we surveyed along streams and along forest trails using visual encounter surveys (Heyer et al. 1994). For each animal we collected the following information to look for potential

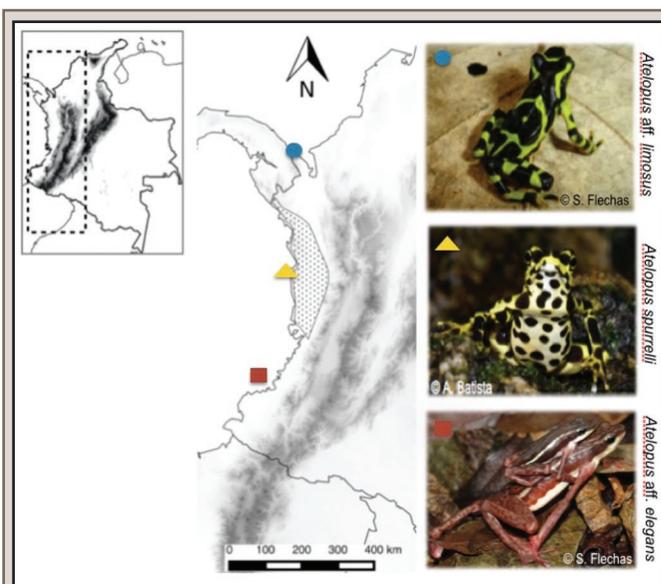


FIG. 1. Colombia locations of the *Atelopus* field sites: (■) *Atelopus* aff. *elegans*; (●) *Atelopus* aff. *limosus*; and (▲) *Atelopus* *spurrelli*. The distribution of *A.* aff. *elegans* is restricted to Gorgona Island near the Pacific Coast of Colombia, the distribution of *A.* aff. *limosus* is unknown, and the stippled area is the distribution of *A.* *spurrelli* (IUCN 2015).

TABLE 1. *Batrachochytrium dendrobatidis* prevalence among three lowland *Atelopus* species in Colombia. The number in parenthesis represents the number of *Bd*-positive individuals. p/h = person/hours. Prevalence CI 95% = Prevalence of infection, in parenthesis are the Bayesian Credible Intervals based on 95% confidence. Bold numbers indicate summaries per species.

Species	Date	Sex and Life Stage			Search Efforts (p/h)	Total	Prevalence CI (95%)	Technique
		Female	Male	Juvenile				
<i>Atelopus</i> aff. <i>elegans</i>	Jul 2007	15 (4)	57 (10)	6 (1)	144	78 (15)	19 (11.6–29)	PCR
	Jul 2010	12 (2)	25 (5)	46 (2)	150	83 (9)	10.7 (5.4–18.6)	PCR
	Jun 2012	5 (0)	60 (2)	82 (1)	120	147 (3)	2 (0.5–5.3)	qPCR
						308 (27)	8.7 (5.9–12.2)	
<i>Atelopus</i> <i>spurrelli</i>	May 2009	1 (0)	67 (1)	6 (0)	96	74 (1)	1 (0.1–6.1)	PCR
	Nov 2009	3 (0)	40 (1)	2 (1)	60	45 (2)	4 (0.9–13.5)	PCR
	Jun 2011	0 (0)	9 (1)	0 (0)	72	9 (1)	11 (1.2–41.4)	PCR
	Sep 2011	1 (0)	4 (1)	7 (0)	96	12 (1)	8 (0.9–32.8)	PCR
	Jun 2012	3 (0)	9 (1)	20 (0)	144	32 (1)	3 (0.3–13.6)	qPCR
						172 (6)	3.4 (1.4–7.0)	
<i>Atelopus</i> aff. <i>limosus</i>	Sep 2008	1 (0)	8 (0)	2 (0)	48	11 (0)	0 (0–15.6)	PCR
	Oct 2009	0 (0)	61 (0)	11 (0)	48	72 (0)	0 (0–2.6)	PCR
	Jul 2012	8 (0)	87 (0)	0 (0)	72	95 (0)	0 (0–1.9)	qPCR
						178 (0)	0 (0–1.0)	

TABLE 2. Museum specimens swabbed. The asterisk (*) indicates the year when *Batrachochytrium dendrobatidis* (*Bd*) was first detected.

Species	Year	No. swabbed	No. <i>Bd</i> -positives
<i>Atelopus</i> aff. <i>elegans</i>	1968	54	0
	1977	1	0
	1978	12	0
	1985	24	0
	1986	20	0
	1987	1	0
	2000*	25	3
<i>Atelopus</i> <i>spurrelli</i>	1968	6	0
	1969	91	0
	1983	31	0

patterns in *Bd* occurrence: sex and life-stage (male, female; adult, juvenile), snout–vent length (mm), and weight (g). To examine the hypothesis that lowland conditions may be unsuitable for *Bd* occurrence, body temperature was measured using an infrared thermometer (OAKTON® InfraPro) with a resolution of 0.2°C. From a distance of 50 cm, we pointed the laser to the frog’s dorsal surface or the exact place where the individual was perched before handling.

To determine infection status and to avoid cross contamination, animals were collected using clean, decontaminated equipment, individually handled with fresh disposable gloves, and placed in individual bags prior to obtaining the skin swab samples. Each animal was sampled by running a synthetic cotton swab (skin swabs) over the ventral surface, the inner thigh area and the plantar surface of the hind-feet webbing for a total of 50 strokes. Skin swabs were preserved either in 96% ethanol or were air dried and stored at -20°C until processed. To diagnose *Bd* infection we used two techniques: end-point PCR and Real-time TaqMan PCR – qPCR. When we started the surveys our main goal was simply to determine *Bd* presence and thus we ran end-point PCR and qPCR assay to determine presence/absence

of the pathogen. Starting in 2012 we performed qPCR assay, to test not only for the presence of *Bd* but also for the infection intensity (pathogen load) and report it as the relative number of zoospores (Zoospore Equivalents, ZE) on each animal.

For end-point PCR, DNA was extracted using GeneReleaser® (Bioventures Inc., Carlsbad, California, USA). We used the primers developed by Annis et al. (2004) to amplify the ITS1-ITS2 region specifically in *B. dendrobatidis*: Bd1a (5'- CAGTGTGCCAT-ATGTCACG-3') and Bd2a (5'-CATGGTTCATATCTGTCCAG-3'). Amplifications were performed in an MJ Research Peltier Thermal Cycler (PTC-200), as follows: an initial 2 min denaturation at 95°C, followed by 35 cycles of DNA amplification (45 sec at 95°C, 45 sec at 55°C, and 1 min at 72°C), and a final extension at 72°C for 10 min completed the amplifications. Each reaction consisted of 0.5 µL of each primer (1 µM), 3.0 µL of doubly distilled DNA-free water, 6 µL of GoTaq® Green Master Mix (1X; Promega) and 2 µL of the DNA extract. The amplified fragments were separated by electrophoresis in 1% agarose gels. The qPCR assay was performed using a BIO-RAD PCR machine (to diagnose) and an Applied BioSystems 7300 Real Time PCR System (to quantify zoospore loads). For the qPCR, DNA was extracted using PrepMan Ultra. Extractions and qPCR’s were performed following the methods described by Hyatt et al. (2007) and Boyle et al. (2004). Each reaction consisted of 0.45 µL of each primer (50 µM), 5.975 µL of water, 12.5 µL of TaqMan, 0.625 µL of MGB Probe and 5 µL of the DNA template. To quantify infection intensity we used standards of known concentrations and negative controls.

To determine the historical presence of *Bd*, we swabbed museum specimens of *A. spurrelli* and *A. aff. elegans*. Specimens for *A. aff. limosus* were not available (Table 2). We used the molecular methods and protocols described by Cheng et al. (2011). In order to decrease the chances of contamination we rinsed each frog with 70% EtOH and we used fresh gloves for each frog handled. We ran a synthetic cotton swab (MWE 113) over the ventral surface, inner thighs, abdomen, and between toes for a total of 30 strokes. DNA was extracted using Prepman Ultra. Each sample was run in triplicate by using qPCR assay following Boyle et al. (2004) using an Applied BioSystems 7300 Real Time PCR System.

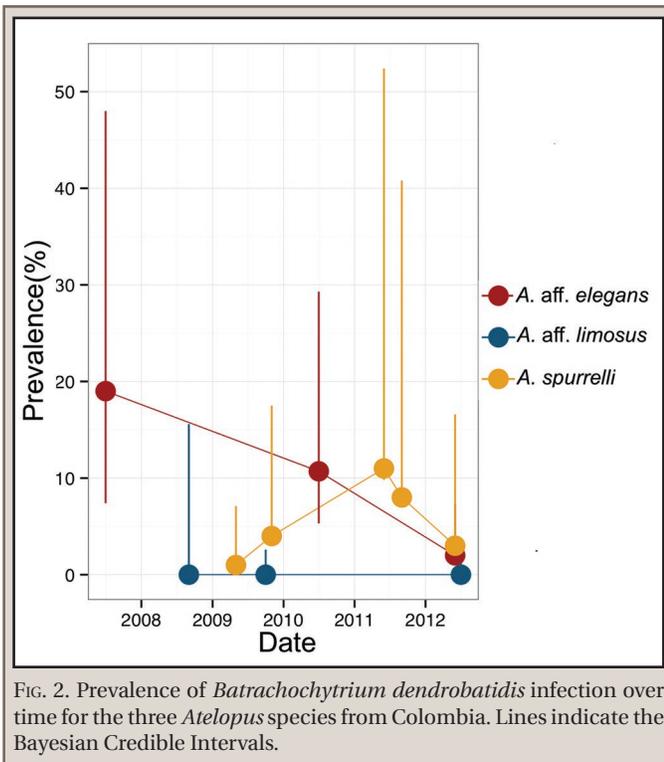


FIG. 2. Prevalence of *Batrachochytrium dendrobatidis* infection over time for the three *Atelopus* species from Colombia. Lines indicate the Bayesian Credible Intervals.

We analyzed skin swabs from a total of 658 individuals collected during the field surveys, including 308 *A. aff. elegans*, 178 *A. aff. limosus*, and 172 *A. spurrelli* (Table 1). We analyzed for *Bd* presence with 384 samples (end-point PCR and qPCR), and *Bd* zoospore equivalents or infection intensity with 274 swabs (qPCR assay). In total we detected *Bd* in 6 (3.5%) *A. spurrelli* and 27 (8.7%) *A. aff. elegans*. We have infection intensity data for four samples taken in 2012: one male *A. spurrelli* (16.9 ZE), two males (63 and 65490 ZE) and one juvenile (726 ZE) *A. aff. elegans*. Per sample date, the prevalence of the infection (i.e., the number of infected animals out of the total number of individuals sampled) was very low, ranging from 1–11% in *A. spurrelli* and from 2–19% in *A. aff. elegans* (no *Bd*-positive *A. aff. limosus*). From 2007 to 2012, the prevalence of *Bd* in *A. aff. elegans* dropped steadily (Table 1) (G-test test of independence with Williams' correction, $\chi^2_{df=2} = 19.792$, $P = 5.037 \times 10^{-5}$); conversely, there was not a discernible pattern in the prevalence of *Bd* in *A. spurrelli* during that same time frame (G-test, $\chi^2_{df=4} = 2.325$, $P = 0.6762$) (Fig. 2). Visual checks of animals that were released after capture did not reveal any signs of chytridiomycosis in any species. We found that *Bd* prevalence was not influenced by body size ($N = 658$, $Z = 1.067$, $P = 0.286$). However, in *A. aff. elegans* the prevalence was higher for males ($\chi^2 = 11.412$, $df = 2$, $P = 0.003$). Frog body temperatures ranged between 23.2–28.2°C ($\bar{x} = 25.1^\circ\text{C}$) in *A. aff. elegans*, 22.6–29.3°C ($\bar{x} = 25.2^\circ\text{C}$) in *A. aff. limosus*, and 22.5–28.5°C ($\bar{x} = 25.4^\circ\text{C}$) in *A. spurrelli*. We detected differences in body temperature among surveys only for *A. aff. limosus* ($F = 136.5$, $P < 0.01$). To provide a retrospective view of *Bd* emergence, we swabbed available museum specimens: 128 formalin-preserved specimens of *A. spurrelli* collected between 1968 and 1983 and 137 *A. aff. elegans* collected between 1968 and 2000 (Table 2). We detected *Bd* presence in three (12%) *A. aff. elegans* collected in 2000.

Visual encounter surveys showed that from 2009 to 2012, *A. spurrelli* were encountered at a much lower rate. We spent approximately 414 h (person hours) surveying for individuals

of *A. aff. elegans*, and 168 h and 468 h for individuals of *A. aff. limosus* and *A. spurrelli*, respectively. By the end of the study, *A. spurrelli* were difficult to find despite intense effort (Table 1). Our findings suggest a decrease in abundance of *A. spurrelli* during the course of our study. We first detected *Bd* in one of 74 frogs sampled in 2009 in the wild, then in the following surveys (2009–2012), *A. spurrelli* became increasingly harder to find. Although we acknowledge that we did not conduct systematic population abundance monitoring, we did not detect chytridiomycosis and *Bd* prevalence from our samples was low, we hypothesize that *Bd* may have contributed to the apparent decline. We cannot rule out the possibility that this drop in abundance over the time frame sampled might be due to other factors, including natural population variation (Pechmann et al. 1991). The apparent decline of *A. spurrelli* is puzzling due to the presence of cutaneous bacteria with antifungal properties (Flechas et al. 2012), the fact that these frogs occur in an environment with relatively high temperatures (22.6–29.3°C) which may be unsuitable for optimal *Bd* growth (Johnson et al. 2003; Piotrowski et al. 2004), and no observed habitat degradation or other stressors. Based on our retrospective study of museum specimens, we found no evidence of *Bd* presence in this species before the first positive record in 2009. Unfortunately, the lack of systematic population surveys makes it hard to compare abundances between years, which would allow for detecting gradual versus sudden population collapse. Other species of *Atelopus* have declined due to habitat destruction and not *Bd* (Tarvin et al. 2014), but we found no indication of habitat alteration or disturbance in our study area. Therefore we suggest that systematic surveys (population monitoring and disease diagnostics) be conducted to assess the current status of *A. spurrelli* from Arusí in order to more accurately estimate future trajectories for this population.

A recent study conducted on the same three *Atelopus* species that are the focus here revealed that *A. aff. elegans*, the species that we found to be most common despite *Bd* infection, harbors the highest proportion of bacterial isolates with anti-*Bd* activity; these bacteria also exhibited the strongest anti-*Bd* effect (Flechas et al. 2012). An alternative explanation for species survival when exposed to lethal strains of *Bd* could be an environmental effect that limits *Bd* growth (Rowley and Alford 2007; Bustamante et al. 2010; Zumbado-Ulate et al. 2014). Various studies have proposed the existence of climatic refugia from chytridiomycosis-driven amphibian declines (Puschendorf et al. 2011; 2013) as a mechanism to allow susceptible species to persist despite infection (Puschendorf et al. 2005). Our three study species occur in tropical coastal forests that exhibit high temperatures, above the optimal physiological range of the pathogen (Johnson et al. 2003; Piotrowski et al. 2004). This temperature regime might limit *Bd* growth causing a decrease in zoospores production, thereby slowing transmission rates and allowing individuals the time to mount an immune response or simply tolerate the pathogen. However, we suggest that when the pathogen first arrives it could cause high mortality in the naïve populations.

Knowing the relative time frame of the emergence of a pathogen in host populations is important for understanding present-day disease dynamics (Briggs et al. 2010; Vredenburg et al. 2010). For example, host pathogen dynamics can move from unstable dynamics soon after pathogens emerge in naïve host populations (e.g., epizootic state) to more stable dynamics long after emergence events (e.g., enzootic state). As part of our study, we provide a retrospective view of *Bd* emergence in two of our

three study species (specimens were not available for the third species). We sampled museum specimens using a molecular technique that has been shown to accurately reflect *Bd* presence or absence in specimens collected up to a century ago (Cheng et al. 2011; Rodríguez et al. 2014). We first detected *Bd* in three of 25 individuals of *A. aff. elegans* collected in 2000. Since there is a lack of sampling between 1987 and 2000, we cannot discard the possibility that *Bd* arrived to the island earlier. The elapsed time could fit an enzootic pattern of host/pathogen dynamic, where *A. aff. elegans* populations remain stable despite the presence of the pathogen.

Atelopus aff. limosus, our third study species, occurs near the border between Colombia and Panamá. This species is believed to have a wider distribution in the Darién region of Panamá (R. Ibáñez, pers. comm.), and this area was considered until recently one of the last *Bd*-free areas in Central America (Rebollar et al. 2014). Our data from 2008 to 2012 did not find any evidence of *Bd*, supporting the idea that the pathogen has not arrived to Capurganá. However, the situation is critical because *Bd* was detected in 2012 in the Darién Province in Panamá (Rebollar et al. 2014) very close (~50 km) to our study site in Colombia. We predict that *Bd* will spread across the Colombian border very soon. The fact that this is a *Bd*-naïve area and the high infection susceptibility reported for the genus, we believe that *Bd* may have a considerable impact on the *Atelopus aff. limosus* population. However, we also expect that the suboptimal environmental conditions (i.e., high temperatures) will limit *Bd* growth and colonization rate, which may allow for host tolerance of the infection or evolution of host resistance allowing for coexistence between host and pathogen.

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