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A CHYTRIDIOMYCOSIS EPIDEMIC AND A SEVERE DRY SEASON PRECEDE THE DISAPPEARANCE OF *ATELOPUS* SPECIES FROM THE VENEZUELAN ANDES

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Chytridiomycosis has been identified as one of the major forces driving global amphibian declines. Between 1988 and 1994, five *Atelopus* species endemic to the Venezuelan Andes disappeared. We examined histological samples of Andean *Atelopus* species available in Venezuelan museum collections for the presence of the chytrid fungus *Batrachochytrium dendrobatidis*. When infection was detected, sympatric species were examined to investigate the occurrence of the pathogen and how widespread it was. Infection with *B. dendrobatidis* is reported for the first time in *Atelopus carbonerensis*, *A. mucubajiensis* and *A. sorianoi*, *Mannophryne cordilleriana* and an undescribed *Leptodactylus* species. The spatio-temporal patterns of prevalence of this pathogen in *Atelopus* individuals, with all infections concentrated in one year but spread over distant locations, suggest that synchronized epidemic outbreaks occurred in populations of these *Atelopus* species in the years prior to their disappearances. Local climate data indicate that one of the most severe dry seasons recorded in the region since 1970 coincided with these epidemic events. The climatic-linked epidemic hypothesis seems a plausible explanation for the coincidence between the observed amphibian declines, the chytridiomycosis outbreaks and the droughts recorded in that area.

Key words: amphibian declines, *Batrachochytrium dendrobatidis*, climate change, emerging diseases

INTRODUCTION

Batrachochytrium dendrobatidis, a pathogenic agent responsible for the fungal disease chytridiomycosis, has been implicated in population crashes and extinctions of many amphibian species around the world. Among the anuran genera endemic to South America and the Caribbean, *Atelopus* appears to be one of the most affected by this disease (La Marca *et al.*, 2005). Declines have affected 61 species in the taxon, a previously unknown degree of biodiversity loss for a single genus (IUCN *et al.*, 2004; Lötters *et al.*, 2004*a*; La Marca *et al.*, 2005). Mass mortalities and declines of *Atelopus chiriquiensis* (Panama and Costa Rica) and *Atelopus varius* (Panama) have been attributed to chytridiomycosis (Lips, 1998; Berger *et al.*, 1998). In Venezuela, *B. dendrobatidis* has been reported only in a museum specimen of *Atelopus cruciger,* a critically endangered endemic species from lowland rainforests and cloud forests of the Cordillera de la Costa, collected in 1986 (Bonaccorso *et al.*, 2003) and live populations of bullfrogs (*Lithobates catesbeianus,* Ranidae), an exotic species introduced recently in disturbed Andean cloud forests, well after the *Atelopus* declines were first observed (Hanselmann *et al.*, 2004).

The Venezuelan Andes hosts eight *Atelopus* species; with the possible exception of *A. tamaense,* all of them are endemic to the country. Six of these species have suffered declines: *A. carbonerensis*, *A. chrysocollarus*, *A. mucubajiensis, A. oxyrhynchus A. pinangoi* and *A. sorianoi* (La Marca & Reinthaler, 1991; La Marca, 1995*a,b*; La Marca & Lötters, 1997; Manzanilla & La Marca, 2004). All were abundant from 1920, the earliest herpetological records available in the country, until the late 1980s. However, regardless of the numerous efforts to find these species in their former habitats, none of them have been recorded after 1994 (La Marca, 2004; La Marca *et al.*, 2005), except for the recent record of a single individual of *A. mucubajiensis* (Barrio-Amorós, 2004). As a result, all of these species are currently critically endangered except for *A. vogli* , which is most probably extinct (IUCN *et al.*, 2004; Lötters *et al.*, 2004*b*).

Habitat destruction, fragmentation and over collection were suggested previously as probable causes for the declines of the Andean *Atelopus* populations (La Marca & Lötters, 1997), but this conclusion was

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reached prior to the discovery of chytridiomycosis in other *Atelopus* species. Although their restricted distribution and small home ranges (Dole & Durant, 2006) makes these species vulnerable to local disturbances, the apparent synchrony between decline events on several countries suggests a more complex interaction of factors (Daszak *et al.*, 1999; Daszak *et al.*, 2003). Pounds *et al.* (1999, 2006), Pounds & Crump (2004) and Pounds & Puschendorf (2004) suggested large scale climate change as the key factor driving epidemic outbreaks of chytridiomycosis and amphibian extinctions. To test whether *B. dendrobatidis* was present prior to the declines reported for the Andean Venezuelan *Atelopus* species between 1988 and 1994, we examined histological samples of most *Atelopus* species, and a selected sample of other amphibian species, available at the major Venezuelan museum collections. In addition, we analyzed local climate data to determine whether unusual climate events in the Andean region coincided with these declines.

METHODS

CHYTRIDIOMYCOSIS DETECTION

Samples were taken from four museum collections: Laboratorio de Biogeografía de la Universidad de Los Andes (ULABG) and Colección de Vertebrados de La

TABLE 1. Number of specimens sampled of each species in each of the four herpetological collections used: Colección de Vertebrados de La Universidad de Los Andes (CVULA), Museo de Biología de la Universidad Central de Venezuela (MBUCV), Museo de Historia Natural La Salle (MHNLS) and Laboratorio de Biogeografía de la Universidad de Los Andes (ULABG).

Species	CVULA	MBUCV	MHNLS	ULABG	Total
Aromobates alboguttatus	13	0	11	0	24
Aromobates leopardalis	31	0	0	0	31
Aromobates molinarii	0	0	0	4	4
Aromobates sp.1	0	0	0	2	2
Aromobates sp.2	0	0	0	2	2
Atelopus carbonerensis	12	0	3	3	18
Atelopus chrysocorallus	0	0	0	4	4
Atelopus mucubajiensis	3	0	0	11	14
Atelopus oxyrhynchus	11	4	2	17	34
Atelopus pinangoi	0	0	0	3	3
Atelopus sorianoi	11	0	0	6	17
Atelopus sp.	0	0	0	2	2
Atelopus tamaense	0	0	0	3	3
Centrolene andinum	7	0	7	0	14
Centrolene venezuelense	0	0	8	0	8
Chaunus marinus	0	4	0	3	7
Dendropsophus meridensis	3	0	0	1	4
Dendropsophus microcephalus	0	0	0	1	1
Eleutherodactylus briceni	0	0	0	1	1
Eleutherodactylus ginesi	0	0	0	2	2
Eleutherodactylus lancinii	0	0	0	2	2
Eleutherodactylus prolixodiscus	0	0	0	1	1
Eleutherodactylus sp.1	0	0	0	3	3
Eleutherodactylus sp.2	0	0	0	3	3
Eleutherodactylus sp.3	0	0	0	1	1
Eleutherodactylus sp.4	0	0	0	1	1
Flectonotus pygmaeus	0	0	0	1	1
Gastrotheca nicefori	0	0	0	2	2
Hyalinobatrachium duranti	0	0	21	0	21
Hyloscirtus jahni	0	0	0	3	3
Hyloscirtus lascinius	0	0	0	1	1
Hyloscirtus platydactylus	0	0	0	2	2
Hypsiboas cf. *crepitans*	22	0	0	24	46
Mannophryne collaris	27	0	3	2	32
Mannophryne cordilleriana	0	0	0	1	1
Mannophryne sp.	0	0	0	12	12
Rhinella sp. ("typhonius" group)	0	0	0	1	1
Scarthyla vigilans	0	0	0	1	1

Universidad de Los Andes (CVULA) in Mérida, and Museo de Historia Natural La Salle (MHNLS) and Museo de Biología de la Universidad Central de Venezuela (MBUCV) in Caracas (Table 1). These collections include most of the *Atelopus* specimens from the Andean region available in Venezuela. Sampling was carried out in two phases. First, we screened a large sample of *Atelopus* specimens from the Andean region housed in these collections. Secondly, at locations and dates where infections were detected in *Atelopus,* other Andean species were also sampled to examine the spatial and temporal distribution of the disease.

Skin tissue was removed from the ventral abdominal and pelvic regions of specimens using a scalpel blade or a tissue biopsy punch (Baker's dermal punch 4 and 6 mm, J. A. Webster, Inc.). In addition, toe clips including interdigital membranes were obtained from some specimens. Tissue samples were washed with phosphate buffer, dehydrated through a graded ethanol series, embedded in paraffin, sectioned at 5 µm and stained with haematoxylin and eosin (Humason *et al.*, 1997) and were examined under a light microscope for the presence of *B. dendrobatidis* or lesions consistent with chytridiomycosis. Lesions were classified as mild, moderate or severe depending on the percentage of skin section with zoosporangia, and the number of epithelial layers showing infected keratinaceous cells (Berger *et al.*, 1999; Pessier *et al.*, 1999; Nichols *et al.*, 2001).

Climate Patterns

Temperature and rainfall data were obtained from the Venezuelan Ministry of Environment and Natural Resources (MARN) for four climatic stations in the Andean region: Santo Domingo (8°52'27"N; 70°40'27"W; 2155 m), La Cuchilla (8°38'00"N, 71°21'10"W; 2280 m), Tovar (8°20'30"N, 71°44'40"W; 952 m) and Mérida-Airport (8°35'21"N,71°09'38"W; 1500 m). We analyzed rainfall data from the first three stations, as these were the closest ones to the sites where chytridiomycosis was detected. Temperature data, however, were obtained from the Mérida-Airport station because this was the only one in the area with a complete temperature series that covered all years between 1970 and 1990. Pearson correlation analyses were performed to assess whether the mean, maximum and minimum monthly air temperature recorded at the Mérida-Airport station showed similar patterns to those from the other three stations (Sokal & Rohlf, 1995).

We examined trends for the mean, maximum and minimum monthly air temperatures for the 1951-1990 period (n=480). Fourier analyses were used to detect periodicities and the series were fitted to linear models with harmonic terms using regression analyses. The series were transformed to remove the location by subtracting each value to the mean temperature for the complete series. Linear trends were removed when present and 95% confidence intervals were estimated. Atypical values were detected by examining the outliers in the mean annual values with respect to the 95% confidence intervals of the transformed series.

Trends in the mean monthly precipitation were also determined for the 1970-1996 period. We used accumulated precipitation for the first five months (January-May) of each year, as these are better estimates of humidity and availability of water during the local dry season. A Fourier analysis was used to detect annual trends and 95% confidence intervals were estimated for the mean accumulated precipitation for the first five months to identify extreme values.

RESULTS

Skin sections from 335 specimens from six families (Bufonidae, Centrolenidae, Dendrobatidae, Hylidae, Leptodactylidae, Plethodontidae) belonging to 13 genera and 39 species of amphibians were examined (Table 1). These included animals collected between 1952-2002. *Batrachochytrium dendrobatidis* was detected in seven of the 95 *Atelopus* specimens examined (Table 2). All seven specimens showed zoosporangia, in different developmental stages, localized in less than 30% of the epithelial surface examined. Many of these zoosporangia were found empty, and some contained septa characteristic of *B. dendrobatidis*. Skin sections showed hyperkeratosis with up to three layers of infected keratinaceous cells present in some regions (Fig. 1).

The museum catalogue information associated with each infected *Atelopus* individuals indicated that the pathogen was present in populations located at three lo-

TABLE 2. Catalogue data for Venezuelan Andean specimens found positive for chytridiomycosis.

Catalog ID	Species	Coordinates	Year of collection
ULABG 2107	*Atelopus carbonerensis*	8°38'42'N y 71°23'24'W	1988
ULABG 2108	*Atelopus mucubajiensis*	8°51'00''N y 70°42'50''W	1988
ULABG 2109	*Atelopus mucubajiensis*	8°51'00''N y 70°42'50''W	1988
ULABG 2005	*Atelopus sorianoi*	08°15'28''N y 71°43'08''W	1988
ULABG 2006	*Atelopus sorianoi*	08°15'28''N y 71°43'08''W	1988
ULABG 2103	*Atelopus sorianoi*	08°15'28''N y 71°43'08''W	1988
ULABG 2104	*Atelopus sorianoi*	08°15'28''N y 71°43'08''W	1988
ULABG 4269	*Leptodactylus* sp.	08°29'27''N y 71°31'30''W	1996
ULABG 4886	*Mannophryne cordilleriana*	8°52'53''N y 70°38'10''W	2002

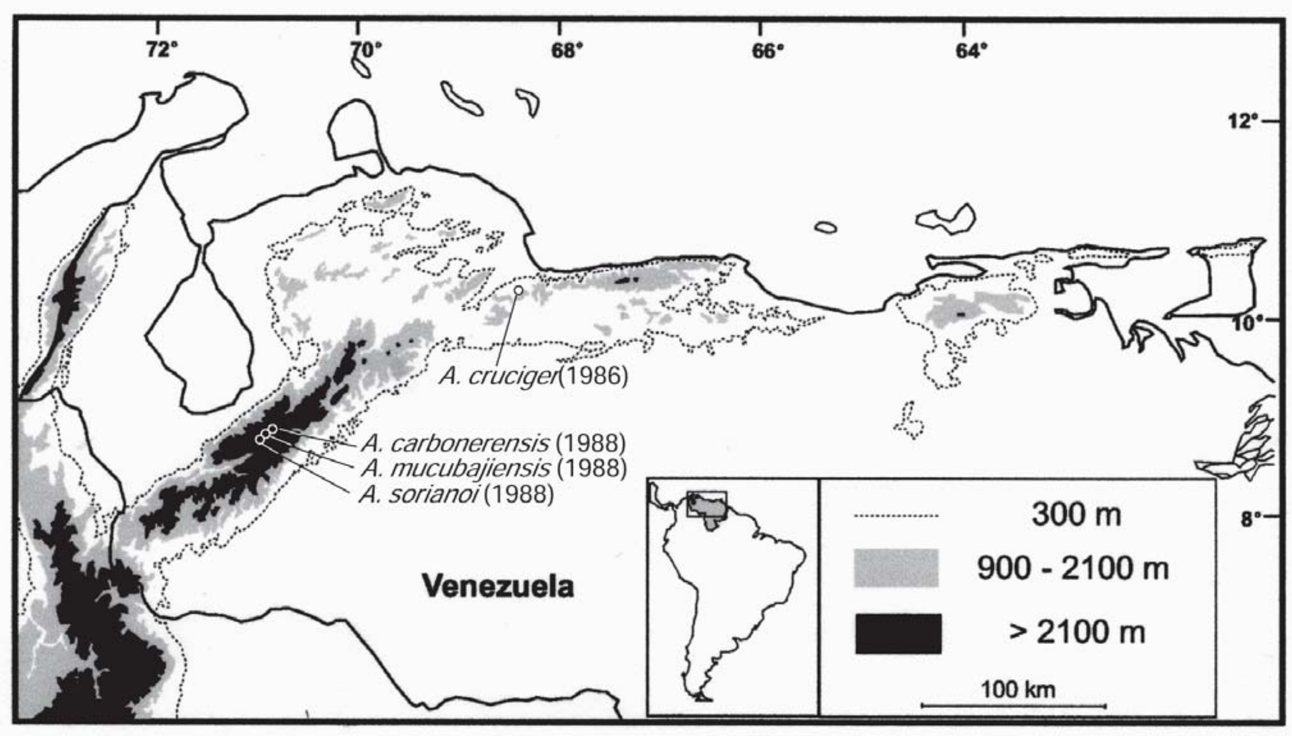

FIG.1. Known locations and dates of infection with *Batrachochytrium dendrobatidis* in *Atelopus* species from Venezuela. The *A. cruciger* record corresponds to Bonaccorso *et al.* (2003).

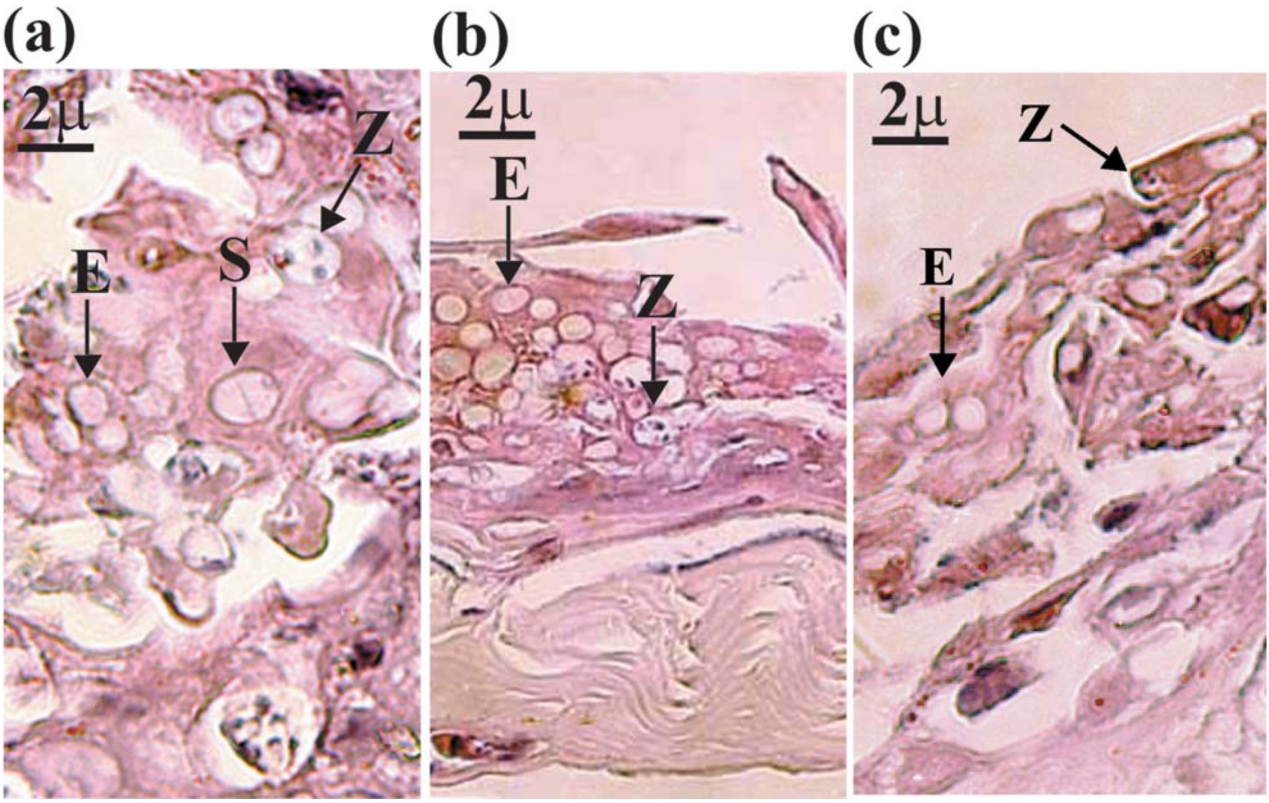

FIG. 2. Infection with *Batrachochytrium dendrobatidis* in native *Atelopus* species from Venezuela:(a) developing sporangia (Z) and empty zoosporangia with (S) and without septa (E) are visible in the stratum corneum of the epithelium of *Atelopus sorianoi*; (b) marked hyperkeratosis and some hyperplasia and developing and empty zoosporangia in a skin section of *Atelopus mucubajiensis*; and (c) *Atelopus carbonerensis.*

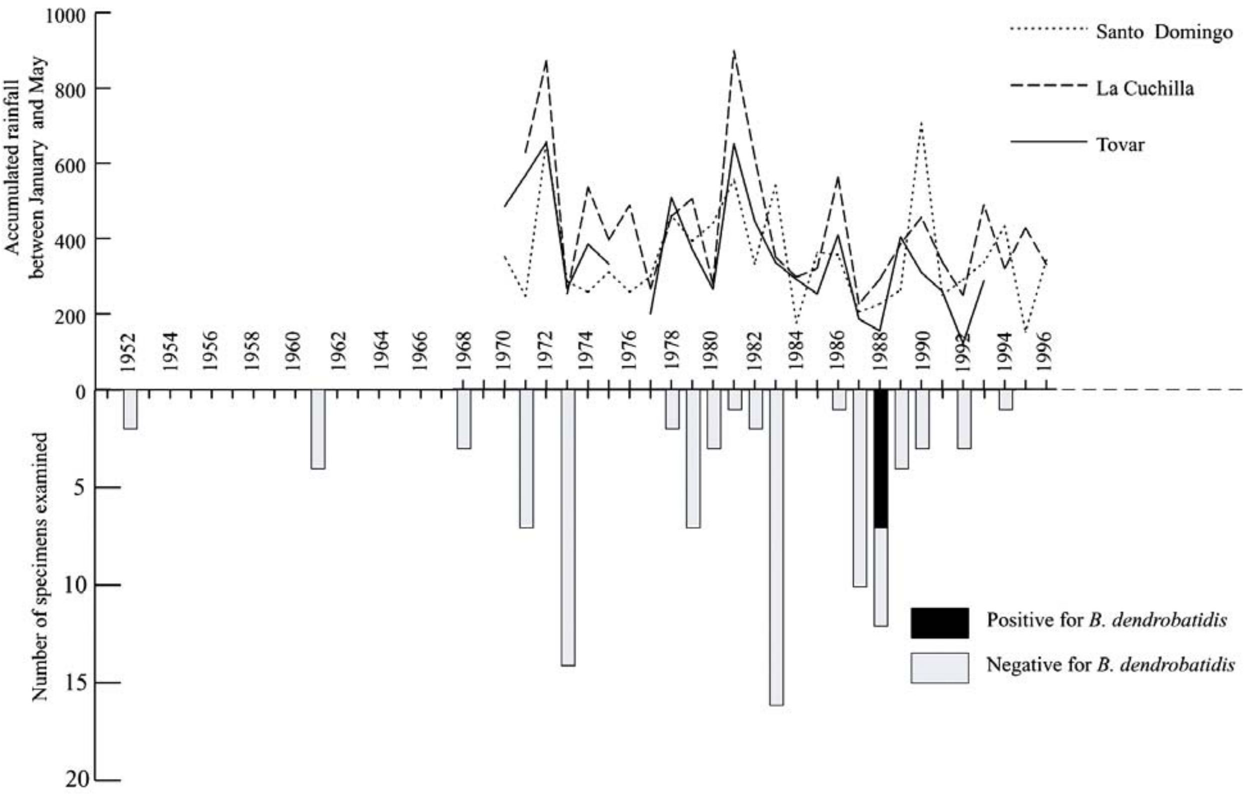

FIG. 3. Annual rainfall trends and distribution of infection with chytridiomycosis in *Atelopus* specimens available from the Venezuelan Andean region. The sample includes *A. carbonerensis*, *A, chrysocorallus*, *A. mucubajiensis*, *A. oxyrhynchus*, *A. pinangoi*, *A. sorianoi*, *A.* sp. and *A. tamaense*.

calities (Table 2, Fig. 2). These sites, separated by more than 60 km, belong to different river basins. Despite the geographic isolation between these sites, all infections ocurred in individuals collected only during 1988. The fact that these frogs, collected by different persons on several dates, were kept apart and fixed separately rules out the possibility of cross contamination prior to fixation. The prevalence of infection during that year appeared to be high; 58 % (7/12) of all *Atelopus* specimens examined had evidence of *B. dendrobatidis* (Fig. 3). In contrast, no infection was detected in any of the 72 *Atelopus* frogs collected between 1952 and 1987, or the 11 frogs collected after 1988. In addition, one *Mannophryne cordilleriana* and one *Leptodactylus* sp. currently under description by one of us (ELM) collected in 2002 and 1996 respectively, were also found to be infected (Table 2). Both of these samples showed less than 5% of the epithelial surface infected, without signs of hyperkeratosis.

CLIMATE ANALYSIS

Mean, maximum and minimum monthly air temperatures from La Cuchilla and Tovar were correlated with those recorded at the Mérida-Airport station (Pearson $R^2 = 0.83$ and 0.95, respectively). All these stations, located on the western slope of the Cordillera de Mérida, are under the influence of a similar climatic regime. In general, the latter station showed greater air temperatures than the Tovar station but lower than La Cuchilla as the result of differences in altitude. In contrast, the mean monthly air temperature data from the Santo Domingo station, nearest to the location where *A. mucubajiensis* was found infected, showed little correlation with their equivalents from the Mérida-Airport station (Pearson $R^2 = 0.2249$). This station is located on the eastern slope of the Cordillera de Mérida and most influenced by a different climatic regime (Vivas, 1992).

Fourier analyses suggested that, besides the annual and biannual cycles associated with the dry and wet seasons, the maximum and mean monthly air temperatures showed periodic cycles about every 20 years. A least square fit of these variables to a linear model with the corresponding harmonic terms showed that the mean and maximum monthly air temperature increased at a rate of 0.02°C per year between 1951 and 1990 (F=56.4, df=472, P<0.001 and F=30.8, df=472, P<0.001, respectively). Also, due to the apparent 20 year cycling, maximum and mean air temperatures during the 1980s were, on average, higher than during the previous decade. However, no significant deviations in the mean, maximum or minimum monthly air temperatures with respect to the complete series mean were observed for 1988, when the infections were detected.

The annual rainfall trends showed no relationship with the number of infected specimens detected. However, two patterns appeared when annual trends in the January-May accumulated rainfall were examined. Before 1988, when the chytridiomycosis epizootic was observed, three distinct peaks in accumulated rainfall (1972, 1981 and 1986) were followed by the three highest numbers of *Atelopus* specimens deposited in the museums (1973, 1983 and 1988; Fig. 3). Since 1988, however, only a fourth peak (1990) in the accumulated rainfall was recorded, but only three *Atelopus* specimens were deposited thereafter. Second, between 1987 and 1988 one of the lowest values in the January-May accumulated rainfall recorded in the region coincided with the 1988 epizootic. At Santo Domingo and Tovar, lower values were recorded during 1984 and 1992, respectively, but no specimens from nearby locations were deposited in the collections during those years.

DISCUSSION

The presence of zoosporangia of *B. dendrobatidis* in museum specimens of *A. carbonerensis, A. mucubajiensis* and *A. sorianoi* show that these species were infected with the causative agent of chytridiomycosis, in the years preceding their local disappearances. Possible explanations for the relationship between the presence of *B. dendrobatidis* and the subsequent declines of the host populations include: *B. dendrobatidis* has been endemic to the region with little effect on *Atelopus* species and other amphibians, and local populations disappeared from the region for reasons different than the presence of this pathogen; alternatively, epidemics of this chytrid were the cause, alone or in combination with other factors, of the declines of these three Andean *Atelopus* species and possibly that of *A. cruciger*. The temporal distribution of prevalence in museum specimens, with all infections occurring in one year, cannot be attributed to random variation, or to a selective sampling of infected individuals as none of them were morbid at the time of collection (see McCallum, 2005). This suggests that *B. dendrobatidis* was either rare or absent prior to 1984, and then increased its prevalence or infected naïve populations thereafter, reaching high levels of infection in 1988. This pattern is more consistent with a significant increase in the prevalence of the pathogen, characteristic of an epidemic event.

The epidemiological and clinical patterns suggest a possible link between the epizootic in 1988 and the severe declines of these three *Atelopus* species from the Cordillera de Mérida. First, the epidemic die-off after 1988 was followed by the abrupt disappearance of two species, *A. carbonerensis* and *A. sorianoi*. There were only two records of *A. carbonerensis* and none of *A. sorianoi* after 1988, despite significant efforts to find these species in the habitats where they used to live (La Marca & Lötters, 1997). For *A. mucubajiensis,* nine specimens were found between 1989 and 1994, but these were collected in sites distant to those where infected individuals were detected in 1988. None of these species were observed in the last ten years, except for a single record of *A. mucubajiensis* in 2004 (Barrio-Amorós, 2004). Second, chytridiomycosis has been linked to mortality events in some bufonid species (Muths *et al.*, 2003) including two other *Atelopus* species (Lips, 1998; Berger *et al.*, 1998). The absence of infected specimens found dead at the time of collection constitutes an important gap in linking the presence of *B. dendrobatidis* with declines (Daszak *et al.*, 1999; McCallum, 2005). However, some of the pathological evidence found in this study appears consistent with fatal chytridiomycosis. Some lesions observed showed a pathology consistent with severe disease (Berger *et al.*, 1998; Pessier *et al.*, 1999). Although it is not possible to determine whether the disease would have progressed and eventually caused mortality in these animals, this evidence shows that some *Atelopus* frogs had clinical signs of the disease prior to the declines of three of these species.

The spatial distribution of prevalence of chytridiomycosis in *Atelopus* species suggests that the increase in prevalence detected during 1988 was not local, but occurred simultaneously in populations separated by more than 60 km in different river basins. The synchronization of epizootics in distant locations can occur if the pathogen spreads rapidly through naïve populations upon its introduction, or if epidemics are driven by regional climatic changes, as suggested by the climate-linked epidemic hypothesis. Severe droughts could drive epidemic outbreaks of chytridiomycosis by increasing the transmission rates of the *B. dendrobatidis* or predisposing the host populations to heightened impact of pathogens (Daszak *et al.*, 2003). Most amphibians control the evaporative water loss by restricting their activities to habitats in which dehydration may be avoided easily (Shoemaker *et al.*, 1992). A contraction in the availability of humid microhabitats as a result of prolonged dry periods could lead to population crowding where transmission rates of *B. dendrobatidis* may increase above the required threshold for an epidemic. Dry periods can also act directly as a stressor in anurans; individuals exposed to dehydration may suffer from hyperosmotic stress that could affect the function of all organ systems, especially the cardiovascular (Shoemaker *et al.*, 1992). Local climatic data indicated that one of the most severe dry seasons recorded in the Cordillera de Mérida between 1972 and 1991 occurred during 1988, coincident with our observed high prevalence of *B. dendrobatidis* in Andean *Atelopus* species. The extinction of *A. ignescens* also coincided with warm and dry periods although no evidence of *B. dendrobatidis* was found in this species (Ron *et al.*, 2003). In the absence of evidence of other epidemic events, it is impossible to draw inference about drought and chytridiomycosis from these data. Climatic data from other regions where chytridiomycosis has been reported is needed to test whether droughts have played a role in driving epidemic outbreaks of this disease.

The rediscovery of one *A. mucubajiensis* (Barrio-Amorós, 2004) and two populations of *A. cruciger* (Eliot, 2003), after many years of intensive search, is heartening. Despite the fact that *A. carbonerensis*, *A. oxyrhynchus* and *A. sorianoi* have not been sighted in the last ten years, we do not consider them to be extinct. The possibility of finding remnant populations of these andean *Atelopus* species remains open, but search effort must be intensified.

The data presented in this paper highlight the need to understand the role of climate as a cause of amphibian declines, whether this is directly (e.g. Daszak *et al.*, 2005), or via changes in the dynamics of a host-pathogen system (e.g. Pounds *et al.*, 2006). Finally, our study shows that a comprehensive understanding of how climatic events interact with chytridiomycosis epidemics will be fundamental to designing effective conservation programs to ensure the long term persistence of *Atelopus* species.

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