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Cite this article: Jenkinson TS, Rodriguez D, Clemons RA, Michelotti LA, Zamudio KR, Toledo LF, Longcore JE, James TY. 2018 Globally invasive genotypes of the amphibian chytrid outcompete an enzootic lineage in coinfections. *Proc. R. Soc. B* **285**: 20181894. http://dx.doi.org/10.1098/rspb.2018.1894

Received: 31 August 2018
Accepted: 19 November 2018

Subject Category:
Ecology

Subject Areas:
ecology, molecular biology

Keywords:
chytridiomycosis, disease ecology, intraspecific competition

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Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.4320824.

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Globally invasive genotypes of the amphibian chytrid outcompete an enzootic lineage in coinfections

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Competition between genotypes is likely to be a key driver of pathogen evolution, particularly following a geographical invasion by distant strains. Theory predicts that competition between disease strains will result in the most virulent strain persisting. Despite its evolutionary implications, the role of strain competition in shaping populations remains untested for most pathogens. We experimentally investigated the *in vivo* competitive differences between two divergent lineages of the amphibian-killing chytrid fungus (*Batrachochytrium dendrobatidis*, *Bd*). These *Bd* lineages are hypothesized to have diverged in allopatry but been recently brought back into secondary contact by human introduction. Prior studies indicate that a panzootically-distributed, global lineage of *Bd* was recently introduced into southern Brazil, and is competitively excluding enzootic lineages in the southern Atlantic Forest. To test for differences in competitive ability between invasive and enzootic Brazilian *Bd* isolates, we coinfected a model host frog system which we developed for this study (*Hymenochirus curtipes*). We tracked isolate-specific zoospore production over the course of the coinfection experiment with chip-based digital PCR (dPCR). The globally invasive panzootic lineage had a competitive advantage in spore production especially during the first one to four weeks of infection, and on frogs that eventually succumbed to *Bd* infection. Our study provides new evidence that competitive pressure resulting from the human movement of pathogen strains can rapidly alter the genetics, community dynamics and spatial epidemiology of pathogens in the wild.

1. Introduction

Competition is a key factor structuring ecological communities [1–3]. Over time, competition and competitive exclusion contribute to the selection pressure shaping organismal evolution [4]. The competitive exclusion principle predicts that two species (or lineages) should not be able to occupy the same ecological niche indefinitely [5,6]. While this principle is well supported in numerous plant and animal systems, its ecological implications for pathogens remain unclear [7]. Some authors suggest that strain competition will result in pathogen coexistence through host partitioning [8], while others argue that competing strains will be selected for faster growth and higher virulence leading to the exclusion of all but the most virulent lineages [9,10]. Theoretical models support the idea that competing pathogen strains will be under selection for growth rate and virulence, despite an accompanying trade-off in increased host mortality [11,12]. This 'shortsighted evolution' scenario predicts

the competitive exclusion of all but the most virulent disease strains [13]; however, the prediction remains untested for most diseases (a notable exception being in malaria coinfections) [9]. Here, we examined the outcomes of strain coinfection, and its implications on the disease ecology of a recently emerged fungal pathogen.

Chytridiomycosis is the emerging fungal disease implicated in population declines and extinctions of amphibians worldwide [14–16]. Caused by the chytridiomycete fungus *Batrachochytrium dendrobatidis* (*Bd*) [17], this disease has emerged as one of the most significant contemporary threats to global amphibian biodiversity [18]. In many regions of the globe *Bd* is newly arrived and actively spreading [19–21]. In other regions *Bd* appears to be enzootic and in stable equilibrium with its associated hosts [22]. This is the case in the Atlantic Forest of southeastern Brazil where two deeply divergent lineages of *Bd* co-occur in a number of contact zones [23,24]. In the Atlantic Forest region, the globally distributed, panzootic clone of *Bd* (termed *Bd*-GPL—*Global Panzootic Lineage*) [25] and a regional enzootic lineage (*Bd*-Brazil, also known as *Bd*-Asia-2/Brazil) [23,26] occupy overlapping ranges.

In addition to *Bd*-GPL and *Bd*-Brazil, two additional lineages of *Bd* have now been recognized from southern Africa (*Bd*-Cape) and east Asia (*Bd*-Asia-1; which includes *Bd*-CH) [25–27]. We now know that differences in virulence exist among these lineages [25,26,28,29]. We also know that multi-lineage coinfections by diverse *Bd* genotypes occur in natural populations (e.g. the *Bd*-GPL isolate CLFT024/0 and hybrid isolate CLFT024/2 were both collected from the same *Hylodes cardosoi* tadpole host) [23,30]. Coinfections in nature provide opportunities for competition which can occur directly or indirectly. In the case of direct competition, individuals directly exclude one another from a limited resource. Alternatively, competition may be indirect, where competitive interactions are mediated through the antagonistic response of a resource species, or host immune response in the case of pathogens. Indirect competition is well documented in fungi [31,32], and in pathogen species in general [33]; and both modes of competition can result in the exclusion of a weaker competitor [34,35]. The possibilities for diverse *Bd* coinfections to occur are growing continually as disease lineages are increasingly transported away from their native ranges by anthropogenic activity [26,36]. Despite the potential for competitive dynamics to shape disease genotypes present in a pathogen population over time, little work to date has explored the nature of competition between any of the divergent *Bd* lineages or considered the population-level outcomes of genotype competition.

Our study was motivated in-part by the striking geographical distribution of enzootic *Bd*-Brazil we observed in the field. Despite ample surrounding habitat predicted to be highly suitable for *Bd* growth in the southern Atlantic Forest [37], *Bd*-Brazil is found only from a small handful of sampling sites. The population structure of the two *Bd* lineages in the Brazilian Atlantic Forest suggests that *Bd*-GPL has been rapidly expanding [24], and excluding *Bd*-Brazil from its former range. To evaluate whether competitive differences are shaping the genetic structure of this expanding pathogen population, we conducted a coinfection experiment using a novel amphibian host model. The specific goals of this study were to quantify the relative performance of *Bd*-GPL and *Bd*-Brazil strains when inoculated onto the same host resource, and to develop a model *Bd* host system suitable for standardizing future laboratory-based virulence and transmission studies. Our results support strain competition as an ecological force capable of shifting pathogen genotype frequencies in mixed populations and have wide-ranging implications for understanding the evolution of pathogenicity traits following human-mediated pathogen introductions.

2. Material and methods

(a) Experimental design

To investigate if fitness differences exist among *Bd* strains, we tracked the temporal population genetics of *Bd*-GPL × *Bd*-Brazil coinfections in experimental amphibian populations. We measured differences in zoospore production at four time points over the course of a 10-week experiment, and used chip-based digital PCR (dPCR) to quantify a mitochondrial SNP (Bdmt26360) that distinguishes *Bd*-GPL from *Bd*-Brazil in a mixed sample.

We used the aquatic, western dwarf clawed frog (*Hymenochirus curtipes*, Pipidae) as the host for this study. *Hymenochirus curtipes* is tolerant to *Bd* infection at low levels but may succumb to disease at high pathogen loads [38,39]. *Hymenochirus curtipes* is also available from commercial suppliers in the aquarium trade, allowing for the iteration of this study by separate research groups. This species is completely aquatic and is amenable to housing in groups [40], which allowed our study to simulate the dynamics of active strain transmission within a host population. Finally, *H. curtipes* is native to the Congo basin of central Africa. Having been recently collected from Brazil, these *Bd* isolates will not have encountered the *H. curtipes* host environment in their recent ecological histories. This common garden design provides a competitive landscape distinct from the host diversity available to either strain within their current ranges in the Atlantic Forest.

We selected two *Bd*-GPL isolates and two *Bd*-Brazil isolates to coinfect in the four possible combinations between the represented lineages (figure 1*a*). This design addressed competitive differences between *Bd*-GPL and *Bd*-Brazil while accounting for possible isolate-to-isolate differences within each lineage. The study isolates were all recently collected from the lineage contact zones in the Brazilian Atlantic (figure 1*b*). We passaged the experimental isolates minimally in culture (table 1) to prevent major genetic or phenotypic changes under laboratory conditions [41,42]. Our experimental units consisted of 20 aquarium tanks (35 l of water) housing small populations ($n = 5$) of western dwarf clawed frogs. Each experimental population was randomly assigned to one of the four infection treatments, and each treatment was replicated in five experimental tanks ($n = 100$ animals total) (electronic supplementary material, table S1). Full details of our animal care procedures and *Bd* culture methods are available in the electronic supplementary material.

For the coinfections, our goal was not to induce or measure mortality as an effect, but rather to inoculate hosts with a dose of *Bd* that would result in the maintenance of infection over the study period. Each host population ($n = 5$) was exposed to a 20 ml zoospore bath containing 10^7 infective zoospores from each of two competing isolates (combined inoculum concentration = 10^6 zoospores ml^{-1}; total exposure with both isolates = 2×10^7 zoospores). The exposure baths were incubated at 19°C to 20°C for 6 h. After the exposures, all animals were released back to their respective tanks along with the inoculation bath and monitored daily for mortality or signs of morbidity.

(b) Data collection

We monitored infection progress for 70 days (10 weeks) after inoculation by swabbing the animals weekly with sterile skin

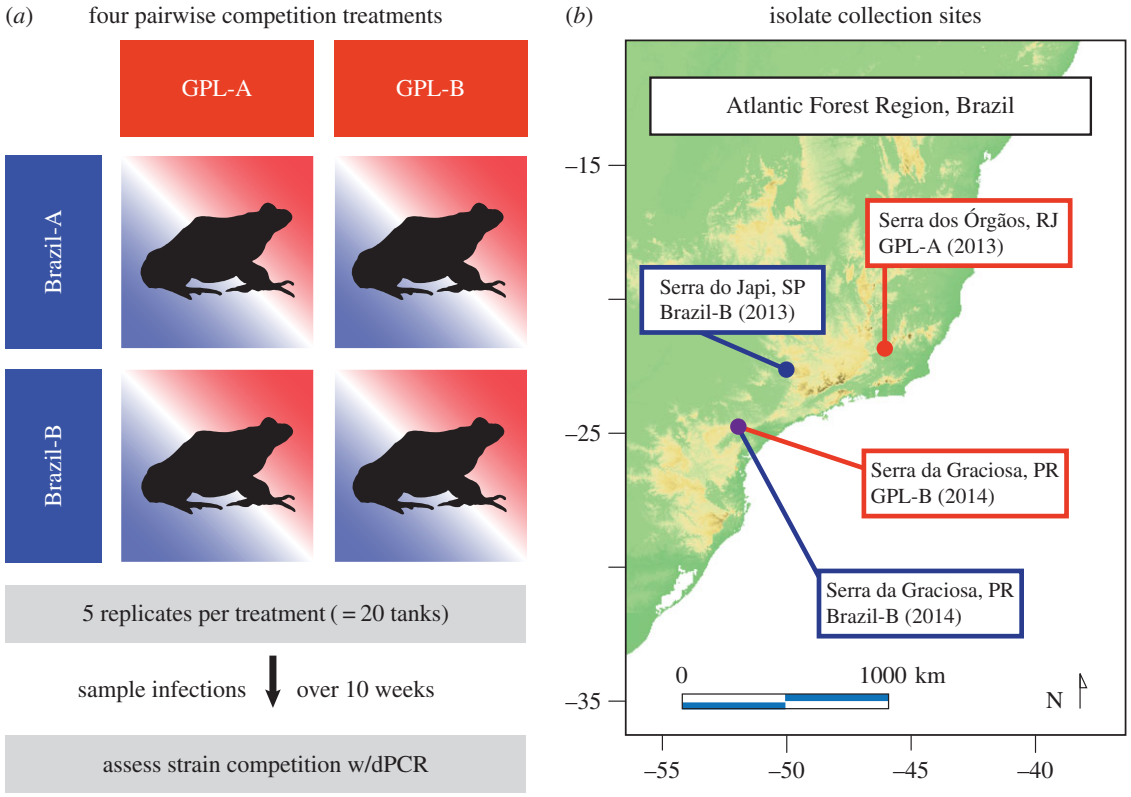

Figure 1. Experimental design of the pairwise strain competition experiment. (*a*) Two *Bd*-GPL and two *Bd*-Brazil strains were coinfected in all four possible GPL × Brazil treatment combinations. We repeated each treatment five times for a total of 20 tanks. (*b*) Collection localities, dates and isolate codes for the strains examined in this study.

Table 1. *Bd* isolates used for this experiment.

designation	isolate	year	passages	host	locality
GPL-A	CLFT073	2013	9	*Aplastodiscus* sp.	Serra dos Órgãos National Park, Rio de Janeiro
Brazil-A	CLFT070	2013	9	*Hylodes japi*	Serra do Japi, Jundiai, São Paulo
GPL-B	CLFT137	2014	6	*Hylodes cardosoi*	Serra da Graciosa, Morretes, Paraná
Brazil-B	CLFT150	2014	6	*Hylodes cardosoi*	Serra da Graciosa, Morretes, Paraná

swabs (MW 113, Medical Wire and Equipment Co.). We swabbed the interdigital webbing of each limb five times and both lateral surfaces of the abdomen five times (30 passes total). We employed the same procedure to swab any dead or moribund individuals ($n = 38$) encountered during daily health checks. We extracted genomic DNA from skin swabs with 50 µl of PrepMan Ultra sample preparation reagent (Thermo Fisher Inc.). For routine monitoring of *Bd* infection through the course of the experiment, we performed qPCR assays on the weekly skin swabs [43]. Reaction conditions and cycling parameters are presented in electronic supplementary material, table S2.

To assess the outcome of coinfection, we chose four equally spaced, temporal samples (weeks 1, 4, 7, and 10) for isolate identification and quantification with the chip-based, QuantStudio 3D digital PCR system (Thermo Fisher Inc.) following the manufacturer's protocols. Chip-based dPCR is ideal for detecting rare allelic variation in mixed samples [44,45]. The dPCR system partitions a duplexed, lineage-specific TaqMan assay into a PCR reaction chip composed of 20 000 nanowells. We used qPCR-estimated quantification of total DNA to adjust each sample dilution so that some wells received target DNA, while others did not. Any well that received a target sequence which was successfully amplified in the PCR step released a specific fluorescent signal. Probe sequences, dPCR reaction conditions and cycling parameters are presented in electronic supplementary material, table S3. Post-dPCR, we used the QuantStudio 3D digital PCR chip reader to detect the fluorescence signal of reporter dyes from each nanowell (from either the VIC-tagged *Bd*-GPL probe or the FAM-tagged *Bd*-Brazil probe), and used the dPCR measured zoospore density (GE μl^{-1}) of each isolate as our indicator of competitive ability.

At week 7 we euthanized a sample of five individuals from the coinfection experiment to isolate *Bd* cultures. We microscopically scanned interdigital tissue for evidence of infection, and isolated cultures following Longcore *et al.* [46]. These re-isolations provided one additional way to assess the genotypic winners of competitive coinfections. We obtained five isolates and determined their genotypes using two multilocus sequence type markers known to differentiate the major *Bd* lineages (see electronic supplementary material, Supplementary methods).

(c) Data analyses

Even closely related isolates of *Bd* can vary significantly in molecular marker copy number [47,48]. We addressed this by

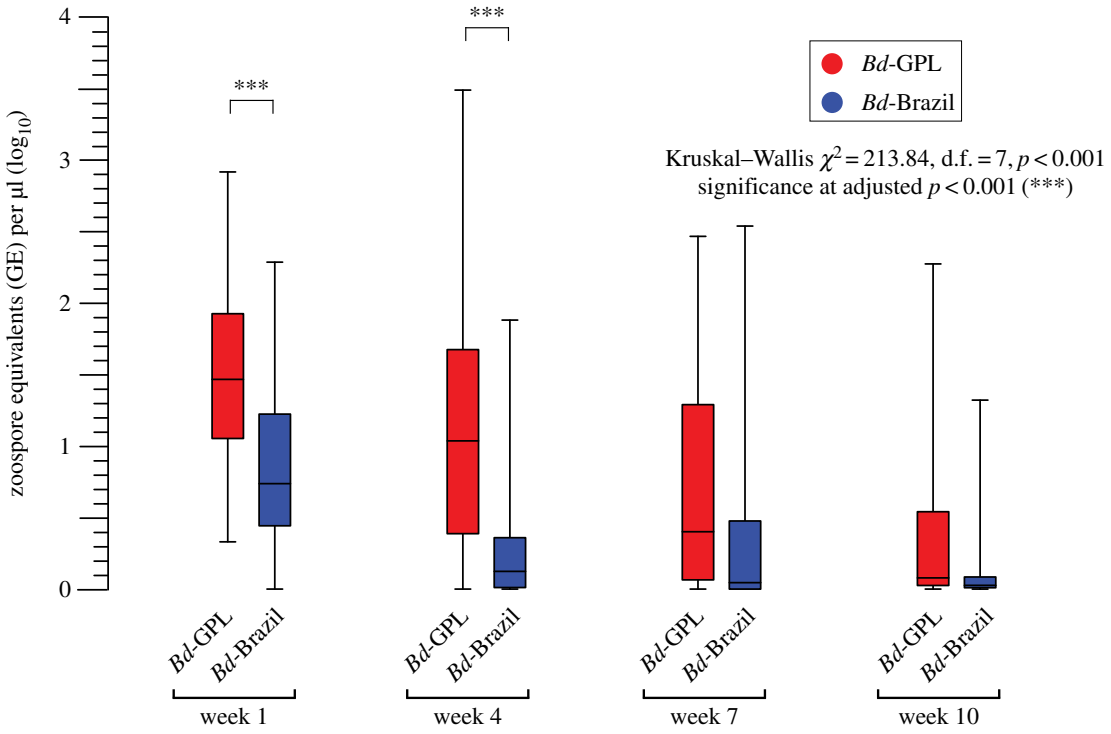

Figure 2. Zoospore production by *Bd*-GPL is more robust than *Bd*-Brazil. *Bd*-GPL produces significantly more spores in the early stages of the 10-week coinfection experiment. Box plots show the median and interquartile zoospore densities (log (GE $\mu l^{-1} + 1$)) for *Bd*-GPL (red) and *Bd*-Brazil (blue) at four sampled time points. Whiskers show the range for all observations.

first determining the degree of copy number variation among isolates for the mitochondrial assay marker. We made isolate-specific 10^6 zoospore standards for each of the experimental isolates by counting zoospores with a haemocytometer as before. We performed the same dPCR assays on a serial dilution of each isolate-specific standard (10^3, 10^4, 10^5 and 10^6). We constructed standard curves, and calculated the slope of the linear relationship between marker concentrations (copies μl^{-1}). For each isolate, we multiplied the slope of its dPCR standard curve by observed copy concentrations to determine zoospore density (GE μl^{-1}). To improve the variance homogeneity in zoospore density across partitions (by lineage, by isolate, by time point), we log transformed ($\log_{10}(x+1)$) observed zoospore densities before the analyses. Because of the non-normal distributions of these data, we used non-parametric methods for statistical hypothesis testing, which were performed in R v. 3.4.2 [49].

3. Results

(a) *Bd* infection over 10 weeks

From week 1 to week 6 post-inoculation, mean infection loads in all tanks (assessed by qPCR) plateaued at 996–5770 GE per swab. After the sixth week, infection loads dropped rapidly, continuing to the end of the experiment (mean: 891–50 GE per swab; electronic supplementary material, figure S3). Infection loads dropped by an overall rate of 311.5 GE per week (linear regression: slope $= -311.5$, $r^2 = 0.557$, $p = 0.008$). Many of the *H. curtipes* hosts suppressed (or fully recovered from) *Bd* infection over the 10-week period; however, individual host outcomes ranged widely. Of the 100 starting animals, 21% of individuals tested negative for *Bd* infection by the end of 10 weeks, while 38% of individuals died or were euthanized because of disease signs. The time to death of succumbing individuals was not associated with any of the four treatments (Kruskal–Wallis rank-sum test $\chi^2 = 0.63$, d.f. $= 3$, $p = 0.889$).

(b) Competitive effects between *Bd*-GPL and *Bd*-Brazil

We partitioned the weekly zoospore densities by lineage to test for differences in spore production. *Bd*-GPL produced higher spore densities than *Bd*-Brazil at all time points (figure 2). We observed differences in zoospore density between lineages as a function of time (Kruskal–Wallis rank-sum test $\chi^2 = 213.84$, d.f. $= 7$, $p < 0.001$). We assessed pairwise differences between these lineage partitions *post hoc* using Dunn's test of multiple comparisons with a Bonferroni correction. Our Dunn's test comparisons showed that *Bd*-GPL produced more spores than *Bd*-Brazil early in the infection, during week 1 (corrected $p < 0.001$) and week 4 (corrected $p < 0.001$). By week 7, *Bd*-GPL spore densities were reduced from previous weeks, whereas mean *Bd*-Brazil spore density increased from the previous time point. *Bd*-GPL still produced more spores on average than *Bd*-Brazil, but the differences in density were statistically indistinguishable (corrected $p = 0.080$). Finally, by week 10, spore densities continued to drop for both strains. Again, by week 10 mean *Bd*-GPL spore densities were greater than those of *Bd*-Brazil, but not significantly so (corrected $p = 0.370$). Based on DNA sequences of two population-informative markers, the five cultures we isolated at seven weeks post-inoculation were all *Bd*-GPL.

(c) Differences in competitive fitness among isolates

We partitioned spore density data to test for fitness differences between our four individual *Bd* isolates. Within each lineage, we observed significantly superior and inferior competitor isolates (figure 3). Both GPL-B and Brazil-B were significantly

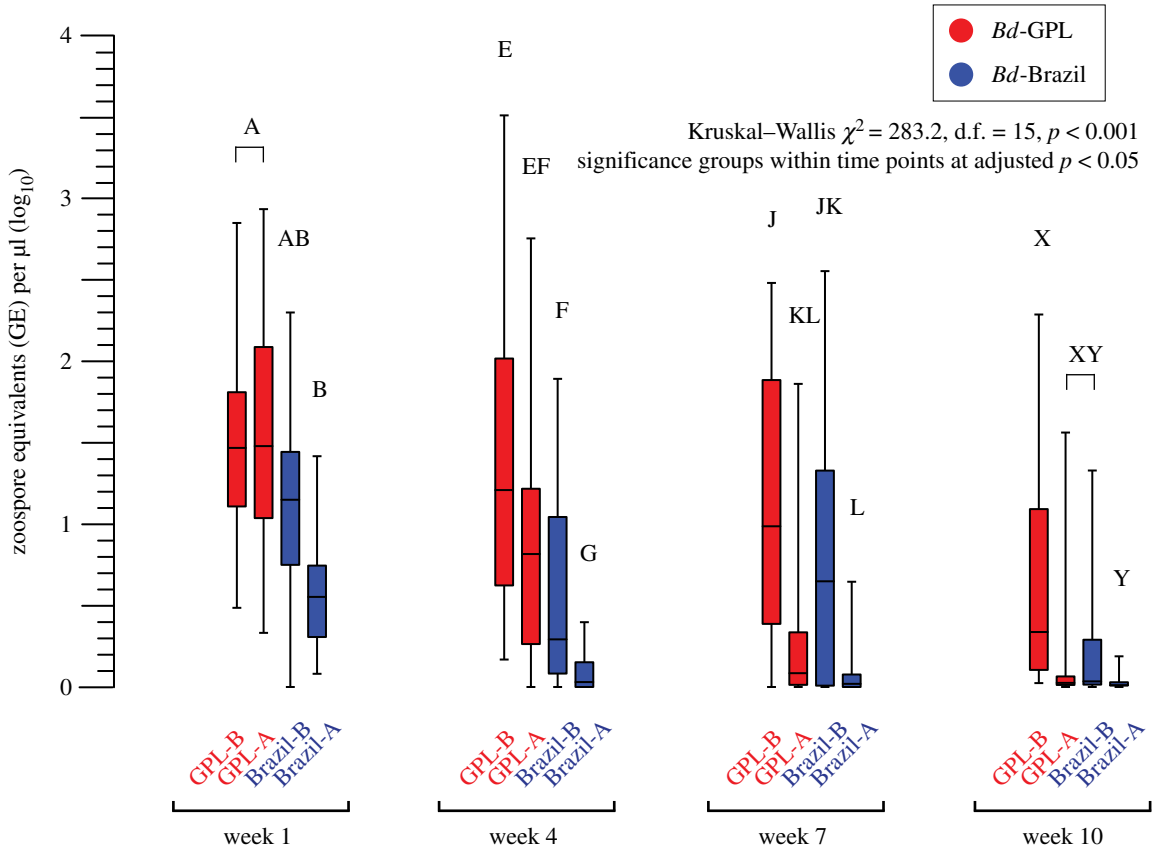

Figure 3. Zoospore production over 10 weeks varies significantly by isolate strain. Box plots show the median and interquartile zoospore densities (log (GE μl^{-1} + 1)) at sampled time points for the two *Bd*-GPL (red) and the two *Bd*-Brazil (blue) isolates studied. Whiskers show the range for all observations.

better spore producers than their co-lineage counterparts GPL-A and Brazil-A (Kruskal–Wallis rank-sum test $\chi^2 = 283.2$, d.f. = 15, $p < 0.001$). We again assessed differences *post hoc* between isolate partitions using Dunn's test. In week 1, both *Bd*-GPL isolates and the competitively superior Brazil-B isolate produced higher zoospore densities than the inferior Brazil-A. In week 4, the competitively inferior GPL-A produced a lower density of spores than GPL-B, but this difference was not significant (corrected $p > 0.999$).

The average increase in *Bd*-Brazil spore density at week 7 was driven entirely by the competitively superior Brazil-B isolate. It was at week 7 that the competitive differences among isolates were most pronounced. For both lineages, the competitively superior isolates (GPL-B and Brazil-B) produced higher zoospore densities than their competitively inferior counterparts in the same lineage (GPL-A and Brazil-A). However, the competitively superior GPL-B isolate still produced higher average spore densities than the competitively superior Brazil-B (although not significantly; corrected $p > 0.999$). By week 10, the most competitive of the four isolates (GPL-B) produced higher median spore densities than the other isolates; however, this trend was only significant compared to the least competitive of the isolates (Brazil-A; corrected $p = 0.011$).

(d) Competitive outcomes differed by treatment tank

To visualize the magnitude of competitive differences at the level of individual tanks, we plotted the difference between *Bd*-GPL and *Bd*-Brazil spore densities (Δ = (*Bd*-GPL GE μl^{-1}) − (*Bd*-Brazil GE μl^{-1})) taken from each host animal (figure 4). A positive delta indicated a greater density of *Bd*-GPL spores, while negative deltas indicated greater densities of *Bd*-Brazil spores. Overall, mean deltas through the experiment were positive (mean week 1 $\Delta = 69.98$ GE μl^{-1}, mean week 4 $\Delta = 93.65$ GE μl^{-1}, mean week 7 $\Delta = 7.87$ GE μl^{-1}, mean week 10 $\Delta = 11.84$ GE μl^{-1}).

We observed that competitive differences were greatest in the first part of the infection experiment (week 1 and 4) and grew weaker as the infection was cleared (week 7 and 10). The variation in competitive outcomes was largely shared across individuals in a tank. For example, all living individuals in Tank R during week 4 had strong negative Δs, showing that Brazil-B was the dominant isolate across host individuals at that time point. In the less common case where the *Bd*-Brazil strain had the advantage at the end of the experiment (figure 4; tanks Q and R, week 10), treatments were inoculated with a GPL-B × Brazil-B pairing (electronic supplementary material, table S1). This pairing, however, did not always yield a *Bd*-Brazil advantage (figure 4; tanks S and T, week 10). Brazil-B was also outperformed by GPL-A in all replicates (figure 4; tanks K through O), underscoring the complex nature of strain hierarchy.

(e) Zoospore densities at host death

The zoospore densities from post-mortem skin swabs ranged an order of magnitude greater than those from live animals (spore densities from surviving animals: approximately 0.0–10^3 GE μl^{-1}; post death: approximately 0.0–2.0×10^4 GE μl^{-1}). Because of this discrepancy in range, we analysed zoospore densities from the post-mortem swabs separately from the data presented above. *Bd*-GPL zoospore

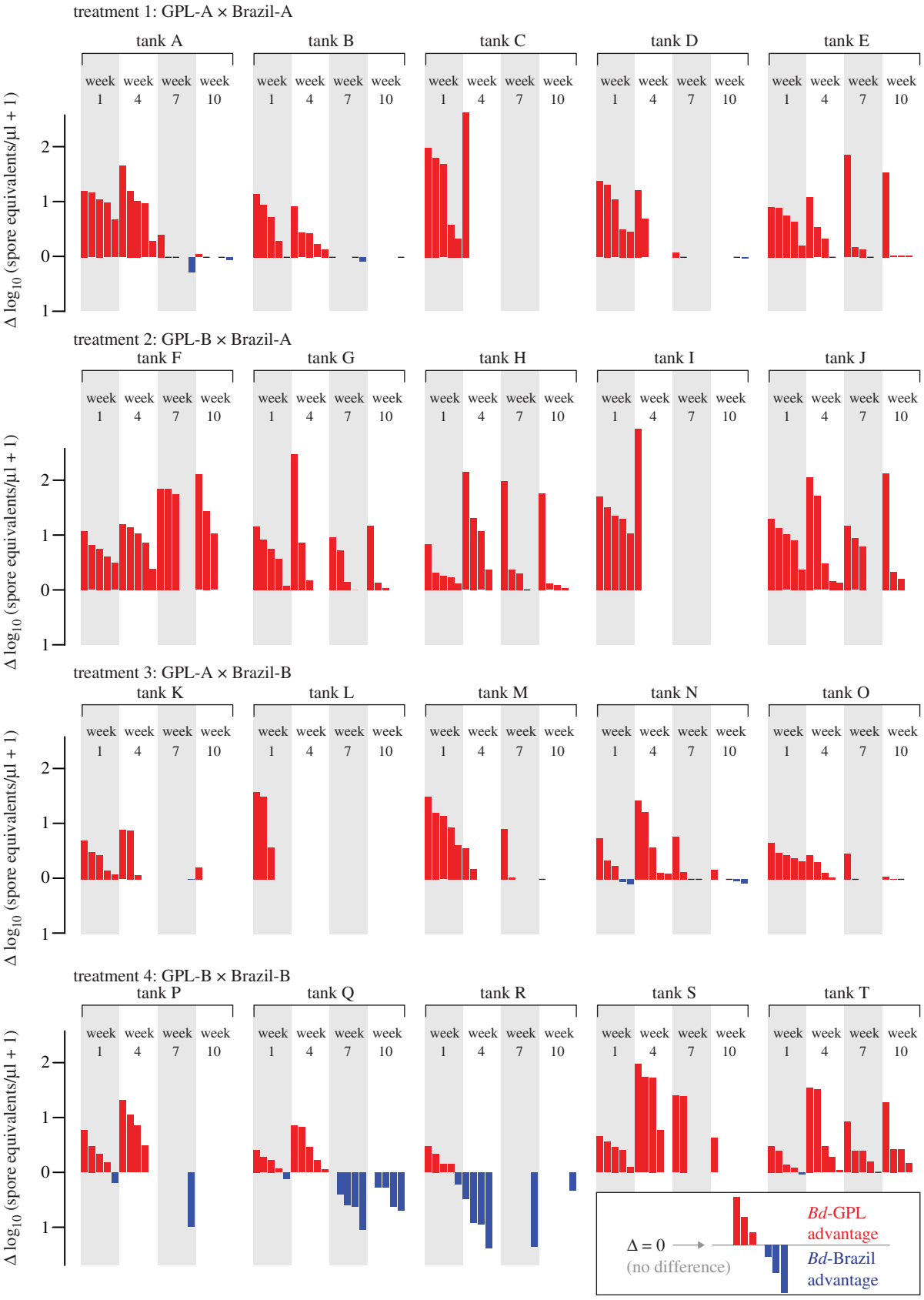

Figure 4. Differences in zoospore densities by individuals and tanks. Bar plots represent the difference (Δ) between *Bd*-GPL and *Bd*-Brazil zoospore densities [Δ = (log (*Bd*-GPL (GE μl^{-1}) + 1) − log (*Bd*-Brazil (GE μl^{-1}) + 1))] for each host. Bars with positive values (red) show individuals in which *Bd*-GPL spore densities are greater than *Bd*-Brazil. Bars with negative values (blue) show the degree of *Bd*-Brazil advantage. Missing bars indicate individuals that have either died or cleared the disease over the course of this experiment.

densities were higher than *Bd*-Brazil spore densities on dead individuals (Wilcoxon rank-sum test $W = 1309$, $p < 0.001$; figure 5). As with spore densities on live individuals, we observed differences in average spore density between the more and less competitive isolates, but the differences were not significant at $\alpha = 0.05$. A linear regression showed a slight decrease (slope = −0.184) in post-mortem *Bd*-GPL zoospore densities over the course of 10 weeks ($r^2 = 0.140$,

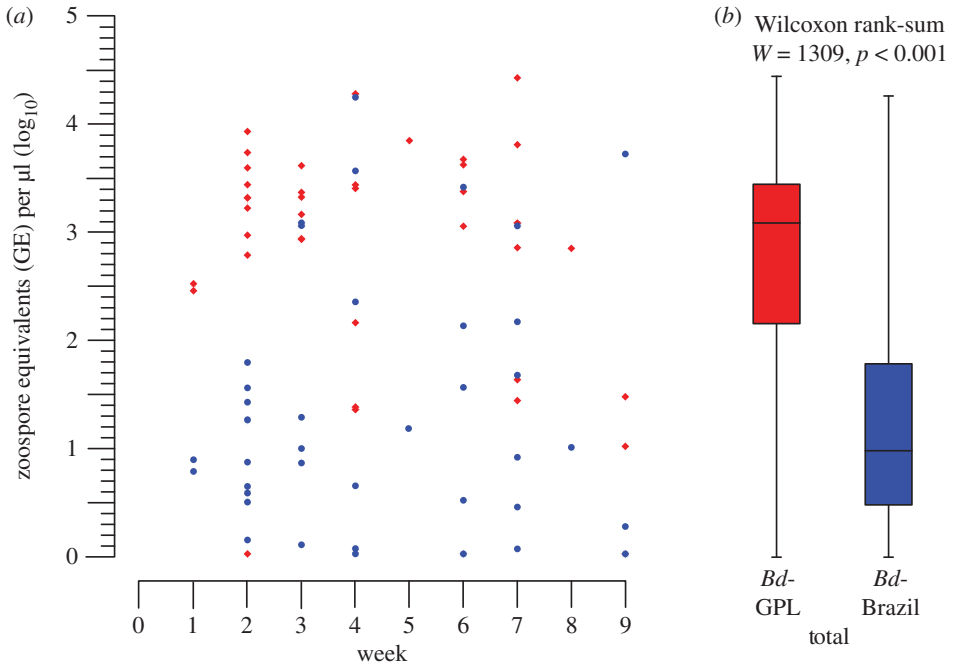

Figure 5. Zoospore densities in post-mortem skin swabs. (*a*) Spore densities (log (GE $\mu l^{-1} + 1$)) over time. *Bd*-GPL spore density is shown in red diamonds, and *Bd*-Brazil spore density is shown in blue circles. (*b*) Difference in all *Bd*-GPL and *Bd*-Brazil zoospore densities at death. Box plots show the median and interquartile zoospore densities (log (GE $\mu l^{-1} + 1$)) for *Bd*-GPL (red) and *Bd*-Brazil (blue). Whiskers show the range for all observations.

$p = 0.016$) and no temporal trend in post-mortem *Bd*-Brazil spore densities ($r^2 = 0.001$, $p = 0.854$).

4. Discussion

Our results demonstrate that *Bd* genotypes differ in reproductive ability when coinfecting the same hosts. We found differences in competitive fitness between major lineages of *Bd* (*Bd*-GPL and *Bd*-Brazil) and a hierarchical relationship of competitive ability within the lineages. We suggest that strain to strain competition can result in the eventual replacement of existing pathogen diversity resulting in populations of the most reproductively competitive genotypes. Given that the international amphibian trade is closely associated with the long-distance transport of major *Bd* lineages [23,25,26], we predict a future escalation of pathogen transmissibility though the competitive dynamics we describe here. The implications of our results grow in urgency as diverse *Bd* strains are increasingly transported between continents, which expands opportunities for secondary contact between divergent genotypes. The rapid genetic turnover of pathogen populations though competition holds the potential to alter disease outcomes at regional scales—potentially presenting a conservation risk where an earlier, enzootic strain did not [50].

Our study showed significant differences in relative fitness between *Bd*-GPL and *Bd*-Brazil at the critical phase of infection for successful transmission—the peak of zoospore production and host mortality in the first one to four weeks after inoculation [51,52]. It is clear that the ecological and evolutionary consequences of strain competition depend crucially on whether competitive dynamics affect transmission to new hosts [53]. Our measure of competitive success in this study—zoospore production—is directly tied to the mode of transmission for *Bd*, making the competitive advantage we observed in the laboratory likely to translate to increased transmission success in the field.

Under coinfection, each *Bd* strain is also in conflict with the host's immune system. Most (62%) of our *H. curtipes* hosts in this experiment either suppressed or completely cleared *Bd* infections after 10 weeks. In an open, natural system with an available pool of new, susceptible individuals, we expect that the probability of propagating infection through a host community will favour strains with the ability to persist and produce greater spore densities than their conspecific competitors. Based on our data, we predict that a *Bd*-GPL epizootic is more likely to spread in a susceptible population owing to differences in propagule output over the infection cycle. Because the host immunological landscape in our experiment is ecologically novel to the isolates collected in southeastern Brazil, the patterns of competitive survivorship we observed may be relevant to inferring outcomes of *Bd* strain coinfection generally as *Bd* lineages invade news habitats—a scenario repeated in diverse habitats worldwide through anthropogenic pathogen transport [26].

The precise connection between increased spore production and its implications for virulence evolution in *Bd* remains an open area of research. Prior studies have shown that *Bd* phenotypes with greater rates of spore production cause increased host mortality [41]. This suggested a simple relationship between virulence (host tissue damage) and the production of reproductive propagules. Though not the main focus of our coinfection study, our pilot experiments on single isolate virulence preliminarily suggest that the most reproductively successful *Bd*-GPL isolates were not necessarily the most lethal. Zoospore production in our single isolate infections did not directly relate to host mortality (electronic supplementary material, figure S2, host survival versus spore production), nor did host mortality in single isolate inoculations coincide with reproductive success under coinfection (figure 3 versus electronic supplementary material, figure S2). While this pilot study should be repeated with a

larger sample size, the initial results suggest that *Bd* isolates may vary in traits that interact in yet unknown ways with the host immune system. Our results point to the evolutionary links between isolate virulence and spore transmission as an intriguing area of future investigation in *Bd*.

At present, the evolutionary ecology of genetically diverse pathogen populations remains poorly understood. This is especially true for eukaryotic pathogens such as fungi [54]. For many bacterial and viral pathogens, a theoretical constraint on virulence is imposed by the inherent trade-offs between host exploitation and transmission [7]. Does this constraint hold true for emerging mycoses with broad host ranges such as *Bd*? Our understanding of the link between transmission and virulence in fungal pathogens—and in eukaryotic pathogens more broadly—still must be inferred from the few eukaryotic systems studied thus far.

Among the few well-studied examples in the eukaryotes, *Plasmodium chabaudi* (Apicomplexa; causing rodent malaria) is constrained by the reproduction/transmission trade-off [55]. In this example, the dominance of a specific clone in a mixed infection did not translate to increased transmission success of that clone to new hosts. Like this example, our results showed that the better spore producer was not the deadliest. These observations provide support for the hypothesis that extreme *Bd* virulence may come at a cost to transmissibility. Ultimately, the primary trait allowing *Bd*-GPL to rise to global prominence may in fact be its transmissibility through reproductive characteristics rather than virulence. In previous studies [28], the virulence of *Bd*-GPL isolates were not always greater than those of *Bd*-Brazil as measured by host survival. Therefore, strain competition between divergent lineages may be one situation where the virulence/transmission trade-off becomes more critical to determining disease outcomes.

In addition to the differences in zoospore production we observed between *Bd* lineages, we also found that zoospore production by specific isolates varied within each lineage. These results agree with previous studies showing phenotypic variation among closely related genetic isolates of *Bd* [28,56,57]. Although genomic changes and virulence attenuation can occur in *Bd* isolates serially passaged over long periods of time [41,42,58], we do not believe that laboratory passage was a contributing factor in the results we observed. Prior studies documenting *in vitro* changes were based on cultures passaged over 30 times in the span of 6 years. When our inoculations took place, all experimental isolates had been passaged either six or nine times. We also controlled for passage history by selecting paired *Bd*-GPL and *Bd*-Brazil isolates collected contemporaneously and passaged an equal number of times in culture. Our results showed that the *Bd*-GPL isolate with nine passages significantly outperformed its *Bd*-Brazil counterpart with nine passages. The same pattern held for the *Bd*-GPL and *Bd*-Brazil pair with six passages.

Along with the ecological and evolutionary implications of these results, our study serves as a proof of concept for two new tools to improve our understanding of amphibian chytrid ecology and evolution. First, dPCR is a viable tool for the detection of rare genotypes in mixed pathogen populations. The ability to simultaneously quantify and genotype samples with high accuracy makes this tool extensible to a range of studies of mixed infections within a pathogen population. For example, the dPCR probes could be readily adapted to distinguish between *Bd* and its congeneric sister taxon *Batrachochytrium salamandrivorans* [59]. The ability to detect one variant copy in 1–5 µl of sample outperforms traditional duplexed qPCR, which is prone to allele drop out at these template concentrations [60]. We also describe a potential model host in which to test *Bd* phenotype across a standardized host species, *Hymenochirus curtipes*. At present, virulence studies are typically conducted on haphazard assemblages of locally available species [61], which makes the replication and comparison of experiments across research groups difficult. *Hymenochirus curtipes* has a number of traits that make it a practical model host to explore virulence phenotype across *Bd* lineages. It is susceptible to chytridiomycosis, unlike *Xenopus laevis*, and its fully aquatic nature and small body size simplifies husbandry while allowing for facile disease transmission. *Hymenochirus curtipes* is also commercially available and easy to breed [40]. Finally, the ability to expose animals to a prolonged 30°C incubation period in order to clear incoming chytridiomycosis is a major advantage not present in many amphibian species.

The experimental system we describe here provides a base upon which to test more complex questions about the ecology of pathogen communities. For example, our current study only explored the outcomes of simultaneous coinfection. Priority effects are known to play a large role in ecological structuring [62], especially within the fungi [63,64]. An immediate next area of research is to explore the effects of sequentially inoculated strains and address the roles of priority and contingency on the competitive structuring of microbial populations. Another potential elaboration on this system could be introducing new susceptible hosts at various time points in the experiment to better understand transmission dynamics over longer experimental time scales. Together, these types of studies will allow a better understanding of how pathogen populations assemble, and provide empirical parameters to tailor disease dynamic predictions to emerging fungal pathogens.

Ethics. We performed all investigations involving live vertebrate animals following protocols approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC protocols PRO00005605 and PRO00007691).

Data accessibility. The datasets analysed for this article are available from the Dryad Digital Repository at http://dx.doi.org/10.5061/dryad.1qh6772 [65].

Authors' contributions. T.S.J., D.R., L.F.T., K.R.Z., and T.Y.J. conceived of and designed the study. T.S.J., D.R., R.A.C., L.A.M., J.E.L., and T.Y.J. performed laboratory work. T.S.J., D.R., and T.Y.J. analysed the data. T.S.J. and T.Y.J. wrote the article. All authors gave their final approval for publication.

Competing interests. We declare we have no competing interests.

Funding. This study was supported by a Doctoral Dissertation Improvement Grant from the United States National Science Foundation (DEB-1601259), the São Paulo State Research Foundation (FAPESP no.2014/25358-3), the Brazilian National Council for Scientific and Technological Development (CNPq no.405285/2013-2, no.302589/2013-9, no.300896/2016-6), and the Research Enhancement Program at Texas State University (no.9000001537).

Acknowledgements. We would like to acknowledge Joice Ruggeri, Anat M. Belasen and the veterinary team from the University of Michigan Laboratory Animal Medicine Unit for support and advice on animal care. We also thank two anonymous reviewers for their helpful suggestions on improving this manuscript.

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