

Reptiles as potential vectors and hosts of the amphibian pathogen *Batrachochytrium dendrobatidis* in Panama

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ABSTRACT: Chytridiomycosis, the disease caused by *Batrachochytrium dendrobatidis*, is considered to be a disease exclusively of amphibians. However, *B. dendrobatidis* may also be capable of persisting in the environment, and non-amphibian vectors or hosts may contribute to disease transmission. Reptiles living in close proximity to amphibians and sharing similar ecological traits could serve as vectors or reservoir hosts for *B. dendrobatidis*, harbouring the organism on their skin without succumbing to disease. We surveyed for the presence of *B. dendrobatidis* DNA among 211 lizards and 8 snakes at 8 sites at varying elevations in Panama where the syntopic amphibians were at pre-epizootic, epizootic or post-epizootic stages of chytridiomycosis. Detection of *B. dendrobatidis* DNA was done using qPCR analysis. Evidence of the amphibian pathogen was present at varying intensities in 29 of 79 examined *Anolis humilis* lizards (32%) and 9 of 101 *A. lionotus* lizards (9%), and in one individual each of the snakes *Pliocercus euryzonus*, *Imantodes cenchoa*, and *Nothopsis rugosus*. In general, *B. dendrobatidis* DNA prevalence among reptiles was positively correlated with the infection prevalence among co-occurring anuran amphibians at any particular site ($r = 0.88$, $p = 0.004$). These reptiles, therefore, may likely be vectors or reservoir hosts for *B. dendrobatidis* and could serve as disease transmission agents. Although there is no evidence of *B. dendrobatidis* disease-induced declines in reptiles, cases of coincidence of reptile and amphibian declines suggest this potentiality. Our study is the first to provide evidence of non-amphibian carriers for *B. dendrobatidis* in a natural Neotropical environment.

KEY WORDS: *Batrachochytrium dendrobatidis* · Chytridiomycosis · Amphibian pathogen · Reservoir host · Lizard · Snake · Reptilia · Neotropics

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INTRODUCTION

The pathogenic chytrid fungus *Batrachochytrium dendrobatidis* has been implicated in declines and extirpations of amphibian populations worldwide (Collins & Crump 2009). *B. dendrobatidis* invades the epidermal tissue of amphibian skin and causes the disease chytridiomycosis, evidently by disrupting the physiological function of the skin in transporting

electrolytes and water (Voyles et al. 2007, 2009, Carver et al. 2010). Chytridiomycosis is fatal to amphibians and is considered to be exclusively an amphibian disease (Berger et al. 1998, Wake 2007). Although it had previously been thought that *B. dendrobatidis* likely feeds on keratin in amphibian skin (Berger et al. 1998, Longcore et al. 1999, Pessier et al. 1999, Green & Kagarise Sherman 2001), recent work has shown this to be unlikely (Voyles et al. 2011) and

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that proteases secreted by *B. dendrobatidis* apparently degrade proteins such as elastin in the extracellular matrix of skin tissue (Moss et al. 2010).

Batrachochytrium dendrobatidis is highly contagious among amphibians. It is generally held to be a water-borne pathogen, with its motile, flagellated zoospores comprising the infective stage, and can be transmitted via physical contact or zoospore-contaminated water (Berger et al. 1999). Some amphibian species, particularly cane toads *Rhinella marina*, American bullfrogs *Lithobates catesbeianus*, African clawed frogs *Xenopus laevis* and coquis *Eleutherodactylus coqui*, can harbour high infections of *B. dendrobatidis* without succumbing to chytridiomycosis. These invasive species may be important reservoir hosts or vectors of the pathogen (Berger et al. 1999, Daszak et al. 2004, Weldon et al. 2004, Beard & O'Neill 2005) but they do not occur in all areas where native amphibian populations have been stricken with chytridiomycosis. On the contrary, some native Panamanian amphibians such as *Centrolene prosoblepon* also show some resistance to *B. dendrobatidis* and could act as a reservoir hosts for this pathogen (Woodhams et al. 2006).

It appears that *Batrachochytrium dendrobatidis*, unlike other chytrids, is probably incapable of living as a saprobe in the natural environment (Johnson & Speare 2003, Lips et al. 2006, Briggs et al. 2010), though this possibility cannot be ruled out (Longcore et al. 1999, Briggs et al. 2010). *B. dendrobatidis* zoospores, though, are capable of survival away from an amphibian host. Zoospores are known to persist in the natural environment on rock and boulder substrates, in stream water and in arboreal bromeliads at epizootic sites (Lips et al. 2006, Cossel & Lindquist 2009, Richards-Zawacki 2010). Under sterile conditions, zoospores can persist on moist bird feathers or boiled snake skin and can be cultivated on tryptone agar or other media (Piotrowski et al. 2004, Symonds et al. 2008). Zoospores have been found to survive for up to 4 wk in tap water, as long as 7 wk in lake water, and 3 mo in sterile moist river sand with no added nutrients (Johnson & Speare 2003, 2005).

Since it is possible that *Batrachochytrium dendrobatidis* may not necessarily require an amphibian host for survival, there may exist reservoir hosts or vectors for the pathogen even though none, as yet, have been positively identified. Freshwater shrimp were thought to maintain *B. dendrobatidis* on their carapaces, but this was later found not to be the case (Rowley et al. 2006, 2007). Nevertheless, experimental and genetic evidence has shown that the transfer

of pathogens between fish and amphibians is possible (Mao et al. 1999, Kiesecker et al. 2001), with introduced exotic fishes, such as rainbow trout *Oncorhynchus mykiss* or goldfish *Carassius auratus*, as the culprits (Gillespie & Hero 1999, Lips et al. 2005a).

Considering the survivability of *Batrachochytrium dendrobatidis* outside of aquatic media or amphibian skin, it may not be necessary for a host to be aquatic. *B. dendrobatidis* infection has been observed in completely terrestrial amphibian species (Lips et al. 2006). Although Phillott et al. (2009) were unable to detect *B. dendrobatidis* in a small sample of eastern water dragons *Physignathus lesueurii* in Australia, reptiles in other localities could nevertheless be vectors or reservoir hosts of *B. dendrobatidis*, particularly those small lizards and forest floor snakes that live in close proximity to amphibians, share similar habitat and microhabitat requirements and have comparable foraging behaviours and prey preferences or, in the case of snakes, that prey upon amphibians. If such reptiles are vectors or reservoir hosts, they should harbour infections of *B. dendrobatidis* that may mirror levels of infection prevalence and intensity by the pathogen among syntopic amphibians.

MATERIALS AND METHODS

Sampling

Sampling was conducted in western and central Panama intermittently over a 9 mo period, from February 18 to October 28, 2006, at 8 sites of varying elevation and at pre-epizootic, epizootic or post-epizootic stages of chytridiomycosis among anuran amphibians (Table 1, Fig. 1). While seeking out anurans, we opportunistically collected all observed reptiles that were present at the same time and in the same habitats. We subsequently examined epidermal skin swabs from 211 lizards of 13 species and 8 snakes, each of a different species (Table 1). All work with animals was conducted under the auspices of McGill University Animal Use Protocol #4569 to D. M. Green. Most reptiles examined were identified to species, except for some individuals in the lizard families Sphaerodactylidae and Teiidae, and the snake family Colubridae, as well as one species of arboreal *Anolis* lizard and one species each of *Leptophis* and *Dipsas* snakes. Sampling was conducted by day and night in forest and stream habitats in both the wet and dry seasons to ensure sampling of the

Table 1. Sample sites in central and western Panama and reptile species examined for *Batrachochytrium dendrobatidis* DNA. Species that tested positive are in **bold**

| Site | Elevation (m) | Reptile species examined (n) | |
|------------------|---------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------|
| | | Lizards | Snakes |
| Cuatro Callitas | 45 | <i>Anolis</i> sp. (1) <i>Echinosaura panamensis</i> (2) Sphaerodactylidae (2) <i>Anolis humilis</i> (6) <i>Anolis limifrons</i> (7) <i>Anolis lionotus</i> (25) | <i>Imantodes cenchoa</i> (1) |
| Palmarazo | 135 | <i>Lepidoblepharis xanthostigma</i> (1) <i>Anolis limifrons</i> (3) <i>Anolis lionotus</i> (16) | |
| La Rica | 250 | <i>Anolis humilis</i> (2) <i>Anolis lionotus</i> (24) | <i>Nothopsis rugosus</i> (1) <i>Dipsas</i> sp. (1) |
| Cerro Trinidad | 540 | <i>Basiliscus basiliscus</i> (1) <i>Anolis lionotus</i> (17) | <i>Leptophis</i> sp. (1) |
| El Copé | 760 | <i>Anolis limifrons</i> (1) <i>Echinosaura panamensis</i> (3) <i>Anolis humilis</i> (5) <i>Anolis lionotus</i> (16) | <i>Geophis bellus</i> (1) <i>Rhadinaea decorata</i> (1) |
| Altos de Campana | 860 | <i>Echinosaura panamensis</i> (1) <i>Anolis apletophallus</i> (1) <i>Anolis lionotus</i> (1) <i>Anolis humilis</i> (6) | Colubridae (1) |
| Altos del María | 890 | <i>Echinosaura panamensis</i> (1) <i>Ptychoglossus plicatus</i> (1) <i>Anolis lionotus</i> (2) <i>Anolis humilis</i> (8) | <i>Pliocercus euryzonus</i> (1) |
| Fortuna | 1215 | <i>Anolis</i> sp. (1) <i>Anolis capito</i> (1) Teiidae (2) <i>Anolis humilis</i> (53) | |

maximum number of diurnal and nocturnal species. To minimize the possibility of resampling individuals, we did not sample any transect more than once. To

assay for *Batrachochytrium dendrobatidis*, all reptiles encountered were caught by hand in new individual plastic bags and were swabbed 10 times each on the ventral surface, thighs, and feet using the same sterile technique developed for amphibians and described by Hyatt et al. (2007). Samples were stored at -20°C prior to use.

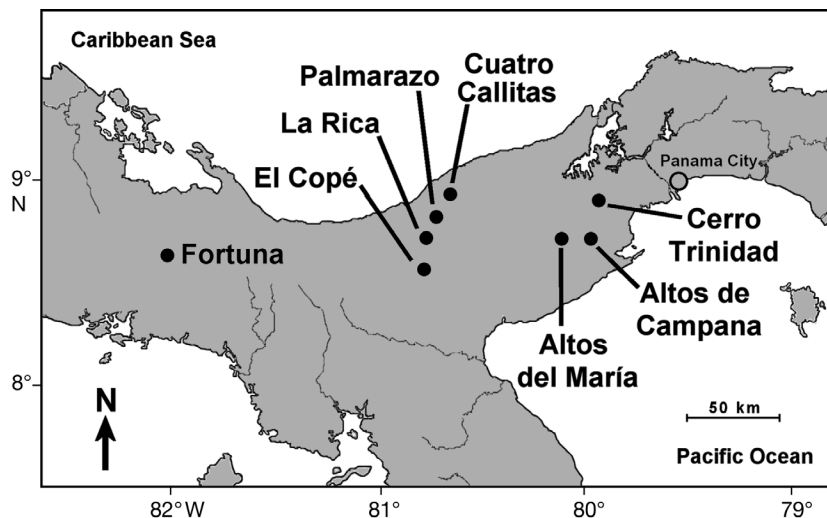


Fig. 1. Sample sites in central and western Panama

DNA extraction

All DNA extractions and purifications of samples were done as described by Kilburn et al. (2011) according to the protocol of Hyatt et al. (2007). After DNA extraction and purification was complete, samples were sent to the RNomics Platform of Genome Quebec for qPCR analysis, as developed by Boyle et al. (2004)

and adapted for a singlicate assay as described by Kriger et al. (2006). Following Kilburn et al. (2011), we were satisfied that triplicate assays provided no greater quality control over singlicate assays with these methods.

Statistical analyses

We used raw binary (positive or negative) data on *Batrachochytrium dendrobatidis* occurrence to determine prevalence, which was calculated as: number of individuals testing positive ÷ total number of individuals examined. *B. dendrobatidis* DNA intensity was quantified as the log₁₀ transformed arithmetic mean zoospore equivalent per individual. With these data, we tested for significant differences in infection prevalence and intensity between snakes and lizards at each individual site and between sites. We also compared prevalence and intensity of *B. dendrobatidis* DNA between reptiles and co-occurring amphibians sampled over the same period of time. The relatively small sample sizes for reptiles yielded insufficient power to discriminate between seasons or habitats for any analyses. For comparisons with co-occurring amphibians, we used data on *B. dendrobatidis* DNA prevalence and intensity as presented by Kilburn (2008) and Kilburn et al. (2011).

Statistical analyses comparing *Batrachochytrium dendrobatidis* prevalence among samples ($\alpha = 0.05$) were done employing Minitab 15.0 Statistical Software for multiple contingency Pearson chi-squared tests for multiple comparisons and Fisher's exact tests for pair-wise comparisons, and software developed by Wessa (2011) for tests employing Pearson product moment correlation. We calculated 95% confidence intervals (CI) based on a binomial distribution for all

prevalence estimates. One-tailed randomization tests were run using the Resampling Stats add-in for Microsoft Excel for all DNA intensity comparisons. For statistical tests involving intensity, we used only positive samples (mean zoospore equivalents ≥ 1.0), with 10 000 data permutations for each test, $\alpha = 0.05$ and 95% bootstrap confidence intervals (BCI).

RESULTS

Prevalence of *Batrachochytrium dendrobatidis* on reptiles

Evidence of possible infection by *Batrachochytrium dendrobatidis* was found in 2 of the 13 species of lizards sampled (Table 1). Affected individuals of *Anolis humilis* (ground anole) and/or *A. lionotus* (lion anole) were identified at all sites studied except Palmarazo, where *A. humilis* was not found. From a total of 79 *A. humilis* individuals, 25 were found to test positive for *B. dendrobatidis* DNA (Table 2), for a prevalence of 32% (95% CI: 22 to 43%), whereas only 9 of 101 *A. lionotus* tested positive, for a prevalence of 9% (4 to 16%). The higher prevalence of *B. dendrobatidis* DNA found in *A. humilis* was significantly different ($p < 0.001$, Fisher's exact test) compared to its prevalence in *A. lionotus*, although intensity did not differ (Table 2). The overall prevalence of *B. dendrobatidis* DNA among all lizards sampled from all sites was 16% (11 to 22%, $n = 211$).

Batrachochytrium dendrobatidis DNA was also found on 3 individual snakes belonging to 3 of the 8 species examined (Table 1). One *Pliocercus euryzonus* (Cope's false coral snake) that tested positive was found at the high elevation site of Altos del María (890 m), whereas an individual *Imantodes cen-*

Table 2. Prevalence (%) and intensity (mean zoospore equivalents) of *Batrachochytrium dendrobatidis* DNA among reptiles, with 95% CI and probability of difference (prevalence: Fisher's exact test; intensity: randomization test; $\alpha = 0.05$) between reptiles and syntopic amphibians. *Difference between anurans and reptiles is significant at $\alpha = 0.05$

| Site | Anuran amphibians | | | Reptiles | | | p | |
|--------------------|-------------------|-----------------------|------|------------------|------------------|-----|-------------------|---------------|
| | Prevalence | Intensity | n | Prevalence | Intensity | n | Prevalence | Intensity |
| Cuatro Callitas | 17.9 (9.6–29.1) | 694.0 (9.4–2008.8) | 67 | 6.8 (1.4–18.7) | 18.7 (1.2–65.0) | 44 | 0.154 | 0.568 |
| Palmarazo | 10.6 (6.1–16.9) | 156.0 (3.0–419.3) | 141 | 0.0 (0.0–13.9) | – | 20 | 0.220 | – |
| La Rica | 27.6 (18.0–39.1) | 69.6 (6.2–175.3) | 76 | 6.9 (0.8–22.8) | 1.8 (1.1–2.4) | 29 | 0.032* | 0.334 |
| Cerro Trinidad | 15.1 (10.8–20.2) | 40.2 (14.3–73.8) | 245 | 21.1 (6.1–45.6) | 34.7 (1.2–101.2) | 19 | 0.510 | 0.310 |
| El Copé | 15.8 (6.0–31.3) | 2.6 (1.7–3.8) | 38 | 7.4 (0.9–24.3) | 1.4 (1.1–1.8) | 27 | 0.452 | 0.104 |
| Altos de Campana | 13.1 (9.3–17.8) | 3.7 (2.5–5.2) | 267 | 20.0 (2.5–55.6) | 3.4 (2.6–4.2) | 10 | 0.628 | 0.644 |
| Altos del María | 67.3 (61.2–73.1) | 1211.0 (329.2–2338.0) | 254 | 69.2 (38.6–90.0) | 11.3 (2.6–24.5) | 13 | 1.000 | 0.137 |
| Fortuna | 42.3 (30.6–54.6) | 441.0 (4.2–1305.2) | 71 | 26.3 (15.5–39.7) | 9.5 (1.6–23.9) | 57 | 0.066 | 0.127 |
| All sites combined | 28.2 (25.6–30.9) | 715.7 (230.6–1303.0) | 1159 | 16.9 (12.2–22.5) | 13.1 (4.9–22.8) | 219 | <0.001* | 0.008* |

choa (blunt-headed tree snake) and one *Nothopsis rugosus* (rough coffee snake) that tested positive came from the low elevation sites of Cuatro Callitas (45 m) and La Rica (250 m), respectively. Prevalence of *B. dendrobatidis* DNA among snakes, with all sites combined, was 38% (9 to 76%, n = 8). Overall *B. dendrobatidis* prevalence did not differ statistically between lizards and snakes (Table 2).

A considerably greater prevalence of *Batrachochytrium dendrobatidis* DNA was detected in reptiles (lizards and snakes) at Altos del María compared to other sites. Prevalence of *B. dendrobatidis* DNA among all examined reptiles at Altos del María was 69% (39 to 91%, n = 13) whereas reptiles at other sites showed prevalences of $\leq 26\%$ (Table 2). Altos del María is a highland site that at the time of sampling was experiencing an epizootic of chytridiomycosis disease among amphibians. Significant differences in prevalence of *B. dendrobatidis* DNA among reptiles between sites were found when data from Altos del María were included in the analysis ($\chi^2 = 40.3$, $p < 0.001$) but not between the other 7 sites with Altos del María removed ($\chi^2 = 16.0$, $p = 0.014$).

In general, *Batrachochytrium dendrobatidis* DNA prevalence among reptiles was positively correlated with the infection prevalence among co-occurring anuran amphibians at any particular site ($r = 0.88$, p [2-sided] = 0.004, Pearson product moment correlation). Both amphibians and reptiles showed their lowest prevalence of *B. dendrobatidis* DNA at Palmarazo and highest prevalence at Altos del María. In 5 of the 8 study sites, prevalence of *B. dendrobatidis* DNA was slightly higher among the anuran amphibians, whereas in 3 study sites it was slightly higher among the reptiles (Table 2). A significant difference in prevalence of *B. dendrobatidis* DNA between reptiles and amphibians was detected only at La Rica, where the reptiles exhibited a significantly lower prevalence than did the syntopic anuran amphibians ($p = 0.032$, Fisher's exact test). Overall, however, comparing all reptiles at all sites to co-occurring anuran amphibians, the overall prevalence of 17% (95% CI: 12 to 23%, n = 219) found among reptiles was significantly lower than the 28% (26 to 31%, n = 1159) overall prevalence detected among syntopic anuran amphibians ($p < 0.001$, Fisher's exact test).

Intensity of *Batrachochytrium dendrobatidis* DNA on reptiles

Mean intensity of *Batrachochytrium dendrobatidis* DNA among lizards (Table 2) was 10.6 zoospore

equivalents per sample (95% CI: 3.0 to 21.0, n = 34). There was no significant difference in mean intensity between *Anolis lionotus* (16.7 mean zoospore equivalents; 1.5 to 74.8, n = 9) and *A. humilis* (8.2 mean zoospore equivalents; 3.4 to 24.5, n = 25). However, mean intensity of *B. dendrobatidis* DNA among snakes was 43.4 zoospore equivalents (1.1 to 65.0, n = 3), which was statistically higher than intensity in lizards ($p = 0.035$, Randomization test). With lizards and snakes combined, no significant difference in intensity of *B. dendrobatidis* DNA was found between sites.

We found no significant difference in *Batrachochytrium dendrobatidis* DNA intensity between reptiles and syntopic anuran amphibians at any site, including Altos del María (Table 2). However, when data from all sites were combined, reptiles showed an overall mean intensity of 13.1 zoospore equivalents per sample (4.9 to 22.8, n = 37), which was significantly lower than the infection intensity of 715.7 mean zoospore equivalents (230.6 to 1303.0, n = 327) found among co-occurring anuran amphibians ($p = 0.008$, Randomization test).

No individual reptile that tested positive for *Batrachochytrium dendrobatidis* DNA showed either the symptoms of chytridiomycosis disease or the elevated levels of infection intensity notable in anuran amphibians stricken with chytridiomycosis (Kilburn et al. 2011).

DISCUSSION

Batrachochytrium dendrobatidis is clearly not exclusive to amphibians. We have shown that, in Panama, lizards and snakes will harbour *B. dendrobatidis* DNA at non-pathological levels of prevalence and intensity that are comparable to those found among anuran amphibians that are not showing signs of chytridiomycosis disease (Kilburn et al. 2011). Without histology, we cannot say that we have proven the presence of *B. dendrobatidis* infection or the presence of infective zoospores on the reptiles, but the assays we used are routinely considered diagnostic for *B. dendrobatidis* infection in amphibians and, therefore, our findings indicate that infection is highly plausible.

Batrachochytrium dendrobatidis DNA is not uniformly present in those species of reptiles that may carry it. For example, we detected *B. dendrobatidis* DNA in *Anolis lionotus* from 4 sites, but not among 16 specimens from Palmarazo or 24 specimens from La Rica (Table 1). *Anolis humilis* tested positive for *B.*

dendrobatidis DNA in 5 of the 6 sites where the species was present. Compared to *A. lionotus* and *A. humilis*, our sample sizes were small for those species of lizards that did not test positive for *B. dendrobatidis* DNA. Thus, Phillott et al. (2009) may not have detected *B. dendrobatidis* DNA in Australian eastern water dragons simply because they sampled only 15 lizards from a single site, and not necessarily due to any inability of *B. dendrobatidis* DNA to persist on water dragon skin.

The species of reptiles we found to carry the DNA signature of *Batrachochytrium dendrobatidis* infection have habitat preferences and/or behaviours that may readily put them in contact with the pathogen. *Anolis humilis* is a terrestrial lizard found on the wet leaf litter of the forest floor (Savage 2002) and *A. lionotus* is semiaquatic, exclusively found along streams (Campbell 1973). Both *Nothopsis rugosus* and *Pliocercus euryzonus* are terrestrial leaf litter snakes (Savage 2002, Solórzano 2004), and *Imantodes cenchoa*, though primarily arboreal, sometimes is seen on the forest floor and is known to sleep hidden in hollow sections of trees and other plants, in bromeliads or in leaf litter and fallen vegetation (Myers 1982). Unlike the 2 insectivorous lizards, the snakes could also become infected with *B. dendrobatidis* in the act of subduing and ingesting their prey, which consists of salamanders, frogs and lizards.

By potentially maintaining the pathogen in the environment without succumbing to the disease, these reptiles may be important vectors or reservoir hosts for *Batrachochytrium dendrobatidis*. As carriers of the pathogen, they may allow virulent strains of *B. dendrobatidis* to spread. Significantly, reptiles at Altos del María exhibited elevated levels of *B. dendrobatidis* DNA prevalence at a time when syntopic amphibians were succumbing to epizootic chytridiomycosis disease. The observed enzootic persistence of *B. dendrobatidis* in the environment even after susceptible amphibian populations have succumbed to chytridiomycosis (Lips et al. 2005b, Kilburn et al. 2011) may also be abetted by reptiles serving as reservoir hosts.

Although the skin of reptiles is evidently capable of carrying *Batrachochytrium dendrobatidis* DNA under natural field conditions, it remains to be seen whether *B. dendrobatidis* can complete its life cycle on reptile integument, or whether it can persist on reptiles indefinitely in the absence of amphibian hosts. Reptile skin is quite unlike the skin of amphibians. It is highly keratinised, non-respiratory, relatively impermeable to water and has little physiological role in maintaining electrolyte balance.

Batrachochytrium dendrobatidis produces an assortment of proteolytic enzymes (Symonds et al. 2008, Moss et al. 2010). Though there is, as yet, no evidence among reptiles of the lethal, epizootic chytridiomycosis that has afflicted amphibian populations in many parts of the world, it does not follow that *B. dendrobatidis* infection in reptiles, should it occur, is necessarily benign.

Like amphibians, populations of lizards and snakes are in decline throughout the world (Gibbons et al. 2000), including locations in Central America (Pounds et al. 1999, Greenbaum & Komar 2005, Whitfield et al. 2007) and at sites where *Batrachochytrium dendrobatidis*, and chytridiomycosis, has been detected in co-occurring amphibians (Reading et al. 2010, Sinervo et al. 2010). Declines in species of snakes that rely on adult frogs and frog egg masses as their primary prey may be related to chytridiomycosis-induced losses of amphibian biomass (Whiles et al. 2006). Reasoning, though, that chytridiomycosis was strictly a disease of amphibians, Whitfield et al. (2007), Wake (2007) and Sinervo et al. (2010) attributed declines in reptile abundance in Central America, in general, to the effects of climate change. We cannot conclude from our results that *B. dendrobatidis* is a virulent reptile pathogen, or that it causes disease-induced decline in reptile populations, but our evidence that reptiles do carry *B. dendrobatidis* DNA in neotropical localities where both reptiles and amphibians have declined warrants some concern.

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