

Disassembly of a tadpole community by a multi-host fungal pathogen with limited evidence of recovery

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Abstract. Emerging infectious diseases can cause host community disassembly, but the mechanisms driving the order of species declines and extirpations following a disease outbreak are unclear. We documented the community disassembly of a Neotropical tadpole community during a chytridiomycosis outbreak, triggered by the generalist fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*). Within the first 11 months of *Bd* arrival, tadpole density and occupancy rapidly declined. Species rarity, in terms of tadpole occupancy and adult relative abundance, did not predict the odds of tadpole occupancy declines. But species losses were taxonomically selective, with glassfrogs (Family: Centrolenidae) disappearing the fastest and tree frogs (Family: Hylidae) and dart-poison frogs (Family: Dendrobatidae) remaining the longest. We detected biotic homogenization of tadpole communities, with post-decline communities resembling one another more strongly than pre-decline communities. The entire tadpole community was extirpated within 22 months following *Bd* arrival, and we found limited signs of recovery within 10 years post-outbreak. Because of imperfect species detection inherent to sampling species-rich tropical communities and the difficulty of devising a single study design protocol to sample physically complex tropical habitats, we used simulations to provide recommendations for future surveys to adequately sample diverse Neotropical communities. Our unique data set on tadpole community composition before and after *Bd* arrival is a valuable baseline for assessing amphibian recovery. Our results are of direct relevance to conservation managers and community ecologists interested in understanding the timing, magnitude, and consequences of disease outbreaks as emerging infectious diseases spread globally.

Key words: amphibians; *Batrachochytrium dendrobatidis*; disease; extinction; Neotropics; Panama; site-occupancy model.

INTRODUCTION

Emerging infectious diseases can cause community disassembly (Zavaleta et al. 2009, Fisher et al. 2012), defined as the predictable loss of species and population declines. During community disassembly, the first species extirpated are generally rare species: species with small geographic ranges, small population size, or a narrow habitat tolerance (Rabinowitz 1981, Larsen et al. 2005, Gehring et al. 2014, Rader et al. 2014). Subsequent losses tend to include common, generalist species that have declined since the initial disturbance (e.g., Wright et al. 2007, Larsen et al. 2008). The last remaining species reduce patterns of community turnover, increasing biotic homogenization (McKinney and Lockwood 1999).

In the case of tropical amphibian declines and extirpations caused by the fungal pathogen *Batrachochytrium*

dendrobatidis (hereafter *Bd*), many amphibian communities experience rapid, widespread population declines and species extirpations following pathogen arrival (Berger et al. 1998, Lips 1998, 1999, Lips et al. 2006). A species' susceptibility to *Bd* is correlated with several host characteristics (Lips et al. 2003). First, phylogeny can predict species susceptibility to *Bd*. For example, family-level amphibian phylogenies suggest that families with similar traits share the same vulnerabilities to threats (Corey and Waite 2008), but a species-level phylogenetic analysis on the amphibian fauna of El Copé, Panama showed no evidence that species with similar traits shared the same susceptibility to *Bd* (Crawford et al. 2010). The discrepancy between the results in each study are likely caused by differences in taxonomic, spatial, and temporal scales, where rapid, widespread, amphibian losses produced no species-level phylogenetic variation to *Bd* susceptibility in El Copé, Panama. Second, species ecology (i.e., traits) and rarity (i.e., abundance, geographic distribution, and habitat specialization; Rabinowitz 1981) affects a host's vulnerability to disturbance-related

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declines (Lips et al. 2003, Rachowicz and Briggs 2007, Briggs et al. 2010). For example, species with small geographic distributions experience larger *Bd*-related occupancy declines than widespread species (Smith et al. 2009). A species' vulnerability to *Bd*-related declines can be a product of either environmental or demographic stochasticity (Lande 1993) or species-specific traits that predispose them to disease (Lips et al. 2003). Host density can have the opposite effect, where higher host densities increase the number of infections, making population declines worse (e.g., Rachowicz and Briggs 2007, Briggs et al. 2010). For example, density-dependent *Bd* pathogen transmission of *Rana muscosa* has led to species extirpations and population declines across its range. Given that species' *Bd* susceptibility is correlated to these variables, we expect that the community disassembly of tadpoles may be correlated to phylogeny, ecological traits, and/or rarity.

Distinguishing between ecological rarity (i.e., low density, low occupancy, habitat specialization) and observational rarity (i.e., cryptic, fossorial, secretive) of hard-to-find species is largely overlooked in community disassembly studies. For instance, in a Neotropical forest, colorful dendrobatid amphibians are much easier to find than cryptic species of the genus *Eleutherodactylus* (Duellman and Trued 1986). If imperfect detection is not accounted for, species occupancy (i.e., the proportion of sites where the species is present) will be underestimated (i.e., MacKenzie et al. 2006, Kéry 2010). By underestimating species occupancy pre- and post- outbreaks, population occupancy declines will be overestimated and extirpations will be biased towards difficult to find species, leading to false inference with regards to the drivers of occupancy declines.

Here, we describe the disassembly of a stream-dwelling tadpole community in response to a *Bd* outbreak, while taking into account imperfect species detection. In this system, stream tadpoles occupy semi-isolated microhabitats (e.g., leaf packs, isolated pools, and in-stream pools and riffles) that allow for the consistent quantification of tadpole occupancy, density, and species richness. Prior to the arrival of *Bd*, these tadpole assemblages were diverse (McDiarmid and Altig 1999, Crawford et al. 2010), abundant (McDiarmid and Altig 1999), and structured spatially (Inger et al. 1986) and temporally (Heyer 1976), creating an opportunity to compare several species characteristics simultaneously that have been shown to contribute to species losses caused by disease. We address the following questions in this study: (1) What are the patterns of community disassembly following an outbreak? (2) What factors correlate to the order of species losses? (3) And how can we improve the sampling of species rich communities when species' detection is variable and imperfect?

We expected that tadpole occupancy would decline following the mass mortality of adult amphibians in El Copé, Panama (Lips et al. 2006), and that the magnitude of tadpole occupancy declines would depend on their

microhabitat use and the season of survey. Like most other community disassembly studies, we predicted that rare species and relatives that share similar traits would be extirpated first. We predicted that the tadpole communities that remained following the *Bd* outbreak would be more similar in species composition, and would be mainly comprised of common, generalist species. Finally, we expected that *Bd* arrival would cause rapid changes to the tadpole community that would persist several years post-invasion. Our results are relevant to conservation managers trying to understand and predict community disassembly following outbreaks, especially as generalist fungal pathogens spread globally.

MATERIALS AND METHODS

Study site

The study site was located within Parque Nacional G. D. Omar Torrijos Herrera in Coclé Province, approximately 8 km north of the town of El Copé, Panama (8°40' N, 80°37'17" W; Lips et al. 2003). The park spans elevations between 500 and 1000 m, and our study sites are located at ~775 m elevation. This site experiences both a dry (December–April) and wet (May–November) season. Mean annual air temperature at the park during 2003–2005 ranged from 16° to 23°C, and mean annual rainfall was ~3709 mm (McCaffery and Lips 2013; *KRL unpublished data*).

Study system

Starting in 1998, we started monitoring adult amphibian populations in El Copé, Panama (Lips et al. 2006), and we were consistently capturing amphibians until September 2004 when *Bd* was first detected in El Copé. We started monitoring tadpole populations starting July 2003, 15 months prior to the September 2004 die-off. The El Copé amphibian fauna experienced rapid species losses and declines. We, therefore, expect minimal compensatory or evolutionary dynamics to interfere with community disassembly inference. This project is part of the larger Tropical Amphibians Declines in Streams (TADS) project to quantify the consequences of amphibian losses on ecosystem structure and function.

The original El Copé amphibian community consisted of 74 species (Lips et al. 2003, Crawford et al. 2010), of which ~22 had stream-dwelling tadpoles. The amphibian community was diverse with respect to life history (e.g., habitat use, reproductive mode), demography (e.g., survivorship, longevity), and ecology (e.g., clutch size, body size, dispersal distance). By 2008, only 44 species remained at low population densities (Crawford et al. 2010).

Field surveys

We surveyed tadpole communities in four 200-m stream transects: Loop, Silenciosa, Cascada, and Guabal

(see map in McCaffery and Lips 2013, Angeli et al. 2015). We mapped and measured the area covered by each of four microhabitats (riffle, pool, isolated pool, and leaf pack) at the beginning of the wet and dry seasons. We defined riffles as fast-flowing, shallow sections with gravel and cobble substrates, pools as areas of calm water deeper than 20 cm in the main channel, isolated pools as small, shallow pools spatially separated from the main stream channel, and leaf packs as detritus accumulations at the bottom of pools. We used a *k*-means clustering analysis to divide streams into segments that were repeatedly visited each month throughout the study. To determine the appropriate number of stream segments, we calculated the within group sum of squares by the number of clusters extracted and examined this plot for a bend, similar to a scree plot in factor analysis, indicating the minimum number of clusters. The *k*-means analysis divided each stream transect into four segments for a total of 16 stream sites per microhabitat. Each segment was sampled either once, twice, or three times per month using the random sampling method described below.

To sample riffles, we used 250- μ m D-nets and disturbed substrate with our feet while holding nets immediately downstream (Barbour et al. 1999). To sample leaf packs, we used a modified stovepipe benthic corer (22 cm diameter) with a base of rubberized flaps that kept the sampler sealed against rough and uneven substrates. We drove the corer into the substrate and searched through the contents for tadpoles (Colón-Gaud et al. 2010). We used a dip net to exhaustively sample pools and isolated pools until three consecutive scoops produced no tadpoles (Heyer et al. 1994, Ranvestel et al. 2004). We also measured the length, width, and depth of all microhabitats across each 200-m transect to account for variability in survey area.

We expected minimal differences in individual- and species-level detection probabilities within microhabitats because each microhabitat was searched until no new tadpoles were found. Within a given microhabitat, this guaranteed that although tadpole species vary in size and coloration (e.g., *Lithobates warszewitschii*, 115 mm, dark and heavily mottled [Villa 1990], *Espadarana prosoblepon*, 12.3 mm, bright red dorsal, and pale brown ventral [Savage 2002], species detection probability is close to 1.0; but within a stream segment, tadpole detection probability is less than 1.0 because, tadpoles are present in some microhabitat sites and not others. The variables that caused the largest difference in detection probability among tadpole species were likely to be differences in abundance and distribution across microhabitats, rather than within microhabitat differences.

For leaf packs, isolated pools, and riffles, we randomly sampled three microhabitat sites per stream each month for 15 months before (June 2003–August 2004) and 11 months following (October 2004–August 2005) *Bd* arrival in September 2004. For pools, we randomly sampled between four and eight pool sites per stream each month before *Bd* arrived. We resampled all

microhabitats in at least one stream annually between 2006 and 2011 and again in 2014 (Appendix S1: Table S1). All our analyses are based on the first two years of intensive sampling (2003–2005) of leaf pack, isolated pools, and riffles because no individuals were found in the majority of subsequent annual surveys.

We excluded pools from all analyses because logistical difficulties prevented the sampling of pools post-decline (2004–2005) and the sampling of leaf packs from September to December 2003. We report data of pools pre-decline to provide baseline data of these understudied communities. We also did not include September 2004 in analyses to limit biases between pre- and post- *Bd* samples because *Bd* arrived mid-September 2004 (Lips et al. 2006).

Statistical analyses

Patterns of community disassembly.—1. Tadpole density.—To determine if the magnitude of tadpole density declines differed among microhabitats or between seasons, we calculated tadpole habitat-weighted density (HWD) before and after *Bd* arrival in each microhabitat and stream by pooling monthly tadpole abundances across species. We used HWD to adjust for spatiotemporal variations in microhabitat availability caused by differences among streams and between seasons. HWD was calculated by dividing total tadpole abundance per microhabitat in each stream each month by the total area sampled and multiplying by the percent area each microhabitat covered in each stream that season. Our reformatted data consisted of tadpole HWD per microhabitat per stream per month from 2003 to 2005.

To determine if tadpole HWD differed among microhabitats or between seasons following *Bd* arrival, we used a generalized linear mixed effects model, with monthly tadpole HWD as the response variable and microhabitat, season, disease state (*Bd* present or absent), all two-way interactions, and the three-way interaction as the explanatory variables. We included month as a fixed effect to account for repeated measures of density across months and included stream as a random effect to account for pseudo-replication of microhabitats within streams (Gillies et al. 2006). We used a negative binomial distribution to account for over dispersion of the response variable, and we assessed model fit by visually inspecting the residuals. We fit this model using package *glmmADMB* (Fournier et al. 2012, Skaug et al. 2015) in R version 3.2.1 (R Core Team 2015).

We could not account for biases in tadpole abundance caused by imperfect detection because the parameters of the species-specific hierarchical *N*-mixture models we tried to fit did not converge. We used stream segments as sites and monthly repeated visits to stream segments as replicate surveys. The lack of parameter convergence was likely caused by large differences in tadpole densities between the replicate surveys at each site. These

differences could be caused by variations in species microhabitat use within a given stream segment and not necessarily a violation of the closure assumption (i.e., no births, deaths, immigration, or emigration). Density estimates that are not adjusted for imperfect detection are often underestimates of the true abundance (e.g., Banks-Leite et al. 2014) and inappropriately using N -mixture models causes abundance overestimates (i.e., Dail and Madsen 2011).

2. Species occupancy.—To determine if species occupancy differed before and after *Bd* arrival, we used a hierarchical occupancy model to quantify changes in species-specific occupancy. In this analysis, we were able to account for imperfect detection, by estimating microhabitat specific detection rates. We define occupancy as the probability a species occupied a stream segment, and we define detection probability as the probability we detect a species in a given stream segment, given that the species is present. We included data for all species that were detected in three or more microhabitat samples within a season (Ferraz et al. 2007, Ruiz-Gutiérrez et al. 2010; Appendix S1: Table S2). We had sufficient data to estimate occupancy for eight of the 13 species identified to species level.

We used different microhabitat samples as repeated surveys for stream segments (Hines et al. 2010). Since we did not know a priori species microhabitat use or breeding season, we ran each species occupancy model with the full set of microhabitat and season covariates.

For a selected species, we estimated the occupancy probability (ψ) of tadpoles as

$$z_{i,m} \sim \text{Bernoulli}(\psi_{i,m})$$

where $z = 1$ when the m th species occupies the i th stream segment, and $z = 0$ otherwise. We investigated the association between species tadpole occupancy and the covariates microhabitat, season, disease state, and their interactions using an effects-parameterized generalized linear mixed model where

$$\begin{aligned} \text{logit}(\psi_{i,m}) = & \alpha_{0,n,m} + \beta_{0,m} \text{Wet}_i + \beta_{1,m} \text{LeafPack}_i + \beta_{2,m} \text{Riffle}_i \\ & + \beta_{3,m} \text{Wet}_i \text{LeafPack}_i + \beta_{4,m} \text{Wet}_i \text{Riffle}_i \\ & + \beta_{5,m} \text{Post}_i + \beta_{6,m} \text{Post}_i \text{Wet}_i + \beta_{7,m} \text{Post}_i \text{LeafPack}_i \\ & + \beta_{8,m} \text{Post}_i \text{Riffle}_i + \beta_{9,m} \text{Post}_i \text{Wet}_i \text{LeafPack}_i \\ & + \beta_{10,m} \text{Post}_i \text{Wet}_i \text{Riffle}_i + \gamma_{i,m} + \eta_{d,m} \end{aligned}$$

We included $\alpha_{0,n,m}$ to account for spatial variations of the m th stream for the m th species, $\gamma_{i,m} \sim \text{normal}(0, \sigma_\gamma^2)$ was included as a random effect to account for variation in stream segment use for the m th species, and $\eta_{d,m} \sim \text{normal}(0, \sigma_\eta^2)$ was included as a random effect to account for pseudo-replication of stream segments across d months.

We estimated detection probability (p) as

$$y_{i,j,m} \sim \text{Bernoulli}(p_i z_{i,m})$$

where

$$\text{logit}(p_i) = \alpha_1 + \beta \text{Hab}_i.$$

When tadpoles of the m th species were observed during the j th survey at the i th stream segment site then $y = 1$, and $y = 0$, otherwise. Detection was modeled as the product of p_i , the probability of detecting a species, given that it is present at the i th stream segment site (i.e., $z = 1$).

To reduce the number of parameters estimated and to increase precision, we combined the detection probability of leaf packs and isolated pools as the intercept of the model and added riffles as the covariate *Hab*, since previous runs of the model showed very similar detection probability estimates between leaf packs and isolated pools (*unpublished model runs*). We assumed that tadpole detection probability was constant between seasons and years because sparse post-decline data prevented us from estimating detection probability.

We fit all models using Bayesian methods and estimated the posterior distributions for all parameters using Markov chain Monte Carlo (MCMC) methods implemented in JAGS 3.4.0 in R version 3.2.1 (R Development Core Team 2015) using the *rjags* package (Plummer 2015). For all parameters, we used priors following the recommendation of Lunn et al. (2012; i.e., $\text{normal}[0, 0.368]$, $\text{gamma}[0.01, 0.01]$, $\text{uniform}[0, 1]$). We ran three chains for each parameter, and ran each chain for 100,000 iterations with a burn-in period of 5,000 iterations. We evaluated convergence of chains by visual inspecting trace plots and using the diagnostics of Gelman (Brooks and Gelman 1998). We also assessed model fits using posterior predictive checks (Appendix S1: Fig. S1; Gelman et al. 2014).

To determine how much more likely a species was to successfully occupy a microhabitat before *Bd* than after *Bd* arrival, we calculated the odds ratio, OR (i.e., $\text{OR} = \text{odds}_{\text{post}}/\text{odds}_{\text{pre}}$), by dividing the post-*Bd* logit output of the occupancy model by the pre-*Bd* logit output of the occupancy model. If the OR is close to 1, then it suggests that there is no change in occupancy. If the OR is < 1 , then it suggests that the odds of occupancy is greater pre-*Bd* than post-*Bd*. And if the OR is > 1 , then the odds of occupancy is lower pre-*Bd* than post-*Bd*. We considered the effect of *Bd* biologically meaningful if the 95% credible interval fell below or above 1, interpreted as a 95% probability that the OR significantly changed.

3. Community composition.—To determine if tadpole communities post-*Bd* invasion were more similar to one another than tadpole communities before *Bd* arrival, we used a permutational analysis of multivariate dispersion (PERMDISP2; Anderson et al. 2006) in R version 3.2.1 (R Development Core Team 2015). We used the Bray-Curtis metric, which allows dispersion distance to reflect variability in community structure. We visualized the data using non-metric multidimensional scaling (NMDS). We defined communities as the tadpole assemblages sampled in each microhabitat-stream-season-year

combination, for a total of 48 communities (3 microhabitats \times 4 streams \times 2 seasons \times 2 years). We only included data between 2003 and 2005.

The order of species losses.—1. Species relatedness.—To determine if the order of species disappearances was correlated with their phylogenetic relationship, we fit several macroevolutionary likelihood models to the last day a species was seen in El Copé. We fit Brownian, Ornstein-Uhlenbeck, Lambda, and white noise models using the package *geiger* in the R version 3.2.1 (Harmon et al. 2008, R Development Core Team 2015). We used our observational field data to determine the last day each species was detected in El Copé. We did not interpret results as the true date of species extirpations because our data likely reflect the date of last species detection.

We set 1 January 2003 as day 0, and 31 December 2005 as day 1095. *Bd* likely arrived between days 609 and 638 in September 2004 (Lips et al. 2006). We used a rooted, time-calibrated, El Copé amphibian tree (Crawford et al. 2010). All species differed from sister lineages by a genetic distance at the COI gene or 16S gene by at least 8% or 2%, respectively. We excluded any individuals that were not classified to species level (e.g., *Centrolene* spp. and *Colostethus* spp.). We also did not include any pool samples or pool habitat specialist (i.e., *Atelopus zeteki*) because the last day those species were seen reflects the last day pools were sampled. We included all species that were found pre-*Bd* arrival for a total of 11 species, representing four families (i.e., Ranidae, Centrolenidae, Hylidae, Dendrobatidae), and we compared the fit of each model using AIC_c . We considered the model with the lowest AIC_c as the model of best fit.

2. Rarity.—To determine if species ecological rarity was a predictor of occupancy decline, we used two metrics of rarity: (1) tadpole seasonal microhabitat occupancy from the species occupancy model outlined above and (2) raw field data from transects of adult relative abundance (Crawford et al. 2010). We used both tadpole occupancy and adult relative abundance to reflect species' variations in rarity across life stages. We calculated the species-specific habitat-weighted OR as the product of the odds ratios for each microhabitat in each season from the occupancy model outlined above and the average percent habitat available to adjust for variations in microhabitat cover among streams. To quantify the strength of the relationships between adult relative abundance vs. OR and between tadpole pre-*Bd* occupancy vs. OR, we tested for an association between paired samples by calculating Pearson's correlation coefficient using the function `cor.test()` in R.

Imperfect detection and sampling biases.—1. Not adjusting for imperfect detection in occupancy models.—We compared all the results from our detection-adjusted occupancy model (i.e., species occupancy declines and

rarity analyses) to the results of a logistic regression, which does not adjust for detection probability, using a slightly modified data set and the model outlined above. We modified the data set by collapsing the stream segment site by visit matrix for each species, such that if a species was ever detected at a stream segment site, it was considered present. We assigned the detection probability, p , for all microhabitats equal to 1. We then used the same statistical approach using Markov chain Monte Carlo (MCMC) methods implemented in JAGS 3.4.0 in R version 3.2.1. (R Development Core Team 2015) using the *rjags* package (Plummer 2015).

2. Optimizing species sampling.—To determine how to improve the sampling of species rich communities when species' detection is variable and imperfect, we used a single-season, single-species, occupancy model to analyze simulated data under different scenarios (Data S1; Metadata S1). We simulated occupancy data for a single species under scenarios spanning high to low detection and occupancy probabilities (range 0.1–0.9) and varied the number of sites sampled (range 5–200 sites by 20) and the number of surveys per site (range 1–9 surveys per site by 2). We generated a total of 5000 unique scenarios to test how variations in occupancy, detection, number of sites sampled, and number of surveys per site affected the precision of occupancy estimates.

We fit all models using the same Bayesian methods outlined above for species occupancy. For each unique combination of occupancy, detection, sites, and surveys, we simulated 25 occupancy data sets and analyzed each data set under a Bayesian framework. For each analysis, we ran three chains for each parameter, and ran each chain for 10,000 iterations with a burn-in period of 1,000 iterations.

To determine how well models performed under different sampling schemes, we calculated the root mean square error between true and recovered occupancy estimates for each of the 25 data sets per scenario. The root mean square error represents the sample standard deviation of the difference between predicted and true estimates. Based on occupancy and the degree of precision we wanted in model estimates, we decided a priori that our maximum acceptable root mean square error was 0.10 (Guillera-Arroita et al. 2010, Guillera-Arroita 2011).

RESULTS

Field summary

We captured 2,021 individuals of 14 species across four microhabitats 15 months prior to *Bd*'s arrival. Of those, 1,123 individuals were found in pools. We found 11 species during the wet season and 12 species during the dry season, with 9 species common to both (Appendix S1: Table S2).

Before *Bd* arrival, average monthly HWD per microhabitat ranged from 0.00 to 20.08 individuals/m² during

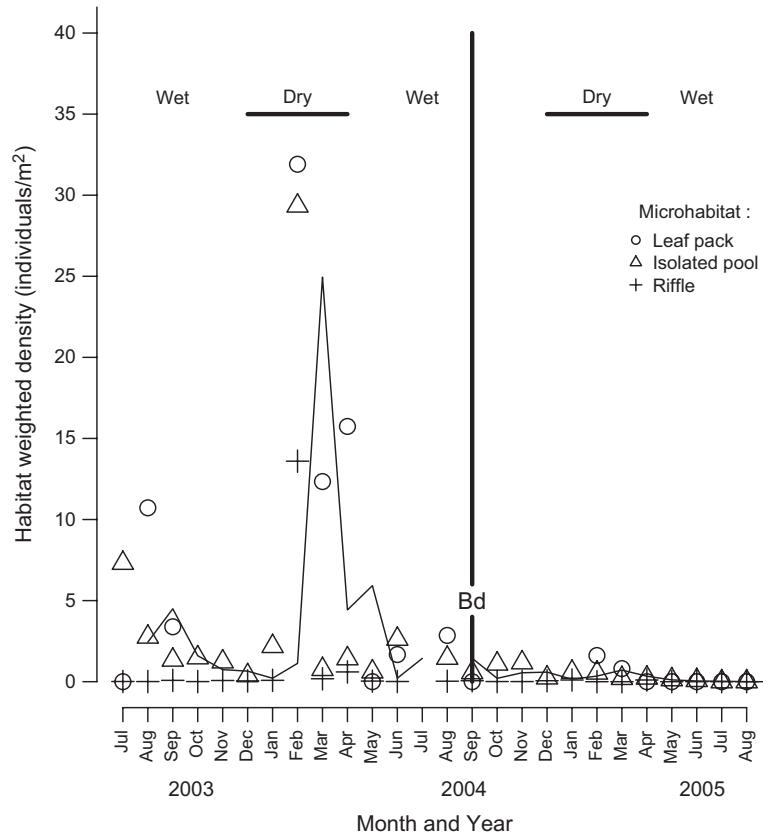


FIG. 1. Effects of *Batrachochytrium dendrobatidis* (*Bd*) arrival on habitat weighted density of tadpoles in each of three microhabitats (leaf pack, isolated pool, riffle) for 15 months before and 11 months after *Bd* arrival in September 2004 (Lips et al. 2006). The solid black line represents the rolling average of tadpole habitat weighted density for the entire tadpole community. The heavy black horizontal lines represent the dry season, and the heavy black vertical line represents the arrival of *Bd* in September 2004.

the dry season and 0.00–6.69 individuals/m² during the wet season (Fig. 1). Species that had >75% of captures during the dry season included *Atelopus varius*, *Colostethus panamensis*, *Lithobates warszewitschii*, *Espadarana prosoblepon*, *Sachatamia albomaculata*, *Hyloscirtus colymba*, and *Hyalinobatrachium colymbiphylum*. Species with >75% of captures in the wet season included *Colostethus* spp., *Hyloscirtus palmeri*, *Sachatamia illex*, and *Teratohyla spinosa*. Species with >98% of captures in a single microhabitat included *Atelopus varius* (pools), *L. warszewitschii* (pools), most centrolenid species (leaf packs), and *Colostethus* spp. (isolated pools; Appendix S1: Table S2).

During the 11 months following *Bd*'s arrival, we captured 249 individuals of eight species across three microhabitats, representing a 72% decrease in captures and a 43% decrease in species richness. Within 11 months of *Bd* arrival, habitat-weighted density decreased from an average HWD of 4.53 ± 1.19 individuals/m² (mean \pm SE) to 0.34 ± 0.08 individuals/m² after *Bd* ($z = 4.12$, $P < 0.001$; Fig. 1). The magnitude of density declines did not differ between microhabitats or seasons ($P > 0.05$). Post-decline, the highest densities of tadpoles were found in isolated pools, mostly of the families

Dendrobatidae (*Silverstoneia flotator*, *C. panamensis*, *S. nubicola*, *Allobates talamancae*, and *Colostethus* spp.) or Hylidae (*H. palmeri*, *H. colymba*). Only two species had >75% of captures in the dry season: *Allobates talamancae* and *L. warszewitschii*, and only *Hyalinobatrachium colymbiphylum* had >75% of captures during the wet season (Appendix S1: Table S2). Five species were never seen post-decline (*Atelopus varius*, *E. prosoblepon*, *S. albomaculata*, *S. illex*, and *T. spinosa*; Appendix S1: Table S2).

We did not detect any tadpoles during any of the annual surveys conducted from 2006 to 2011, precluding further analyses. In April 2014, we found several pools and isolated pools with tadpoles of *Silverstoneia nubicola* and an unidentified species, ranging in HWD between 0.95 and 4.49 individuals/m².

Patterns of community disassembly

Species occupancy.—One-half of the species in any microhabitat and season (24 of 48) declined in occupancy after *Bd* arrival (Fig. 2). Detection probability was significantly higher for tadpoles in leaf packs and isolated pools

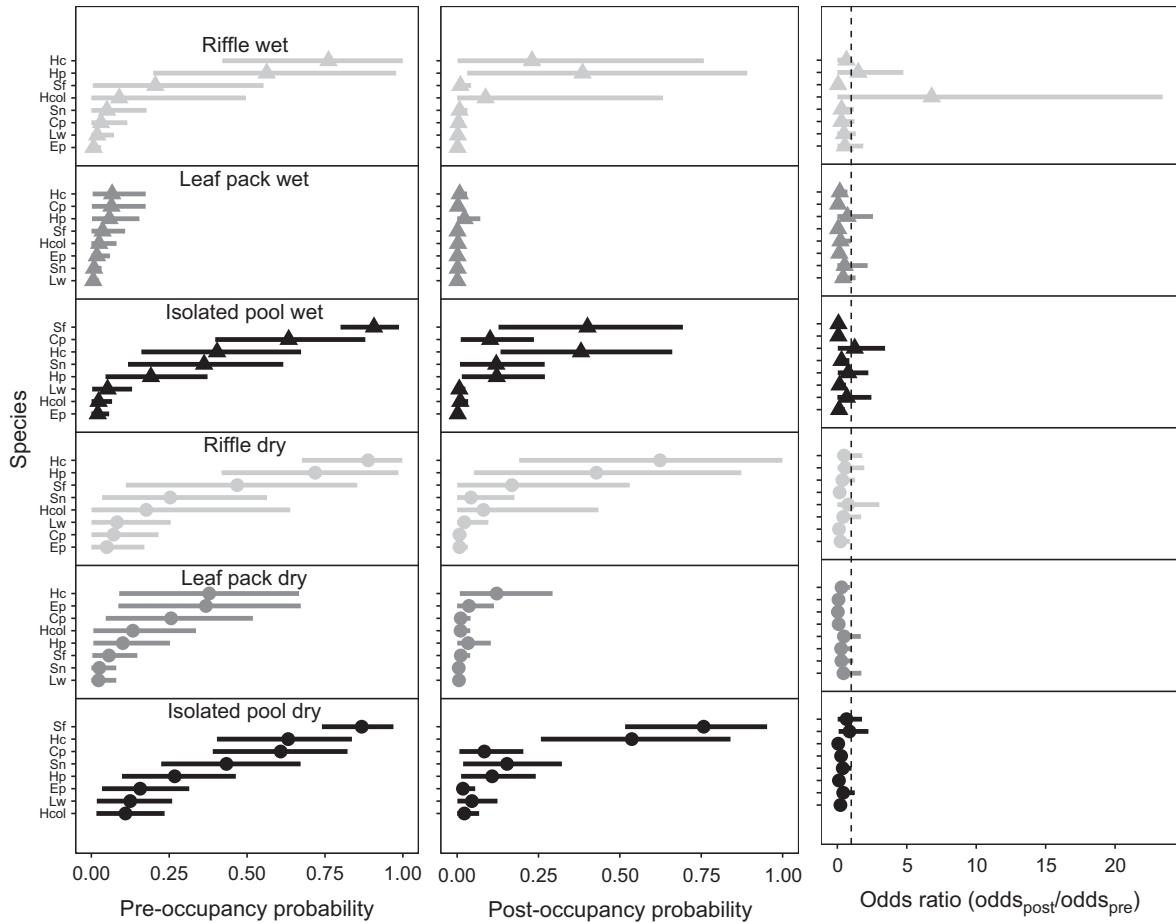


Fig. 2. Patterns of occupancy by species, microhabitat, and season pre- (left) and post- (middle) *Bd* arrival with the odds ratio (OR, i.e., $OR = odds_{post}/odds_{pre}$; right). All points represent the mean and 95% credible interval. Tadpole pre-*Bd* occupancy rarity was not a significant predictor of decline (Pearson’s correlation coefficient = -0.01 , $t = -0.05$, $df = 46$, $P = 0.95$). Odds ratios <1 indicate occupancy declines post-*Bd*. Species codes: Hc, *Hyloscirtus colymba*; Hp, *Hyloscirtus palmeri*; Sf, *Silverstoneia flotator*; Sn, *S. nubicola*; Cp, *Colostethus panamensis*; Lw, *Lithobates warszewitschii*; Hcol, *Hyalinobatrachium colymbiphylum*; Ep, *Espadarana prosoblepon*.

(0.41 ± 0.20), than for tadpoles found in riffles during the entire study (0.13 ± 0.03 ; Appendix S1: Table S3).

Community composition.—Tadpole communities were homogenized, where post-decline tadpole communities were more similar to one another than the pre-decline tadpole communities were to each other (Fig. 3; PERMDISP2, $F_{1,46} = 15.02$, $P < 0.001$). Pre-decline tadpole community dissimilarity among microhabitat and between seasons was 65% greater than their post-decline counterparts (pre-decline average distance to median = 0.35; post-decline average distance to median = 0.12).

The order of species losses

Species relatedness.—We found that the Brownian model best fit the timing of species disappearance dates, indicating a taxonomic signal to the order of species losses

and taxonomic homogenization (Fig. 4; Appendix S1: Table S4), with centrolenids disappearing first, sometimes without ever being seen post-*Bd* arrival, and hylids, dendrobatids, and the ranids still seen several months post-*s* arrival. All other models increased the AIC_c score by at least three points (Appendix S1: Table S4). No tadpoles were seen during the survey in 2006.

Rarity.—Neither tadpole occupancy nor adult relative abundance predicted the odds of occupancy decline among tadpole species (Figs. 2 and 5; Pearson’s correlation coefficient = -0.01 , $t = -0.05$, $df = 46$, $P = 0.95$; Pearson’s correlation coefficient = 0.08 , $t = 0.20$, $df = 6$, $P = 0.84$, respectively).

Imperfect detection and sampling biases

Not adjusting for imperfect detection in occupancy models.—Using the logistic regression, we found that

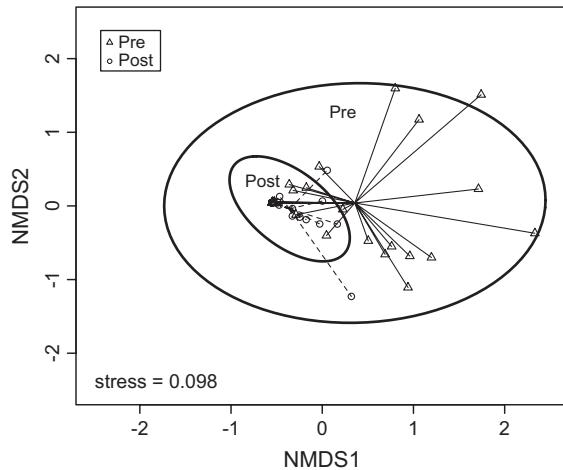


FIG. 3. Nonmetric multidimensional scaling (NMDS) ordination of tadpole communities (tadpole samples from each microhabitat–stream–season combination) pre- and post-*Bd* using Bray-Curtis dissimilarity. After *Bd* arrival, tadpole communities became more similar to one another, represented by the nested circles. Lines connect communities to the centroid of each group (i.e., pre- or post-*Bd*). Ellipses represent 95% confidence intervals around group centroids.

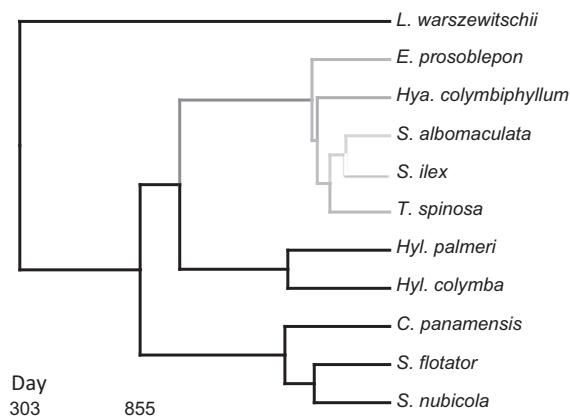


FIG. 4. Phylogenetic patterns of order of species losses in stream-dwelling tadpoles with the last day that a species was seen mapped onto branches. Our rooted, time-calibrated, and trimmed phylogenetic tree comes from the El Copé amphibian tree (Crawford et al. 2010). Day 0 corresponds to 1 January 2003 and day 1095 corresponds to 31 December 2005. *Bd* arrived between day 609 and 638. Most glassfrog species were the first to disappear from El Copé, with many not seen post-*Bd* arrival, while treefrogs and poison-dart frogs remained detectable after *Bd* arrival (model of best fit: Brownian; Appendix S1: Table S4).

over one-half (~58%) of tadpole species, regardless of microhabitat and season, declined following *Bd* arrival (Appendix S1: Table S5). Similar to the detection-adjusted model results, we found no relationship between adult relative abundance and the odds of species decline (Pearson's correlation coefficient = -0.11 , $t = -0.27$, $df = 6$, $P = 0.78$). But, in contrast to the

detection-adjusted model, we found that as tadpole pre-*Bd* occupancy increased, the likelihood of species decline also increased (Pearson's correlation coefficient = 0.96 , $t = 25.69$, $df = 46$, $P < 0.001$).

Optimizing species sampling.—When sampling a species-rich community with variable and imperfect detection, our simulations suggest that the minimum number of sites a surveyor should sample is 25 microhabitat sites at least three times to obtain an occupancy estimate with a maximum error of 0.10 (Appendix S1: Table S6 and Fig. S2).

DISCUSSION

Bd caused rapid, widespread abundance and occupancy declines in the tadpole community that was immediate and persistent. Tadpoles declined in abundance and occupancy rapidly within the first 11 months of the adult outbreak, and by the second year, all tadpoles had been extirpated. Sampling between 2006 and 2011 produced no tadpoles, even for species with adults that persisted post-*Bd* invasion. In 2014, the first tadpoles were detected but at very low densities and in few microhabitats.

Within 11 months of *Bd* invasion, tadpole community disassembly, the order of species declines and losses, was marked by taxonomic and ecologic homogenization with the disappearance of Centrolenid habitat-specialists, resembling the regional pattern of adult community

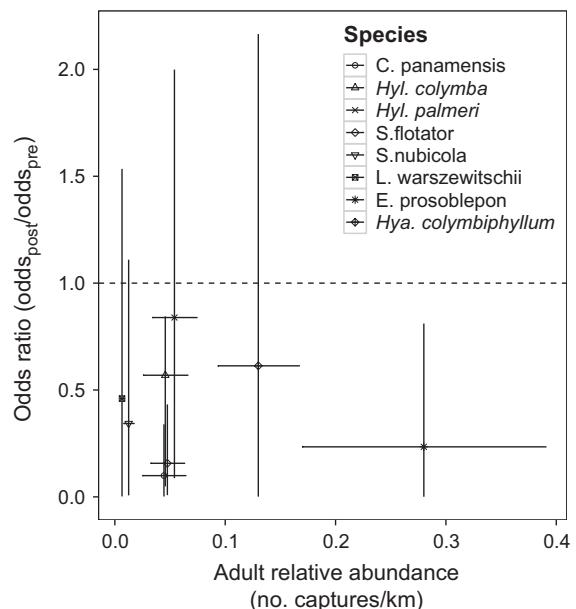


FIG. 5. Adult relative abundance pre-*Bd* arrival (Crawford et al. 2010) by habitat-weighted odds ratio of tadpole declines. Values plotted are means and 95% credible intervals. Adult relative abundance was not a significant predictor of the odds of tadpole occupancy decline (Pearson's correlation coefficient = 0.08 , $t = 0.20$, $df = 6$, $P = 0.84$).

disassembly (Smith et al. 2009). Centrolenids were mainly found in leaf packs and were the first ones that disappeared, likely driving the homogenizing pattern across El Copé, whereas at the regional scale, geographically restricted endemic species drove the homogenizing pattern of adult amphibians (Smith et al. 2009). Homogenization in both adults and tadpoles resulted in higher than expected taxonomic and ecological similarity among communities post-*Bd*.

The biotic homogenization detected in both adults and tadpoles at regional and local scales, respectively, has implications for future ecological and evolutionary processes (Olden et al. 2004). Ecologically, across space, *Bd* has dissolved historical biogeographical patterns, such that increasing distance between sites, even >500 km, is not correlated with community composition dissimilarity (Smith et al. 2009), essentially erasing information on why species are in their present locations. These species losses also decrease functional stability of communities and ecosystems, where food webs may be restructured (Barnum et al. 2015) or ecosystem resilience degraded (Petchey and Gaston 2009). Evolutionarily, biotic homogenization can decrease genetic variability within species, compromising individual fitness by disrupting local adaptation and decreasing the genetic variability of isolated populations and speciation (Olden et al. 2004). Therefore, via the mechanism of biotic homogenization, the world may be losing more species than appreciated when we combine observed species extirpations and decreased speciation rates.

Species rarity, both in terms of adult relative abundance and tadpole occupancy, did not predict the odds of species occupancy declines caused by *Bd*, indicating that species rarity does not predict community disassembly by a generalist pathogen. Rare and common species experienced comparably large occupancy declines from *Bd* invasion. Rarity is a widely accepted indicator of species vulnerability for many taxa (Zavaleta et al. 2009), but the mechanism (i.e., species ecology vs. low numbers) driving species susceptibility can vary by disturbance. In this system, where *Bd* is highly virulent and hosts are naïve to infection, species rarity, of either tadpoles or adults, did not influence vulnerability to *Bd*. Host susceptibility to pathogen-related declines is more complicated than relating them to host population size, where aspects of host ecology may also contribute to species vulnerability (Lips et al. 2003). For example, riparian species are more vulnerable to abundance and occupancy declines than terrestrial species (Lips et al. 2003, Brem and Lips 2008). In our system, we only examined stream-dwelling tadpoles; if we had surveyed the entire landscape for tadpoles (i.e., bromeliads, canopy, refuges, etc.), we may have detected more pronounced variations in susceptibility. Within a single habitat type though, we did not find that rarity of tadpole occupancy or adult relative abundance predicted the odds of species occupancy declines.

For some threats, such as disease, species extinction risk may not be predicted by the usual explanations, such as rarity (i.e., small geographic range size, low abundance, and ecological specialization; Rabinowitz 1981) or species traits (e.g., Lips et al. 2003, Langwig et al. 2012), because of spatial scale. In this study, we focused on stream-dwelling species, which are among the most susceptible to *Bd* (Brem and Lips 2008), and we did not find that rarity or species traits correlated to extinction risk because all tadpoles declined and disappeared within 22 months of *Bd* arrival. These results strongly reinforce the hypothesis that, for Neotropical amphibian species that live in streams, very little else matters besides whether a species survives the initial *Bd* outbreak or not.

We hypothesize that low adult abundance, low reproductive output, and high metamorph mortality are preventing tadpole community recovery. We found little evidence that tadpole communities were recovering within the decade after *Bd* invasion, although we likely did not sample enough to detect all species of tadpoles. For tadpole abundance to increase, adult abundance and reproductive output needs to increase. It is possible that infected tadpoles have reduced growth rates (Parris and Cornelius 2004, Garner et al. 2009) and higher disease-related mortality, or that metamorphs and subadults have high mortality rates (Berger et al. 1998, Rachowicz et al. 2006, Langhammer et al. 2014) but evidence for the latter is lacking.

In this system, recovery almost certainly does not mean that the tadpole community will return to their pre-*Bd* state. Stochastic (ecological drift) and deterministic (niche-selection) driven processes, as well as priority effects, will likely restructure the tadpole community as it reassembles, where the relative importance of each process may depend on the harshness of the ecological filter, in this case *Bd* (e.g., Chase 2007). In 2014, we detected a few individuals of *Silverstoneia nubicola*, which were among the last tadpole species detected in 2005. Although there is a parallel between species disassembly and reassembly order, the tadpole community is unlikely to reassemble in the reverse disassembly order because community disassembly was triggered by species' pathogen naivety, whereas reassembly will likely reflect the combined effects of amphibian dispersal and ability to cope with *Bd* infection and persistence. We propose that more reliable metrics to quantify tadpole community resilience and stream ecosystem function is the comparison of algal community composition (Connelly et al. 2008), macroinvertebrate assemblages (Colón-Gaud et al. 2010), or nitrogen cycling rates (Whiles et al. 2013) before and after the *Bd* outbreak.

The 100% tadpole abundance declines reported here are larger than the adult abundance declines described at El Copé, Panama (Crawford et al. 2010). The higher tadpole rate of loss is likely driven by both decreased recruitment and lower detection probability than adults. Tadpoles have naturally high mortality rates (Calef 1973, Heyer et al. 1975) and when the additional

chytrid-related mortality (Garner et al. 2009) is added to the system, the likelihood of tadpole survival is slim, explaining the discrepancy between tadpole and adult relative abundance declines. It may also be that tadpoles were still present but we did not detect them, especially given that some centrolenid adults are present at El Copé and we did not find their tadpoles (Crawford et al. 2010).

By not sampling pools after *Bd* arrival, we were unable to quantify the impact of *Bd* invasion on that microhabitat. However, our main conclusions would not have changed because amphibian adult mass mortality was widespread across El Copé (Lips et al. 2006). We resampled pools in 2006, and we found no individuals, similar to the patterns in the other microhabitats.

Sampling recommendations

We provide the first estimates of Neotropical tadpole detection probabilities, which could replace vague priors traditionally used in Bayesian analyses to make more precise occupancy estimates. Most Neotropical regions have experienced widespread losses of amphibians from *Bd* (James et al. 2015), making it difficult to estimate unbiased tadpole detection probabilities. Tadpoles are cryptic, secretive, and difficult to detect (Heard et al. 2006, Smith et al. 2007), but monitoring tadpoles may provide a better solution to monitoring amphibian community dynamics post-*Bd* because stream-dwelling tadpoles are spatially constrained, whereas amphibian adults are not.

Our study was not designed with the intent of using *N*-mixture or occupancy models, but we were able to analyze the majority of species using hierarchical occupancy models. If we had not accounted for imperfect species detection in this analysis, we would have likely overestimated occupancy declines and inaccurately interpreted the correlation between species odds of decline to tadpole pre-*Bd* occupancy (but see Welsh et al. 2013). Observational error, in this case, can lead to misclassifying species as extirpated or having greater odds of decline. We recommend that future researchers survey at least 25 sites, three times each per season, to adequately sample a species-rich community for both rare and common species.

Occupancy studies should be designed carefully to ensure efficient use of available resources. To avoid wasted effort, biologists should anticipate the quality of their data (Mackenzie and Royle 2005, Guillera-Arroita et al. 2011). The precision and bias of occupancy estimates will also depend highly on the species biology and the system in general. For example, when working with rare species, the best sampling designs will tend to have more replication than in cases where the precision of occupancy estimates is of interest. Therefore, thought and care should be given to designing sampling schemes before collecting data to prevent loss of time, money, and resources.

CONCLUSIONS

Phylogeny, ecological traits, and rarity have been associated with adult amphibian declines (but see Crawford et al. 2010), but we only detected evidence that one of these three characteristics predicted tadpole declines, where closely related species did share susceptibility to *Bd*. The discrepancy between the order of extirpations and declines of adult and tadpoles may be attributed to when in the life cycle hosts are gaining infection and dying. If hosts are dying before reproduction, the patterns of species abundance and occupancy declines will be greater than after they reproduce. For example, the mountain yellow-legged frog, *Rana muscosa*, develops fatal *Bd* infection post-metamorphosis, creating the illusion of healthy abundant tadpole populations but severely declined juvenile and adult populations.

We found that tadpole communities were taxonomically homogenized within 11 months of *Bd* invasion and communities collapsed within 22 months. *Bd* drove hosts to extirpation, and we have not seen signs or evidence of substantial tadpole community recovery within 10 years post-outbreak. Our results are directly relevant to researchers interested in improving sampling methods of diverse communities, disease ecologists interested in understanding how multi-host fungal pathogens impact different life stages, community ecologists interested in pathogen-driven community disassembly of vertebrates, and conservation practitioners in charge of culling, vaccinating, and sterilizing wild populations experiencing declines and extirpations caused by multi-host fungal pathogens.

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LITERATURE CITED

- Anderson, M. J., K. E. Ellingsen, and B. H. McArdle. 2006. Multivariate dispersion as a measure of beta diversity. *Ecology Letters* 9:683–693.

- Angeli, N. F., G. V. DiRenzo, A. Cunha, and K. R. Lips. 2015. Effects of density on spatial aggregation and habitat associations of the glass frog *Espadarana (Centrolene) prosoblepon*. *Journal of Herpetology* 49:388–394.
- Banks-Leite, C., R. Pardini, D. Boscolo, C. R. Cassano, T. Püttker, C. S. Barros, and J. Barlow. 2014. Assessing the utility of statistical adjustments for imperfect detection in tropical conservation science. *Journal of Applied Ecology* 51:849–859.
- Barbour, M. T., J. Gerritsen, B. D. Snyder, and J. B. Stribling. 1999. Rapid bioassessment protocols for use in wadeable streams and rivers: periphyton, benthic macroinvertebrates, and fish. United States Environmental Protection Agency, Office of Water, Washington, D.C., USA.
- Barnum, T. R., J. M. Drake, C. Colón-Gaud, A. T. Rugenski, T. C. Frauendorf, S. Connelly, S. S. Kilham, M. R. Whiles, K. R. Lips, and C. M. Pringle. 2015. Evidence for the persistence of food web structure after amphibian extirpation in a Neotropical stream. *Ecology* 96:2106–2116.
- Berger, L., et al. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences USA* 95:9031–9036.
- Brem, F. M. R., and K. R. Lips. 2008. *Batrachochytrium dendrobatidis* infection patterns among Panamanian amphibian species, habitats and elevations during epizootic and enzootic stages. *Diseases of Aquatic Organisms* 81:189–202.
- Briggs, C. J., R. A. Knapp, and V. T. Vredenburg. 2010. Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proceedings of the National Academy of Sciences USA* 107:9695–9700.
- Brooks, S. P., and A. Gelman. 1998. General methods for monitoring convergence of iterative simulations. *Journal of Computational and Graphical Statistics* 7:434–455.
- Calef, G. W. 1973. Natural mortality of tadpoles in a population of *Rana aurora*. *Ecology* 54:741–758.
- Chase, J. M. 2007. Drought mediates the importance of stochastic community assembly. *Proceedings of the National Academy of Sciences USA* 104:17430–17434.
- Colón-Gaud, C., M. R. Whiles, K. R. Lips, C. M. Pringle, S. S. Kilham, S. Connelly, R. Brenes, and S. D. Peterson. 2010. Stream invertebrate responses to a catastrophic decline in consumer diversity. *Journal of the North American Benthological Society* 29:1185–1198.
- Connelly, S., C. M. Pringle, R. J. Bixby, R. Brenes, M. R. Whiles, K. R. Lips, S. S. Kilham, and A. D. Huryn. 2008. Changes in stream primary producer communities resulting from large-scale catastrophic amphibian declines: Can small-scale experiments predict effects of tadpole loss? *Ecosystems* 11:1262–1276.
- Corey, S. J., and T. A. Waite. 2008. Phylogenetic autocorrelation of extinction threat in globally imperiled amphibians. *Diversity and Distributions* 14:614–629.
- Crawford, A. M., K. R. Lips, and E. Bermingham. 2010. Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. *Proceedings of the National Academy of Sciences USA* 107:13777–13782.
- Dail, D., and L. Madsen. 2011. Models for estimating abundance from repeated counts of an open metapopulation. *Biometrics* 67:577–587.
- Duellman, W. E., and L. Trueb. 1986. *The biology of amphibians*. McGraw-Hill Books, New York, New York, USA.
- Ferraz, G., J. D. Nichols, J. E. Hines, P. C. Stouffer, R. O. Bierregaard Jr., and T. E. Lovejoy. 2007. A large-scale deforestation experiment: effects of patch area and isolation on Amazon birds. *Science* 315:238–241.
- Fisher, M. C., D. A. Henk, C. J. Briggs, J. S. Brownstein, L. C. Madoff, S. L. McCraw, and S. J. Gurr. 2012. Emerging fungal threats to animal, plant and ecosystem health. *Nature* 484:186–194.
- Fournier, D. A., H. J. Skaug, J. Ancheta, J. Ianelli, A. Magnusson, M. Maunder, A. Nielsen, and J. Sibert. 2012. AD Model Builder: using automatic differentiation for statistical inference of highly parameterized complex nonlinear models. *Optimization Methods and Software* 27:233–249.
- Garner, T. W. J., S. Walker, J. Bosch, S. Leech, J. Marcus Rowcliffe, A. A. Cunningham, and M. C. Fisher. 2009. Life history tradeoffs influence mortality associated with the amphibian pathogen *Batrachochytrium dendrobatidis*. *Oikos* 118:783–791.
- Gehring, C. A., R. C. Mueller, K. E. Haskins, T. K. Rubow, and T. G. Whitham. 2014. Convergence in mycorrhizal fungal communities due to drought, plant competition, parasitism, and susceptibility to herbivory: consequences for fungi and host plants. *Frontiers in Microbiology* 5:1–9.
- Gelman, A., J. B. Carlin, H. S. Stern, and D. B. Rubin. 2014. *Bayesian data analysis*. Volume 2. Chapman and Hall/CRC, Boca Raton, Florida.
- Gillies, C. S., M. Hebblewhite, S. E. Nielsen, M. A. Krawchuk, C. L. Aldridge, J. L. Frair, D. J. Saher, C. E. Stevens, and C. L. Jerde. 2006. Application of random effects to the study of resource selection by animals. *Journal of Animal Ecology* 75:887–898.
- Guillera-Arroita, G. 2011. Impact of sampling with replacement in occupancy studies with spatial replication. *Methods in Ecology and Evolution* 2:401–406.
- Guillera-Arroita, G., M. S. Ridout, and B. J. T. Morgan. 2010. Design of occupancy studies with imperfect detection. *Methods in Ecology and Evolution* 1:131–139.
- Guillera-Arroita, G., B. J. T. Morgan, M. S. Ridout, and M. Linkie. 2011. Species occupancy modeling for detection data collected along a transect. *Journal of Agricultural, Biological, and Environmental Statistics* 16:301–317.
- Harmon, L. J., J. T. Weir, C. D. Brock, R. E. Glor, and W. Challenger. 2008. GEIGER: investigating evolutionary radiations. *Bioinformatics* 24:129–131.
- Heard, G. W., P. Robertson, and M. P. Scroggie. 2006. Assessing detection probabilities for the endangered growling grass frog (*Litoria raniformis*) in southern Victoria. *Wildlife Research* 33:557–564.
- Heyer, W. R. 1976. *Studies in larval amphibian habitat partitioning*. Smithsonian Contributions in Zoology 242:1–27.
- Heyer, W. R., R. W. McDiarmid, and D. L. Weigmann. 1975. Tadpoles, predation and pond habitats in the tropics. *Biotropica* 7:100–111.
- Heyer, W. R., M. A. Donnelly, R. W. McDiarmid, L. Hayek, and M. S. Foster. 1994. *Measuring and monitoring biological diversity. Standard methods for amphibians*. Smithsonian Institution Press, Washington, D.C., USA.
- Hines, J. E., J. D. Nichols, J. A. Royle, D. I. MaKenzie, A. M. Gopalaswamy, and N. S. Kumar. 2010. Tigers on trails: occupancy modeling for cluster sampling. *Ecological Applications* 20:1456–1466.
- Inger, R. F., H. K. Voris, and K. J. Frogner. 1986. Organization of a community of tadpoles in rain forest streams in Borneo. *Journal of Tropical Ecology* 2:193–205.
- James, T. Y., et al. 2015. Disentangling host, pathogen, and environmental determinants of a recently emerged wildlife disease: lessons from the first 15 years of amphibian chytridiomycosis research. *Ecology and Evolution* 5:4079–4097.
- Kéry, M. 2010. *Introduction to WinBUGS for ecologists: Bayesian approach to regression, ANOVA, mixed models and related analyses*. Academic Press, Burlington, MA.

- Lande, R. 1993. Risks of population extinction from demographic and environmental stochasticity and random catastrophes. *American Naturalist* 142:911–927.
- Langhammer, P. F., P. A. Burrowes, K. R. Lips, A. B. Bryant, and J. P. Collins. 2014. Susceptibility to the amphibian chytrid fungus varies with ontogeny in the direct-developing frog, *Eleutherodactylus coqui*. *Journal of Wildlife Diseases* 50:438–446.
- Langwig, K. E., W. F. Frick, J. T. Bried, A. C. Hicks, T. H. Kunz, and A. M. Kilpatrick. 2012. Sociality, density-dependence and microclimates determine the persistence of populations suffering from a novel fungal disease, white-nose syndrome. *Ecology Letters* 15:1050–1057.
- Larsen, T. H., N. M. Williams, and C. Kremen. 2005. Extinction order and altered community structure rapidly disrupt ecosystem functioning. *Ecology Letters* 8:538–547.
- Larsen, T. H., A. Lopera, and A. Forsyth. 2008. Understanding trait-dependent community disassembly: dung beetles, density functions, and forest fragmentation. *Conservation Biology* 22:1288–1298.
- Lips, K. R. 1998. Decline of a tropical montane amphibian fauna. *Conservation Biology* 12:106–117.
- Lips, K. R. 1999. Mass mortality and population declines of anurans at an upland site in western Panama. *Conservation Biology* 13:117–125.
- Lips, K. R., J. D. Reeve, and L. R. Witters. 2003. Ecological traits predicting amphibian population declines in Central America. *Conservation Biology* 17:1078–1088.
- Lips, K. R., F. Brem, R. Brenes, J. D. Reeve, R. A. Alford, J. Voyles, C. Carey, L. Livo, A. P. Pessier, and J. P. Collins. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academy of Sciences USA* 103:3165–3170.
- Lunn, D., C. Jackson, N. Best, A. Thomas, and D. Spiegelhalter. 2012. *The BUGS book: a practical introduction to Bayesian analysis*. CRC Press, Boca Raton, Florida, USA.
- Mackenzie, D. I., and J. A. Royle. 2005. Designing occupancy studies: general advice and allocating survey effort. *Journal of Applied Ecology* 42:1105–1114.
- MacKenzie, D. I., J. D. Nichols, J. A. Royle, K. H. Pollock, L. L. Bailey, and J. E. Hines. 2006. *Occupancy estimation and modeling: inferring patterns and dynamics of species occurrence*. Elsevier Academic Press, London, UK.
- McCaffery, R., and K. R. Lips. 2013. Survival and abundance in males of the glass frog *Espadarana (Centrolene) prosoblepon* in Central Panama. *Journal of Herpetology* 46:213–220.
- McDiarmid, R. W., and R. Altig. 1999. *Tadpoles: the biology of the anuran larvae*. University of Chicago Press, Chicago, Illinois, USA.
- McKinney, M., and J. Lockwood. 1999. Biotic homogenization: a few winners replacing many losers in the next mass extinction. *Trends in Ecology and Evolution* 14:450–453.
- Olden, J. D., N. Leroy Poff, M. R. Douglas, M. E. Douglas, and K. D. Fausch. 2004. Ecological and evolutionary consequences of biotic homogenization. *Trends in Ecology and Evolution* 19:18–24.
- Parris, M. J., and T. O. Cornelius. 2004. Fungal pathogen causes competitive and developmental stress in larval amphibian communities. *Ecology* 85:3385–3395.
- Petchey, O. L., and K. J. Gaston. 2009. Effects on ecosystem resilience of biodiversity, extinctions, and the structure of regional species pools. *Theoretical Ecology* 2:177–187.
- Plummer, M. 2015. *rjags: Bayesian Graphical Models using MCMC*. R package version 3-15. <http://CRAN.R-project.org/package=rjags>
- R Development Core Team. 2015. *R: a language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org/>
- Rabinowitz, D. 1981. Seven forms of rarity. Pages 205–217 in H. Synge, editor. *The biological aspects of rare plant conservation*. John Wiley and Sons, Hoboken, New Jersey, USA.
- Rachowicz, L. J., and C. J. Briggs. 2007. Quantifying the disease transmission function: effects of density on *Batrachochytrium dendrobatidis* transmission in the mountain yellow-legged frog *Rana muscosa*. *Journal of Animal Ecology* 76:711–721.
- Rachowicz, L., R. Knapp, and J. Morgan. 2006. Emerging infectious disease as a proximate cause of amphibian mass mortality. *Ecology* 87:1671–1683.
- Rader, R., I. Bartomeus, J. M. Tylianakis, and E. Laliberté. 2014. The winners and losers of land use intensification: pollinator community disassembly is non-random and alters functional diversity. *Diversity and Distributions* 20:908–917.
- Ranvestel, A. W., K. R. Lips, C. M. Pringle, M. R. Whiles, and R. J. Bixby. 2004. Neotropical tadpoles influence stream benthos: evidence for the ecological consequences of decline in amphibian populations. *Freshwater Biology* 49:274–285.
- Ruiz-Gutiérrez, V., E. F. Zipkin, and A. A. Dhondt. 2010. Occupancy dynamics in a tropical bird community: unexpectedly high forest use by birds classified as non-forest species. *Journal of Applied Ecology* 47:621–630.
- Savage, J. M. 2002. *The amphibians and reptiles of Costa Rica*. The University of Chicago Press, Chicago, Illinois, USA.
- Skaug, H., D. Fournier, B. Bolker, A. Magnusson, and A. Nielsen. 2015. *Generalized Linear Mixed Models using 'AD Model Builder'*. R package version 0.8.3.2. <http://glmmadmb.r-forge.r-project.org/>
- Smith, M. J., E. S. G. Schreiber, M. P. Scroggie, M. Kohout, K. Ough, J. Potts, R. Lennie, D. Turnbull, C. Jin, and T. Clancy. 2007. Associations between anuran tadpoles and salinity in a landscape mosaic of wetlands impacted by secondary salinisation. *Freshwater Biology* 52:75–84.
- Smith, K. G., K. R. Lips, and J. M. Chase. 2009. Selecting for extinction: nonrandom disease-associated extinction homogenizes amphibian biotas. *Ecology Letters* 12:1069–1078.
- Villa, J. D. 1990. *Rana warszewitschii* (Schmidt). *Catalogue of American amphibians and reptiles*. Society for the Study of Amphibians and Reptiles 459:1–459.2.
- Welsh, A. H., D. B. Lindenmayer, and C. F. Donnelly. 2013. Fitting and interpreting occupancy models. *PLoS ONE* 8:e52015.
- Whiles, M. R., et al. 2013. Disease-driven amphibian declines alter ecosystem processes in a tropical stream. *Ecosystems* 16:146–157.
- Wright, D. H., A. Gonzalez, and D. C. Coleman. 2007. Changes in nestedness in experimental communities of soil fauna undergoing extinction. *Pedobiologia* 50:497–503.
- Zavaleta, E., J. Pasari, J. Moore, D. Hernández, K. B. Suttle, and C. C. Wilmers. 2009. Ecosystem responses to community disassembly. *Annals of the New York Academy of Sciences* 1162:311–333.

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Imperfect pathogen detection from non-invasive skin swabs biases disease inference

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Abstract

1. Conservation managers rely on accurate estimates of disease parameters, such as pathogen prevalence and infection intensity, to assess disease status of a host population. However, these disease metrics may be biased if low-level infection intensities are missed by sampling methods or laboratory diagnostic tests. These false negatives underestimate pathogen prevalence and overestimate mean infection intensity of infected individuals.
2. Our objectives were two-fold. First, we quantified false negative error rates of *Batrachochytrium dendrobatidis* (*Bd*) on non-invasive skin swabs collected from an amphibian community in El Copé, Panama. We swabbed amphibians twice in sequence, and we used a recently developed hierarchical Bayesian estimator to assess disease status of the population. Second, we developed a novel hierarchical Bayesian model to simultaneously account for imperfect pathogen detection from field sampling and laboratory diagnostic testing. We evaluated the performance of the model, using simulations and varying sampling design to quantify the magnitude of bias in estimates of pathogen prevalence and infection intensity.
3. We show that *Bd* detection probability from skin swabs was related to host infection intensity, where *Bd* infections <10 zoospores have <95% probability of being detected. If imperfect *Bd* detection was not considered, then *Bd* prevalence was underestimated by as much as 71%. In the *Bd*-amphibian system, this indicates a need to correct for imperfect pathogen detection in enzootic host populations persisting with low-level infections. More generally, our results have implications for study designs in other disease systems, particularly those with similar objectives, biology, and sampling decisions.
4. Uncertainty in pathogen detection is an inherent property of most sampling protocols and diagnostic tests, where the magnitude of bias depends on the study system, type of infection, and false negative error rates. Given that it may be difficult to know this information in advance, we advocate that the most cautious approach is to assume all errors are possible and to accommodate them by adjusting sampling designs. The modelling framework presented here improves the accuracy in estimating pathogen prevalence and infection intensity.

KEYWORDS

amphibians, *Batrachochytrium dendrobatidis*, chytrid, hierarchical Bayesian model, occupancy model, Panama

1 | INTRODUCTION

Epidemiologists and wildlife managers rely on accurate estimates of disease parameters, such as pathogen prevalence and infection intensity, to assess the risk of disease emergence in wild host populations (e.g. Langwig et al., 2015). Traditionally, disease ecologists have recognised that imperfect host detection (i.e., false negatives) affects the inferences made on disease dynamics, leading to the adoption of capture-mark-recapture methods to correct for imperfect host detection (e.g., Cooch, Conn, Ellner, Dobson, & Pollock, 2011). More recently, however, there has been growing awareness that imperfect pathogen detection biases the estimation of pathogen prevalence and infection intensity (e.g. Lachish, Gopalaswamy, Knowles, & Sheldon, 2012; Miller, Talley, Lips, & Campbell Grant, 2012). Pathogen prevalence tends to be underestimated, whereas mean infection intensity is overestimated when sampling methods or diagnostic tests miss low-level pathogen infections, causing the misclassification of infected hosts as uninfected (e.g. Lachish et al., 2012; Miller et al., 2012). This growing awareness has led to new quantitative methods that provide a platform to correct for disease state misclassification, improving the quality of inference by reducing bias (e.g. Lachish et al., 2012; Miller et al., 2012).

Imperfect pathogen detection has been widely acknowledged in both veterinary and medical fields and is likely present in most sampling and diagnostic methods used by disease ecologists. Veterinary and medical fields have long used statistical tools to adjust pathogen prevalence estimates by correcting for the accuracy of diagnostic tests (reviewed in Enoe, Georgiadis, & Johnson, 2000; Greiner & Gardner, 2000; Toft, Jørgensen, & Højsgaard, 2005). However, the stringent assumptions and requirements of these statistical tools make them impractical for disease ecologists. For example, most methods used in veterinary and medical fields involve determining the accuracy of a diagnostic test by comparing it to an independent reference test (e.g. Drewe, Dean, Michel, & Pearce, 2009; Greiner & Gardner, 2000). In the realm of disease ecology, most disease diagnostics are obtained from a single test or from visual inspections when no diagnostic tools are available (e.g. facial tumour disease of Tasmanian devils, *Sarcophilus harrisii*; Lachish, Jones, & McCallum, 2007).

In the case of pathogen presence, uncertainty is related to the specificity (i.e. the probability an uninfected individual is correctly classified as uninfected) and sensitivity (i.e. the probability an infected host is correctly classified as infected) of the sampling and diagnostic methods. Typically, specificity is assumed maximised when strict protocols are used in the field and lab to decrease the odds of contaminating samples that lead to false positives. False negatives, alternatively, occur during a survey event when the pathogen is present but is not detected (e.g. Colvin, Peterson, Kent, & Schreck, 2015; Thompson,

2007). Sensitivity, therefore, is the product of two processes: (1) sampling methods (e.g. blood, swab, histology sample, etc.) and (2) laboratory diagnostic testing (e.g. qPCR, ELISA, etc.). For example, the causative agent of whirling disease, *Myxobolus cerebralis*, infects the brain of a fish, and infections can be missed when an uninfected area of the brain is examined (Thompson, 2007). In this case, it is also likely that imperfect pathogen detection is related to pathogen infection intensity (e.g. Valkiunas et al., 2008), where low-level infections are more likely missed than high-level infections. Few field studies, however, consider false negative error rates of sampling methods, and even fewer directly estimate them (e.g. Colvin et al., 2015; Thompson, 2007).

Thus far, the primary focus of disease ecologists investigating false negative error rates of pathogens has occurred with respect to laboratory diagnostic tests. For example, several studies have investigated how the sensitivity of quantitative PCR depends on host infection intensity; as host infection intensity increases, the probability of detecting the pathogen also increases (e.g. Gómez Díaz, Doherty, Duneau, & McCoy, 2010; Lachish et al., 2012; Miller et al., 2012). This pattern has been detected across several disease systems using different diagnostic tests, including: qPCR to detect the causative agent of malaria, *Plasmodium* sp., in birds (Knowles et al., 2011; Lachish et al., 2012); qPCR to detect *Batrachochytrium dendrobatidis* on amphibian skin (Miller et al., 2012); γ interferon and ELISA tests to detect the causative agent of tuberculosis, *Mycobacterium bovis*, in cattle (Ritacco et al., 1991); and qPCR to detect the causative agent of Lyme disease, *Borrelia* species complex, in *Ixodes uriae* ticks (Gómez Díaz et al., 2010). Cumulatively, this evidence strongly suggests that host infection intensity affects the probability of detecting the pathogen using several different diagnostic tests, but it remains unclear if host infection intensity affects the probability of detecting the pathogen during sampling.

As a motivating example, we focus on the emerging infectious fungal pathogen *Batrachochytrium dendrobatidis* (hereafter *Bd*; Longcore, Pessier, & Nichols, 1999), the causative agent of chytridiomycosis in amphibians. *Bd* is one of the greatest threats to amphibian biodiversity; it has been detected on over 700 amphibian species; and it has been found on every continent where amphibians occur (Cheng, Rovito, Wake, & Vredenburg, 2011; Fisher, Garner, & Walker, 2009; Lips et al., 2006; Olson et al., 2013; Wake & Vredenburg, 2008). To date, the most sensitive sampling and diagnostic methods to test for the presence of *Bd* are non-invasive skin swabs and qPCR (Kriger, Hero, & Ashton, 2006). While it has been shown that, like most other diagnostic tests, qPCR sensitivity to *Bd* is <1 and correlates with host infection intensity (e.g. Miller et al., 2012), it remains unclear if host infection intensity also impacts *Bd* sampling sensitivity of non-invasive skin swabs (i.e., replication frequency, number of swab strokes, pressure of swab, etc.). Abundance-induced detection heterogeneity is

well-known to affect the estimation of occurrence and abundance (e.g. Royle & Nichols, 2003), so it is expected that if two swabs were collected in sequence from the same amphibian, the likelihood that both swabs would detect the pathogen and quantify the same infection intensity should be lower at low-level infection intensities (e.g. Lachish et al., 2012; Miller et al., 2012).

In this paper, our objectives were two-fold. First, we quantified false negative error rates from imperfect host sampling (via non-invasive skin swabbing) of *Bd* in an amphibian community in El Copé, Panama. To do this, we swabbed amphibians twice in sequence, and we used a recently developed hierarchical Bayesian estimator formulated by Miller et al. (2012), originally used to examine qPCR false negative rates of *Bd* on amphibians. We expected that as host infection intensity increased, the probability of detecting *Bd* on a skin swab would increase, similar to the relationship between *Bd* infection intensity and qPCR *Bd* detection probability (Miller et al., 2012). We also assessed the variation in *Bd* prevalence and infection intensity between habitats (stream vs. trail) and seasons (wet vs. dry) because previous studies have shown that these variables explain differences in host disease susceptibility (e.g. Brem & Lips, 2008; Kriger & Hero, 2006). Second, we developed a novel hierarchical Bayesian model that simultaneously accounted for imperfect pathogen detection from both field sampling and diagnostic tests. We simulated and analysed data under a variety of sampling design scenarios to quantify the magnitude of parameter bias of pathogen prevalence and infection intensity estimates.

Our modelling approach and results provide tools for disease ecologists to refine and optimise pathogen-sampling procedures and reduce the bias of parameter estimates, which will improve inference and the application of epidemiological models to understand and forecast host–pathogen dynamics. We provide an appendix with R code to facilitate the application of these methods. However, we highlight that the biases introduced in estimating parameters of interest and methodological recommendations is highly dependent on details of the study system and objectives. Our driving motivation in the development and applications of this method is to understand how imperfect pathogen detection from samples and diagnostic tests contribute to biases in population-level inferences, which should guide the efficient allocation of resources in epidemiological studies.

2 | MATERIALS AND METHODS

2.1 | Field surveys

We sampled four 200 m stream and three 400 m trail transects in Parque Nacional G. D. Omar Torrijos Herrera, Coclé Province, El Copé, Panama (8°40' N, 80°37'17" W; Lips, Reeve, & Witters, 2003) during two wet seasons (2012, 2013) and one dry season (2013). The park spans elevations between 500 and 1,000 m and is located on the Continental Divide. This site experiences both dry (December–April) and wet (May–November) seasons.

We surveyed each transect six to eight times during each season. Field teams of two to three people conducted nocturnal visual

encounter surveys by slowly walking each transect and using visual and audio cues to locate amphibians within two metres of the stream bank or trail. Upon capture, we swabbed the abdomen and each limb five times (total = 30 strokes) per animal following the swabbing protocol by Hyatt et al. (2007) using a sterile cotton-tipped swab (Dry Swab MW113, Medical Wire). We collected at least one swab per individual captured, and a subset of individuals was swabbed twice in sequence from the same location on the animal's body (see Table 1a in Appendix S1). We used a fresh pair of latex powder-free gloves when handling each individual. We stored all swabs in individually capped 2 ml tubes with 30 μ l of 70% ethanol. Because we did not uniquely mark all individuals that we captured and swabbed, it is possible that we repeatedly swabbed the same individual within a season, making some samples pseudo-replicates. Pseudo-replicates, in this case, will decrease the variability around our reported naïve and adjusted estimates of pathogen prevalence and infection intensity. All individuals were released at the original point of capture. We include *Bd* infection intensity data from all amphibian species captured (see Table 1a in Appendix S1) without discriminating among species in the model because <10 individuals per species were captured for c. 56% of the 39 total species detected.

2.2 | Molecular analysis

We used PrepMan Ultra[®] for DNA preparation of swabs tested for *Bd*. We tested swabs for *Bd* in singlicate, using Taqman qPCR (Boyle, Boyle, Olsen, Morgan, & Hyatt, 2004; Hyatt et al., 2007) running 50 cycles. We ran each plate with JEL 423 standards of 0.1, 1, 10, 100, and 1,000 *Bd* zoospore genomic equivalents (ZGE) to determine *Bd* presence and infection intensity. Isolate JEL 423 was originally isolated at El Copé, Panama during the epizootic of 2004. We categorised individuals as *Bd*-positive when infection intensity was greater than zero (Briggs, Knapp, & Vredenburg, 2010). The qPCR assay consistently detects very small *Bd* infections (0–1 ZGE), likely representing very low levels of infection. To ensure that false positives were negligible, we included multiple negative controls in each qPCR plate.

2.3 | Sampling detection-adjusted model

We used a slightly modified version of the hierarchical Bayesian estimator developed by Miller et al. (2012) to account for heterogeneity in pathogen detection probability due to host infection intensity. We assumed that there was no error associated with the diagnostic test. Instead, we focus on the sensitivity of the swabbing procedure. We did not use the multi-season formulation of the occupancy model because we did not track the individuals that were swabbed across seasons. Alternatively, we assumed that infection intensity solely depended on habitat and season and not an individual's previous infection history.

We modelled the true, but unobservable, *Bd* infection state (z_i) on the i th individual as:

$$z_i \sim \text{Bernoulli}(\Psi_{\text{habitat}, \text{season}_i}) \quad (1)$$

where an individual was either infected ($z_i = 1$) or not ($z_i = 0$), and Ψ , the probability of the i th individual being infected, depends on habitat type (trail vs. stream) and season (dry vs. wet). Note that the parameter Ψ gives rise to the estimate of pathogen prevalence, i.e., the proportion of hosts infected, which is the outcome from the repeated Bernoulli process.

We modelled the observed *Bd* infection on the i th individual and the j th swab, y_{ij} , as:

$$y_{ij} \sim \text{Bernoulli}(p_i \times z_i), \quad (2)$$

where an infection was either detected ($y_{ij} = 1$) or not ($y_{ij} = 0$). We modelled the detection probability, p_i , as a function of true, but unobservable, *Bd* infection intensity, x_i , on the i th individual as:

$$\text{logit}(p_i) = \alpha + \beta \times x_i \quad (3)$$

where α is the log odds of pathogen detection when infection intensity x_i is zero, and β is the scaling coefficient representing how detection log odds changes with respect to host infection intensity. We modelled the true log *Bd* infection intensity, x_i , on the i th host as:

$$\log(x_i) \sim \text{normal}(\mu_i, \sigma^2) \quad (4)$$

where the true mean log infection intensity, μ_i , was a function of habitat, season, and true *Bd* infection state, z_i , for the i th host:

$$\mu_i = \log(0.001 + \omega_{\text{habitat}_i, \text{season}_i} \times z_i) \quad (5)$$

Above, σ^2 represents the standard deviation in *Bd* infection intensities across the host population.

Lastly, we modelled the observed infection intensity, w_{ij} , on the i th individual and the j th swab as:

$$\log(w_{ij}) \sim \text{normal}(\log(x_i + 0.001), \sigma_{\text{error}}^2) \quad (6)$$

In this case, σ_{error}^2 represents the measurement error of the estimates for *Bd* infection intensity produced by the non-invasive swabbing technique.

2.4 | Unadjusted model

To estimate the parameter bias caused by pathogen non-detection, we fit the same model outlined above after removing the detection probability and measurement error portions of the model (Equations 2 and 6). We modified our data by collapsing the host by swab matrix in two key ways: (1) if *Bd* was detected on any swab collected for an individual then that individual was considered infected, and (2) we averaged the infection intensities across all swabs for each host that was considered infected.

2.5 | Model fit

We fit all models using Bayesian methods and estimated the posterior distributions for all parameters and latent states using Markov chain Monte Carlo (MCMC) implemented in JAGS 4.0.0 with the JAGSUI package (Kellner, 2015) in the R environment (R Core Team, 2015). We used vague priors (i.e., $\text{normal}(0, 0.01)$ or $\text{normal}(0, 0.368)$; Lunn, Jackson, Best, Thomas, & Spiegelhalter, 2012) for all parameters. We computed

three chains for each random variable with diffuse initial values. After a burn-in of 10,000 iterations, we accumulated 40,000 samples from each chain, keeping every 50th sample. We assessed convergence by visually inspecting trace plots and using the diagnostics of Gelman (Brooks & Gelman, 1998). We used a posterior predictive check (hereafter Bayesian p -value) to compare the observed data to simulated datasets generated from the parameter estimates at each step in the MCMC algorithm. We confirmed that the Bayesian p -value, defined as the probability that the simulated data were more extreme than the observed data, was indicative of a good model fit (e.g. Gelman et al., 2013; Kéry & Schaub, 2012). Our observed data fit both the sampling detection-adjusted and unadjusted models well (see Figure 1a, Bayesian p -value = .79; Figure 2a, Bayesian p -value = .41 in Appendix S1).

To quantify effects, we calculated the differences between parameters of interest at each MCMC iteration following Ruiz-Gutiérrez, Zipkin, and Dhondt (2010). We computed the proportion of iterations where one parameter was greater than the other, which is directly interpreted as the probability (Pr) that one parameter is greater than the other. We considered effects with large credible intervals to be either unimportant to the process being modelled, or to have been estimated too imprecisely to draw conclusive inference.

2.6 | *Bd*-specific methodological guidance

Given that *Bd* infection may be overlooked on an infected host, we followed Kéry (2002) and calculated the probability of detecting *Bd*, P^* , on n identical and independent swabs or qPCR runs using the binomial argument:

$$P^* = 1 - (1 - p)^n, \quad (7)$$

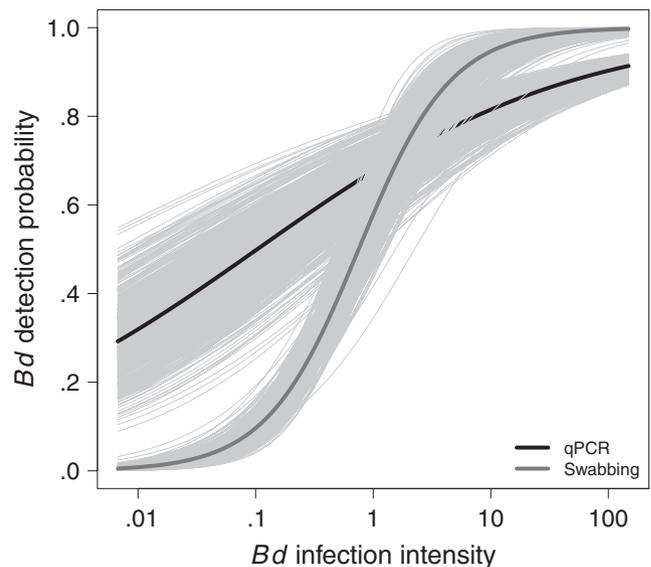


FIGURE 1 The relationship between *Batrachochytrium dendrobatidis* (*Bd*) detection probability and host infection intensity caused by laboratory (black line; qPCR error; Miller et al., 2012) and swabbing (dark grey line) methods. This graph indicates that as host infection intensity increases, pathogen detection probability also increases. The dark lines are mean posterior distribution estimates, and light grey lines represent the 95% credible interval around the mean

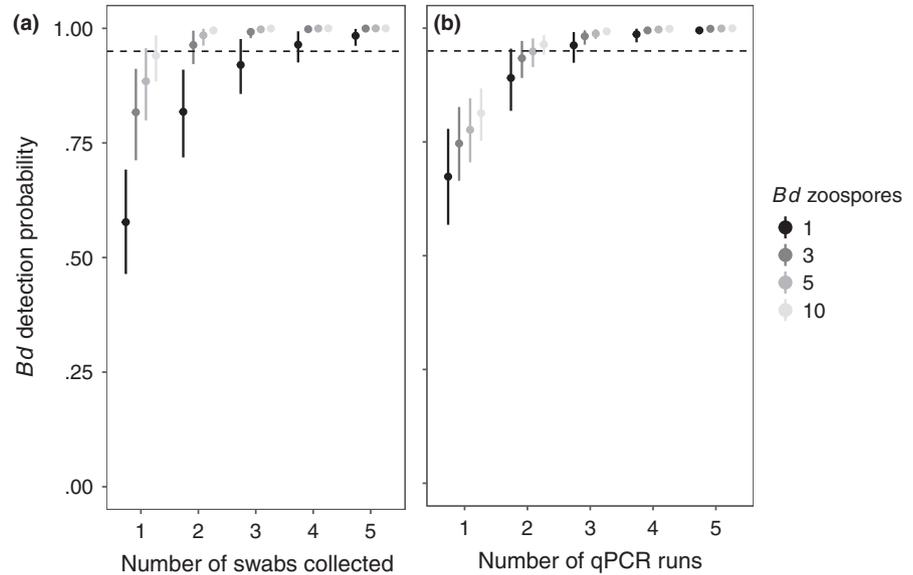


FIGURE 2 Given that pathogen infection may be overlooked on an infected individual, we calculated the probability of detecting *Batrachochytrium dendrobatidis* (*Bd*) on n identical and independent (a) swabs or (b) qPCR runs, using a binomial argument, as $P^* = 1 - (1 - p)^n$ (Kery, 2002). We model the probability of detecting 1 ZGE, 3 ZGE, 5 ZGE, and 10 ZGE. The dashed line indicates 95% certainty of detecting the pathogen when it is present

where p was either obtained from the sampling detection-adjusted model outlined above (i.e., imperfect *Bd* detection from swabbing) or from Miller et al. (2012; i.e., imperfect *Bd* detection from qPCR). We modelled the probability of detecting 1 ZGE, 3 ZGE, 5 ZGE, and 10 ZGE. Our recommendations were based on the minimum number of swabs and qPCR runs to be 95% certain that if *Bd* were present then it would be detected.

2.7 | Sampling and diagnostic detection-adjusted model

To provide general methodological guidance, we developed a novel Bayesian hierarchical model that simultaneously accounts for imperfect sampling and diagnostic detection of the pathogen (see Appendix S2 for the full model outline and R code). We performed a simulation study to explore the ability of this hierarchical model to estimate pathogen prevalence and infection intensity under scenarios of imperfect sampling and diagnostic testing for a pathogen. We set average infection intensity as either low ($\mu = 2$) or high ($\mu = 20$) and the probability of pathogen infection as either low ($\Psi = 0.20$) or high ($\Psi = 0.80$). We assumed low population infection intensity standard deviation ($\sigma^2 = 1$), infection intensity measurement error in sampling ($\sigma_{\text{error}}^2 = 1$), and infection intensity measurement error of the diagnostic test ($\sigma_{\text{diagnostic}}^2 = 1$). We set the log odds scaling coefficient of pathogen detection for sampling and diagnostic testing as either low (-1.38) or high (1.38). We did not consider the case where detection probability for either pathogen sampling or diagnostic testing varied with respect to true infection intensity (i.e., changing the slopes of the relationships). We explored how varying the number of samples collected per host (i.e., 1, 2, 3, 4) and the number of diagnostic runs per sample (i.e., 1, 2, 3, 4) affected the bias in estimated parameters of interest (i.e. pathogen prevalence [Ψ] and average infection intensity [μ]). This resulted in 256 parameter combinations (see Table 2a in Appendix S1). For each parameter combination, we simulated 50 datasets of 500 individuals each before fitting the model using the same methods outlined above. To

TABLE 1 Summary of the posterior distributions from the sampling detection-adjusted model. All parameters were back transformed to their original scale, except detection probability (logit scale) and error estimates

Definition	Mean	95% credible interval	
ω			
Stream dry	0.13	0.04	0.43
Trail dry	0.40	0.11	1.39
Stream wet	0.13	0.02	1.12
Trail wet	0.16	0.07	0.39
Process error (σ^2)	3.17	2.66	3.63
Measurement error (σ_{error}^2)	1.03	0.81	1.29
Ψ			
Stream dry	0.86	0.57	0.98
Trail dry	0.67	0.46	0.91
Stream wet	0.64	0.31	0.95
Trail wet	0.92	0.73	0.99
Detection probability			
α	0.22	-0.23	0.68
β	0.93	0.67	1.20

quantify the magnitude of parameter bias under each scenario, we calculated the root mean squared error between the posterior mean and the actual parameter value.

3 | RESULTS

3.1 | Field summary

We captured and swabbed 865 individuals of 39 species at least once (see Table 1a in Appendix S1). We collected 148 and 99 swabs on streams and trails, respectively, during the dry season, and 288 and

TABLE 2 Summary of the posterior distribution from the unadjusted model. All parameters were back transformed to their original scale

Definition	Mean	95% credible interval	
ω			
Stream dry	0.71	0.38	1.34
Trail dry	1.77	1.18	2.65
Stream wet	1.33	0.50	3.36
Trail wet	1.32	0.91	1.96
Ψ			
Stream dry	0.30	0.23	0.37
Trail dry	0.28	0.23	0.34
Stream wet	0.21	0.14	0.30
Trail wet	0.33	0.28	0.39

302 swabs on streams and trails, respectively, during the two wet seasons. Of the 865 individuals, we double swabbed 205 individuals, where 102 were double swabbed during the dry season and 103 were double swabbed during the two wet seasons. Of these 205 double swabbed individuals, we detected *Bd* DNA on only one swab from 51 individuals, and on both swabs from 25 individuals.

3.2 | Sampling detection-adjusted model

Under the sampling detection-adjusted model, the probability of being infected with *Bd* did not differ between streams and trails during the wet season ($\Pr(\Psi_{\text{stream,wet}} > \Psi_{\text{trail,wet}}) = 0.10$) nor the dry season ($\Pr(\Psi_{\text{stream,dry}} > \Psi_{\text{trail,dry}}) = 0.93$; Table 1). When comparing the probability of being infected with *Bd* in particular habitat types between seasons, the probability of being infected with *Bd* did not differ between wet and dry seasons for streams ($\Pr(\Psi_{\text{stream,wet}} > \Psi_{\text{stream,dry}}) = 0.38$), but it did differ for trails ($\Pr(\Psi_{\text{trail,wet}} > \Psi_{\text{trail,dry}}) = 0.98$).

Average infection intensity did not differ between streams and trails during the wet season ($\Pr(\omega_{\text{stream,wet}} > \omega_{\text{trail,wet}}) = 0.85$) nor the dry season ($\Pr(\omega_{\text{stream,dry}} > \omega_{\text{trail,dry}}) = 0.14$). When comparing average infection intensity in particular habitat types between seasons, average infection intensity differed between wet and dry seasons for streams ($\Pr(\omega_{\text{stream,wet}} > \omega_{\text{stream,dry}}) = 0.99$) but not for trails ($\Pr(\omega_{\text{trail,wet}} > \omega_{\text{trail,dry}}) = 0.49$).

Bd detection probability increased as host infection intensity increased (Figure 1). *Bd* detection probability was c. 99.99% at an infection intensity of 10 ZGE (Figure 1).

3.3 | Unadjusted model

Contrary to the sampling detection-adjusted model, the unadjusted model revealed that the probability of being infected with *Bd* differed between streams and trails only during the wet ($\Pr(\Psi_{\text{stream,wet}} > \Psi_{\text{trail,wet}}) = 0.03$) but not during the dry season ($\Pr(\Psi_{\text{stream,dry}} > \Psi_{\text{trail,dry}}) = 0.84$; Table 2). In contrast, when comparing habitat types between seasons,

the probability of being infected with *Bd* did not differ between wet and dry seasons for streams ($\Pr(\Psi_{\text{stream,wet}} > \Psi_{\text{stream,dry}}) = 0.12$) nor trails ($\Pr(\Psi_{\text{trail,wet}} > \Psi_{\text{trail,dry}}) = 0.92$), which is similar to the sampling detection-adjusted model.

Again, similar to the sampling detection-adjusted model, average infection intensity from the unadjusted model did not differ between streams and trails during the wet season ($\Pr(\omega_{\text{stream,wet}} > \omega_{\text{trail,wet}}) = 0.91$) nor the dry season ($\Pr(\omega_{\text{stream,dry}} > \omega_{\text{trail,dry}}) = 0.49$). In contrast to the sampling detection-adjusted model, average infection intensity did not differ between wet and dry seasons for streams ($\Pr(\omega_{\text{stream,wet}} > \omega_{\text{stream,dry}}) = 0.93$) nor trails ($\Pr(\omega_{\text{trail,wet}} > \omega_{\text{trail,dry}}) = 0.60$).

3.4 | Sampling detection-adjusted model vs. unadjusted model

All four of the Ψ parameters estimates, quantifying the probability of being infected with *Bd*, from the unadjusted model were lower than the parameter estimates from the sampling detection-adjusted model (all $\Pr(\Psi_{\text{adjusted}} > \Psi_{\text{unadjusted}}) < 0.05$). Likewise, all of the parameter estimates for average infection intensity, ω , from the unadjusted model were higher than the sampling detection-adjusted model (all $\Pr(\omega_{\text{adjusted}} > \omega_{\text{unadjusted}}) > 0.95$).

3.5 | *Bd*-specific methodological guidance

To be 95% certain that 1 ZGE is present on a host and that it is detected using non-invasive skin swabs, at least four swabs need to be collected. While, to be 95% certain that 3 or 5 ZGE are present and detected, at least two swabs need to be collected (Figure 2). On the contrary, non-invasive skin swabs can detect 10 ZGE with greater than 95% certainty using only a single skin swab. To be 95% certain that 1, 3, 5, or 10 ZGE are present on a host and detected using qPCR, at least two qPCR runs need be performed per sample collected (Figure 2).

3.6 | Sampling and diagnostic detection-adjusted model

The estimated probability of pathogen infection, Ψ , was less biased and more precise when average infection intensity, μ , was high and when both contributors to pathogen detection probability—sampling methods and laboratory diagnostic testing—were high (Figure 3; see Figure 3a in Appendix S1). In general, the root mean squared error of pathogen prevalence decreased more rapidly when the number of samples increased rather than the number of diagnostic tests in most scenarios (Figure 3).

Similarly, estimated average infection intensity, μ , was less biased and more precise when the probability of pathogen infection, Ψ , was high and at high values of pathogen detection probability (Figure 4; see Figure 4a in Appendix S1). In general, the root mean squared error of the estimated infection intensity was similarly impacted if either the number of samples collected or the number of diagnostic runs increased (Figure 4).

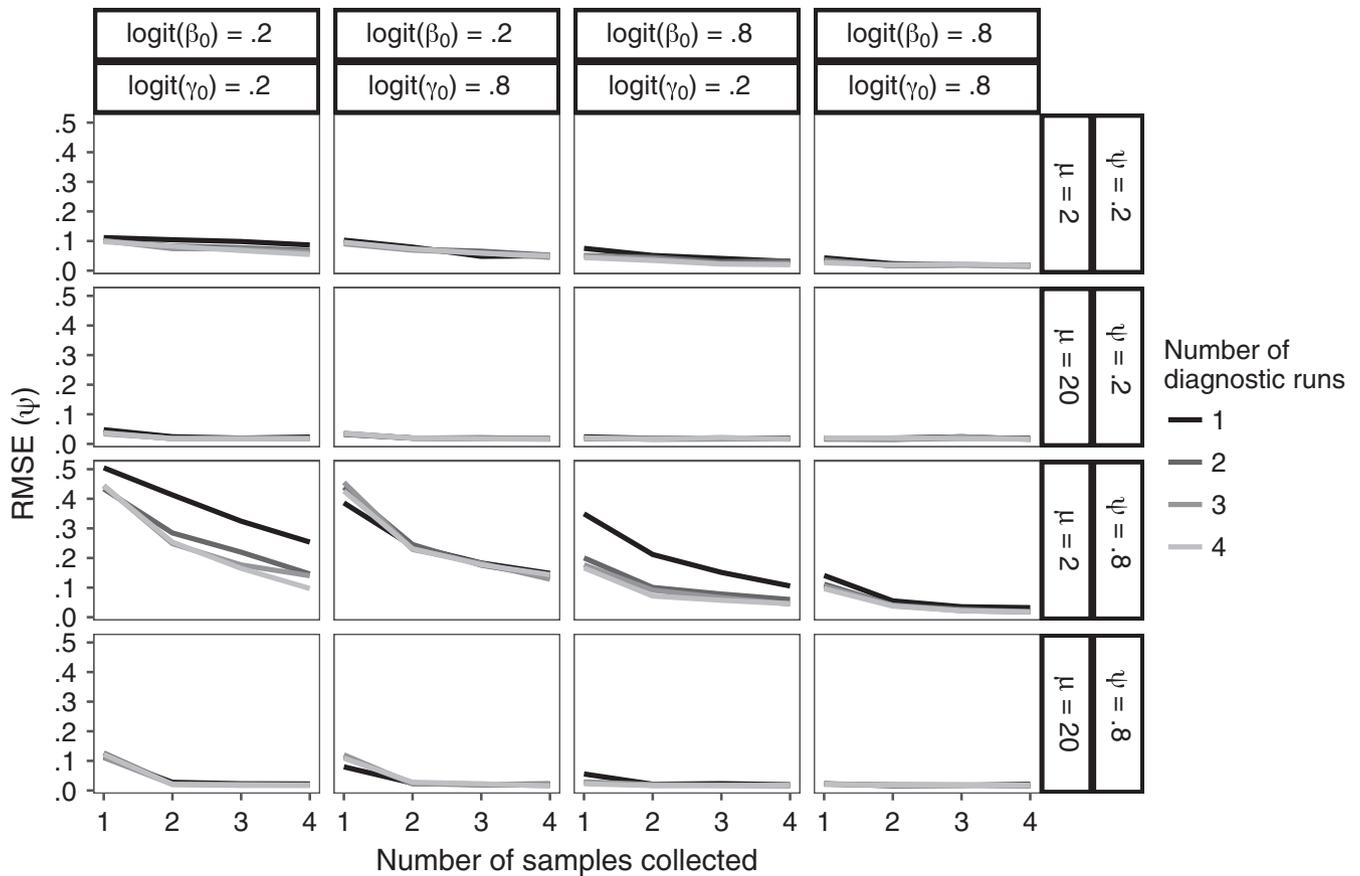


FIGURE 3 Root mean square error of estimated pathogen prevalence (Ψ) over different scenarios of known high and low pathogen prevalence (Ψ), average infection intensity (μ), pathogen log odds of detection by sampling method (β_0), and pathogen log odds of detection by laboratory diagnostics (γ_0) as the number of samples (1–4) and number of diagnostic runs (1–4) vary

4 | DISCUSSION

4.1 | Implications for wildlife disease ecology

Our results demonstrate that non-invasive skin swabbing imperfectly detects *Bd* and that Bayesian hierarchical models can adjust prevalence and average infection intensity for low-level pathogen infections that are missed. The bias caused by pathogen non-detection can affect disease inference, especially in regions where hosts harbour low-level *Bd* infections, such as in enzootic populations.

Imperfect pathogen detection threatens the success of disease monitoring programs intended to prevent pathogen invasion (e.g. Langwig et al., 2015). For example, the recent emergence of *Batrachochytrium salamandrivorans* (hereafter *Bsal*; Martel et al., 2013), the only known sister taxa of the amphibian-killing fungus, *Bd* (Longcore et al., 1999), threatens salamander biodiversity worldwide and is lethal to some of the New World salamandrid species (genera: *Taricha* and *Notophthalmus*; Martel et al., 2014). The United States has taken precautionary measures to prevent the arrival of *Bsal* into its borders by restricting the movement of salamanders under the Lacey Act (18 U.S.C. 42). The techniques used to sample and diagnose *Bsal* are similar to those used to test for *Bd*, such as non-invasive skin swabs tested by qPCR (Hyatt et al., 2007; Martel

et al., 2013). At some point, a second precautionary step would be to require that salamanders obtain health certificates to move across borders. But, given the results of this study, there is a chance that low-level *Bsal* infections will be missed. If *Bsal* detection probability is similar to *Bd*, then we expect that *Bsal* infections less than 5 ZGE will likely be missed up with a certainty of 95% by a single non-invasive skin swab; similarly, qPCR will detect infections less than 10 ZGE approximately 81% of the time if only one qPCR run were performed. This is especially concerning when importing salamanders from Eastern Asia, where salamanders typically have *Bsal* infection intensities less than 30 zoospores (Martel et al., 2014) and *Bsal* prevalence is low (i.e., <10%; Laking, Ngo, Pasmans, Martel, & Nguyen, 2017; Martel et al., 2014).

In El Copé, Panama, where *Bd* is now enzootic, most individuals were infected (average *Bd* prevalence c. 64%–92%) and carried low-level infections (<10 ZGE), which is similar to other regions in the Americas (James et al., 2015). In the 1990s, as *Bd* spread worldwide, many amphibian populations experienced mass mortality events and population declines (e.g. Berger, Hyatt, Speare, & Longcore, 2005; Lips et al., 2006; Muths, Corn, Pessier, & Green, 2003; Vredenburg, Knapp, Tunstall, & Briggs, 2010). In many of these areas today, amphibians persist with enzootic *Bd* infections, and disease ecologists are interested in explaining the ecological patterns of infection and

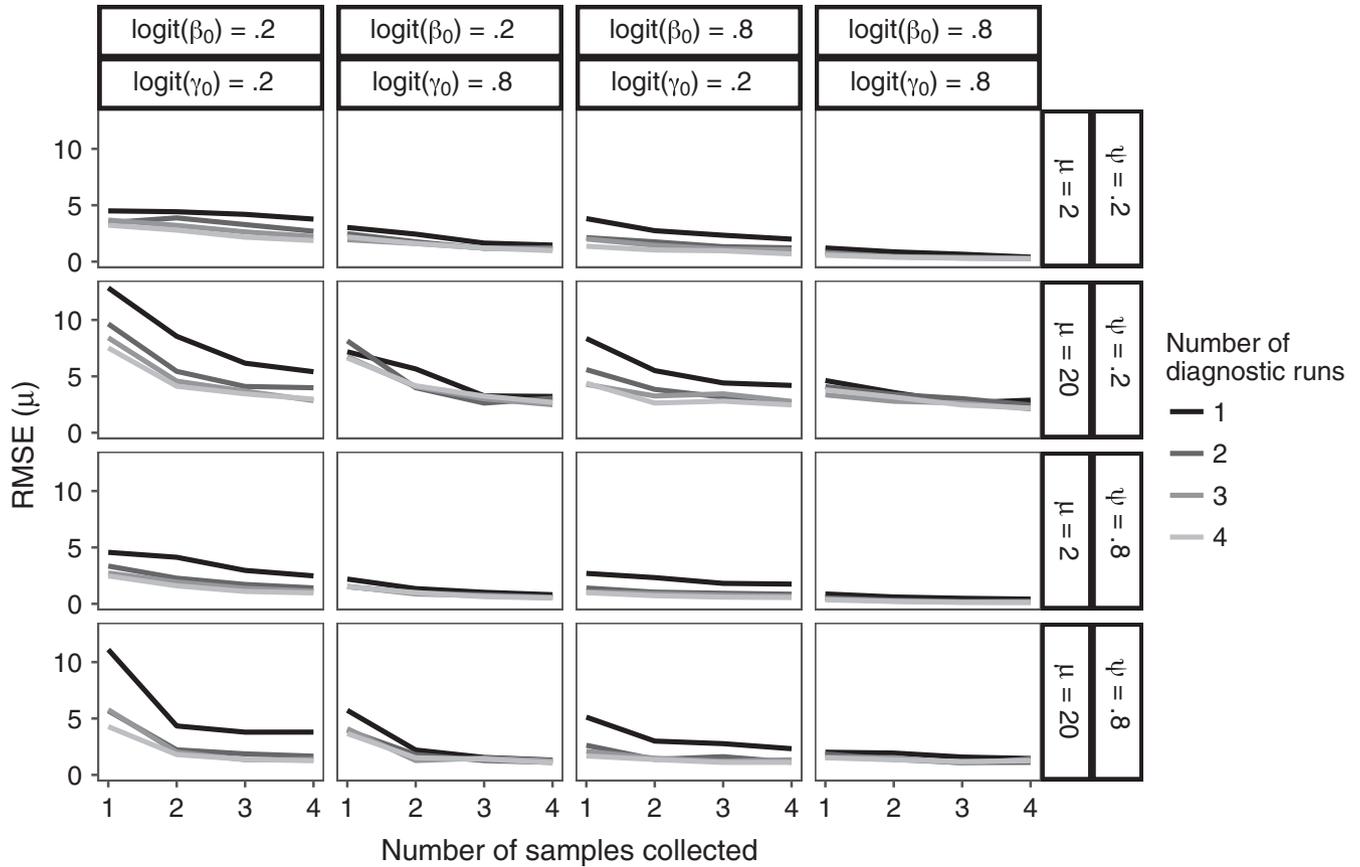


FIGURE 4 Root mean square error of estimated average infection intensity (μ) over different scenarios of known high and low pathogen prevalence (Ψ), average infection intensity (μ), pathogen log odds of detection by sampling methods (β_0), and pathogen log odds of detection by laboratory diagnostics (γ_0) as the number of field samples (1–4) and number of diagnostic runs (1–4) vary

host–pathogen coexistence. If disease ecologists do not correct for imperfect pathogen detection, then estimates of disease will be biased, and in some cases, covariates that affect the sampling process may end up in the ecological process model (e.g. Kéry & Schaub, 2012). In this study, our conclusions on *Bd* probability of infection and infection intensity with respect to habitat and seasons were predominately similar regardless of whether we accounted for imperfect pathogen detection, which is not surprising given the “noisy” estimates for these covariates. However, the parameter estimates from the sampling detection-adjusted and unadjusted model differed significantly with respect to precision and bias, which emphasises the importance of accounting for imperfect pathogen sampling. If overlooked, even small frequencies of false negatives can lead to inaccurate inference and biased conclusions.

4.2 | *Bd*-specific methodological guidance

In the case of *Bd*, we found that the greatest pathogen detection bias was caused when host infection intensity was low, as expected. We highlight that pathogen detection probability is lower than reported from only the double swab data because total imperfect pathogen detection depends on both pathogen detection probability of the laboratory diagnostic tests (i.e., qPCR; Lachish et al., 2012; Miller et al., 2012) and sampling methods (i.e., swabbing; e.g. Thompson, 2007;

Figure 1). These results indicate that replication of samples in both sampling and laboratory methods are critical to minimise observational uncertainty, especially when pathogen prevalence and infection intensity are expected to be low. This is the case in both enzootics and in the invasion phase of an epizootic (Langwig et al., 2015).

We recognise the increase cost and effort needed to analyse more swab samples in replicate; therefore, we suggest collecting replicate swabs when possible because if the results from the first swab set shows few pathogen detections, low pathogen prevalence, and low host infection intensity, it may be worth analysing the second set to calculate false negative error rates.

4.3 | General methodological guidance

Applying the sampling and diagnostic detection-adjusted model, we find that there are trade-offs, for a fixed effort, in precision and accuracy of pathogen prevalence and average infection intensity estimates. Although our simulation study provides general methodological guidance under different sampling scenarios, these results must be considered in combination with common sense and expert knowledge of the study system. For example, researchers must consider the cost and time constraints of collecting multiple samples per individual and running multiple diagnostic tests per sample, as well as the trade-offs between sampling breadth and accuracy. Given that the magnitude of bias depends on the study

system, type of infection, and false negative error rates, we advocate that the most cautious approach is to assume all errors are possible and to accommodate them by adjusting sampling designs. Using the R code provided in Appendix S2 as a foundation, it may be worthwhile to simulate study-specific scenarios to understand the trade-offs between efficiency and robustness of particular study designs.

The models we present here can be applied to designing studies and analysing data for other emerging infectious fungal diseases, such as white-nose syndrome (Langwig et al., 2015), snake fungal disease (Tetzlaff, Allender, Ravesi, & Smith, 2015), and the salamander fungus (*Bsal*; Martel et al., 2014). Similar to detecting *Bd* on amphibian skin, each of these study systems is typified by the collection of non-invasive skin samples and using qPCR analysis to test for pathogen presence and infection intensity, subjecting them to similar kinds of detection errors as the *Bd*-amphibian system. Should these infectious diseases spread from their initial distributions, it will be critical to be able to compare disease status and dynamics across studies with respect to biotic and abiotic covariates. This will require unbiased and precise estimates of key epidemiological parameters, such as pathogen prevalence and infection intensity. By accounting for detection errors arising from sampling and diagnostic tests, we can more readily compare disease inference among systems and species.

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AUTHORS' CONTRIBUTIONS

G.V.D. helped in designing the study, collected and analysed samples in the laboratory, formulated the models, and wrote the first draft of the paper. K.R.L. helped design the study and collect samples. A.V.L. and K.R.Z. helped analyze the samples in the laboratory. C.C.C. helped formulate the models. E.H.C.G. helped design the study and formulate the models. All authors contributed substantially to manuscript revisions.

DATA ACCESSIBILITY

The data used in the analysis for the main text can be found online at the Dryad Digital Repository <https://doi.org/10.5061/dryad.p1006>

(DiRenzo et al., 2017). Simulation data can be generated using code in Appendix S2, and all the R scripts used to analyse the data and create figures can be found on the Github repository https://github.com/Grace89/ImperfectPathogenDetection_MEES. This repository is citable using: <https://doi.org/10.5281/zenodo.840132>.

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REFERENCES

- Berger, L., Hyatt, A. D., Speare, R., & Longcore, J. E. (2005). Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms*, *68*, 51–63.
- Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A. T., & Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis. *Diseases of Aquatic Organisms*, *60*, 141–148.
- Brem, F., & Lips, K. (2008). *Batrachochytrium dendrobatidis* infection patterns among Panamanian amphibian species, habitats and elevations during epizootic and enzootic stages. *Diseases of Aquatic Organisms*, *81*, 189–202.
- Briggs, C. J., Knapp, R. A., & Vredenburg, V. T. (2010). Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proceedings of the National Academy of Sciences of the United States of America*, *107*, 9695–9700.
- Brooks, S. P., & Gelman, A. (1998). General methods for monitoring convergence of iterative simulations. *Journal of Computational and Graphical Statistics*, *7*, 434–455.
- Cheng, T. L., Rovito, S. M., Wake, D. B., & Vredenburg, V. T. (2011). Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. *Proceedings of the National Academy of Sciences of the United States of America*, *108*, 9502–9507.
- Colvin, M. E., Peterson, J. T., Kent, M. L., & Schreck, C. B. (2015). Occupancy modeling for improved accuracy and understanding of pathogen prevalence and dynamics. *PLoS ONE*, *10*, e0116605.
- Cooch, E. G., Conn, P. B., Ellner, S. P., Dobson, A. P., & Pollock, K. H. (2011). Disease dynamics in wild populations: Modeling and estimation: A review. *Journal of Ornithology*, *152*, 485–509.
- DiRenzo, G. V., Campbell Grant, E. H., Longo, A. V., Che-Castaldo, C., Zamudio, K. R., & Lips, K. R. (2017). Data from: Imperfect pathogen detection from non-invasive skin swabs biases disease inference. *Dryad Digital Repository*, <https://doi.org/10.5061/dryad.p1006>
- Drewe, J. A., Dean, G. S., Michel, A. L., & Pearce, G. P. (2009). Accuracy of three diagnostic tests for determining *Mycobacterium bovis* infection status in live-sampled wild meerkats (*Suricata suricatta*). *Journal of Veterinary Diagnostic Investigation*, *21*, 31–39.
- Enoe, C., Georgiadis, M. P., & Johnson, W. O. (2000). Estimation of sensitivity and specificity of diagnostic tests and disease prevalence when the true disease status is unknown. *Preventive Veterinary Medicine*, *45*, 61–81.
- Fisher, M. C., Garner, T. W. J., & Walker, S. F. (2009). Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annual Review of Microbiology*, *63*, 291–310.
- Gelman, A., Carlin, J. B., Stern, H. S., Dunson, D. B., Vehtari, A., & Rubin, D. B. (2013). *Bayesian data analysis*. Boca Raton, FL: CRC Press.
- Gómez Díaz, E., Doherty, P. F., Duneau, D., & McCoy, K. D. (2010). Cryptic vector divergence masks vector-specific patterns of infection: An example from the marine cycle of Lyme borreliosis. *Evolutionary Applications*, *3*, 391–401.

- Greiner, M., & Gardner, I. A. (2000). Epidemiologic issues in the validation of veterinary diagnostic tests. *Preventive Veterinary Medicine*, 45, 3–22.
- Hyatt, A. D., Boyle, D. G., Olsen, V., Boyle, D. B., Berger, L., Obendorf, D., ... Colling, A. (2007). Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms*, 73, 175–192.
- James, T. Y., Toledo, L. F., Rödder, D., da Silva Leite, D., Belasen, A. M., Betancourt-Román, C. M., ... Longcore, J. E. (2015). Disentangling host, pathogen, and environmental determinants of a recently emerged wild-life disease: Lessons from the first 15 years of amphibian chytridiomycosis research. *Ecology and Evolution*, 5, 4079–4097.
- Kellner, K. (2015). jagsUI: A wrapper around 'rjags' to streamline 'JAGS' analyses. R package version 1.3.7. <http://CRAN.R-project.org/package=jagsUI>.
- Kery, M. (2002). Inferring the absence of a species – A case study of snakes. *Journal of Wildlife Management*, 66, 330–338.
- Kéry, M., & Schaub, M. (2012). *Bayesian population analysis using WinBUGS: A hierarchical perspective*. Oxford, UK: Elsevier.
- Knowles, S. C. L., Wood, M. J., Alves, R., Wilkin, T. A., Bensch, S., & Sheldon, B. C. (2011). Molecular epidemiology of malaria prevalence and parasitaemia in a wild bird population. *Molecular Ecology*, 20, 1062–1076.
- Kruger, K. M., & Hero, J. M. (2006). Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. *Journal of Zoology*, 271, 352–359.
- Kruger, K. M., Hero, J., & Ashton, K. J. (2006). Cost efficiency in the detection of chytridiomycosis using PCR assay. *Diseases of Aquatic Organisms*, 71, 149–154.
- Lachish, S., Gopalaswamy, A. M., Knowles, S. C. L., & Sheldon, B. C. (2012). Site-occupancy modelling as a novel framework for assessing test sensitivity and estimating wildlife disease prevalence from imperfect diagnostic tests. *Methods in Ecology and Evolution*, 3, 339–348.
- Lachish, S., Jones, M., & McCallum, H. (2007). The impact of disease on the survival and population growth rate of the Tasmanian devil. *Journal of Animal Ecology*, 76, 926–936.
- Laking, A. E., Ngo, H. N., Pasmans, F., Martel, A., & Nguyen, T. T. (2017). *Batrachochytrium salamandrivorans* is the predominant chytrid fungus in Vietnamese salamanders. *Scientific Reports*, 7, 44443.
- Langwig, K. E., Frick, W. F., Reynolds, R., Parise, K. L., Drees, K. P., Hoyt, J. R., ... Kilpatrick, A. M. (2015). Host and pathogen ecology drive the seasonal dynamics of a fungal disease, white-nose syndrome. *Proceedings of the Royal Society B*, 282, 20142335.
- Langwig, K. E., Voyles, J., Wilber, M. Q., Frick, W. F., Murray, K. A., Bolker, B. M., ... Kilpatrick, A. M. (2015). Context-dependent conservation responses to emerging wildlife diseases. *Frontiers in Ecology and the Environment*, 13, 195–202.
- Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., ... Collins, J. P. (2006). Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 3165–3170.
- Lips, K. R., Reeve, J. D., & Witters, L. R. (2003). Ecological traits predicting amphibian population declines in Central America. *Conservation Biology*, 17, 1078–1088.
- Longcore, J. E., Pessier, A. P., & Nichols, D. K. (1999). *Batrachochytrium dendrobatidis* gen et sp nov, a chytrid pathogenic to amphibians. *Mycologia*, 91, 219–227.
- Lunn, D., Jackson, C., Best, N., Thomas, A., & Spiegelhalter, D. (2012). *The BUGS book: A practical introduction to Bayesian analysis*. Boca Raton, FL: CRC Press.
- Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M. C., ... Pasmans, F. (2014). Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science*, 346, 630–631.
- Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M. C., ... Pasmans, F. (2013). *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences*, 110, 15325–15329.
- Miller, D. A. W., Talley, B. L., Lips, K. R., & Campbell Grant, E. H. (2012). Estimating patterns and drivers of infection prevalence and intensity when detection is imperfect and sampling error occurs. *Methods in Ecology and Evolution*, 3, 850–859.
- Muths, E., Corn, P. S., Pessier, A. P., & Green, D. E. (2003). Evidence for disease related amphibian decline in Colorado. *Biological Conservation*, 110, 357–365.
- Olson, D. H., Aanensen, D. M., Ronnenberg, K. L., Powell, C. I., Walker, S. F., Bielby, J., ... Fisher, M. C. (2013). Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. *PLoS ONE*, 8, e56802.
- R Core Team. (2015). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. <http://www.R-project.org/>.
- Ritacco, V., Lopez, B., Dekantor, I. N., Barrera, L., Errico, F., & Nader, A. (1991). Reciprocal cellular and humoral immune-responses in bovine tuberculosis. *Research in Veterinary Science*, 50, 365–367.
- Royle, J. A., & Nichols, J. D. (2003). Estimating abundance from repeated presence-absence data or point counts. *Ecology*, 84, 777–790.
- Ruiz-Gutiérrez, V., Zipkin, E. F., & Dhondt, A. (2010). Occupancy dynamics in a tropical bird community: Unexpectedly high forest use by birds classified as non-forest species. *Journal of Applied Ecology*, 47, 621–630.
- Tetzlaff, S., Allender, M., Ravesi, M., & Smith, J. (2015). First report of snake fungal disease from Michigan, USA involving Massasaugas, *Sistrurus catenatus* (Rafinesque 1818). *Herpetology Notes*, 8, 31–33.
- Thompson, K. G. (2007). Use of site occupancy models to estimate prevalence of *Myxobolus cerebralis* infection in trout. *Journal of Aquatic Animal Health*, 19, 8–13.
- Toft, N., Jørgensen, E., & Højsgaard, S. (2005). Diagnosing diagnostic tests: Evaluating the assumptions underlying the estimation of sensitivity and specificity in the absence of a gold standard. *Preventive Veterinary Medicine*, 68, 19–33.
- Valkiunas, G., Iezhova, T. A., Krizanauskiene, A., Palinauskas, V., Sehgal, R. N. M., & Bensch, S. (2008). A comparative analysis of microscopy and PCR-based detection methods for blood parasites. *Journal of Parasitology*, 94, 1395–1401.
- Vredenburg, V. T., Knapp, R. A., Tunstall, T. S., & Briggs, C. J. (2010). Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 9689–9694.
- Wake, D. B., & Vredenburg, V. T. (2008). Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 11466–11473.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Fungal Infection Intensity and Zoospore Output of *Atelopus zeteki*, a Potential Acute Chytrid Supershedder

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Abstract

Amphibians vary in their response to infection by the amphibian-killing chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*). Highly susceptible species are the first to decline and/or disappear once *Bd* arrives at a site. These competent hosts likely facilitate *Bd* proliferation because of ineffective innate and/or acquired immune defenses. We show that *Atelopus zeteki*, a highly susceptible species that has undergone substantial population declines throughout its range, rapidly and exponentially increases skin *Bd* infection intensity, achieving intensities that are several orders of magnitude greater than most other species reported. We experimentally infected individuals that were never exposed to *Bd* ($n=5$) or previously exposed to an attenuated *Bd* strain (JEL427-P39; $n=3$). Within seven days post-inoculation, the average *Bd* infection intensity was 18,213 zoospores (SE: 9,010; range: 0 to 66,928). Both average *Bd* infection intensity and zoospore output (i.e., the number of zoospores released per minute by an infected individual) increased exponentially until time of death ($t_{50}=7.018$, $p<0.001$, $t_{46}=3.164$, $p=0.001$, respectively). Mean *Bd* infection intensity and zoospore output at death were 4,334,422 zoospores (SE: 1,236,431) and 23.55 zoospores per minute (SE: 22.78), respectively, with as many as 9,584,158 zoospores on a single individual. The daily percent increases in *Bd* infection intensity and zoospore output were 35.4% (SE: 0.05) and 13.1% (SE: 0.04), respectively. We also found that *Bd* infection intensity and zoospore output were positively correlated ($t_{43}=3.926$, $p<0.001$). All animals died between 22 and 33 days post-inoculation (mean: 28.88; SE: 1.58). Prior *Bd* infection had no effect on survival, *Bd* infection intensity, or zoospore output. We conclude that *A. zeteki*, a highly susceptible amphibian species, may be an acute supershedder. Our results can inform epidemiological models to estimate *Bd* outbreak probability, especially as they relate to reintroduction programs.

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Introduction

Differences in amphibian susceptibility to *Batrachochytrium dendrobatidis* (*Bd*) infection were evident since the pathogen was first described [1,2]. Species-specific responses to infection range from tolerant [3,4] or resistant [5] to highly susceptible [6,7], suggesting that a subset of species can disproportionately affect pathogen spread and disease transmission [8,9]. Yet, we know relatively little about contact rates, infectivity, and zoospore output of *Bd*'s amphibian hosts in either the field or laboratory.

Differences in species transmission rates can cause variations in pathogen spread and dispersal in the wild [10–12]. One illustration of the potential effects of variable inter-specific interactions are superspreaders [8], individuals or species responsible for a greater than average number of secondary infections [8,12,13]. Superspreading occurs under two scenarios: (1) supercontacters transmit more disease by making more contacts in the population per individual, or (2) supershedders transmit more disease per contact (reviewed by [14]). To date, the primary evidence for superspreading stems from supercontacters (e.g., [15–17]); but growing evidence shows that species vary consistently in

pathogen infection intensities (e.g., [18,19]), especially in the amphibian-*Bd* system (e.g., [20,21]).

An amphibian's *Bd* infection intensity likely determines its infectivity (i.e., an individual's ability to infect another individual) and survival time [6,22,23]. A host's *Bd* infection intensity increases via reinfection by zoospores released onto the surface of the skin or by infection from zoospores in the environment. Quantifying host-specific *Bd* zoospore output, the number of zoospores released per minute by an infected individual [4], is critical to understanding differences in infectivity across species and species-specific contributions to the environmental zoospore pool.

Highly susceptible amphibian species typically die at high *Bd* infection intensities (e.g., [7,22]), suggesting that highly susceptible species may act as supershedders for a short period of time. In several cases across Central America [24,25], *Bd* has caused the decline and extirpation of harlequin frog (genus: *Atelopus*) populations. Of the 113 *Atelopus* species, as many as 30 species have been declared Extinct in the Wild [24], and according to the IUCN, 80% of *Atelopus* species are Critically Endangered and 70% have declining populations. *Atelopus* experience rapid widespread population declines upon *Bd* site invasion, demonstrating high

susceptibility. Here, we refer to *Atelopus* as a candidate acute supershedder to better describe the phenomena of high susceptibility and pathogen shedding.

Our goals in this study were to: (1) quantify *Bd* infection intensity and zoospore output of *Atelopus zeteki*, (2) determine the daily percent increase of *Bd* infection intensity and zoospore output on *A. zeteki*, and (3) determine if prior *Bd* exposure affects infection intensity and zoospore output. Our results are important in understanding species and community responses to *Bd* invasion and are relevant to future reintroduction programs.

Methods

Ethics statement

Our research strictly followed the guidelines of and was approved by the University of Maryland Institute for Animal Care and Use Committee (protocol #R-12-98) and the Maryland Zoo in Baltimore Institutional Animal Care and Use Committee.

Experimental procedures

We obtained 13 captive-bred *A. zeteki* individuals, 15 months post-metamorphosis, used in an earlier *Bd* experiment [26]. Ten animals were uninfected controls, and three were previously inoculated with JEL 427-P39 23 weeks before the start of our experiment. During the course of the earlier experiment [26], individuals were swabbed once every two weeks for 130 days. One individual consistently tested *Bd* negative for the duration of that experiment. The other two individuals tested *Bd* positive three and four times, respectively. The last swabbing event was five weeks before the start of our experiment where two of the three individuals were mildly infected.

We matched individuals by weight into two groups of five. We found no difference in weight between the infected and control groups at the start of the experiment ($p > 0.05$). The three individuals previously exposed to *Bd* strain JEL 427-P39 were assigned to the infected treatment. All individuals were sexed by examination for eggs, ovaries, or testicles at time of death (12 female and 1 male). The single male had been placed in the control treatment.

Animals were housed in plastic boxes filled with sphagnum moss, a hide, and a water dish, in a laboratory maintained at 21–22°C with a 12:12 light: dark photoperiod. We replaced all housing materials every seven days, changed water dishes every three days, fed frogs vitamin-dusted crickets or fruit flies (*Drosophila melanogaster ad libitum* every three days, and misted terraria daily. We monitored individuals daily for clinical symptoms of *Bd* and euthanized all individuals once they lost righting abilities by applying Benzocaine 20% gel to the venter. All control individuals were euthanized when the last infected individual was euthanized.

We inoculated individuals with *Bd* strain JEL 423, a member of the hypervirulent *Bd*GPL lineage, originally isolated from an infected *Hylomantis lemur* during the epidemic at El Copé, Panama in 2004 [27]. We grew *Bd* strain JEL 423 on 1% tryptone agar plates for seven days, flooded plates with 1% tryptone broth, filtered the liquid to obtain a pure zoospore stock solution, and diluted the pure stock solution with water to achieve the desired concentration [26]. We individually inoculated the eight infected treatment frogs with 30,000 *Bd* zoospores for 10 hours. The five control individuals were exposed to a sham solution of water and <1% tryptone broth, roughly the same amount that had been used for the *Bd* treatment minus the zoospores, for the same period.

We used a fresh pair of latex powder-free gloves when handling each individual. We followed the swabbing protocol of Hyatt et al.

[28]. Immediately post-swabbing, we individually soaked each frog in 50 mL of distilled water for 15 minutes and added 50 μ L of bovine serum albumen (BSA) to the water solution after removing each frog [4]. We immediately filtered the solution using a 60 mL sterile syringe and 0.45 μ m filter for each sample. Filters were plugged with syringe caps and stored in a 4°C refrigerator. Swabbing individuals before soaking could reduce the number of *Bd* zoospores estimated from the soak, thus our estimates are minimum zoospore output estimates.

We swabbed and soaked all individuals starting on day seven post-inoculation, thereafter every three to four days, and immediately prior to euthanasia. We extracted DNA from samples using PrepMan Ultra and analyzed samples using the standard real-time quantitative polymerase chain reaction assay [28,29]. *Bd* infection intensity was defined as the number of *Bd* genomic equivalents detected on a single swab [7]. We categorized individuals as *Bd*-positive when *Bd* infection intensity was greater than or equal to one zoospore genomic equivalent [30].

We performed all statistical analyses in R [31]. We modeled the change in *Bd* infection intensity (N) with respect to time (t) using $dN/dt = y_0 e^{rt}$, where y_0 is the initial infection intensity, r is the daily rate of increase of infection intensity, and t is time in days. We used the same equation to model the change in zoospore output with respect to time. To calculate parameter estimates, we fitted two linear mixed models with a first order autoregressive correlation term to ln transformed response variables (i.e., *Bd* infection intensity and zoospore output; package *nlme*, [32]). We included prior infection history as an independent variable to determine if prior *Bd* exposure affected either response variable. We used AIC to compare model fit.

To determine if *Bd* infection intensity and zoospore output were correlated, we used a generalized linear mixed model with a first order autoregressive correlation term and a lognormal error distribution. To determine if survival curves of frogs with different infection histories differed, we used a logrank-test (package *survival*, [33]).

Results

All frogs exposed to *Bd* lost righting abilities and were euthanized within 33 days post-inoculation (Figure 1; 100% mortality, mean: 28.88 days, SE: 1.58). All control animals tested negative at all sampling events, and no control animal experienced mortality during the course of the experiment.

At time of death, infected frogs had an average *Bd* infection intensity of 4,334,422 zoospores (SE: 1,156,576; range = 520,436 to 9,584,158) and an average zoospore output of 23.55 zoospores per minute (SE: 22.78; range = 0.00 to 172.61; Table 1).

Bd infection intensity and zoospore output increased exponentially over time ($t_{50} = 7.018$, $p < 0.001$; $t_{46} = 3.164$, $p = 0.001$, respectively). Including prior exposure or higher order polynomials did not improve model fit. The daily percent increase in *Bd* infection intensity and zoospore output were 35.4% (SE: 0.05) and 13.1% (SE: 0.04), respectively. *Bd* infection intensity and zoospore output were positively correlated (Figure 2; $t_{43} = 3.926$, $p < 0.001$). Prior *Bd* exposure did not affect *Bd* infection intensity or zoospore output ($t_6 = 1.896$, $p = 0.106$; $t_6 = 0.624$, $p = 0.555$, respectively). Survival rates also did not differ between naïve and previously exposed individuals ($p > 0.05$).

Filtered water from frog soaks produced more false negatives than skin swabs. Seventeen soaks tested negative, even though skin swabs tested positive. Only three swabs tested negative during the entire experiment. At time of death, three individual soaks tested *Bd* negative, although swab infection intensity from the same

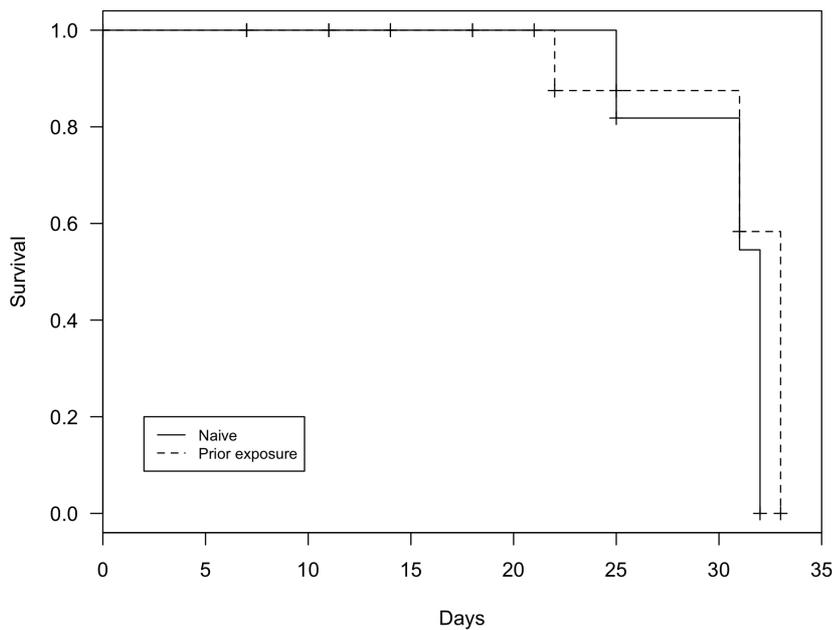


Figure 1. Survival curves of *Atelopus zeteki* with (n=3) and without (n=5) prior *Bd* exposure (log-rank test: $\chi^2 = 0.7, p = 0.40$).
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sampling period was extremely high (Table 1), suggesting either zoospores were trapped in the filters or the PCR reaction was inhibited.

Discussion

Exposing *Atelopus zeteki* to *Bd* strain JEL 423 produced individuals with *Bd* infection intensities among the highest reported for any species to date (Table 2). Individuals also had high zoospore output, indicating *A. zeteki* were highly infectious and may contribute disproportionately to the environmental *Bd* zoospore pool. Other experimental infections [26,34] and field studies [35] also show that *Atelopus* spp. develop high *Bd* infection intensities, further suggesting that the genus *Atelopus* may be acute supershedders.

Other *Atelopus* studies have shown similarly high *Bd* infection intensities. Experimental infections of *A. zeteki* with other *Bd* strains (another Panamanian isolate JEL408 and a Puerto Rican isolate JEL427) showed *Bd* infection intensities ranging between 7.2×10^4

and $>10^6$ zoospores at death (Table 2; [26,34]). Field studies also show high infection intensities in other species of *Atelopus*. Lampo et al. [35] reported the *Bd* infection intensity of a single dying *Atelopus crucifer* individual as high as 244,000 zoospores. We cannot rule out *Bd* identity as the cause of variable high infection intensities at death because *Atelopus* were exposed to different *Bd* strains. Yet, the infection intensities in all lab and field studies were very high and caused rapid mortality.

Although we used an unnaturally high inoculation dose in this experiment, our results and conclusions are applicable to field scenarios because they mimic late stage infections. Carey et al. [22] showed that all individuals of *Bufo [Anaxyrus] boreas* died of infection at the same *Bd* infection intensity, those receiving lower doses only took longer to build infections and die. We used a high inoculation dose to minimize the duration of the experiment. Further studies are needed to document *Bd* infection intensities of *Atelopus* in the field and to determine whether *Atelopus* drives disease dynamics in other species.

Table 1. Summary of *Atelopus zeteki* infection intensity (number of zoospores on skin swabs) and zoospore output (number of zoospores released per minute) at death.

Prior exposure	Total days survived post-inoculation	<i>Bd</i> infection intensity at death	Zoospore output at death
Naïve	21	520,436	3.5
Naïve	28	1,697,306	0.0
Naïve	18	4,454,759	4.9
Naïve	31	8,781,016	0.2
Naïve	25	9,584,158	170.6
Previous	18	2,291,631	7.1
Previous	33	2,960,916	0.0
Previous	31	4,385,154	0.0

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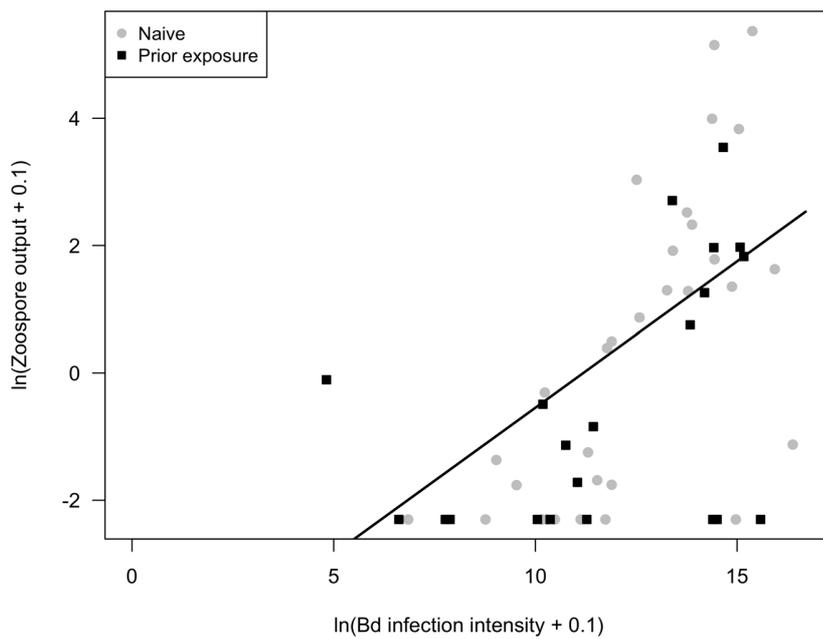


Figure 2. Relationship between *Bd* infection intensity and zoospore output. The solid black line corresponds to the linear regression fitted to all points ($t_{43} = 3.926, p < 0.001$). *Bd* infection intensity and zoospore output were positively correlated and not influenced by prior *Bd* exposure of the amphibian.

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We not only found that *Bd* infection intensity in *A. zeteki* at time of death was $>10^6$, but that *A. zeteki* had a high daily rate of increase in *Bd* infection intensity and zoospore output. We are only aware of a few studies that have quantified the daily rate of increase in *Bd* infection intensity [22,36] or zoospore output [28]. *Bufo* [*Anaxyrus*] *boreas* had daily percent increases in *Bd* infection intensity of 68% and produced individuals with $>10^7$ zoospores at death (Table 2). Interestingly, *Rana* [*Lithobates*] *muscosa/sierra* had daily percent increases in *Bd* infection intensity of only 8% and infection intensities at death were approximately 10^4 zoospores [36]. Meanwhile, *Litoria caerulea* had a daily rate of increase in zoospore output of 15.43% (SE: 2.29; [28]), but we were unable to compare the *Bd* infection intensity at death or mortality rate of this species to others because it was not reported. Yet, the first three species mentioned (*A. zeteki*, *B. boreas*, and *R. muscosa/sierra*) have

experienced mass mortality and widespread population declines [6,7,24,25,37–39], suggesting that where infections build rapidly, frogs die with higher burdens.

Our study also provides evidence that *Bd* pre-exposure is insufficient to change the outcome of infection. This suggests that either (1) *A. zeteki* can not mount an effective adaptive immune response or (2) *Bd* possibly evades [40] and/or suppresses the immune system [41–43]. For example, Fites et al. [43] showed that *Bd* cells and supernatant impaired lymphocyte proliferation and induced apoptosis. The three individuals that were inoculated with JEL427-P39 may have persisted with mild infections during the first experiment because of several mechanisms acting singly or in concert: (1) their immune system was able to minimize infections, (2) the attenuated strain did not reproduce well, or (3) the inoculation was ineffective. We have no data to inform the first or

Table 2. Average *Bd* infection intensity of adult amphibians at death by several experimental studies.

Species	Study	<i>Bd</i> strain	Average <i>Bd</i> infection intensity at death
<i>Bufo boreas</i>	Carey et al. [22]	JEL 275*	10^7 to 10^8
<i>Atelopus zeteki</i>	Becker et al. [34]	JEL 408*	$>10^6$
<i>Atelopus zeteki</i>	This study	JEL 423*	$>10^6$
<i>Litoria booroolongensis</i>	Cashins et al. [47]	Native*	10^4 to 10^5
<i>Pseudacris regilla</i>	Reeder et al. [4]	Unknown	2.2×10^5
<i>Atelopus zeteki</i>	Langhammer et al. [26]	JEL 427-P9	1.2×10^5
<i>Atelopus zeteki</i>	Langhammer et al. [26]	JEL 427-P39	7.2×10^4
<i>Rana sierrae</i>	Rosenblum et al. [48]	Sierra Nevada- <i>Bd</i> *	5.6×10^4
<i>Rana muscosa</i>	Rosenblum et al. [48]	Sierra Nevada- <i>Bd</i> *	2.2×10^4
<i>Rana muscosa/sierrae</i>	Stice and Briggs [36]	LJR119*	5.1×10^3

* indicates the *Bd* strain used occurs within the amphibian species native range.
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second possibility, although the first possibility seems unlikely given the eventual mortality of those individuals; and the third possibility can be eliminated, given that all individuals, except one, tested *Bd* positive during the experiment.

Ex situ captive assurance *Atelopus* colonies are used as conservation tools to prevent extinction of the genus, with the ultimate goal of returning individuals to their native habitats. Yet, high *Bd* infection intensities and zoospore output of *A. zeteki* may create challenges for reintroduction programs. Not only do *Atelopus* experience high mortality rates when exposed to *Bd*, but there is substantial cause for concern if *Atelopus* are acute supershedders. To determine the feasibility of *Atelopus* reintroductions, future studies should examine *Bd* infection intensity, zoospore output, and immune function of *Atelopus* under different environmental conditions (e.g., [44–46]). Understanding infectivity, duration of infectiveness, and transmission heterogeneity among amphibian

species and populations will lead to a more comprehensive understanding of factors leading to different disease outcomes among populations following *Bd* invasion.

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Author Contributions

Conceived and designed the experiments: GVD PFL KRZ KRL. Performed the experiments: GVD PFL. Analyzed the data: GVD. Contributed reagents/materials/analysis tools: KRZ KRL. Wrote the paper: GVD PFL KRZ KRL.

References

- Lips KR, Reeve JD, Witters LR (2003) Ecological traits predicting amphibian population declines in Central America. *Conserv Biol* 17: 1078–1088.
- Crawford AM, Lips KR, Bermingham E (2010) Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. *Proc Natl Acad Sci U S A* 107: 13777–13782.
- Daszak P, Strieby A, Cunningham AA, Longcore JE, Brown CC, et al. (2004) Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. *Herpetol J* 14: 201–207.
- Reeder NMM, Pessier AP, Vredenburg VT (2012) A Reservoir Species for the Emerging Amphibian Pathogen *Batrachochytrium dendrobatidis* Thrives in a Landscape Decimated by Disease. *PLoS ONE* 7: e33567.
- Bishop PJ, Speare R, Poulter R, Butler M, Speare BJ, et al. (2009) Elimination of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* by Archey's frog *Leiopelma archeyi*. *Dis Aquat Organ* 84: 9–15.
- Briggs CJ, Knapp RA, Vredenburg VT (2010) Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proc Natl Acad Sci U S A* 107: 9695–9700.
- Vredenburg VT, Knapp RA, Tunstall TS, Briggs CJ (2010) Dynamics of an emerging disease drive large scale amphibian population extinction. *Proc Natl Acad Sci U S A* 107: 9689–9694.
- Lloyd-Smith JO, Schreiber SJ, Kopp PE, Getz WM (2005) Superspreading and the effect of individual variation on disease emergence. *Nature* 438: 355–359.
- Streicker DG, Fenton A, Pedersen AB (2013) Differential sources of host species heterogeneity influence the transmission and control of multihost parasite. *Ecol Lett* 16: 975–984.
- Dwyer G, Elkinton JS, Buonaccorsi JP (1997) Host heterogeneity in susceptibility and disease dynamics: tests of a mathematical model. *Am Nat* 150: 685–707.
- Keeling MJ, Rohani P (2008) Modeling infectious diseases in humans and animals. Princeton, NJ: Princeton University Press.
- Kemper JT (1980) On the identification of superspreaders for infectious disease. *Math Biosci* 48: 111–127.
- Galvani AP, May RM (2005) Epidemiology—dimensions of superspreading. *Nature* 438: 293–295.
- McCaig C, Begon M, Norman R, Shankland C (2011) A symbolic investigation of superspreaders. *Bull Math Biol* 73: 777–794.
- Altizer S, Nunn CL, Thrall PH, Gittleman JL, Antonovics J, et al. (2003) Social organization and parasite risk in mammals: integrating theory and empirical studies. *Annu Rev Ecol Syst* 34: 517–547.
- Small M, Tse CK, Walker DM (2006) Super-spreaders and the rate of transmission of the SARS virus. *Physica D* 215:146–158.
- Alexander KA, McNutt JW (2010) Human behavior influences infectious disease emergence at the human–animal interface. *Front Ecol Environ* 8: 522–26.
- Gopinath S, Hotson A, Johns J, Nolan G, Monack D (2013) The Systemic Immune State of Super-shedder Mice Is Characterized by a Unique Neutrophil-dependent Blunting of TH1 Responses. *PLoS Pathog* 9: e1003408.
- Jankowski MD, Williams CJ, Fair JM, Owen JC (2013) Birds Shed RNA-Viruses According to the Pareto Principle. *PLoS ONE* 8: e72611.
- Searle CL, Gervasi SS, Hua J, Hammond JL, Relyea RA, et al. (2011) Differential Host Susceptibility to *Batrachochytrium dendrobatidis*, an Emerging Amphibian Pathogen. *Conserv Biol* 25: 963–974.
- Gervasi S, Gondhalekar C, Olson DH, Blaustein AR (2013) Host identity matters in the amphibian-*Batrachochytrium dendrobatidis* system: Fine-scale patterns of variation in responses to a multi-host pathogen. *PLoS ONE* 8: e54490.
- Carey C, Bruzgul JE, Livo IJ, Walling ML, Kuehl KA, et al. (2006) Experimental exposures of Boreal Toads (*Bufo boreas*) to a pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*). *EcoHealth* 3: 5–21.
- Voyles J, Young S, Berger L, Campbell C, Voyles WF, et al. (2009) Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* 326:582–585.
- La Marca E, Lips KR, Lotter S, Puschendorf R, Ibanez R, et al. (2005) Catastrophic population declines and extinctions in neotropical Harlequin frogs (Bufonidae: *Atelopus*). *Biotropica* 37:190–201.
- Lips KR, Diffendorfer J, Mendelson JR, Sears MW (2008) Riding the wave: reconciling the roles of disease and climate change in amphibian declines. *PLoS Biol* 6: e72.
- Langhammer PF, Lips KR, Burrows PA, Tunstall TS, Palmer CM, et al. (2013) A fungal pathogen of amphibians, *Batrachochytrium dendrobatidis*, attenuates in pathogenicity with in vitro passages. *PLoS ONE*: e77630.
- Lips KR, Brem F, Brenes R, Reeve JD, Alford RA, et al. (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proc Natl Acad Sci U S A* 103: 3165–3170.
- Hyatt AD, Boyle DG, Olsen V, Boyle DB, Berger L, et al. (2007) Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Dis Aquat Organ* 73: 175–192.
- Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Dis Aquat Organ* 60: 141–148.
- Kruger KM, Hero JM, Ashton KJ (2006) Cost efficiency in the detection of chytridiomycosis using PCR assay. *Dis Aquat Organ* 71: 149–154.
- R Core Team (2012) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org/>.
- Pinheiro J, Bates D, DebRoy S, Sarkar D, the R Development Core Team (2012) nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1–105.
- Therneau T (2012) A Package for Survival Analysis in S. R package version 2.37-2, <URL: <http://CRAN.R-project.org/package=survival>>.
- Becker MH, Harris RN, Minbiole KPC, Schwantes CZ, Rollins-Smith LA, et al. (2010) Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. *EcoHealth* 8:501–506.
- Lampo M, Celsa SJ, Rodriguez-Contreras A, Rojas-Runjaic F, Garcia CZ (2011) High Turnover Rates in Remnant Populations of the Harlequin Frog *Atelopus cruciger* (Bufonidae): Low Risk of Extinction? *Biotropica* 0: 1–7.
- Stice MJ, Briggs CJ (2010) Immunization is ineffective at preventing infection and mortality due to the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *J Wildl Dis* 46: 70–77.
- Muths E, Stephen P, Pessier AP, Green DE (2003) Evidence for disease-related amphibian decline in Colorado. *Biol Conserv* 110: 357–365.
- Scherer RD, Muths E, Noon BR, Corn PS (2005) An Evaluation of Weather and Disease As Causes of Decline in Two Populations of Boreal Toads. *Ecol Appl* 15: 2150–2160.
- Pilliod DS, Muths E, Scherer RD, Bartelt PE, Corn PS, et al. (2010) Effects of amphibian chytrid fungus on individual survival probability in wild boreal toads. *Conserv Biol* 24: 1259–1267.
- Berger L, Speare R, Kent A (1999) Diagnosis of chytridiomycosis in amphibians by histological examination. *Zoos Print J* 15: 184–190.
- Ribas L, Li MS, Doddington BJ, Robert J, Seidel JA, et al. (2009) Expression profiling the temperature-dependent amphibian response to infection by *Batrachochytrium dendrobatidis*. *PLoS ONE* 4: e6494.
- Rosenblum EB, Poorten TJ, Settles M, Murdoch GK, Robert J, et al. (2009) Genome-wide transcriptional response of *Silurana (Xenopus) tropicalis* to infection with the deadly chytrid fungus. *PLoS ONE* 4: e6494.
- Fites JS, Ramsey JP, Holden WM, Collier SP, Sutherland DM, et al. (2013) The invasive chytrid fungus of amphibians paralyzes lymphocyte responses. *Science* 342: 366–369.
- Piotrowski JS, Annis SL, Longcore JE (2011). Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia* 96: 9–15.

45. Raffel TR, Rohr JR, Kiesecker JM, Hudson PJ (2006) Negative effects of changing temperature on amphibian immunity under field conditions. *Funct Ecol* 20: 819–828.
46. Bustamante HM, Livo LJ, Carey C (2010) Effects of temperature and hydric environment on survival of the Panamanian Golden Frog infected with a pathogenic chytrid fungus. *Integr Zool* 5:143–53.
47. Cashins SD, Grogan LF, McFadden M, Hunter D, Harlow PS, et al. (2013) Prior infection does not improve survival against the amphibian disease Chytridiomycosis. *PLoS ONE* 8:e56747.
48. Rosenblum EB, Poorten TJ, Settles M, Murdoch GK (2012) Only skin deep: shared genetic response to the deadly chytrid fungus in susceptible frog species. *Mol Ecol* 21: 3110–3120.