INTRODUCTION

Puerto Rico has lost 3 species of endemic anurans in the last 3 decades: *Eleutherodactylus karlschmidtii* (1976), *E. jasperi* (1981), and *E. eneidae* (1990) (Joglar & Burrowes 1996, Burrowes et al. 2004). In addition, long-term amphibian monitoring data revealed that 8 populations of 6 different species declined at El Yunque, a mountain forest reserve in eastern Puerto Rico, between 1990 and 2000 (Burrowes et al. 2004). Declines in populations of *E. coqui* have been linked to increments in the number of dry periods, defined as 5 or more consecutive days with \( \leq 3 \) mm of precipitation (Stewart 1995). Burrowes et al. (2004) reported an increase of 1°C in the minimum temperature at El Yunque from 1970 to 2000, and found that the number of dry periods during the dry season increased significantly in the mid-1970s and early 1990s. These climatic changes are correlated with the years of anuran disappearances in Puerto Rico, and the first occurrence of *Batrachochytrium dendrobatidis* (*Bd*) in several declining species at El Yunque (Burrowes et al. 2004, 2008). *Bd* infections have been associated with mass mortality and declines of amphibians around the world (Berger et al. 1998, Lips et al. 2006, Skerratt et al. 2007). Laboratory studies have shown that amphibian susceptibility to *Bd* infection varies across taxonomic, ontogenetic, and thermal conditions (Lamirande &
Nichols 2002, Davidson et al. 2003, Berger et al. 2004, Daszak et al. 2004), but little is known about the interactions of this pathogen and its hosts in the wild. Retallick et al. (2004) showed a seasonal peak of infections during the cooler months of the year in 1 geographic region in Australia, and Kriger & Hero (2006) found that frogs that were infected during the winter could clear the infection during the warmer months. However, studies of this type in the Neotropics, and particularly among direct-developing frogs that appear to be more resistant to Bd (Lips et al. 2003, Bustamante et al. 2005), have not been conducted. Amphibian communities devastated by Bd in Central America have been left with few resistant species, although several are terrestrial direct developers (Lips et al. 2003, 2006). Thus, it is important to understand the interactions between Bd and Eleutherodactylus species, because these may be the only amphibians left at some places after a Bd epidemic. Bd is present throughout Puerto Rico in 9 species of Eleutherodactylus and 1 Leptodactylus at elevations above 600 m (Burrowes et al. 2008).

Here we present the results of 2 yr of consecutive monitoring of Eleutherodactylus coqui and E. portoricensis in 2 upland forests in Puerto Rico, characterized by historic population declines in the presence of chytridiomycosis. We tested the hypothesis of a synergistic interaction between climate and Bd as suggested by Burrowes et al. (2004), by monitoring prevalence of Bd (number of infected frogs per total sampled), and level of infection (number of zoospores) in individuals from populations in the wild. In addition, we conducted an ex situ experiment where we simulated seasonal climatic conditions in the laboratory, inoculated frogs with Bd, and observed their behavior and susceptibility to infection. Previous experiments showed that E. coqui frogs exposed to drought conditions in the laboratory clumped in humid areas of the terraria, while frogs that had unlimited moisture did not clump (Joglar et al. 2007). Thus, we expected that if host-pathogen dynamics are influenced by climatic conditions, Bd infection loads of individuals in the wild would be higher during the dry season when temperatures are lower, and drought-stressed frogs would clump in humid pockets of the forest where the fungus is more likely to occur (Burrowes et al. 2004). In addition, we expected that under laboratory conditions of drought, frogs would be more likely to die from chytridiomycosis. However, if seasonal climatic variables do not affect vulnerability and/or pathogenicity of Bd, we expected to find no differences in infection load, prevalence, or experimental survivorship of inoculated frogs between seasons or treatments. Results of this work are important in order to understand the dynamics of Bd when it is endemic in populations of presumably less vulnerable terrestrial direct-developing frogs.

MATERIALS AND METHODS

Study sites. This study took place at El Yunque Caribbean National Forest in eastern Puerto Rico at 2 different elevations, Palo Colorado Forest (18.30°N, 65.79°W) at 661 m, and Bosque Enano Forest (18.30°N, 65.79°W) at 850 m. Palo Colorado Forest is part of the subtropical wet forest association (Ewel & Whitmore 1973), and is characterized by abundant Cyrilla racemiflora. Bosque Enano Forest is an elfin forest near Mt. Britton classified as lower montane wet forest by Holdridge Life Zones (Ewel & Whitmore 1973) and is characterized by the abundance of Ocotea spathulata and Eugenia borinquensis.

Field work. Amphibian populations were monitored for Bd infection every month for 2 yr (starting in September 2005). Visual encounter surveys were conducted in 300 m² (100 m long × 3 m wide) transects at the 2 study sites. Frogs were captured in individual plastic bags worn as gloves and inverted to prevent cross contamination. A fresh pair of powder-free nitrile gloves was worn every time a different frog was manipulated. Individuals were sampled for Bd by swabbing their ventrum, feet, and toes as described by Kriger et al. (2006). Swabs were stored at –20°C upon arrival at the laboratory. Each frog was toe clipped following a modification of Twitty’s (1966) code for an ongoing mark-recapture study, and toes were used for Bd diagnostics. This code allowed us to take tissue from different limbs of the frog minimizing the impact of toe-clipping (P. Burrowes & A. Longo unpubl. data). Toe clips were stored in 70% ethanol and kept at room temperature until DNA was extracted for Bd diagnosis.

Batrachochytrium dendrobatidis diagnosis. In this study, only frog toe clips were used as tissue for disease diagnosis. Toe clippings were preferred over swabs, because when comparing results from swabs and toes from the same frog, we found that swabs did not detect low-level infection (A. Longo & P. Burrowes unpubl. data). Bd pathogen load was diagnosed following the Taqman quantitative-PCR (qPCR) method to determine the number of Bd zoospores per individual. Samples were run on singlicates, but repeated if the quantity was <1 zoospore equivalent (Boyle et al. 2004). To correct for potential underestimation of Bd zoospores in toe clips, and to make our data more comparable to that reported from swabs of similar size frogs, the number of zoospore equivalents obtained from qPCR detection was multiplied by the snout-vent length (SVL) of each individual. This number is also an indication of intensity of infection, and we refer to it as the Bd load. The mean ± SE SVL for Eleutherodactylus coqui was 34.9 ± 0.39 mm in males and 43.7 ± 0.39 mm in females; for E. portoricensis, it was 32.2 ± 0.36 mm in males and 37.8 ± 0.43 mm in females.
**Bd inoculation ex situ experiment.** Adult *Eleutherodactylus coqui* were collected from El Yunque at Palo Colorado forest, very close but not in our transects. Every individual was tested for *Bd*, and only *Bd*-negative frogs were used in the experiments. Infected frogs were treated with Itraconazole® (Nichols & Laraminde 2001), held in a separate room, and released in the wild when they had cleared the infection. Healthy frogs were acclimated in 110 l terraria for 2 wk before experiments began. Terraria were prepared with potting soil and contained 4 potential retreat sites consisting of parts of rolled yagrumo *Cecropia peltata* leaves, bamboo shoots, palm fronds, and 2 large branches (Fig. 1). Four adult *E. coqui* frogs of the same sex were held in each terrarium with equal numbers of refugia and sufficient food, and kept at a 12:12 h night:day cycle at 20.5°C to simulate nocturnal temperatures at El Yunque during the dry season. By the time the experiments were started, all frogs were in healthy condition, *Bd*-free, eating well, and distinguishable through unique toe-clip codes. The experiment consisted of 12 terraria (110 l) comprising 3 replicates each of 4 different treatments where frogs were (1) inoculated with *Bd* under drought conditions (experimental positives); (2) not exposed to *Bd*, but under drought conditions (experimental negatives); (3) inoculated with *Bd* under humid conditions (control positives); and (4) not exposed to *Bd* or drought (control negatives). To simulate drought conditions in the experimental terraria, the substrate was divided in 2 halves with aluminum trays that prevented diffusion of water from the side that was kept humid to the side that was kept dry (Fig. 1A). During the acclimation period and throughout the experiments, experimental terraria were hydrated every 4 d by applying water directly (not sprayed) to the wet side only, while the controls received water throughout the entire substrate every other day. The purpose of this water treatment in the experimental terraria was to simulate the understory forest conditions during drought, when frogs tend to clump in humid areas and share retreat sites (Joglar et al. 2007).

*Bd* isolate JEL427 was obtained from a sick *Eleutherodactylus coqui* collected at our study site in El Yunque, and sent to J. Longcore at the University of Maine (USA) for initial isolation and culture. Zoospores were harvested by flooding *Bd*-rich plates with distilled water for 15 min and using a pipette to collect them. The specific zoospore concentration for inocula-
...was estimated by counting the zoospores under a light microscope at 400× with a hemocytometer, then diluted to $10^3$ Bd zoospores ml$^{-1}$. Frogs in experimental positive and control positive treatments were inoculated with $Bd$ for 4 consecutive days. On inoculation days, frogs were removed from their terraria and housed individually in a plastic container with 10 ml of the $Bd$ zoospore solution during 10 h of daytime (from 8:00 to 18:00 h), and then returned to their respective terrarium. Plastic containers were covered with large perforated carton boxes to simulate relatively dark diurnal retreat sites. Experimental negative and control negative frogs were treated in the same manner, except that the water in the containers was not inoculated with $Bd$ zoospores. Frog behavior, sickness symptoms, use of retreat sites, and death were monitored daily for 15 d. When frogs were found dead, tissue samples were taken to confirm infection status via qPCR. Koch's postulates were fulfilled to confirm cause of death by chytridiomycosis in 6 of 12 dead frogs in 6 of 12 dead frogs. All surviving frogs that had been inoculated with $Bd$ were euthanized after the experiment. This experiment was done twice to confirm repeatability.

**Data analysis.** To test for differences in $Bd$ infection level of individuals (measured in zoospores and $Bd$ load) between seasons in Puerto Rico, we used a Mann-Whitney ($W$) test. Prevalence (number of infected frogs/total sampled) of $Bd$ in frog populations between seasons was compared using a $t$-test. To assess the status of $Bd$ in frog populations over time, we compared prevalence and level of infection between years (2005–2006; 2006–2007) using 2-proportion ($Z$) and Mann-Whitney tests, respectively. A Logrank test on censored survival data was performed to examine differences in frog survival per treatment in ex situ experiments. All statistical tests were done with Minitab Release 15. Weather data on daily precipitation and minimum temperature were taken from the Long Term Ecological Research Station (LTER) of El Verde, Puerto Rico, located 4.2 km NW of our study sites.

**RESULTS**

**Infection level and prevalence in wild populations**

Analysis of climatic variables with respect to daily precipitation and minimum temperature, which are important variables both in amphibian and $Bd$ biology (Carey & Alexander 2003, Piotrowski et al. 2004, Pounds et al. 2006), were analyzed for the 2 yr of monitoring starting in September 2005 through September 2007. Long-term analysis of climatic variables have shown that the climate of Puerto Rico is characterized by 2 seasons: one with higher precipitation and warmer temperatures, and another that is drier and cooler (Colón 1983, Burrowes et al. 2004). Fig. 2 shows that despite a few anomalies, the years of this study reflect this seasonality. We will refer to these seasons as wet-warm and dry-cool, respectively.

The level of $Bd$ infection in positively diagnosed frogs from wild populations was significantly different across seasons, independent of the type of measure used (Table 1). In *Eleutherodactylus coqui*, both the mean number of zoospores (from toe clips) and the mean $Bd$ load (zoospores × SVL) were significantly higher in the dry-cool season ($W = 14,085$, $N = 212$, $p = 0.0004$; $W = 14,087$, $N = 212$, $p = 0.0004$, respectively). The same pattern was observed for *E. portoricensis* a few meters higher in El Yunque forest, where the mean number of zoospores ($W = 3991.5$, $N = 125$, $p = 0.0165$) and the mean $Bd$ load ($W = 4040$, $N = 125$, $p = 0.0311$) were higher during the dry-cool months. However, monthly prevalence of $Bd$ in the populations showed the opposite pattern. From a total of 321 individuals of *E. coqui* sampled in the Palo Colorado forest, 212 were positively diagnosed with $Bd$. More frogs were found infected during the wet-warm season (Table 1), where prevalence was approximately 25% higher in dry-cool months ($Z = 4.80$, $N = 321$, 95% CI = 0.15–0.36, $p < 0.000$). This trend was also significant for *E. portoricensis* ($Z = 3.07$, $N = 204$, 95% CI = 0.07–0.34, $p = 0.004$) for which the prevalence during the wet-warm season was 30% greater than in the dry-
cool months. Thus, data on \( Bd \) prevalence suggest a different response to seasonality than data on the intensity of infection. While prevalence is a population-level measure of infection status, zoospore counts provide information on individual susceptibility to chytridiomycosis. A comparison of the status of \( Bd \) in populations over time showed that while infection level in adults of both species decreased significantly from the first year of this study (2005–2006) to the next (2006–2007), prevalence in the populations increased. Thus, more frogs had \( Bd \) but at lower levels of infection (Table 2).

### Ex situ inoculation experiments

Results of our laboratory experiment showed that after 4 consecutive days of \( Bd \) inoculations at \( 10^6 \) zoospores per individual, only frogs subjected to \( Bd \) and drought died (Fig. 3). Only frogs in the drought treatments clumped under refugia in the humid side of the terraria, as reported by Joglar et al. (2007). Frog health changed drastically after inoculations with \( Bd \). These frogs started showing signs of chytridiomycosis such as skin sloughing, lethargy, inability to respond to stimulus, and necrosis around the snout and abdomen after the second day of inoculation (Fig. 4). Although frogs in the control positive terraria contracted \( Bd \) (detected by qPCR), symptoms of chytridiomycosis were not as severe, and they persisted throughout the experiment. From Days 3 to 11 after \( Bd \) exposure, 50% (12) of the frogs died of chytridiomycosis in the experimental positive treatment. At the time of death, all frogs had \( Bd \) present on their skin, confirmed by qPCR analyses. Frogs did not die in any of the other treatments; neither \( Bd \) alone (control positive) nor drought

<table>
<thead>
<tr>
<th>Species</th>
<th>Forest</th>
<th>Year</th>
<th>Zoospores (range)</th>
<th>( Bd ) load (range)</th>
<th>Prevalence (%), (N infected/total sampled)</th>
<th>95% CI of prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coqui</em></td>
<td>PC</td>
<td>2005–2006</td>
<td>258 ± 1292 (6–9450)</td>
<td>13116 ± 70705 (277–527972)</td>
<td>38.5 (55/143)</td>
<td>30.5–47</td>
</tr>
<tr>
<td></td>
<td>PC</td>
<td>2006–2007</td>
<td>48 ± 398 (1–4926)</td>
<td>1418 ± 10286 (37–12095)</td>
<td>87.7 (157/179)</td>
<td>82–92.1</td>
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<td>( W = 8963.0, p &lt; 0.000 )</td>
<td>( W = 8926.5, p &lt; 0.000 )</td>
<td>( 95% \text{ CI} = -0.50 \text{ to } -0.40, p &lt; 0.000 )</td>
<td>( Z = -10.37 )</td>
</tr>
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| *E. portoricensis* | BE     | 2005–2006  | 180 ± 715 (1–3335) | 5765 ± 23388 (40–128572) | 41.6 (42/101) | 31.9–51.8 |
|                   | BE     | 2006–2007  | 158 ± 1236 (1–11253) | 5072 ± 39030 (30–357839) | 80.6 (83/103) | 71.6–87.7 |
|                   |        |            | \( W = 3264.0, p < 0.001 \) | \( W = 3229.5, p = 0.002 \) | \( 95\% \text{ CI} = -0.51 \text{ to } -0.27, p < 0.000 \) | \( Z = -6.23 \) |
alone (experimental negative) resulted in deaths. These results show that *Eleutherodactylus coqui* adults are significantly more likely to die from chytridiomycosis when subjected to limited water treatments that induce clumping at humid retreat sites (Logrank test, p < 0.000, N = 24 per treatment), and support the hypothesis that vulnerability to chytridiomycosis is associated with seasonality.

**DISCUSSION**

Our *ex situ* experiments provide evidence that drought conditions under cool temperatures increased vulnerability of frogs to chytridiomycosis. In the laboratory, 50% of the frogs exposed to *Bd* and drought died (experimental positives), while all those exposed to other treatments, including *Bd* and unlimited water, survived (Fig. 3). The fact that clumping in refugia on humid sides of the terraria occurred only under drought treatments suggests that a drought-induced behavioral change (i.e. clumping) increases susceptibility of these terrestrial frogs to *Bd*.

Patterns of *Bd* infection in wild populations are much more difficult to discern, especially in this genus of frogs, since they do not congregate in ponds or streams to reproduce. If an *Eleutherodactylus* is sick from chytridiomycosis, it is likely to stay in its retreat site where herpetologists cannot find it. This 2 yr study allowed us to monitor enough frogs in the field to show that infection levels of *Bd* were significantly higher during the dry-cool season (Table 1). Thus, we provide *ex situ* and *in situ* evidence in favor of the climate-linked epidemic hypothesis as originally described by Pounds & Crump (1994) and proposed for Puerto Rican frogs by Burrowes et al. (2004). This hypothesis proposes that under the environmental stress that drought brings about to amphibians (Carey et al. 2003) *E. coqui* change their random distribution in the forest and clump in humid patches where the chytrid fungus is more likely to occur. Drought and clumping at retreat sites enhance the vulnerability of frogs to *Bd* infection at a time when environmental temperatures (mean minimum temperature = 20.52°C, Fig. 2) favor its growth (Piotrowski et al. 2004). Thus, under extreme drought conditions such as those described for the years of drastic declines in Puerto Rico (1976 to 1978 and 1981; Burrowes et al. 2004), an epidemic may drive the extirpation of populations even when *Bd* is enzootic in a geographical area.

The fact that the prevalence of infection is higher during the wet-warm months leads us to propose a cyclic synergistic effect between seasonality and infection that explains *Bd* persistence in populations of these 2 resistant, yet vulnerable, species of *Eleutherodactylus* (Fig. 5). During the dry-cool season, fewer frogs are active in the forest because they spend more time in retreat sites to reduce water loss (Pough et al. 1983, Joglar 1998). Thus, the frogs that are sampled during these times are those that have overcome lower temperatures and drier conditions successfully. Frogs that were positive for *Bd* during the dry-cool season had significantly higher infection levels than those in the wet season, but prevalence of *Bd* was lower because very sick frogs remain inactive in their retreat sites, and the probability of capturing an infected frog decreases. During the wet season, as temperature increases, survivors recuperate and persist but still carry a low *Bd* load. Prevalence of *Bd* in the population is higher because frogs are not that sick, and the probability of sampling an infected individual increases. With lower infection levels in the wet-
warm season, frogs are able to reproduce, and therefore the population recovers. This cycle (Fig. 5) describes a possible mechanism that allows Bd to thrive without decimation of its hosts. This synergistic interaction between Bd and seasonal temperature and precipitation may explain why species in this ecological guild (terrestrial direct-developers) are less vulnerable to Bd in tropical forest communities as found by Lips et al. (2003) and Bustamante et al. (2005) in Central and South America.

The disparity observed between measures of Bd load in individuals versus prevalence of Bd in populations at a given time indicates that differences between prevalence and level of infection parameters are important. Prevalence describes the status of infection in a population at a given time, while level of infection (number of zoospores or Bd load) provides information on the response of a subgroup of individuals (the infected) to this pathogen. When Bd is enzootic, and environmental conditions are not stressful to the amphibians or favor Bd physiology, prevalence may be high while Bd load in infected individuals is low, allowing frogs to persist. On the other hand, when environmental conditions become stressful, as during droughts in Puerto Rico, prevalence does not change significantly, but infected individuals have a higher Bd load. Therefore, low prevalence of Bd does not necessarily imply low risk of infection or low susceptibility to this pathogen in terrestrial frogs as has been suggested by Kriger & Hero (2007).

In this paper we show that declining populations of 2 species of Eleutherodactylus in Puerto Rico respond to Bd in a similar way with respect to climatic variables that determine seasonality. The fact that Bd infection levels of adult individuals decreased significantly between the 2 yr of this study, while prevalence increased in the populations (Table 2), suggests that E. coqui and E. portoricensis are persisting with a stable infection typical of enzootic conditions (Retallick et al. 2004, Briggs et al. 2005). Although E. coqui and E. portoricensis may be able to survive through the cyclic seasonal mechanism proposed (Fig. 5), they can serve as carriers of Bd to other species that are rare, have higher degrees of local endemism, are more ecologically specialized, and may be more vulnerable to this pathogen. Thus, even under enzootic conditions, Bd can still be a threat to anuran biodiversity. Three species are extinct in Puerto Rico possibly due to chytrid-mycosis (Burrowes et al. 2004, 2008), and there are other at-risk species, such as E. locustus, E. richmondi, and E. wightmanae, whose survival may be further compromised by E. coqui and E. portoricensis that act as reservoirs of Bd.

At a time when more data are needed to explain the link between climate change and amphibian declines (Rohr et al. 2008), our work provides evidence of increased vulnerability of direct-developing frogs to Bd under drought conditions. Climate change models predict increased episodes of drought in the Caribbean for the next 50 yr (Christensen et al. 2007). Under this scenario, persisting populations of Eleutherodactylus in Puerto Rico could be at major risk. This study contributes to our understanding of how Bd affects persistent populations, but further studies on Bd movement patterns, environmental interactions, defense mechanisms, and disease physiology are needed in order to devise conservation measures to curtail global amphibian declines and extinctions.

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