Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community

Karen R. Lips*†, Forrest Brem*, Roberto Brenes*, John D. Reeve*, Ross A. Alford‡, Jamie Voyles§, Cynthia Carey§, Lauren Livo§, Allan P. Pessier¶, and James P. Collins‖

*Department of Zoology, Southern Illinois University, Carbondale, IL 62901-6501; ‡School of Tropical Biology, James Cook University, Townsville, Queensland 4811, Australia; §Department of Integrative Physiology, University of Colorado, Boulder, CO 80309-0354; ¶Division of Pathology, Conservation and Research for Endangered Species, Zoological Society of San Diego, San Diego, CA 92112-0551; and ‖School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501

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Pathogens rarely cause extinctions of host species, and there are few examples of a pathogen changing species richness and diversity of an ecological community by causing local extinctions across a wide range of species. We report the link between the rapid appearance of a pathogenic chytrid fungus *Batrachochytrium dendrobatidis* in an amphibian community at El Copé, Panama, and subsequent mass mortality and loss of amphibian biodiversity across eight families of frogs and salamanders. We describe an outbreak of chytridiomycosis in Panama and argue that this infectious disease has played an important role in amphibian population declines. The high virulence and large number of potential hosts of this emerging infectious disease threaten global amphibian diversity.

extinction | fungus | tropics | chytridiomycosis | Panama

Understanding the causes and consequences of diminishing biodiversity and understanding the ecology and evolution of infectious diseases are two of eight grand challenges in environmental sciences (1). Emerging infectious diseases may reduce biodiversity (2) and may account for at least some species extinctions (3), although, traditionally, infectious disease has not been considered a cause of extinction, because, in many cases, the ability of pathogens to be transmitted between hosts (most specifically R_0) is expected to reduce as hosts become rare (4). Recent modeling of pathogens with multiple hosts, small host-population sizes, and biotic or abiotic reservoirs, however, has shown that extinctions of rare species caused by disease may be more common than realized (4, 5). We provide evidence showing how chytridiomycosis, an emerging infectious disease, is strongly correlated with decreased population sizes and species richness of a vertebrate community.

In recent decades, at least 43% of amphibian species have declined, 32.5% are globally threatened, 34 are extinct, and an additional 88 are possibly extinct (6). Rapidly declining species are commonly found in upland Neotropical or Paleotropical riparian habitats, often in protected areas. These declines were characterized (6) as "enigmatic" for lack of obvious cause (e.g., deforestation or introduced predators), although chytridiomycosis was suspected in many of these cases.

The best documented declines of amphibians are from Central America, where species richness is high, and endemism is concentrated in upland areas (7). Whenever mass die-offs were observed, population declines were rapid (4–6 months), >50% of species were extirpated, remaining species persisted at ≈20% of normal abundance, and recovery time exceeded 15 y postdecline (8). In most cases where dead and dying frogs were observed and subsequent population declines occurred, *Batrachochytrium dendrobatidis*, a pathogenic fungus known to kill amphibians, was found to be the cause of death for almost all individuals (8–9). This fungus causes chytridiomycosis, an emerging infectious disease of amphibians (3, 9, 10) associated with declines in at least 43 amphibian species in seven Latin American countries and 93 species worldwide (www.jcu.edu.au/school/phtm/PHTM/frogs/chyglob.htm), but conclusive evidence of the status of the fungus at sites before die-offs and declines has been lacking. Several studies (refs. 8 and 11; and K. R. L., unpublished data) suggested that, for over a decade, the species richness of amphibian communities was collapsing in a series of habitats along a north-to-south transect in Central America (Fig. 1). Our goal was to take advantage of this pattern and document the prevalence of *B. dendrobatidis* at a site we believed should have lacked the pathogen, because it was ahead of the postulated epidemic wave. Should the pathogen continue to appear at sites to the southeast, we could then describe the full pattern of change in pathogen and disease prevalence during an epidemic and the subsequent change in amphibian diversity.

We present evidence that this emerging infectious disease of amphibians was absent, at very low prevalence, or present saprobically in the environment before it abruptly increased in prevalence in many amphibian species and was followed by widespread mortality and local population extirpations.

Results

Chytridiomycosis was not detected at our study site during the initial 4 y of sampling until we found the first infected frog on September 23, 2004. The first dead amphibian positive for *B. dendrobatidis* was found October 4, 2004 (Fig. 2). Subsequent mortality was high (1–19 dead frogs found per day) until mid-January, 2005, when overall amphibian abundance was much reduced (Fig. 3). We found 346 dead anurans and 5 dead salamanders between October 4, 2004 and February 15, 2005; 340 animals on the four riparian transects and 6 (5 frogs and 1 salamander) on two terrestrial transects (see *Supporting Text*, which is published as supporting information on the PNAS web site). There were no dead caecilians, but these largely subterranean species are difficult to sample. The dead frogs included 38 species [57% of the amphibian species from this site (K.R.L., unpublished data)] from all seven families at the site (Table 1). All but 3 of the 318 dead amphibians examined were moderately to heavily infected (prevalence, 0.98; 95% confidence interval = 0.964–0.995); those 3 were too decomposed to confidently diagnose infection, whereas the skin of the other 315 was heavily infected with *B. dendrobatidis*. Another 9 species were positive for *B. dendrobatidis* from PCR assays of swabs from live frogs for a total of 47 (70%) infected species of the 67 known from the site (Table 1; and see *Supporting Text*). Six of seven samples from substrates associated with dead frogs were positive for *B. dendrobatidis*, as was one of nine haphazardly chosen stream boulders.

None of the 1,566 individuals of 59 species sampled before September, 2004 was infected with *B. dendrobatidis* (Table 1).

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†To whom correspondence should be addressed. E-mail: klips@zoology.siu.edu.

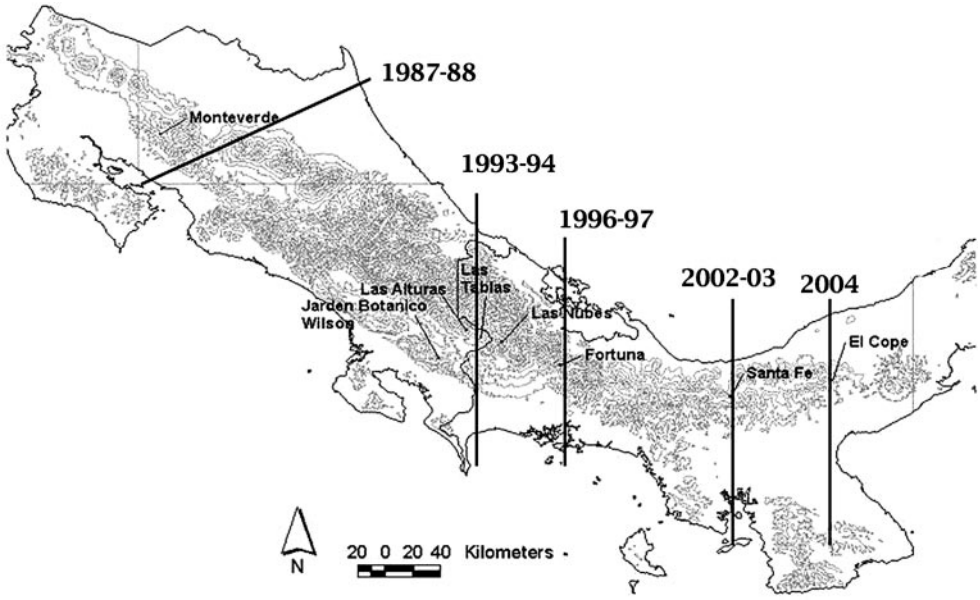

Fig. 1. Map of Central America, with sites of published population declines; lines represent date and location of reported declines (36).

Large-scale sampling in October, 2004 showed that prevalence was already >10% in 21 of 27 species sampled that month, and, by late December, 2004, *B. dendrobatidis* was infecting 40 species.

We analyzed our data in three ways to ensure that our results truly demonstrate that the pathogen increased dramatically in prevalence between the pre- and post-September, 2004 samples. The first analysis is highly conservative. We assumed that species could differ in "normal" prevalence of *B. dendrobatidis* and calculated one-tailed binomial tests for seven species (see *Supporting Text*). Within each species, pooling all samples collected before September, 2004 yielded sample sizes large enough (>58 individuals) to provide the statistical power to reject the null hypothesis that the true prevalence of *B. dendrobatidis* was >5%. For each of these species, we rejected this hypothesis; if *B. dendrobatidis* were present in these species before September, 2004, it occurred at very low prevalence. In the second conservative approach, we compared the upper 95% binomial confidence limits for prevalence before September, 2004 with the lower 95% binomial confidence limits for prevalence after August, 2004 for all species that had samples available in both periods. For 17 species (see *Supporting Text*) these confidence limits do not overlap, indicating that prevalence in these species increased significantly after September, 2004. In our final conservative analysis, we examined pooled data before September, 2004 for only 22 species that were sampled both before and after September, 2004 and for which the lower 95% binomial confidence limit for the prevalence of *B. dendrobatidis,* calculated for all samples of each species taken after September, 2004, was >10% (see *Supporting Text*). These species form a set in which all species are susceptible to infection by *B. dendrobatidis,* and each had a relatively high prevalence during the period of the outbreak. Pooling across these species to estimate the maximum prevalence across species before the outbreak, therefore, appears to be justified. For these species, all 1,217 individuals sampled before September, 2004 tested negative for the presence of *B. dendrobatidis*; the upper 95% binomial confidence limit for prevalence in this aggregated sample is 0.0030, or 0.30%.

In September, 2004, 1 mo before we first detected chytridiomycosis, amphibian density and species richness declined abruptly in both diurnal and nocturnal riparian amphibian communities; this decline followed six consecutive years of high and generally increasing population abundances (Figs. 3 and 4). In the same period, density and species richness of terrestrial transects showed little evidence of a decline (Figs. 3 and 4). Diurnal and nocturnal riparian transects (Figs. 3 and 4) displayed a similar pattern of decline, although amphibian density and species richness were consistently lower in diurnal surveys.

Epidermal hyperplasia and hyperkeratosis associated with chytridiomycosis were the only consistent findings in tissues of all 10 wild frogs found dead and examined histologically. Most animals were in good body condition, with readily identifiable fat stores. Chytridiomycosis lesions were diffuse to multifocally extensive, especially on skin sections from the ventral body. These lesions were significantly more severe than the relatively minimal focal lesions described in infected, but apparently healthy, bullfrogs (12) and were of a level consistent with those associated with mortality in natural and experimental infections (13). Molecular analyses of tissue collected from 38 individuals of 11 species were negative for ranavirus (A. Picco, personal communication). These findings, with the lack of evidence for other significant underlying or concurrent disease, suggest that death was likely caused by chytridiomycosis. We fulfilled Koch's postulates for chytridiomycosis by reisolating *B. dendrobatidis* from *Colosthethus panamensis* that had previously been exposed to isolate JEL 408 (see *Supporting Text*). Four animals that were PCR-positive for *B. dendrobatidis* were examined histologically. Three were found to have *B. dendrobatidis* infections, and one was too decomposed to accurately interpret histologic findings. In one of the positive cases, the extent of lesions was consistent with a lethal infection typical of wild animals. Tissues of nine individuals analyzed by PCR for ranavirus proved to be negative (A. Picco, personal communication).

Discussion

Our results demonstrate that the prevalence of *B. dendrobatidis* increased from zero to high prevalence very rapidly at our site, suggesting that *B. dendrobatidis* invaded the region, causing an epizootic. We suggest that chytridiomycosis was associated with mass mortality and the subsequent decline of amphibian populations at this site. The high prevalence of *B. dendrobatidis* in dead animals, no detection of other diseases, and fulfillment of Koch's postulates supports high disease-induced mortality. Our

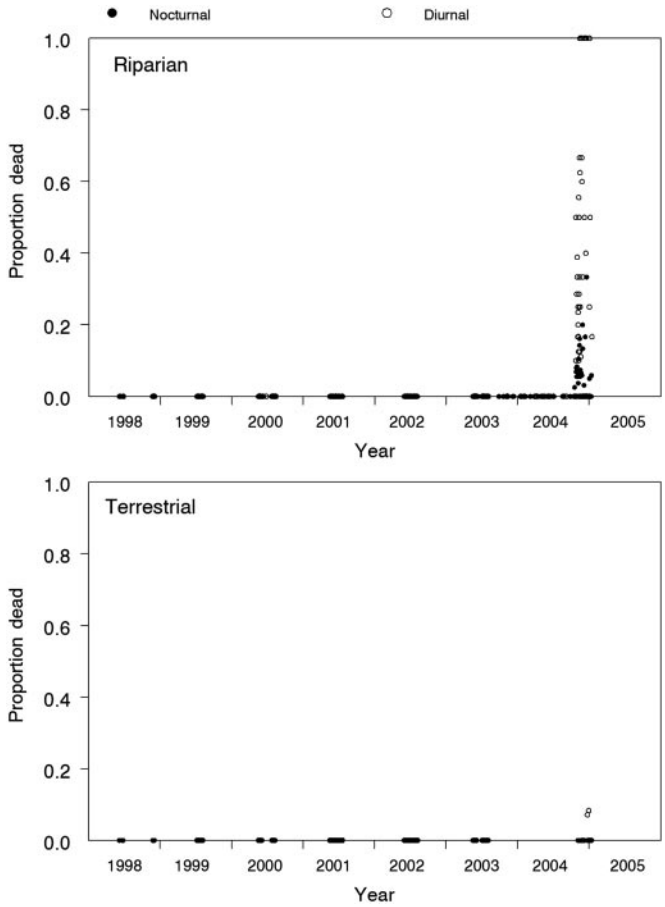

Fig. 2. Mortality rates for riparian and terrestrial transects, calculated as the proportion of dead frogs in all captures for both night and day transects (1998–2005). No dead animals were found on transects until October 4, 2004, at which time mortality increased until January, 2005, by which time abundance was significantly reduced.

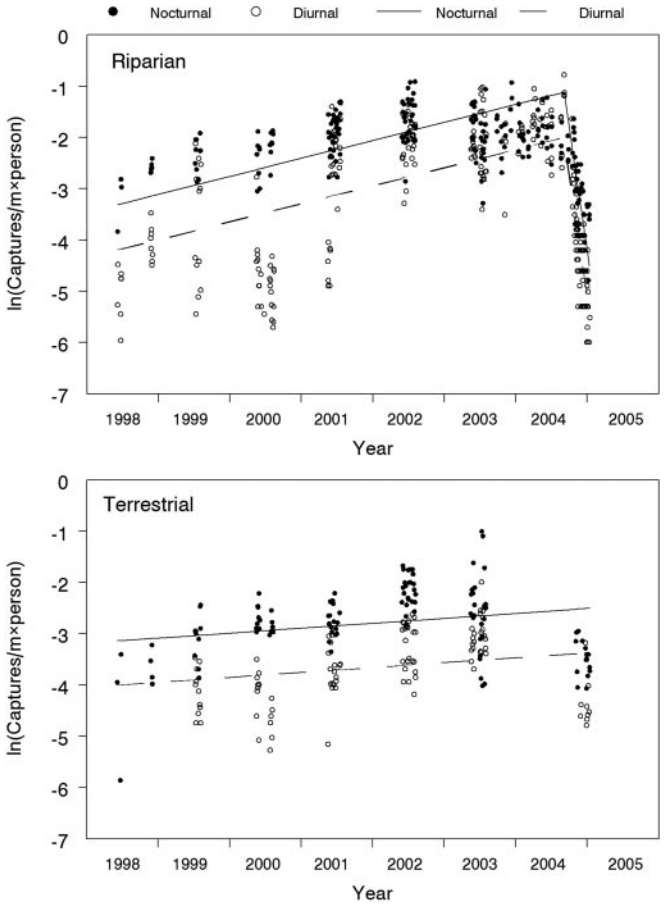

Fig. 3. Amphibian densities and statistical models for riparian and terrestrial transects (1998–2005). By using a segmented linear model for the riparian transects, we found a highly significant difference (θ_2) in slope ($t = -24.44$, df = 486, $P < 0.0001$), with the estimated time of change (α) being September 4, 2004 (95% confidence interval, September 1–6). We could fit a linear model to only the terrestrial transects (see text for details). Diurnal transects were significantly lower in density than nocturnal ones ($t = -13.05$, df = 486, $P < 0.0001$ for riparian transects; $t = -9.11$, df = 212, $P < 0.0001$ for terrestrial transects).

sampling did not detect the ascending phase of the epidemic curve, which probably occurred during August or September. The timing of this outbreak is consistent with the hypothesis that, first in Costa Rica and then in Panama, chytridiomycosis is moving southeastward, thus allowing us to predict its entry into communities in central Panama. Other survey and monitoring efforts to the east of El Copé showed no amphibian population declines (ref. 14 and K.R.L., unpublished data), and our samples of 120 amphibians from a site 100 km east of El Copé were negative for chytridiomycosis in January, 2005.

The high species richness at our study site provides a large pool of potential reservoir taxa that could promote long-term persistence of chytridiomycosis that could drive rare or less resistant species to extinction (4, 5). Likewise, similar sampling of species at Santa Fé, a postdecline site to the west of El Copé, identified six species (*Agalychnis callidryas*, *Bufo marinus*, *Hyla microcephala*, *Leptodactylus pentadactylus*, *Smilisca phaeota*, and *Rana "pipiens* sp. E") with high population abundance, high prevalence of chytridiomycosis, and potential for long-distance dispersal; these characteristics make them likely disease reservoirs.

Although many diseases can impact host populations by causing temporary or permanent (2–5) declines in abundance, only recently has disease been seen as a possible major cause of species extinctions. Theoretical work on disease ecology predicts that, as an epidemic infectious disease reduces the abundance of its hosts, there is an increase in the relative abundance of immune individuals, and disease transmission is reduced to zero, such that the pathogen becomes extinct before the host (4). Theoretically, conditions such as the presence of biotic or abiotic reservoirs (15, 16), high transmissibility (15), or impacts on host fecundity (17) could promote extinctions (5). At El Copé, many species are rare, at least 60% of species are susceptible to infection, and we have identified potential abiotic (this study) and biotic reservoirs.

We propose that chytridiomycosis operates similarly to a typical susceptible, exposed, infectious, recovered (SEIR) model with delayed density dependence (18). That is, chytridiomycosis emerges at a site and spreads by a combination of frog-to-frog and environment-to-frog transmission, as was shown in the laboratory (19) and in field mesocosms (20). As prevalence increases within the amphibian community, diseased frogs shed zoospores into the environment or directly pass them to other amphibians by contact. In the laboratory, *B. dendrobatidis* zoospores remain viable for at least 3 mo in sterile sand or bird feathers (21), and infected frogs and salamanders can carry an infection 24–220 days before dying, with animals at cooler temperatures shedding zoospores over a longer period (refs. 22, 23, and V. Miera, unpublished data). If positive environmental samples from El Copé were also infectious, then environmental transmission would be possible. All filtered water samples were

Table 1. Sampling effort for *B. dendrobatidis* by time period

Date	Technique	No. of species	No. of individuals	No. infected	Prevalence (95% CI)
2000	Histology	3	10	0	0 (0–0.309)
2002	Histology	5	11	0	0 (0–0.285)
May–Jul 2003	Histology	43	125	0	0 (0–0.029)
May–Jul 2003	Toes–PCR	7	100	0	0 (0–0.036)
Jan 2004	Swabs–PCR	36	400	0	0 (0–0.009)
Mar 2004	Swabs–PCR	32	282	0	0 (0–0.013)
May 2004	Swabs–PCR	43	311	0	0 (0–0.012)
Jul 2004	Swabs–PCR	38	327	0	0 (0–0.011)
Pre die-off totals		43	1,566	0	0 (0–0.0030)
23 Sep–2 Oct 2004	Swabs–PCR	2	86	9	0.10 (0.049–0.189)
Oct 2004	Swabs–PCR	21	216	127	0.59 (0.519–0.654)
Nov 2004	Swabs–PCR	31	456	240	0.53 (0.479–0.573)
Dec 2004	Swabs–PCR	16	121	56	0.34 (0.372–0.556)
Post die-off totals	Swabs–PCR	48	879	432	0.49 (0.498–0.569)

Columns indicate technique used for assessing *B. dendrobatidis,* number of species and individuals examined, and number found infected. Prevalence includes 95% confidence intervals (CI).

negative, suggesting that, if zoospores are in flowing water, they occur at very low density. We hypothesize that the presence of *B. dendrobatidis* in the environment and the long period of infectivity of many amphibians promote saturation of the environment with zoospores, enabling transmission among species that partition habitat spatially or temporally and producing the pattern we observed in which prevalence quickly changed from very low to very high, followed by widespread mortality.

We used three separate analyses to estimate prevalence, because simply presenting 95% binomial confidence limits for samples pooled across species (e.g., Table 1) might be criticized if species were differentially susceptible to chytridiomycosis; prevalence estimated for pooled samples would inevitably understate the true prevalence in more-susceptible species and overstate it in less-susceptible species. If samples taken before September, 2004, in which we did not detect chytridiomycosis, included mostly species with low susceptibilities or species which are constitutively immune to the disease, using the 95% binomial confidence limit to estimate the upper bound for true prevalence in susceptible species would overstate the actual statistical power available. All analyses demonstrated that prevalence was very low and increased rapidly to high levels.

Initially, it was hypothesized that this disease was endemic and had recently emerged (24); but growing evidence indicates that this fungus has been introduced into other areas (3, 10, 25, 26). This abrupt change in prevalence of infection and subsequent die-offs are consistent with the introduction of *B. dendrobatidis* from infected sites, followed by increasing prevalence and low-level mortality and infection of environmental substrates, until dead frogs occur at detectable levels. This pattern is evident in our data, with a decline in amphibian abundance ≈1 mo before observing the first dead frog.

The die-off at El Copé occurred during the peak of the rainy season, a pattern like that observed at three other sites: Las Tablas, Costa Rica; Fortuna, Panama; and Santa Fé, Panama (ref. 8; and K.R.L., unpublished data). Increased prevalence of chytridiomycosis in wild frogs from Australia during the wet season was also reported (22). Many montane Neotropical frogs breed during the prolonged rainy season, when many individuals are likely to be near water bodies, facilitating rapid proliferation and transmission of the obligate aquatic chytrid zoospores and increasing amphibian population density, which is a predictor for increased transmission rates for a waterborne, directly transmitted pathogen such as *B. dendrobatidis*. Breeding season is also a time when frog behavior facilitates transmission by physical contact during amplexus and defense of reproductive resources. Our data show that even completely terrestrial species become infected and decline, although at reduced rates; this slower decline is likely caused by reduced transmission in the terrestrial environment because of greater variability in temperature and moisture conditions in the forest and canopy. Our report definitively links the appearance of chytridiomycosis in a community with subsequent declines attributable to this disease. We do not have detailed climate data from our study site, but central Panama had no major climatic anomalies in 2004, and temperature and rainfall patterns were similar to long-term means (http://striweb.si.edu/esp). Our results support a model of amphibian declines in which *B. dendrobatidis* enters and quickly spreads through communities with no infected individuals. Our model does not require interactions with climate change to provide a proximate mechanism to account for local extirpations of montane amphibian populations, but we cannot eliminate the possibility that climate change has increased the susceptibility to chytridiomycosis of these many amphibian species or that it has influenced the tempo or mode of spread, especially if susceptibility to chytridiomycosis is affected strongly by microenvironment (27, 28).

We found no evidence that other agents (e.g., exotic predators, land-use changes, or commercial overharvesting) were involved in the massive die-off and population declines at El Copé. Toxic chemicals are an unlikely cause, because none of the 10 dead frogs from the 2004 die-off examined by a pathologist had lesions typical of exposure to contaminants that result in detectable histologic changes. A 1998 survey of air, water, sediment, and frog tissues found no evidence of chemical contaminants of a level likely to cause mortality (V. Beasley, personal communication).

There never were industries or large-scale or intensive uses of agrochemicals in this region, and the study site is at the top of the watershed on the continental divide. Although air currents could transport contaminants into the site (29), prevailing winds do not originate from urban or agricultural areas (http://striweb.si.edu/esp).

We conclude that chytridiomycosis is likely responsible for this mass mortality event and subsequent decline of amphibian populations at this site. Bioclimatic modeling (30) suggests that *B. dendrobatidis* can survive in many other parts of the globe and supports the idea that it has likely caused other enigmatic declines of anurans and salamanders throughout the tropics (6).

It is clear that Neotropical amphibian declines and extinctions are not an artifact of sampling or natural population fluctuations

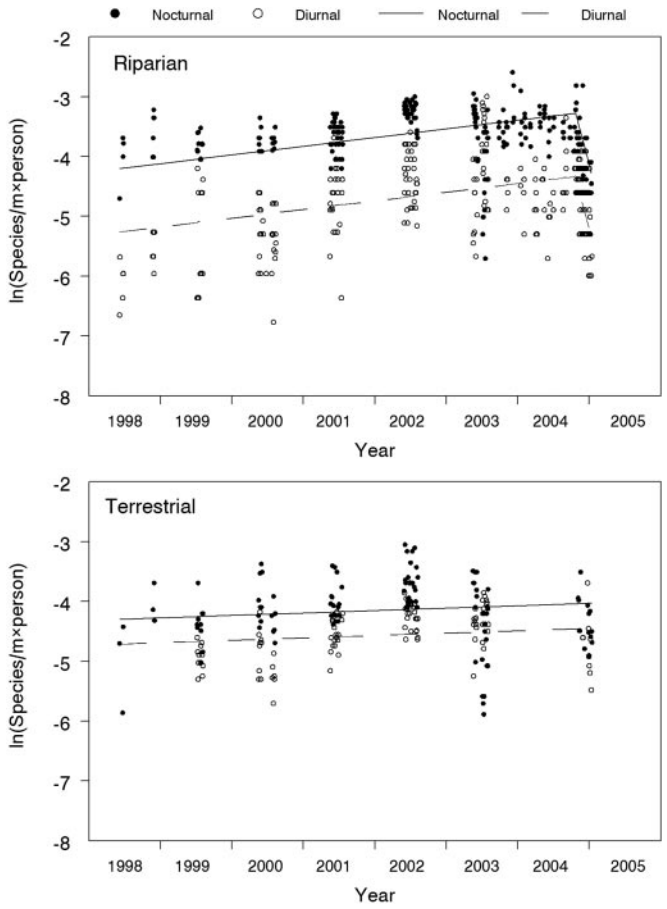

Fig. 4. Species richness and statistical models for riparian and terrestrial transects (1998–2005). By using the segmented linear model for the riparian transects, we found a highly significant difference (θ_2) in slope ($t = -6.97$, df = 486, $P < 0.0001$), with the estimated time of change (α) being October 22, 2004 (95% confidence interval, October 11–November 3, 2004). We could fit a linear model to only the terrestrial transects (see text for details). Diurnal transects were significantly lower in density than nocturnal transects ($t = -21.33$, df = 486, $P < 0.0001$ for riparian transects; $t = -6.14$, df = 212, $P < 0.0001$ for terrestrial transects).

(31) but, in at least some cases, are caused by a pathogen, *B. dendrobatidis*. We predict the loss of many more amphibian species from the Neotropics, most immediately from montane regions directly east of our study site. Chytridiomycosis is an alarming model system for disease-driven extinction of a high proportion of the species richness of a regional fauna, even a significant proportion of an entire class of vertebrates, and, under these circumstances, it is no longer correct to speak of global amphibian declines but, more appropriately, of global amphibian extinctions.

Methods

El Copé (8) is within Parque Nacional G. D. Omar Torríjos H. (8° 40′ N, 80° 37′ 17′′ W) in Coclé Province, Panama (Fig. 1). Mean annual air temperature is between 19°C and 26°C, mean annual precipitation is 3,500 mm, and mean annual water temperatures are between 20°C and 22°C (K.R.L., unpublished data). Meteorological data from Barro Colorado Island (http://striweb.si.edu/esp), a lowland site 100 km east of El Copé, were a proxy for comparing weather in 2004 to long-term means.

We surveyed amphibian populations along permanent transects from 1998 to 2005. Each May to August from 1998 to 2003, we surveyed four stream and three terrestrial transects twice weekly (nocturnal and diurnal). The four stream transects were surveyed twice monthly from August, 2003 to September, 2004. We surveyed streams at 1- to 3-d intervals and increased the frequency of terrestrial surveys after October, 2004, when we began to find dead frogs.

Between June 6, 1998 and January 14, 2005, we carried out 698 transect surveys (187 km) representing >1,301 h and 29,645 captures of amphibians. Transect data were tested for changes in amphibian abundance and species richness by using nonlinear regression. We predicted that an abrupt change in amphibian mortality rates would be detectable by a change in slope of population density vs. time. The change in slope and the time at which it occurred were estimated by fitting a segmented linear model using nonlinear regression techniques (32). The model was of the form $Y_{ij} = \theta_0 + \theta_1(t - \alpha) + \theta_2\sin(t - \alpha) + \delta X + \varepsilon$, where Y_{ij} is amphibian density at time t, t is days elapsed from first sample date, X is an indicator variable for nocturnal vs. diurnal observations, and ε is normally distributed with mean 0 and variance σ^2. The parameter α is the date when population growth rate changes, θ_0 is the population density at this time, θ_1 is the average slope of the two line segments, and θ_2 is half the difference in slopes (32), whereas δ estimates the difference in density between night vs. day. Amphibian density was estimated by using $Y = \ln((C + 1)/(LP))$, where C is total captures, L is the transect length, and P is the number of persons walking a transect. The program NLMIXED (SAS Institute, Cary, NC) was used to fit the model (33). Replacing the sine function with one that smoothed the transition between line segments, in particular the function $\tanh((t - \alpha)/\gamma)$, with $\gamma = 0.1$ (32), improved model convergence. We used a similar model to estimate species richness, with C equal to the total number of species encountered. We tested if θ_2 were different from zero, calculated confidence intervals for α, and tested if δ differed from zero. The segmented linear model readily converged on a solution for only riparian data, so we fitted a simple linear model to the terrestrial data using an indicator variable for nocturnal vs. diurnal observations.

We examined 1,566 individuals sampled between May, 2000 and July, 2004 for the presence of *B. dendrobatidis*. Skin snips from 145 voucher specimens were examined histologically, and 100 toe tips collected from 7 species between May to June 2003 were analyzed by J. Wood (Pisces Molecular, Boulder, CO) using PCR (34). Between January and July 2004 we made bimonthly collections of ≈300 skin swabs from amphibians encountered on transects, and assayed pooled swabs for *B. dendrobatidis* by using PCR. Positive pooled samples were subdivided and each specimen analyzed individually. We estimated probabilities of false negatives for each set of bimonthly samples and for all 1,566 samples collectively (Table 1).

All stream transects were surveyed daily for dead amphibians beginning in October, 2004. Skin from 249 preserved individuals collected dead between October and December, 2004 were microscopically examined for the presence of *B. dendrobatidis.* We used PCR to estimate prevalence of *B. dendrobatidis* in 890 frogs sampled from October to December, 2004 (Table 1).

Between September 6 and December 2, 2004, we collected environmental swabs from rocky substrates surrounding seven dead frogs, and from nine additional boulders without dead frogs. As part of a companion study, we quantified suspended solids in the stream by filtering water through glass fiber filters. To determine whether zoospores of the fungus were present in the water column, we analyzed solids on 59 alcohol-preserved filters.

Between September 23 and October 2, 2004, we collected 80 individuals of *Colostethus panamensis* at El Copé. These individuals were swabbed in the field by investigators wearing fresh latex gloves, and the swabs were tested by Pisces Molecular for *B. dendrobatidis* DNA with PCR. Ten of the moribund and dead amphibians found between October and December in El Copé were chosen haphazardly for histopathology by a veterinary

pathologist (A.P.P.) to determine the likely cause of death (see *Supporting Text*). We also used PCR (35) to test for ranavirus (A. Picco, personal communication) in tissues collected from 38 haphazardly chosen dead frogs belonging to 11 species.

To verify that *B. dendrobatidis* could cause mortality in Panamanian amphibians, we collected animals in January, 2005 for fulfillment of Koch's postulates. We collected 60 *C. panamensis* from Cerro Compana, a site 111 km east of El Copé that was thought to be free of *B. dendrobatidis*. These animals all tested negative for *B. dendrobatidis* by PCR. Koch's postulates were fulfilled by using *B. dendrobatidis* isolate JEL408 cultured from a *C. panamensis* collected in El Copé (see *Supporting Text*). Eleven of the animals dying in these experiments were examined by histopathology for signs of *B. dendrobatidis* infection; *B. dendrobatidis* was reisolated from six individuals, and nine were tested by PCR (35) for ranavirus (A. Picco, personal communication).

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