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Differential patterns of *Batrachochytrium dendrobatidis* infection in relict amphibian populations following severe disease-associated declines

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ABSTRACT: Global amphibian biodiversity has declined dramatically in the past 4 decades, and many amphibian species have declined to near extinction as a result of emergence of the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*). However, persistent or recovering populations of several amphibian species have recently been rediscovered, and such populations may illustrate how amphibian species that are highly susceptible to chytridiomycosis may survive in the presence of *Bd*. We conducted field surveys for *Bd* infection in 7 species of Costa Rican amphibians (all species that have declined to near extinction but for which isolated populations persist) to characterize infection profiles in highly *Bd*-susceptible amphibians post-decline. We found highly variable patterns in infection, with some species showing low prevalence (~10%) and low infection intensity and others showing high infection prevalence (>80%) and either low or high infection intensity. Across sites, infection rates were negatively associated with mean annual precipitation, and infection intensity across sites was negatively associated with mean average temperatures. Our results illustrate that even the most *Bd*-susceptible amphibians can persist in *Bd*-enzootic ecosystems, and that multiple ecological or evolutionary mechanisms likely exist for host–pathogen co-existence between *Bd* and the most *Bd*-susceptible amphibian species. Continued monitoring of these populations is necessary to evaluate population trends (continuing decline, stability, or population growth). These results should inform efforts to mitigate impacts of *Bd* on amphibians in the field.

KEY WORDS: Chytrid fungus · Chytridiomycosis · *Craugastor* · *Lithobates* · *Incilius* · *Agalychnis* · *Ptychohyla* · Tropical frog · Infection rate · Population monitoring · Costa Rica

INTRODUCTION

Amphibians are facing unprecedented rates of biodiversity loss: 31.6% of amphibian species are threatened with extinction, and 59.1% of amphibian species with known population trajectories are experiencing population declines (IUCN 2016). The emerging infectious disease chytridiomycosis, caused by the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (hereafter, *Bd*), has been associated with amphibian population declines on several continents (Berger et al. 1998, Kilpatrick et al. 2010), and along

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with habitat loss is a major driver of amphibian population declines. The emergence of *Bd* has driven mass mortality events, widespread population extirpations, and severe range contractions in *Bd*-susceptible amphibian species (Lips et al. 2008, Smith et al. 2009, James et al. 2015, Whitfield et al. 2016).

Many species of amphibians were presumed extinct after the emergence of *Bd* (Stuart et al. 2004, Skerratt et al. 2007, Alroy 2015), yet isolated populations have been rediscovered years or decades after mass mortality events that caused extirpations of all known populations of their species (Puschendorf et al. 2005, 2013, Rodríguez-Contreras et al. 2008, Abarca et al. 2010, González-Maya et al. 2013, Chaves et al. 2014). Such populations are of high conservation value as the last living representatives of their species, and are also of high scientific value, as they may help identify mechanisms that allow the most *Bd*-threatened amphibian species to persist in the field following emergence of *Bd* (Woodhams et al. 2012, Bletz et al. 2013, Scheele et al. 2014). Several specific mechanisms have been proposed that may explain persistence of relict populations: evolved immunity (Savage & Zamudio 2011, Savage et al. 2015), protection via the amphibian microbiome (Bletz et al. 2013, Woodhams et al. 2014, Madison et al. 2017), or environmental refugia (Puschendorf et al. 2011, 2013). However, few studies have examined host–pathogen interactions in relict amphibian populations, in part because the inherent rarity of relict populations makes sampling efforts difficult.

While some species may persist (have stable or slowly declining populations), other species that declined locally or regionally with the emergence of *Bd* are showing population growth or range expansion (Sapsford et al. 2015, Scheele et al. 2017). Recovery following decline may suggest that ecological or evolutionary shifts in disease dynamics, at least locally, may allow for persistence of hosts and pathogens in an enzootic environment where co-existence was not possible during an epizootic phase. Recovery following *Bd*-associated decline is apparently widespread (Castro-Cruz & Garcia-Fernandez 2012, Whitfield et al. 2016), though few studies have examined host–pathogen interactions in recovering amphibian populations (Sapsford et al. 2015).

Here, we report the results of pathogen surveys among 7 species of highly *Bd*-susceptible amphibians that persisted or appear to be recovering following the emergence of *Bd*, in order to help identify possible mechanisms for host–pathogen co-existence. If species persist in environmental refugia outside the environmental tolerances of *Bd*, we would expect extremely low infection prevalence and low levels of infection intensity, but only for populations in environments hostile to *Bd* (i.e. warm, dry climates). If species show resistance to infection, we expect that rates of infection prevalence and infection intensity would both be low. If species show tolerance to infection, we would expect high rates of infection and possibly high infection intensity, yet expect no apparent signs of disease.

MATERIALS AND METHODS

Species and sampling sites

We sampled 7 populations of amphibians in Costa Rica, each from a different species and site (Fig. 1). We conducted field sampling between March and November 2012, corresponding to the season of peak detectability for each species. Sites ranged across elevations (<10 to 2100 m a.s.l.) and ranged across temperature and precipitation regimes typical of the diverse climates of Costa Rica (Kappelle 2016). We sampled each species over a ≤3 d period. Because each species in this study has a distinct history of population status and disease, we describe each species briefly here.

Craugastor ranoides (IUCN status: Critically Endangered) is a stream-inhabiting, direct developing frog that formerly ranged up to 1300 m on the Pacific

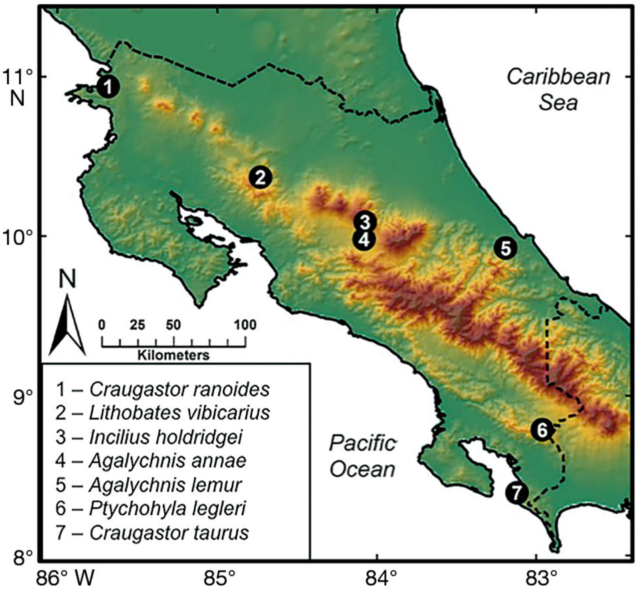

Fig. 1. Each of the 7 relict amphibian populations sampled in this study was from a different site and climate. Sampling sites in this study ranged across elevations, precipitation regimes, and major ecosystems of Costa Rica (see Table 1)

versant of Costa Rica and up to 116 m on the Caribbean versant. This species suffered severe chytridiomycosis-related declines in the 1980s and 1990s, and was presumed extinct until several relict populations were re-encountered in the tropical dry forest of Santa Elena Peninsula, Guanacaste (Puschendorf et al. 2005, Zumbado-Ulate & Willink 2011). To our knowledge, none of the surviving populations was surveyed prior to the emergence of *Bd* in the 1980s or 1990s. Our sampling site (the warm, xeric habitats in the Area de Conservacion Guanacaste in northwestern Costa Rica) has been proposed as an environmental refuge from *Bd* (Puschendorf et al. 2005, Zumbado-Ulate et al. 2011).

Lithobates vibicarius (IUCN status: Vulnerable) is a montane ranid frog that was formerly common throughout the montane forests of Costa Rica's Tilarán, Central, and Talamanca mountain ranges (Savage 2002), but known Tilarán populations had disappeared by 1990 (Pounds et al. 1997, Puschendorf et al. 2006) and the last known Talamanca population had disappeared by 1996 (Lips 1998). These dates coincide with the arrival of *Bd* to each site (Lips et al. 2008, Rohr et al. 2008). This species was subsequently listed as Critically Endangered and possibly extinct by the IUCN. The species was reencountered on a remote, previously unmonitored ridge in the Children's Eternal Rainforest, Monteverde, in 2002. Additional populations have since been found elsewhere in the Children's Eternal Rainforest, in Parque Nacional del Agua Juan Castro Blanco, on private properties in the vicinity of Zarcero, in the Monteverde Cloud Forest Preserve (Castro-Cruz & García-Fernández 2012, M. Wainwright pers. obs.), and in the Reserva Biologica Alberto Manuel Brenes, San Ramon, Alajuela (Morera-Chacón & Sanchez Porras 2015).

Incilius holdridgei (IUCN status: Critically Endangered) is a range-restricted endemic toad known from a very small number of sites on Volcan Barva in Parque Nacional Braulio Carrillo. All known populations (or the single metapopulation) of this species disappeared after the arrival of *Bd* to the site in 1986 (Abarca et al. 2010). Repeated survey efforts in the region failed to detect any individuals of this species, and in 2008 the species was listed by the IUCN as Extinct. However, a single surviving population has since been rediscovered (Abarca et al. 2010).

Agalychnis annae (IUCN status: Endangered) is a phyllomedusine treefrog that was formerly common throughout montane forests and agricultural regions of Costa Rica's Tilarán, Central, and Talamanca mountain ranges. The few remaining populations are found in highly disturbed sites in the Valle Central, which have been proposed to persist in contaminant-mediated refuges (Hoffmann 1995). Our study site, in suburban Santo Domingo (Heredia Province) was a translocation from a persisting population following *Bd* emergence (Hoffmann 1995).

Agalychnis lemur (IUCN status: Critically Endangered) is a phyllomedusine treefrog that was formerly common in montane forests of Costa Rica's Tilarán, Central, and Talamanca mountain ranges. At present only a few relict populations remain, in the Caribbean foothills of Costa Rica's Talamanca mountain range and in Panama (Hertz et al. 2012). To our knowledge, none of the remaining populations were in sites that were surveyed prior to *Bd* emergence.

Ptychohyla legleri (IUCN status: Endangered) is a stream-inhabiting hylid frog that inhabits mid-elevation forests of the Pacific versant of Costa Rica and Western Panama. Most populations of this species declined following the emergence of *Bd* in Central America, yet 2 populations persist in Costa Rica (García-Rodríguez et al. 2012) and one in Panama (Hertz et al. 2012). To our knowledge, *P. legleri* was not detected at our study site (Las Cruces Biological Station) before 2003, although there were some biological surveys at this site (S. M. Whitfield pers. obs.).

Craugastor taurus (IUCN status: Critically Endangered) is a stream-inhabiting direct developing frog that historically ranged from Central to South Pacific Area, with highest abundance in the Osa Peninsula and Golfito (Savage 2002). By the early 1990s, all known populations had been lost in Costa Rica, and the species was presumed extinct. However, several new populations were found in December 2011 in the dry lowlands of Punta Banco in Golfito, southwest Costa Rica, which represented an expansion of the historical distribution (Chaves et al. 2014).

Field sampling and pathogen detection

We systematically searched for amphibians at sites where known persistent populations occurred. Search intensity, time spent searching, number of observers, and area covered varied strongly for each species, as population densities, detectability of individuals, and complexity of habitats varied markedly among species and sites. For stream-associated species (*P. legleri*, *C. ranoides*, and *C. taurus*), we used nocturnal transects of variable lengths along streams. For pond-breeding species, we searched (stream) water margins and surrounding vegetation, either during the day (for *L. vibicarius*) or at night (for *A. annae* and *A. lemur*). For the fossorial species *I. holdridgei*, we conducted diurnal searches in and under leaf litter up to a depth of ~15 cm. We captured each encountered individual using clean, unused plastic bags and swabbed each individual systematically with a cotton applicator (MW-100 cotton-tipped swab; hereafter, 'swab'). We used a quantitative swabbing technique in which we passed the swabs 10 times each along the dorsum, venter, each side of the body, and length of each femur. We swabbed 5 times across each hand and foot. Swabs were stored dry and held at −40°C until DNA extraction and quantitative PCR.

We extracted nucleic acids from swabs using standard protocols from DNEasy Blood and Tissue Kits (Qiagen). We estimated pathogen DNA using published quantitative PCR methods (Kerby et al. 2013). In summary, we used *Bd* primers and probes (Boyle et al. 2004) with 2× TaqMan Master Mix in 10 µl reactions containing 3 µl of DNA template. We ran all reactions in triplicate and with a negative control and set of standards on each plate. Due to variation in number of sequence copies across *Bd* strains (Longo et al. 2013), we used gBlocks (IDT) for standard quantification providing numbers of sequence copies (hereafter, 'SCs') to estimate infection levels.

Data analysis

To compare infection prevalence among species we used generalized linear models with binomial response variables (infected or not infected) and used species as a predictor variable. To compare infection intensity among species we used general linear models using data only from infected individuals, and used species as a predictor and log-transformed *Bd* load (estimated number of sequence copies) as a response variable. To evaluate climatic associations of infection prevalence, we used a generalized linear model with infection prevalence as a response variable and annual mean precipitation and annual mean temperature derived from BIOCLIM (Hijmans et al. 2005) as predictor variables. To evaluate climatic associations with infection intensity, we used a generalized linear model with log-transformed number of sequence copies as a response variable and mean annual precipitation and temperature from BIOCLIM as predictor variables. All analyses were conducted in R v.3.3.1 (R Development Core Team 2015).

RESULTS

Prevalence of infection varied greatly among species (df = 6,122, deviance = 94.666, residual deviance = 169.855, p < 0.0001; Table 1, Fig. 2). No individual of *Incilius holdridgei* was infected, and only 10% of individuals of *Agalychnis lemur* were infected with *Bd*. Two species showed intermediate infection prevalence: *Lithobates vibicarius* (31.8% infected) and *Craugastor ranoides* (60% infected). Finally, a high percentage (≥80% of individuals) of *Agalychnis annae*, *Ptychohyla legleri*, and *Craugastor taurus* were infected with *Bd*. There was a negative relationship between infection prevalence and mean annual precipitation (df = 1,122, deviance = 65.922, residual deviance = 169.855, p = 0.0034), yet there was no relationship between infection prevalence and temperature (df = 1,122, deviance = 0.702, residual deviance = 169.855, p = 0.402).

Infection intensity also varied considerably among species ($F_{5,62}$ = 3.1889, MS = 19.64, p = 0.0126). Average infection intensities were below 100 SCs in *C. taurus*, *A. lemur*, and *P. legleri*, ranged between 100 and 1000 SCs in *C. ranoides* and *A. annae*, yet averaged 7900 SCs in *L. vibicarius*, though this was largely driven by a single individual with an infection load of >50 000. The distribution of individuals among

Table 1. Species, sample sizes, environmental data, and infection information for 7 populations of relict and recovering amphibian populations in Costa Rica. Infection intensity data derived from infected individuals only; SCs: sequence copies

Species	n	Sampling site data (mean) Annual temp. (°C)	 Annual precip. (mm)	 Elevation (m a.s.l.)	Prevalence (%) Mean (95% CI)	Infection intensity (SCs) Mean ± SD
Craugastor ranoides	20	26.5	1655	100	60.0 (36.1–80.8)	122.00 ± 250.7
Lithobates vibicarius	22	17.9	3198	1760	31.8 (13.9–54.9)	7903.34 ± 20736.0
Incilius holdridgei	7	15.0	2718	2280	0.0 (0.0–40.9)	–
Agalychnis annae	20	20.2	2034	1170	90.0 (68.3–98.7)	532.20 ± 1244.8
Agalychnis lemur	20	24.5	3478	300	10.0 (1.2–31.7)	5.46 ± 2.57
Ptychohyla legleri	14	20.6	3359	1200	92.9 (66.1–99.8)	53.34 ± 94.8
Craugastor taurus	20	26.1	3163	20	80.0 (56.3–94.3)	60.67 ± 120.5

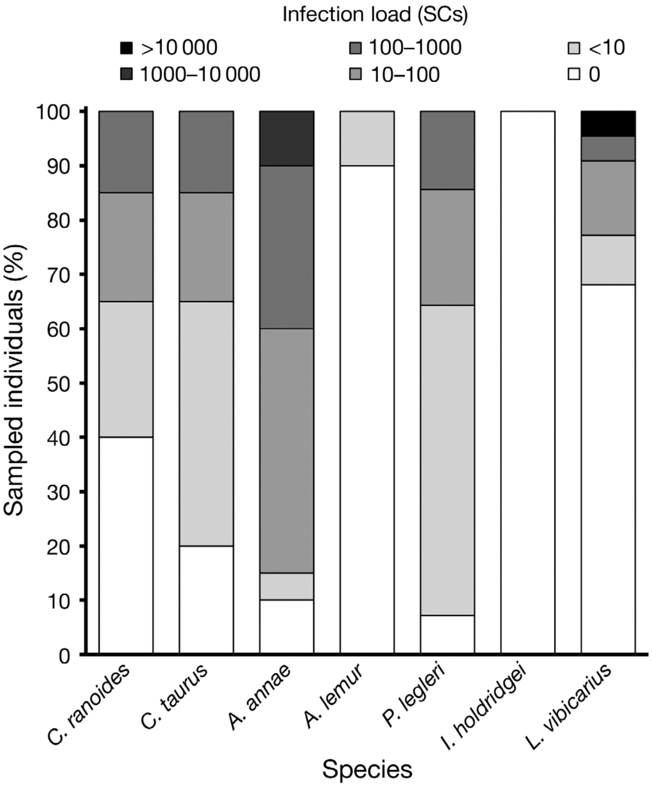

Fig. 2. Results of pathogen surveys among 7 species of amphibians persisting after or recovering following severe *Batrachochytrium dendrobatidis*-associated declines. Infection prevalence and infection intensity (as sequence copies, SCs) are highly variable among species

classes also varied considerably among species (Fig. 2). There was no relationship between infection intensity and precipitation ($F_{1,62}$ = 2.7022, MS = 16.639, p = 0.105; Fig. 3C), but infection intensity was negatively correlated with temperature ($F_{1,62}$ = 11.9744, MS = 73.731, p < 0.001; Fig. 3D).

DISCUSSION

We showed that infection profiles of amphibian populations persisting or recovering following severe chytridiomycosis-associated declines differed strongly among species. Some species (such as *Agalychnis lemur* and potentially *Incilius holdridgei*) showed low infection prevalence (<10%) and infected individuals showed low infection intensity, while other species (*Ptychohyla legleri*, *Agalychnis annae*, *Craugastor taurus*) showed high infection prevalence. While high precipitation among our sites appeared to be linked to high infection prevalence, cool temperatures were linked to high infection intensities.

We found *Bd* infections in all species with the possible exception of *I. holdridgei.* This indicates that while all of the species in our study suffered massive population declines during the *Bd* epizootic stage, all populations now persist or are recovering in the presence of enzootic *Bd*. Our small sample size for *I. holdridgei* results in a large confidence interval for infection estimates for that species. The climatic and environmental conditions at the site where *I. holdridgei* occurs appear suitable for the presence of *Bd*, and it is therefore likely that the apparent absence of infection in *I. holdridgei* is not attributable to an absence of *Bd* at the site, but rather to limited sampling. This species is cryptic and fossorial, and low detection probability is at least partially attributable to its apparent extinction after the emergence of *Bd* in Costa Rica.

We found that sites with higher temperatures showed lower infection intensity than cooler sites, confirming well-established associations between environmental temperatures and the dynamics of chytridiomycosis (Woodhams et al. 2003, Kriger & Hero 2008, Whitfield et al. 2012, Nowakowski et al. 2016). However, we found no relationship between infection prevalence and temperature. We found that more humid sites had lower infection prevalence, counter to expectations, and we found that precipitation regimes had no apparent impact on infection intensity. In any case, because we only sampled one species at each site, any climate trends presented here should be viewed cautiously as they are confounded by species-level effects of disease susceptibility. Community-level evaluations of *Bd* prevalence would be better for establishing climate relations.

For most of the species sampled in our study, there are no data on infection prevalence or intensity by *Bd* either before or after disease-associated declines. Chaves et al. (2014) reported prevalence of 80% (12 of 15 sampled individuals) for *C. taurus*, identical to the 80% prevalence reported herein. Zumbado-Ulate et al. (2014) reported low infection prevalence in *Craugastor ranoides* from 2 populations (0.1 and 9%), which are at odds with the results we report here. While Zumbado-Ulate et al. (2014) had a much larger sample size, our samples were recorded in March, while their study was sampled over a long period between December and July 2007 and between December and July 2008 — thus, potentially seasonal dynamics play a role in this system as in other Costa Rican lowland sites (Whitfield et al. 2012). We are unaware of any previous reports of infection prevalence or intensity for *A. annae*, *A. lemur*, *Lithobates vibicarius*, *P. legleri*, or *I. holdridgei*.

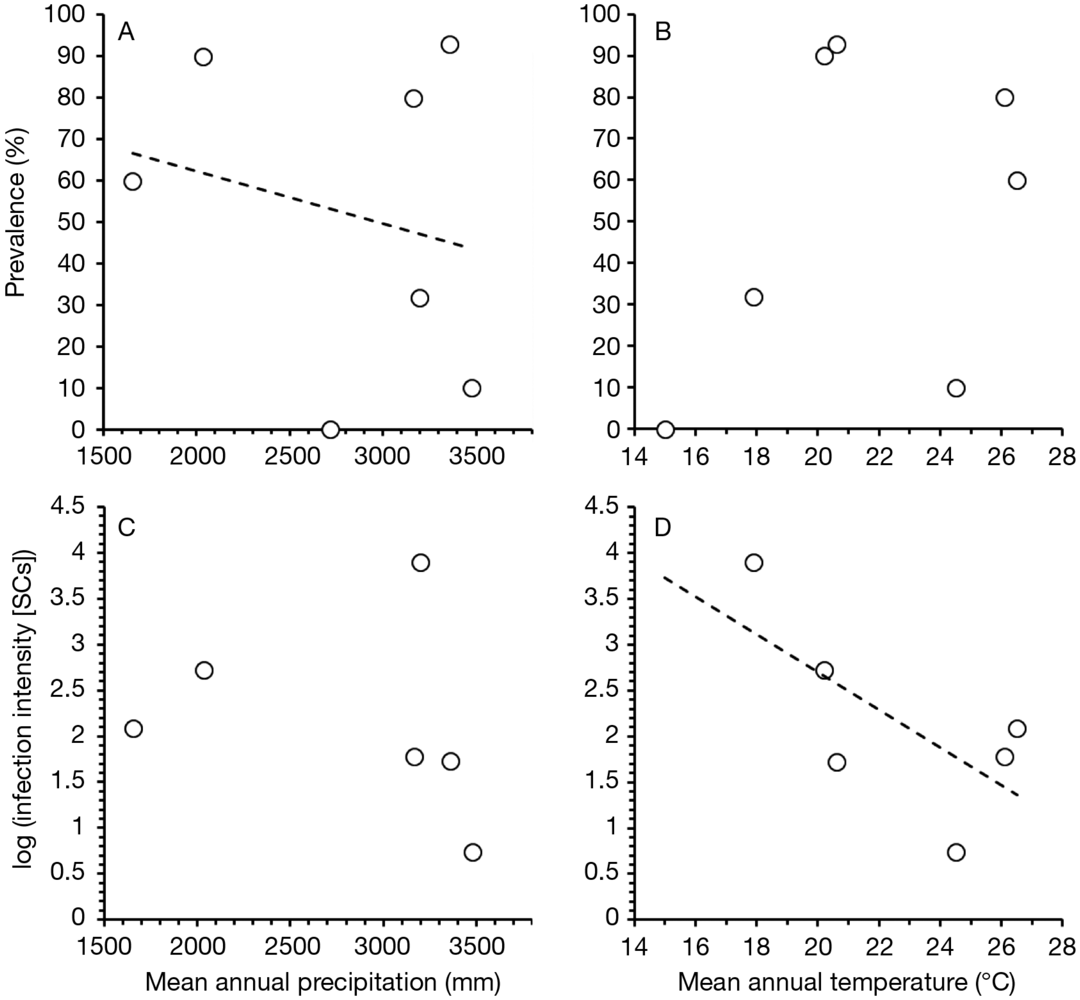

Fig. 3. Associations between climatic variables and *Batrachochytrium dendrobatidis* infection metrics: (A) negative association between infection prevalence and mean annual precipitation; (B) no relationship between prevalence and mean annual temperature; (C) no relationship between infection intensity and mean annual precipitation; and (D) negative relationship between mean annual temperature and infection intensity. Trend lines are shown when there are statistically significant relationships between variables. SCs: sequence copies

Neither the persistence of presumed extinct species nor the recovery of species that had been extirpated following the emergence of *Bd* have been well studied, and few data on *Bd* prevalence or intensity have been published on such populations. Still, the few studies of host–*Bd* interactions following dramatic declines also point to multiple ecological or evolutionary mechanisms in host–pathogen co-existence. In some instances, individuals of severely-impacted species whose populations declined dramatically persist only in sites where *Bd* is absent (Stockwell et al. 2015), and in other cases, highly *Bd*-susceptible amphibians persist in environments hostile to *Bd*, but where *Bd* is still present (Puschendorf et al. 2011). In *Atelopus cruciger* in Venezuela (a species which declined to near extinction following emergence of *Bd*), infection by *Bd* causes mortality, yet the population size remains stable (Lampo & Celsa 2012). Similar observations were made in a study of *Litoria rheocola* in northeastern Australia, a species that declined in upland sites but persisted in lowland sites and subsequently recolonized upland sites. Individuals of this species infected with *Bd* died at higher rates than uninfected individuals, yet *Bd* infection was not found to have any significant effect on the species' survival rate at the population level (Sapsford et al. 2015).

While persistence and recovery of amphibian populations following emergence of *Bd* is encouraging, there are substantial concerns about the long-term viability of these populations. There are no quantitative estimates of population size for any of these species, and population sizes for some species may be under 100 adults (Abarca et al. 2010), very close to extinction and highly susceptible to re-emergence of *Bd* or other stressors. There are also no quantitate data on population trajectory for these species (continuing decline, stable, increasing), though with the exception of *A. lemur*, individuals in each population were detected in 2016. Logging nearby the site occupied by *A. lemur* may have altered the breeding pond or terrestrial habitat for this population, and we have not returned to this site since the sampling in this study. In addition to threats from habitat modification, the presence and possible emergence of ranavirus in Central America (Whitfield et al. 2013, 2016, Stark et al. 2014) may be an understudied threat to amphibian populations throughout the region. For at least 2 of the populations we examined (*C. taurus* and *A. annae*), populations persist in human-impacted areas rather than in protected areas. Only 2 of the species we examined (*A. annae* and *A. lemur*) exist in captive assurance programs.

It remains unclear how these populations have persisted with *Bd* when all other populations of their species declined to extinction, or how recolonization is possible in sites where extirpation from *Bd* has historically occurred. Several hypotheses have been presented to explain the persistence of highly *Bd*-susceptible species post-decline, including *Bd*-free refugia (Puschendorf et al. 2011, García-Rodríguez et al. 2012), adaptive tolerance (Savage & Zamudio 2011), tolerance or resistance via microbial protection (Bletz et al. 2013, Madison et al. 2017), or reduced pathogen virulence (Voyles et al. 2009). Here, we can eliminate the hypotheses that *Bd* is not present in 6 of 7 sites. This includes 2 sites that were plausible environmental refugia inhospitable to *Bd*, though environmental refugia could limit *Bd* growth or transmission rather than prevent its occurrence entirely. Further, we can eliminate the hypothesis that surviving amphibians are now completely capable of preventing infections in these same 6 populations.

The results of our study should be informative for efforts to mitigate *Bd*. For example, one recent and intensive *Bd* mitigation study eliminated *Bd* from Mallorca at great investment of time and resources (Bosch et al. 2015). Our research shows that surviving frog populations can co-exist with *Bd*. Perhaps efforts to engineer resistance to infection (through protection via antimicrobial peptides or the microbiome) may focus on understanding how *A. annae* is able to persist with mild infections, while efforts to engineer tolerance (ability to withstand infections) may focus on *L. vibicarius*. Ultimately, while the exact ecological or evolutionary mechanisms remain unclear, the results of this study show that even the most *Bd*-susceptible amphibian species can (for some set of ecological conditions) persist and recover in areas where *Bd* is now enzootic.

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