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Dead or alive? Viability of chytrid zoospores shed from live amphibian hosts

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ABSTRACT: Pathogens vary in virulence and rates of transmission because of many differences in the host, the pathogen, and their environment. The amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), affects amphibian hosts differently, causing extinction and population declines in some species but having limited effects on others. Phenotypic differences in zoospore production rates among *Bd* lineages likely contribute to some of the variation observed among host responses, although no studies have quantified the viability of zoospores shed from live animals. We compared host survivorship, infection intensity, shedding rates, and zoospore viability between 2 species of endangered tropical frogs, *Hylomantis lemur* and *Atelopus zeteki*, when exposed to a highly virulent lineage of *Bd* (JEL 423). We applied a dye to zoospores 30 to 60 min following animal soaks, to estimate shedding rate and proportion of live zoospores shed by different species. The average infection intensity for *A. zeteki* was nearly 17 times higher (31 455 ± 10 103 zoospore genomic equivalents [ZGEs]) than that of *H. lemur* (1832 ± 1086 ZGEs), and *A. zeteki* died earlier than *H. lemur*. The proportion of viable zoospores was ~80% in both species throughout the experiment, although *A. zeteki* produced many more zoospores, suggesting it may play a disproportionate role in spreading disease in communities where it occurs, because the large number of viable zoospores they produce might increase infection in other species where they are reintroduced.

KEY WORDS: Disease · Transmission · Virulence · Frogs · *Batrachochytrium dendrobatidis* · *Atelopus zeteki* · *Hylomantis lemur*

INTRODUCTION

The amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), has a wide range of effects on amphibian hosts, causing extinction and population declines in some species but having limited effects on others (Lips et al. 2006, Crawford et al. 2010). Higher mortality is typically associated with higher infection loads (Briggs et al. 2010, Vredenburg et al. 2010), and species with high loads are thought to be especially important in spreading disease in natural communities (DiRenzo et al. 2014).

Variation in infection intensity stems from many sources including host genetics (Ellison et al. 2014, 2015), ecology (Lips et al. 2003), skin chemistry and microbiome (Woodhams et al. 2006, Myers et al. 2012), fungal genetics (Rosenblum et al. 2012), and abiotic environmental conditions (Becker et al. 2012). This combination of host defenses and ecological factors may affect the accumulation of zoospores on frog skin and the proportion of zoospores killed by microbes or anti-microbial peptides, in turn shaping the transmission dynamics of *Bd* within ecosystems.

Phenotypic differences in zoospore production rates among differentiated *Bd* strains may also contribute to variation in host infection (Fisher et al. 2009). Likewise, differences in zoospore viability may

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contribute to host infection intensities, especially considering the diverse range of antimicrobial defenses found among amphibians (Woodhams et al. 2006). However, the quantitative PCR (qPCR) method used to quantify infection intensity in amphibian hosts does not distinguish between live and dead zoospores (Boyle et al. 2004, Hyatt et al. 2007, Shin et al. 2014). It has been assumed that all zoospores shed by amphibian hosts are viable, although species have a variety of chemical and biotic defenses that might affect the viability of zoospores as they are shed.

Viability studies of fungal (Levitz & Diamond 1985, Deere et al. 1998, Stockwell et al. 2010, Blooi et al. 2013, McMahon & Rohr 2014) and bacterial (Kaprelyants & Kell 1992, Berney et al. 2007) cells have been designed using fluorescent dyes for highly concentrated cultures. However, no studies have examined viability of *Bd* zoospores produced from live hosts, which is further complicated by the low densities of zoospores typical of natural host communities.

We modified an existing cell-staining protocol originally designed for use with *Bd* cultures (Stockwell et al. 2010) to stain zoospores shed from live animals of 2 highly susceptible amphibian species, *Hylomantis lemur* and *Atelopus zeteki*. To quantify zoospore viability in water baths taken from live hosts, we combined the bath collection method (Hyatt et al. 2007, Shin et al. 2014) and zoospore viability protocol (Stockwell et al. 2010) and added filtration and centrifugation steps. We used fluorescence microscopy to quantify the number of live and dead fungal zoospores. We estimated infection intensity of hosts using qPCR of skin swabs, quantified zoospore shedding rate by quantifying zoospores filtered from water baths of infected animals, and determined zoospore viability using fluorescent dyes. We describe this new approach to quantify zoospore viability at low concentrations from live animals that will be useful to researchers quantifying transmission rates for other species in the lab or field.

MATERIALS AND METHODS

Bd cultures

We obtained a cryopreserved sample of *Bd* isolate JEL 423, a member of the hypervirulent *Bd* Global Pandemic Lineage 2 (GPL-2; Rosenblum et al. 2013), originally isolated from an infected *Hylomantis lemur* during the 2004 epizootic at El Copé, Panama. When we revived the *Bd* isolate from cryopreservation, it had been passaged approximately 5 times. We maintained the *Bd* culture during the experiment in 1% tryptone broth at 4°C and passaged it every 3–4 wk. We cultured *Bd* to use in our experiments by adding 1 ml of the broth culture to 1% tryptone agar plates and allowing them to grow 5–7 d at room temperature (~20°C).

Optimizing zoospore staining protocol

We first tested our ability to accurately quantify the proportion of live and dead zoospores using mixtures made from *Bd* cultures with known proportions (0, 25, 50, 75, and 100%) of dead zoospores. We harvested zoospores from agar plates by flooding them with 5 ml^{-1} of sterilized water and allowing them to sit for 15 min before filtering and collecting the solution (Myers et al. 2012). We used a hemocytometer to quantify the number of zoospores ml^{-1} in the stock solution and diluted it to a concentration of 5000 zoospores ml^{-1}. We took a subset of the zoospore solution and heat-killed it at 47°C for 1 h. Once the solution was completely cooled, we made mixtures of 0, 25, 50, 75, and 100% heat-killed zoospores by mixing it with the diluted stock solution of known concentration so that each final solution was standardized at 10 ml of 5000 zoospores ml^{-1}. We then counted the number of live and dead zoospores in each solution (see Table 1).

Following Stockwell et al. (2010), we used SYBR green nucleic acid stain, a membrane-permeable stain which dyes all cells green, and propidium iodide, a membrane-impermeable stain that dyes dead or dying cells red. We added 50 µl of each of the heat-killed zoospore mixtures into 4 wells of a flat-bottomed 24-well plate. We added 15 µl of 1× SYBR green, followed by a 10 min dark incubation, and then added 2 µl of 2.4 µM propidium iodide. We transferred 20 µl of the mixture to an 8-well chamber slide and used a Zeiss AxioObserver inverted fluorescence microscope at 100× magnification with green and red fluorescence protein (GFP and dsRED) filters to image 10 random pictures of the droplet in each chamber. We identified green cells as alive and red cells as dead (Fig. 1). We used ZEN Microscope Software to capture zoospore images and ImageJ® to count the number of live and dead cells. We determined whether images taken from the top, middle, or bottom parts of the well differed in zoospore viability by counting the number of zoospores seen in 5 images taken from each of those regions.

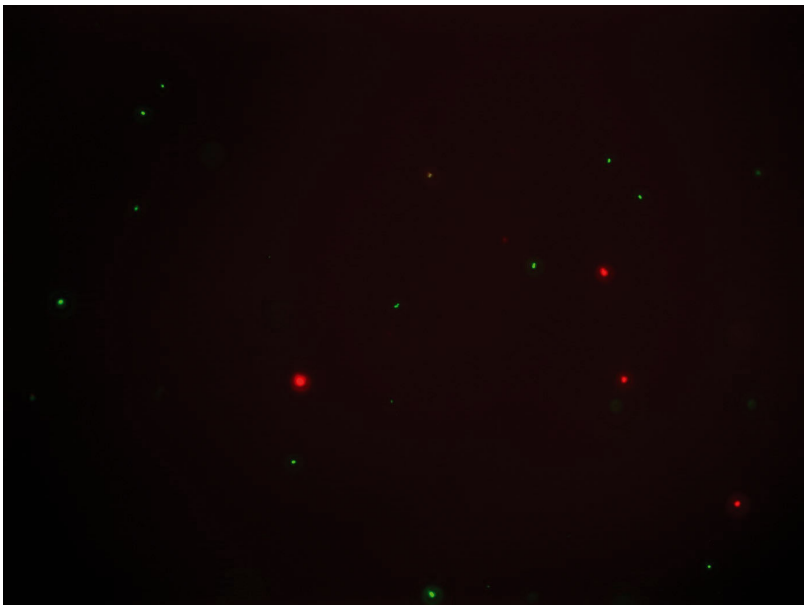

Fig. 1. Example of a microscope image of stained zoospores taken using a Zeiss AxioObserver inverted fluorescence microscope at 100× magnification with GFP and dsRED filters. Red cells are dead zoospores, while green cells are live zoospores

We tested whether centrifuging solutions killed zoospores. We centrifuged a 10 ml sample of 1000 zoospores ml^{-1}, for 10 min at 120 × *g* and then removed 8 ml of supernatant. We lightly vortexed the solution to resuspend the zoospores and centrifuged the remaining 2 ml for another 10 min at 110 × *g*. We dyed the cells using the same protocol (Stockwell et al. 2010) and compared the proportion of dead zoospores in the centrifuged sample to that from the original zoospore suspension.

Animal husbandry and sampling

We obtained 10 captive-bred *Atelopus zeteki* adult individuals from the Maryland Zoo, Baltimore, MD, 41–49 mo post-metamorphosis from 3 clutches. We received 14 captive-bred *H. lemur* juveniles from the Atlanta Botanical Garden, 2–12 mo following metamorphosis from 5 clutches. We randomly selected 8 of the 10 *A. zeteki* and 12 of the 14 *H. lemur* for the inoculation treatment and assigned the remainder as controls. Animals were housed individually in plastic shoeboxes with a moist paper towel, a plastic cover object, and a water dish. Animals were maintained in a laboratory at 21–22°C with a 12 h light: 12 h dark photoperiod and were allowed to acclimate for 4–7 d before the experiment began. We replaced all housing materials every 7 d, changed water dishes every 3 d, fed frogs vitamin-dusted crickets or fruit flies ad libitum every 3 d, and misted terraria daily.

We inoculated each experimental animal for 10 h in a bath of 1 000 000 *Bd* zoospores. We harvested zoospores from plates by flooding tryptone agar plates with 1% tryptone broth following previous methods (DiRenzo et al. 2014, Langhammer et al. 2014). The 4 control individuals were exposed to a sham solution of water and 1% tryptone broth, to match any residual tryptone carried over from the harvest of *Bd* from culture in the infected treatments (DiRenzo et al. 2014, Langhammer et al. 2014). We monitored individuals daily for clinical symptoms of *Bd* and euthanized all individuals once they lost righting reflexes by applying Benzocaine 20% gel to the venter. Control individuals were euthanized when the last infected conspecific was euthanized (*A. zeteki* on Day 18), or at the end of the experiment (*H. lemur* on Day 35). We determined the sex of all individuals at the time of euthanasia by examining for eggs, ovaries, or testes.

Starting on Day 6 (*A. zeteki*) or Day 7 (*H. lemur*) post-inoculation, and twice a week thereafter, we quantified the number of zoospores produced by each frog in 1 of 2 ways: a 15 min soak in a water bath (Reeder et al. 2012), and a 30-stroke swabbing of the venter (Hyatt et al. 2007). We started counts after Day 6 because we estimated that to be enough time to allow *Bd* to embed in the skin and complete a round of zoospore production. We used a fresh pair of nitrile powder-free gloves when handling each individual. For the soak, we placed each individual frog in a container with 10 ml distilled water for *H. lemur* and 20 ml of distilled water for *A. zeteki* to cover the entire ventral area (Reeder et al. 2012) and kept them there for 15 min. We added 10 µl of ≥96% bovine serum albumin to *H. lemur* soaks and 20 µl to *A. zeteki* soaks to prevent zoospores from sticking to the walls of the container (Reeder et al. 2012). We filtered the solution using a sterile syringe and a 11 µm filter (Millipore, NY1104700) housed in a 47 mm filter holder (Millipore, SX0004700) to remove zoosporangia and other large particles.

We transferred the filtered solutions to four 2 ml micro-centrifuge tubes (*H. lemur*) or one 30 ml cen-

trifuge tube (*A. zeteki*). We swabbed the ventral parts of all individuals with a nylon-tipped swab immediately after the soaking procedure (Hyatt et al. 2007). We stored swabs in plastic Nunc tubes at 4°C until processing.

Zoospore collection and quantification

We concentrated the zoospores obtained in the soak solutions by centrifuging the solutions. For *H. lemur*, we centrifuged tubes at 100 × *g* for 10 min and removed 1.5 ml of the supernatant. For the *A. zeteki* samples, we centrifuged the 30 ml tubes at 100 × *g* for 10 min and removed 12 ml of the supernatant. We transferred the remaining 8 ml into four 2 ml micro-centrifuge tubes and centrifuged at 100 × *g* for an additional 10 min before removing 1.5 ml of the supernatant. We followed our optimized staining protocol procedure to quantify the proportion of live and dead zoospores.

We quantified infection intensity using standard qPCR of skin swabs following Boyle et al. (2004) and Hyatt et al. (2007) and running 50 cycles. We extracted DNA from the swabs using a PrepMan Ultra kit and used real-time qPCR to categorize individuals as *Bd*-positive if infection intensity was ≥1 zoospore genomic equivalent (ZGE; Kriger et al. 2006).

Statistical analysis

We performed all statistical analyses in R version 3.2.1 (R Core Team 2015). First, we evaluated the accuracy of our staining protocol. We subtracted mortality of zoospores attributed to handling and processing of cultures (that would not be present in zoospores shed from a live animal) by subtracting the ~7% (Table 1) mortality measured in the stock solution from all mixtures. We used chi-square goodness-of-fit tests to (1) compare expected proportions of viable zoospores to observed proportions, (2) determine if different layers of the droplet differed in the proportion of viable zoospores from the stock concentration, and (3) determine whether the proportion of dead zoospores differed between centrifuged and not centrifuged samples.

We determined the rate at which zoospore viability changed over time and between species by using a mixed effects logistic regression. We used zoospore viability as the response, and species, experiment day, and the interaction between species and experiment day as fixed effects (package *lme4*; Bates et al. 2015). We included frog ID as a random intercept to allow for each individual to differ in initial zoospore viability, and we included experimental day as a random slope to accommodate variation in individual zoospore viability over time.

We modeled the zoospore load and the number of zoospores shed (N_t) on individual frogs with respect to time in days (t) using the exponential growth equation: $N_t = N_0 e^{rt}$, where N_0 is the initial zoospore load or number of zoospores shed, r is the intrinsic daily rate of zoospore increase, and t is time measured in days. To transform the equation to its linear form, we took the natural log of both sides and arrived at $\ln(N_t) = \ln(N_0) + r \times t$. To calculate parameter estimates, we used 2 generalized linear mixed effects model (GLMM) with a Poisson distribution to determine the rate at which zoospores were shed over time from both swabs and soaks. We used the number of zoospores, from either swab or soak data, as the response and used species, experimental day, and the interaction between species and experimental day as fixed effects (package *lme4*; Bates et al. 2015). We included frog ID as a random intercept to allow for each individual to differ in zoospore infection, and we included experimental day as a random slope to accommodate variations in individual zoospore shedding over time. We interpreted the slope of the line, the r parameter, as zoospore production rate (Stice & Briggs 2010, DiRenzo et al. 2014), which we defined as the daily percent increase in either zoospore load (estimated from swab qPCRs) or number of zoospores shed (estimated from the soaks). To determine if survival curves differed between the 2 species, we used a log-rank test (package *survival*; Therneau 2012).

Table 1. Dead zoospores (mean ± SE) were evenly distributed among 3 layers of the experimental mixtures and matched the appropriate stock solution concentration. Percent dead zoospores are not adjusted to account for background mortality of zoospores in the stock solution. Asterisk indicates a significant difference between expected and observed % dead zoospores, where $p < 0.05$

Expected prop. (%)	Observed proportion of dead zoospores (%)		
	Bottom	Middle	Top
0	7.9 ± 1.7	12.1 ± 3.4	10.2 ± 1.8
25	33.5 ± 5.5	41.7 ± 4.9	42.1 ± 5.2
50	53.0 ± 4.0	62.6 ± 3.6	61.6 ± 7.0
75	73.5 ± 4.3*	72.9 ± 6.4*	75 ± 5.1*
100	100 ± 0	100 ± 0	100 ± 0

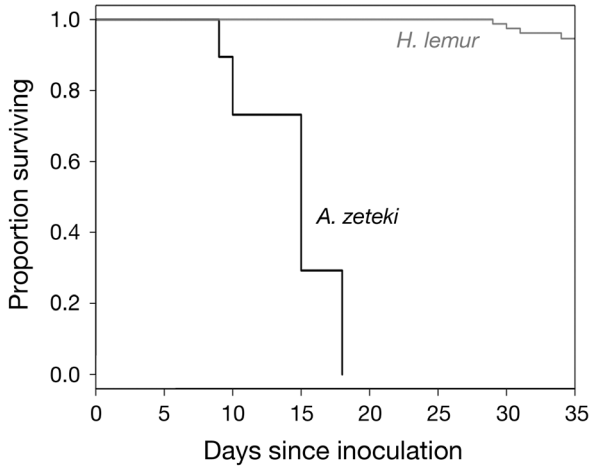

Fig. 2. *Atelopus zeteki* died faster than *Hylomantis lemur*, and survival curves differed between the 2 species (log-rank test: $\chi^2 = 128$, df = 1, $p < 0.0001$)

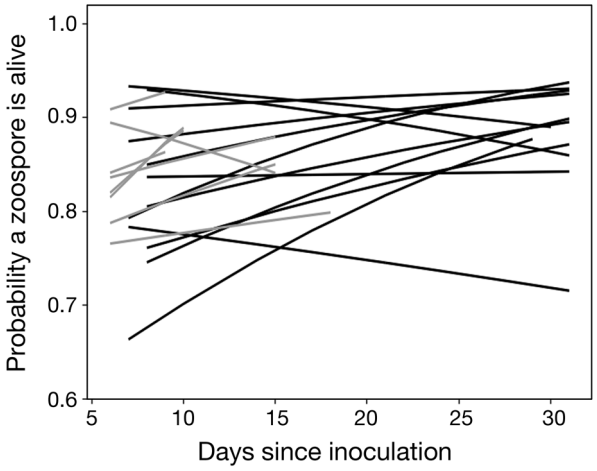

Fig. 3. Change in zoospore viability over time for individual *Atelopus zeteki* (grey lines; $z = 11.84$, $p < 0.001$) and *Hylomantis lemur* (black lines; $z = -4.84$, $p < 0.001$) from the fitted logistic regression model

RESULTS

The proportion of dead zoospores did not differ between samples that were and were not centrifuged ($p > 0.05$). The proportion of dead zoospores did not vary among different layers within a droplet (Table 1; all $p > 0.05$), except for the 75% solution, where the observed proportion of dead zoospores slightly deviated from the expected (Table 1; most $p > 0.05$).

Atelopus zeteki died faster than *Hylomantis lemur* ($\chi^2 = 128$, df = 1, $p < 0.0001$; Fig. 2). All *A. zeteki* died within 18 d of inoculation, while only 4 *H. lemur* died between Day 28 and the end of the experiment on Day 35. The average infection intensity (±SD) at death for *A. zeteki* and *H. lemur* was 86 752 ± 30 512 ZGEs and 13 796 ± 12 311 ZGEs, respectively. The overall average infection intensity for the duration of the experiment was 31 455 ± 10 103 ZGEs for *A. zeteki* and 1832 ± 1086 ZGEs for *H. lemur*.

Bd zoospore viability was ~80% throughout the 35 d study for both species. The odds of zoospore viability slightly increased over time by 1.06 ± 0.01 d^{-1} (GLMM, $z = 4.73$, $p < 0.001$) for *A. zeteki* but did not significantly change for *H. lemur* (Fig. 3, Table 2).

The 2 species differed in the total number of zoospores shed into water baths, as determined by counting the total number of zoospores observed in all images (Fig. 4, Table 3). The first count of zoospores for *A. zeteki* was 6 d post-inoculation, and individuals produced an average of 3640.14 ± 734.41 zoospores in 15 min. The first count of zoospores for *H. lemur* shedding was 7 d post-inoculation, and individuals produced an average of 92.80 ± 28.47 zoospores in 15 min. The number of zoospores shed into water baths by *A. zeteki* individuals increased by an average of 27.7 ± 3.36% daily ($z = 8.23$, $p < 0.001$), and we found no significant increase for *H. lemur*.

Table 2. Summary of fixed effect coefficient estimates of the mixed effects logistic regression model for zoospore viability. Estimates are on the logit scale. Asterisk indicates $p < 0.05$

	Estimate	SE	*z*	p
Intercept	1.29	0.12	10.73	<0.001*
Day	0.05	0.01	4.74	<0.001*
Species	0.2	0.23	0.89	0.37
Day × Species	−0.04	0.01	−2.22	0.02*

The estimates of zoospores sampled by skin swabs were 1 to 2 orders of magnitude greater than the number of zoospores sampled by the water bath soak method (Fig. 5, Table 4). On Day 6 post-inoculation, qPCR of swabs estimated an average of 50 028.19 ± 14 789.14 zoospores per frog for *A. zeteki*, while on Day 7 post-inoculation, *H. lemur* had an average of 902.68 ± 286.85 zoospores per frog. The number of zoospores on the skin of an *A. zeteki* individual increased by an average of 66.57 ± 10.03% daily ($z = 5.11$, $p < 0.001$), and we found no significant increase for *H. lemur*.

DISCUSSION

Both *Atelopus zeteki* and *Hylomantis lemur* shed a large proportion of viable zoospores, although *A.*

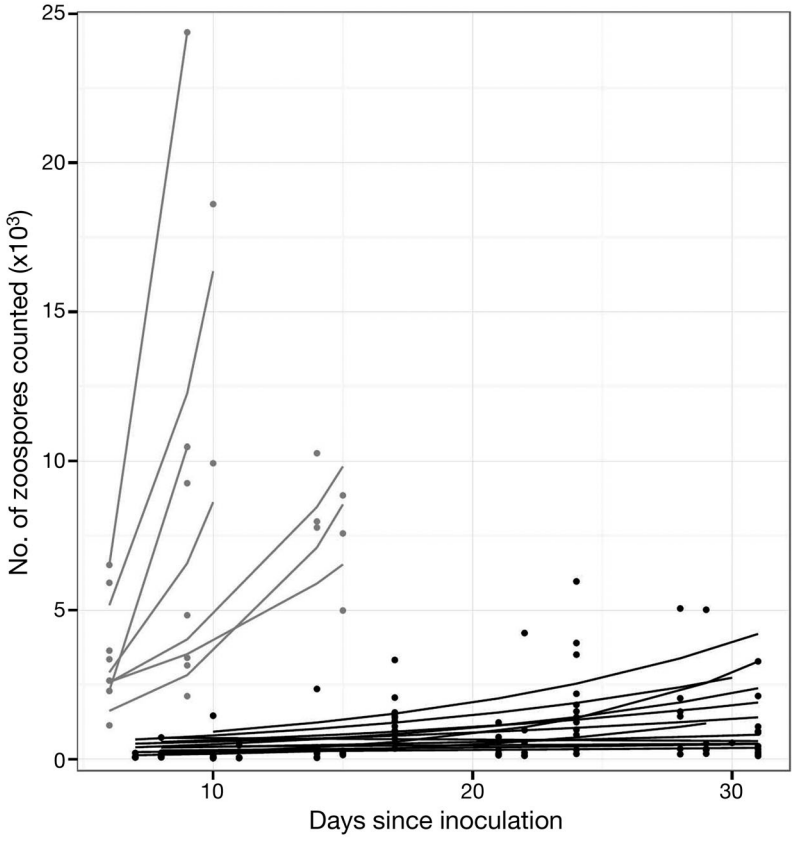

Fig. 4. Differences in the total number of zoospores shed into water baths by *Atelopus zeteki* (grey dots) and *Hylomantis lemur* (black dots). Each line represents the average change (per individual) in zoospores shed into water baths as modeled with a generalized linear mixed effects model (GLMM)

zeteki shed more zoospores overall, had higher infection intensities, and died faster in experimental infections than *H. lemur*. The number of zoospores shed increased with infection intensity. Although variations in infection intensity may result from differences between hosts, pathogens, and the environment, our study found no differences in the viability of zoospores shed from 2 highly susceptible species.

Our focal species overlap in geographic distribution and in habitat use but have different life histories and ecology (Savage 2000) and skin chemistry (Woodhams et al. 2006). The large amounts of antimicrobial peptides produced by *H. lemur* led to the prediction that *H. lemur* would have greater resistance to *Bd* (Woodhams et al. 2006) compared to *A. varius* (the sister taxon of *A. zeteki*), which produced very few peptides. While we know little about the ecological interactions occurring on the amphibian skin between peptides and *Bd*, our results show that these established differences in skin defenses between our 2 study species did not affect zoospore viability. Because approximately 80% of shed zoospores were viable throughout the course of the experiment, we hypothesize that the 20% of dead zoospores were killed by similar mechanisms, either biological or as a result of experimental conditions.

A. zeteki may be an 'acute supershedder' (DiRenzo et al. 2014), and the large number of zoospores shed during infection is hypothesized to affect transmission dynamics. *H. lemur* lived longer but produced far fewer zoospores per soak or swab. Further work establishing the relationship between zoospore load and transmission among host species is necessary as identified by Streicker et al. (2013), but we have established that infected hosts are shedding large numbers of potentially infectious zoospores.

CONCLUSIONS

Our findings demonstrate that most zoospores shed are viable, further supporting the hypothesis that *A. zeteki* may play a disproportionate role in disease dynamics and its presence may drive infection in other members of the community (e.g. Kilpatrick et al. 2010, Streicker et al. 2013, DiRenzo et al. 2014). We found large differences in disease load, numbers of zoospores shed, and mortality rates between the 2 species. Within several days of infection, *A. zeteki* individuals showed morbidity, whereas most *H. lemur* outlived *A. zeteki*. The differences in zoospore loads and survival between species could be due to differences in genetics (Ellison et al. 2015), age differences between the experimental animals, or ecology (Lips et al. 2003). Because both species produced high pro-

Table 3. Summary of fixed effect coefficient estimates of the generalized linear mixed effects model for the number of zoospores counted in water baths over time. Estimates are on the log scale. Asterisk indicates $p < 0.05$

	Estimate	SE	*z*	p
Intercept	6.35	0.27	23.18	<0.001*
Day	0.27	0.03	8.23	<0.001*
Species	−0.76	0.35	−2.22	0.26
Day × Species	−0.22	0.04	−5.36	<0.001*

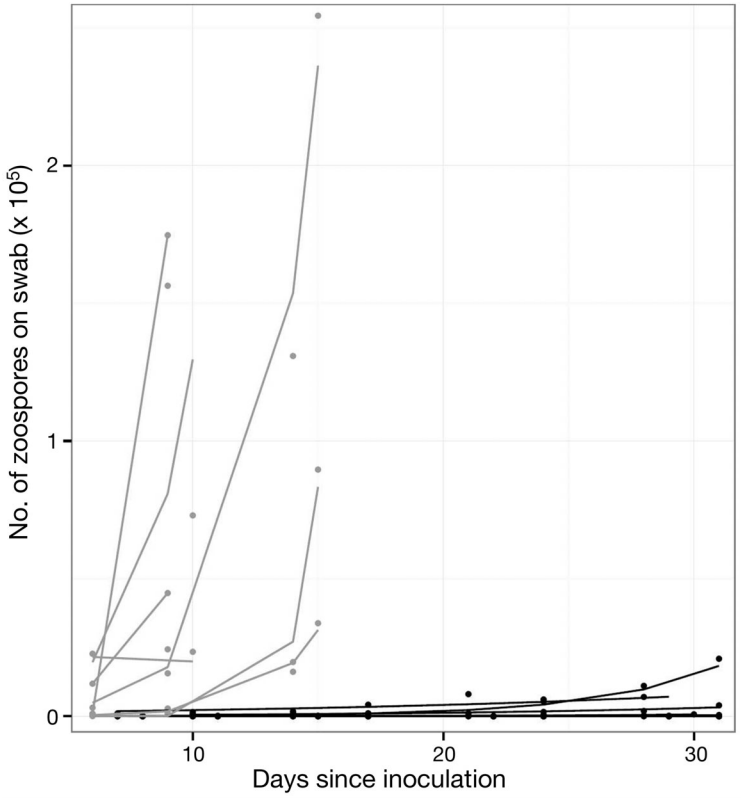

Fig. 5. Differences in the numbers of zoospores on swabs shed by individual *Atelopus zeteki* (grey dots) and *Hylomantis lemur* (black dots). Each line represents the change in zoospore load (per individual) determined by quantitative PCR of skin swabs as modeled with a generalized linear mixed effects model (GLMM)

portions of viable zoospores, we conclude that zoospore viability does not predict host survival or pathogen transmission but that disease load and shedding rate may be better predictors of mortality and infectiousness (Vredenburg et al. 2010, Kinney et al. 2011).

The low mortality rate observed in the lab for *H. lemur* was unexpected given the widespread declines of this species across its range (Puschendorf et al. 2006). The mechanisms underlying mortality are too poorly known to determine why *H. lemur* did not die in our experiments, beyond the general rule that higher intensities usually lead to higher mortality (e.g. Vredenburg et al. 2010). For instance, species vary in the intensity of infection at death (e.g. Vredenburg et al. 2010, DiRenzo et al. 2014), but it is not known how different intensities and durations of infection affect mortality or how this might vary among species. It may be that longer exposure at low pathogen intensity results in death, or these frogs might be able to clear infection with time. Alternatively, this species may have physiological traits, such as a particular skin morphology, chemistry, or microbiome, that prevent *Bd* from growing fast enough to reach lethal levels. The perching behavior in this tree frog might also reduce infection intensity; most individuals perched on the sides of the plastic shoebox, in contrast to *A. zeteki* that spent most of the time on wet paper towels at the bottom of the cage. Alternatively, *H. lemur* may have evolved better defenses against *Bd*, and genomic analyses could provide evidence for differences in the immunogenomic differences in responses of the 2 species (e.g. Rosenblum et al. 2012, Fites et al. 2013, Ellison et al. 2014).

Our protocol is the first to quantify viability of *Bd* zoospores shed from live animals at low concentrations, which are typical of natural infections in the field. We developed a protocol using long, slow centrifuging steps that did not compromise the viability of the zoospores. Although our method undersamples the total number of zoospores because we do not completely quantify the number of zoospores in the water baths, it could help researchers develop a more comprehensive understanding of the disease ecology of *Bd* when coupled with more robust methods such as swabbing or qPCR-based soak extractions where viability is unknown (Hyatt et al. 2007, Shin et al. 2014).

This new method provides key information on understanding disease dynamics in tropical systems where these species are native. It also has potential application for quantifying pathogen growth and survival over the course of infections, which are important parameters for a number of disease dynamic studies in this system. For example, this method will allow for comparisons among species and predictions of transmission rates in frog communities with different species composition (e.g. Blooi et al. 2013).

Table 4. Summary of fixed effect coefficient estimates of the generalized linear mixed effects model for the number of zoospores on swabs over time. Estimates are on the log scale. Asterisk denotes $p < 0.05$

	Estimate	SE	*z*	p
Intercept	3.41	1.56	2.19	0.02*
Day	0.66	0.13	5.11	<0.001*
Species	−1.91	1.95	−0.98	0.32
Day × Species	−0.60	0.16	−3.68	0.002*

Our finding that both study species shed similar proportions of viable zoospores throughout the duration of the experiment was surprising given the species' differences in ecology and known skin defenses. However, this consistent viability proportion could become a useful parameter estimate for future *Bd* transmission modeling projects. Further studies could examine how viability changes over time under various environmental conditions, such as temperature and humidity. Additionally, quantifying the viability of zoospores from different strains of *Bd* might help explain how some strains are more lethal than others.

Our results support the hypothesis that animals with higher zoospore loads shed a greater number of viable zoospores and underscore that *A. zeteki* plays a disproportionate role in community disease transmission. Our findings also help explain how *Bd* moves quickly through some communities (e.g. Lips et al. 2006) and could be extrapolated to explain variation in pathogen spread among species and regions.

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