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# SUSCEPTIBILITY TO THE AMPHIBIAN CHYTRID FUNGUS VARIES WITH ONTOGENY IN THE DIRECT-DEVELOPING FROG *ELEUTHERODACTYLUS COQUI*

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ABSTRACT: Age-related differences in susceptibility to infectious disease are known from a wide variety of plant and animal taxonomic groups. For example, the immature immune systems of young vertebrates, along with limited prior exposure to pathogens and behavioral factors, can place juveniles at greater risk of acquiring and succumbing to a pathogen. We studied the ontogenetic susceptibility of terrestrial direct-developing frogs (*Eleutherodactylus coqui*) to the fungal pathogen, *Batrachochytrium dendrobatidis* (Bd), which is responsible for the decline of amphibian species worldwide. By exposing juvenile and adult frogs to the same dose and strain of Bd, we uncovered ontogenetic differences in susceptibility. Froglets exposed to the pathogen had significantly lower survival rates compared with control froglets, while adult frogs largely cleared infection and had survival rates indistinguishable from control frogs, even when exposed to a much higher dose of Bd. The high disease-induced mortality rate of juveniles may explain ongoing population declines in eastern Puerto Rico, where Bd is endemic and juveniles experience higher prevalence and infection intensity compared to adults. Our results have important implications for understanding and modeling the decline, possibly to extinction, of amphibian populations and species.

*Key words:* Amphibian pathogen, *Batrachochytrium dendrobatidis*, chytridiomycosis, direct-developing frogs, *Eleutherodactylus coqui*, ontogenetic susceptibility.

## INTRODUCTION

Ontogenetic differences in susceptibility to infectious disease are known in a variety of organisms, including plants (Ficke et al. 2002), insects (Bull et al. 2012), amphibians (Rohr et al. 2010), birds (Gavier-Widen et al. 2012), and mammals (Burns-Guydish et al. 2005). Among vertebrates, young animals frequently have immature immune systems and limited prior exposure to pathogens that stimulate adaptive immunity (Solomon 1978), resulting in greater disease susceptibility compared with older juveniles and adults.

Age-related differences in disease susceptibility are of particular interest with chytridiomycosis, an emerging infectious disease implicated in the decline and extinction of amphibian species worldwide (Collins and Crump 2009). The aquatic fungus that causes chytridiomycosis, *Batrachochytrium dentrobatidis* (Bd), is known to infect over 500 amphibian species (Olson et al. 2013). The fungus colonizes the keratinized tissue of amphibian skin (Longcore et al. 1999) and, in at least one species, causes death through osmotic and electrolyte imbalance (Voyles et al. 2007). Species vary in susceptibility (e.g., Searle et al. 2011), with responses ranging from mass mortality (Lips et al. 2006), to tolerance of subclinical infection (Garner et al. 2006), and to resistance (Marquez et al. 2010).

Susceptibility can also vary across amphibian life history stages. Tadpoles of some species often show markedly lower susceptibility than postmetamorphic animals (Rachowicz and Vredenburg 2004; Tobler and Schmidt 2010), presumably because keratinized tissue is restricted to mouthparts in tadpoles (Berger et al.

1998). In contrast, tadpoles of other species show reduced survival from infection, particularly when exposed to high doses of Bd (Blaustein et al. 2005). Fitness impacts of infection on tadpoles include smaller metamorphic body mass, increased larval period length, and reduced foraging performance (Parris and Cornelius 2004; Venesky et al. 2009). These fitness costs can result in mortality of tadpoles or metamorphs, even in the absence of infectious disease (Garner et al. 2009).

Only one study has examined the developmental susceptibility of terrestrial amphibian life stages to Bd (Ortiz-Santaliestra et al. 2013). Researchers exposed juveniles of *Anaxyrus americanus*, a species with larval development, at the end of metamorphosis or 28 days following metamorphosis. Juveniles exposed to Bd showed significantly greater mortality than control animals at day zero but not at day 28, suggesting an ontogenetic difference in immune competence. The authors acknowledge that these results could be also explained by greater body size of juveniles at day 28, as other studies have shown that body size is an important determinant of survival following Bd infection (e.g., Carey et al. 2006).

The ontogeny of the amphibian immune system has been studied in only one anuran, *Xenopus laevis*, a species with larval development (Rollins-Smith 1998). Innate immune responses, such as secretion of antimicrobial peptides, are one of several factors known to influence amphibian susceptibility (Rollins-Smith et al. 2011); other factors include symbiotic skin bacteria that inhibit Bd (Harris et al. 2006), microhabitat preferences (Brem and Lips 2008), and thermoregulatory behavior (Richards-Zawacki 2010). The adaptive immune response of *X. laevis* appears to be inhibited by Bd (Rollins-Smith et al. 2011).

Although direct development of terrestrial eggs is prevalent in the tropics and has evolved independently in at least 12 clades of anurans (Duellman and Trueb 1986), there are no data indicating how and when the immune system of direct-developing frogs develops or matures. Development time of embryos of the common coqui (*Eleutherodactylus coqui*) ranges from 17 to 26 days and is qualitatively different from larval development with at least seven unique morphologic features (Townsend and Stewart 1985). Because direct developers do not undergo metamorphosis with a corresponding reorganization of the immune system (Rollins-Smith 1998), it is unknown if hatchlings of direct-developing anurans are more or less vulnerable to infection as the recently metamorphosed frogs of larval developing species.

We investigated the ontogenetic susceptibility of direct-developing frogs to Bd. Our model species is the common coqui, a terrestrial direct-developing species from Puerto Rico that has been well studied in the laboratory and the field. Bd is suspected in the decline and extinction of three frog species from Puerto Rico in recent decades (Burrowes et al. 2004), and ongoing declines have been reported for coquis and other *Eleutherodacylus* species at midelevations in eastern Puerto Rico (Longo et al. 2010). Recent laboratory work indicates that adult coquis may have low susceptibility to Bd, at least under normal climatic conditions (Longo et al. 2010; Langhammer et al. 2013), and moribund adults are rarely found in the field (Longo et al. 2013). Juvenile coquis show higher prevalence and intensity of Bd infection compared with adults in El Yunque, Puerto Rico (Longo and Burrowes 2010), but it is unclear whether juveniles are susceptible to Bd.

We tested the response of juvenile and adult coquis to Bd infection under identical laboratory conditions, including exposure to the same Bd strain and dose. We hypothesized that the decline of *E. coqui* populations in northeastern Puerto Rico results from high disease-induced mortality of juveniles (Longo and Burrowes 2010), and we predicted that juveniles

exposed to Bd would show significantly lower survival rates than adults. Theoretical work with larval developing species has highlighted the importance of juvenile survivorship in determining population persistence (Hels and Nachman 2002; Hyman 2012). Our work represents an important step in understanding the response of direct-developing juvenile frogs to Bd.

## MATERIALS AND METHODS

The Arizona State University Institutional Animal Care and Use Committee and the University of Maryland Institutional Animal Care and Use Committee approved this research. We obtained permission to collect and export coquis from the Department of Natural and Environmental Resources in Puerto Rico (permits 2009-IC-015, 2009-IC-014) and the Department of Land and Natural Resources in Hawaii (permit EX10-08).

### Frog acquisition and rearing

In 2010, we collected 43 adult (>23-mm snout-vent length [SVL]) coquis from El Yunque, Puerto Rico, and from an introduced population near Hilo, Hawaii. Hawaiian populations of coquis are also affected by Bd (Beard and O'Neill 2005); among 75 frogs collected for this study and other experiments, 14 were Bd-positive (19%). We measured and determined the sex of each animal, toe clipped frogs for individual identification, and swabbed ventral skin surfaces to assess Bd infection status (Hyatt et al. 2007). We used a new pair of disposable nitrile gloves for each frog to prevent cross contamination.

After frogs were housed individually for at least 10 wk and repeatedly found Bd-negative, we placed them in large naturalistic terraria in groups of 4–6 to initiate breeding, keeping the Puerto Rican and Hawaiian coquis separate. More than 15 clutches of 1–34 juvenile froglets were produced by at least nine breeding pairs.

Background survival of froglets raised in the lab was low, similar to that observed by Michael (1995) and has been experienced in captive-breeding attempts with this species in Puerto Rico (P.A.B. unpubl. data). We did not estimate differences in background survival rates (i.e., before the experiment began) between offspring of Puerto Rican and Hawaiian adults. We attribute poor survival of juveniles to the difficulty in maintaining a constant supply of tiny food items. The unavailability of termites, for both juveniles and adults, was particularly problematic. However, enough juveniles survived beyond 6 wk to test overall froglet susceptibility to Bd. At 6 wk of age, most juveniles were large enough so that we could handle them briefly without causing inadvertent mortality. We used 105 juveniles in this experiment (71 from Puerto Rican and 34 from Hawaiian parents). Most juveniles ($n=92$) were 6–10 wk old, and the remaining ($n=13$) were 18–21 wk old. The size range of all experimental juveniles was 6.9–13.9-mm SVL.

### Animal husbandry

For the experiment, we housed adult frogs individually in acrylic terraria (20.3×17.8×12.7 cm) with vented lids. Each terrarium had a moist sphagnum moss substrate with *Cercropia* sp. leaves as hiding places. We sprayed terraria daily with dechlorinated tap water and changed terraria biweekly. Frogs had ad libitum access to crickets twice a week. We maintained the laboratory at a constant temperature of 20–22 C on a 12-hr day-night cycle. We treated juvenile coquis similarly except that we housed them individually in 473-mL plastic Ziploc containers lined with moss and vented lids. We fed springtails to the smallest juveniles and springtails and wingless fruit flies to the older juveniles 5–6 times per week.

### Experimental design

We randomly assigned juvenile coquis to one of two treatment groups: control ($n=54$) or exposure to Bd at a dose of $10^5$ zoospores/mL ($n=51$). Mean SVL (±1 SE) and age of juveniles was 11.1±0.2 mm and 9.6±0.4 wk, respectively. There was no significant difference between treatment groups in length ($F=1.76$; $P=0.19$) or age ($F=0.87$; $P=0.35$).

Because adult coquis were so much larger than juveniles, and potentially better developed immunologically, we exposed some frogs to a higher Bd dose ($10^6$ zoospores/mL) and some to the dose used for the juveniles ($10^5$ zoospores/mL). We also wanted to know if previous Bd infection affected survival, because coquis in the wild are likely to experience frequent reinfection. We thus included a treatment group of frogs that were Bd positive at the time of collection and either remained Bd positive or cleared the infection by the time of the experiment. All other frogs were Bd negative upon collection in the field and remained Bd negative until the start of the experiment. Frogs were randomly assigned to

treatment groups as follows: control ($n=10$), exposure to $10^5$ zoospores/mL ($n=7$), exposure to $10^6$ zoospores/mL ($n=13$), or previously infected frogs exposed to $10^5$ zoospores/mL ($n=13$). Mean length of adults was $37.3\pm0.8$ mm, and there was no significant difference in mean length between treatment groups ($F=2.72$; $P=0.10$).

#### Bd exposure

We obtained a sample of Bd strain JEL427 from Joyce Longcore at the University of Maine, Orono, Maine. This strain was isolated in 2005 from a sick coqui in El Yunque, Puerto Rico, and belongs to the youngest Bd clade, termed the "global pandemic lineage" (Rosenblum et al. 2013). The isolate was continuously maintained at 4 C and transferred into new medium three to four times per year. We held the Bd culture in 1% tryptone broth at 18 C and transferred it into new medium monthly.

One week prior to frog exposure, we transferred fast-growing broth culture to 1% tryptone-agar plates, which we sealed and incubated at 23 C. To harvest zoospores, we flooded plates with 1 mL of sterile 1% tryptone broth and allowed them to sit for 30 min. We collected the zoospore solution into a 50-mL sterile tube and quantified zoospore concentration using two counts on a hemacytometer (Hausser Scientific Bright-Line, Thermo Fisher Scientific, Waltham, Massachusetts, USA), which were then averaged. The zoospore stock solution was diluted with dechlorinated tap water to achieve the desired inoculation concentration of either $10^5$ or $10^6$ zoospores/mL.

For the Bd exposure, we placed adult frogs individually into 236-mL plastic deli cups, with a fresh solution of Bd in 10 mL of zoospore solution for 10 hr per day, on two consecutive days. We treated control frogs identically, except that they were exposed to a sham solution of dechlorinated tap water containing 1% tryptone broth lacking zoospores. Following Bd exposure, adult frogs were returned to individual acrylic terraria ($20.3\times17.8\times12.7$ cm) with vented lids.

We exposed juvenile froglets from both Puerto Rican and Hawaiian parents to 0.5–1.0 mL of zoospore (or sham) solution in 12 mm (height)$\times$32 mm (diameter) SKS Natural Polypro Hinge Top Vials (SKS Bottle & Packaging, Watervliet, New York, USA). These shallow containers prevented tiny froglets from climbing the sides and escaping the Bd solution. We added the Bd inoculate or sham solution to the vial with a pipette through a small hole cut into the lid. We exposed juveniles for 8 hr on one day and for another 8 hr, 1 wk later. We considered the risk of mortality from stress to be too great to expose juveniles on two consecutive days, because they are much more fragile than adults. Following Bd exposure, juvenile frogs were returned to individual 473-mL plastic Ziploc containers with vented lids.

#### Pathogen detection

We detected Bd infection by swabbing ventral skin surfaces of adult and juvenile frogs with fine-tip cotton swabs (Medical Wire & Equipment, number 113, Advantage Bundling SP, Durham, North Carolina, USA) following standardized technique (Hyatt et al. 2007). We swabbed adults on several occasions before the experiment to ensure that they were Bd negative. Following Bd exposure, we swabbed adult frogs on days 9, 17, 30, and upon death or day 77, the end of the experiment; however, day 77 swabs were lost in shipment and could not be analyzed. We swabbed juveniles upon death or at the end of the experiment, and for roughly half of the juveniles, we also assessed Bd infection 20 days postinoculation. We did not swab juvenile frogs prior to the experiment because we found that it was easy to cause mortality through swabbing or merely handling the frogs. We held the juvenile frogs in a pathogen-free section of the laboratory before exposure where they never came into contact with infected frogs or equipment. No control frogs were found positive during the experiment, so we feel confident that juveniles were not unintentionally exposed to Bd.

We stored swabs at room temperature in sterile 2-mL screw-top vials until DNA extraction about 2 wk later. We extracted DNA with PrepMan Ultra (Life Technologies, Grand Island, New York, USA) and determined infection status and intensity using real-time quantitative PCR following a slightly modified version (Hyman and Collins 2012) of the standard protocol for detecting Bd (Boyle et al. 2004).

#### Monitoring disease development

We monitored behavior, morbidity, and mortality of adult and juvenile frogs daily for 77 days. Frogs were observed for clinical signs of chytridiomycosis, including skin sloughing, lesions, loss of appetite, lethargy, and loss of righting reflex. Loss of righting reflex, the only reliable indicator of imminent death that we have observed with *E. coqui*, would have triggered euthanasia to minimize suffering, but this did not occur in our study. At the

conclusion of the experiment, we euthanized adult and juvenile frogs by immersion in 300 mg/L tricaine mesylate (MS-222) and stored frogs in 70% ethanol. We did not necropsy control or pathogen-exposed animals because we chose to bank the specimens for future investigation of Bd load.

## RESULTS

Juvenile coquis exposed to Bd had significantly lower survival than the control group (log-rank test $\chi^2=9.58$; $P=0.002$; Fig. 1a). Only 10% (5/51) of juveniles exposed to Bd survived the 77-day experiment, compared with 35% (19/54) in the control group. There was no overall difference in survival between juveniles descending from Puerto Rican or Hawaiian parents (log-rank $\chi^2=0.21$; $P=0.65$), but within the Bd-exposed group, juveniles of Hawaiian descent had a lower survival rate than juveniles of Puerto Rican decent (log-rank $\chi^2=4.8$; $P=0.023$).

Analysis of skin swabs indicated that 80% (37/46) of juveniles that died in the exposure group were infected with Bd. Clinical signs of chytridiomycosis were not obvious in juveniles, and there were no discernible differences in behavior between exposed and control groups. However, there is little published information on normal behavior of very young juveniles, and adult frogs that die with high Bd loads often show no outward signs of chytridiomycosis (Langhammer et al. 2013; P.F.L unpubl. data). In another study, coquis that died with high Bd loads showed signs of chytridiomycosis upon histologic examination (Longo et al. 2013). Of the five Bd-exposed juveniles that survived the experiment, only one was Bd positive. Three of those animals were infected on day 20, indicating that they had reduced their infections beyond detectable levels by day 77. None of the control juveniles was Bd positive upon death, or at the end of the experiment, confirming no cross contamination.

In contrast to the juveniles, there was no significant difference in survival rate between Bd-exposed and control adult frogs (log-rank $\chi^2=3.4$; $P=0.06$; Fig. 1b), and overall survival across groups was high. There was no difference in survival between adult frogs of Puerto Rican or Hawaiian origin (log-rank $\chi^2=1.5$; $P=0.22$). One adult frog in the previously infected $10^5$ zoospores/mL treatment group died with mild signs of chytridiomycosis, including lethargy and poor appetite. Two control frogs died for unknown reasons.

All but one inoculated adult frog was Bd positive following inoculation (32/33, 97%). Average infection intensity of adult

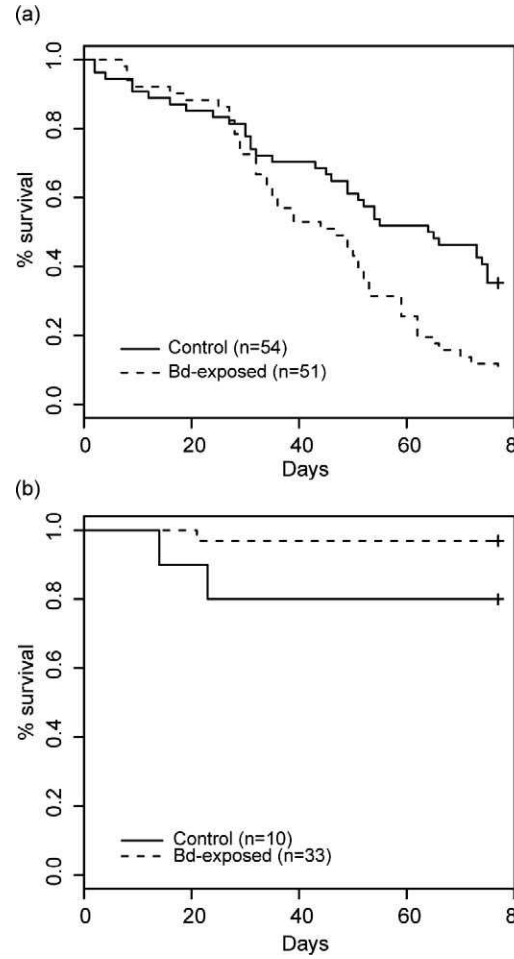


FIGURE 1. Coquis (*Eleutherodactylus coqui*) survival (a) juveniles ($n=105$) and (b) adults ($n=43$) exposed to *Batrachochytrium dendrobatidis* (Bd) strain JEL427 or a sham (control) solution.

frogs on day 9, the first sampling date following inoculation, was 1,336±341 zoospore genomic equivalents. Both average infection intensity and prevalence fell sharply after day 9 (Fig. 2). All control adult frogs were Bd negative at each sampling date analyzed.

## DISCUSSION

A field survey of juvenile and adult coquis in eastern Puerto Rico showed that Bd prevalence and mean infection intensity were greater among juveniles than adults (Longo and Burrowes 2010). Our study corroborates their findings and demonstrates experimentally that juveniles are not only more susceptible to Bd infection but also more likely to die following infection. Juveniles exposed to Bd had a much lower rate of survival than control froglets.

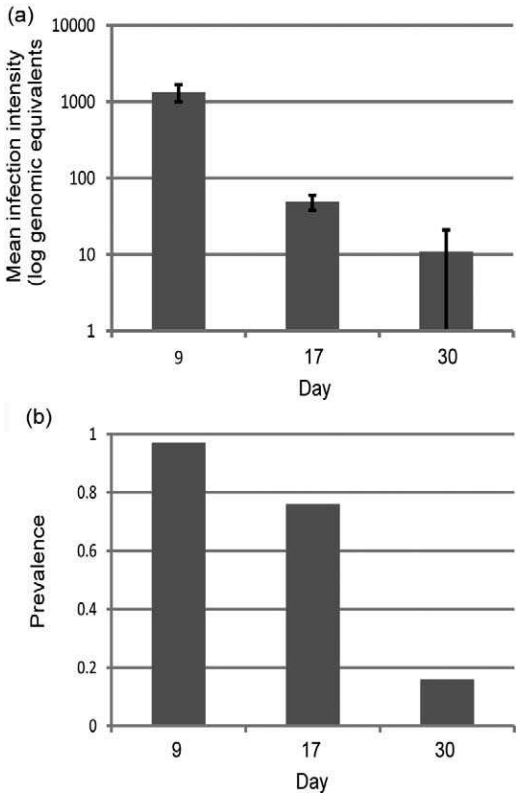


FIGURE 2. (a) Mean *Batrachochytrium dendrobatidis* (Bd) infection intensity and (b) prevalence over time of adult coquis (*Eleutherodactylus coqui*) exposed to Bd strain JEL427. Data for the three groups exposed to Bd are combined. Error bars represent the standard error.

In contrast, adult coquis in our three Bd-exposure treatment groups had survival rates indistinguishable from control frogs. Exposure to a higher Bd dose and prior infection with Bd did not affect survival, and most frogs cleared infections or reduced them to low levels. This finding aligns with other research showing that adult coquis can survive and clear infection when exposed to Bd under normal climate conditions (Longo et al. 2010; Langhammer et al. 2013) and the observation that adult frogs can eliminate natural Bd infections within a few weeks of arriving in the lab (P.F.L unpubl. data).

However, that one adult frog died with mild signs of chytridiomycosis in this study suggests that the ability to clear infection varies among individuals (Tobler and Schmidt 2010). Analysis of capture-mark-recapture data showed that the proportion of recaptured adult frogs was lower for infected adult coquis than for uninfected frogs in the Palo Colorado Forest of eastern Puerto Rico (Longo and Burrowes 2010). Although populations of *E. coqui* persist in the wild with mild Bd infections, some individuals die from chytridiomycosis (Longo et al. 2013). Vulnerability of adult coquis to Bd is thought to be affected by seasonal climatic variables, specifically, a reduced ability to resist the pathogen in cooler, drier conditions or increased transmission in a limited number of humid retreat sites (Longo et al. 2010).

Previous work with two species of *Anaxyrus* toadlets showed that size was an important predictor of survival time following Bd infection, with larger individuals surviving longer (Carey et al. 2006; Garner et al. 2009). Thus, the high susceptibility of froglets that we observed may have been due to their small size (and greater surface area to volume) or to their immature immune systems, as suggested

by Longo and Burrowes (2010). Also, the fact that nearly all Bd-exposed froglets, regardless of size, died in our study indicates that froglets younger than 21 wk are highly vulnerable to Bd. It is unclear how froglets older than 21 wk would respond to Bd. Future work to tease apart size and ontogenetic immune function will require a larger sample of froglets of a given age and locality that vary in size naturally or due to diet manipulation. In addition, models of host-pathogen interactions for Bd and amphibians must account for the possibility of ontogenetic variation in Bd susceptibility.

In our study, coquis were exposed twice to high doses of Bd, were maintained at temperatures favorable to Bd (Piotrowski et al. 2004), and had opportunities for indirect reinfection via zoospores shed onto damp terraria substrate. The rapid clearing of infection in adult frogs suggests a competent immune response that may not characterize juvenile frogs (Rollins-Smith 1998). It is possible that the adults had previously been exposed to Bd in the wild despite being negative upon collection, while the juveniles were encountering a high dose of Bd for the first time. However, recent work shows that Bd can evade the frog immune system by disrupting lymphocyte production, at least in the model amphibian *X. laevis* (Fites et al. 2013). Research is underway (by P.A.B.) to study potential ecoimmunologic mechanisms of coquis, including symbiotic anti-Bd bacteria on the skin (Harris et al. 2006), which could provide insight into how adult coquis clear infection. It is unknown when the immune system of direct-developing frogs matures.

Our study represents an important step toward understanding the ontogenetic susceptibility of direct-developing frogs to Bd, and the results have important ecologic and conservation implications. If froglets are susceptible to lethal Bd infection in the field, or if there is a fitness cost to Bd infection (Burrowes et al. 2008), then the decline of *E. coqui* populations reported by Burrowes et al. (2004) could be a function of reduced recruitment of juveniles despite adult frogs showing resistance (Longo and Burrowes 2010). This possibility is particularly worrying for the eight species of *Eleutherodactylus* occurring in Puerto Rico that are globally threatened with extinction (International Union for Conservation of Nature 2012), assuming they share a similar ontogenetic response to the pathogen.

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