

Original Contribution

# External Reinfection of a Fungal Pathogen Does not Contribute to Pathogen Growth

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**Abstract:** Chytridiomycosis is an emerging infectious disease of amphibians caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), which has led to devastating declines in amphibian populations worldwide. Current theory predicts that *Bd* infections are maintained through both reproduction on the host's skin and reinfection from sources outside of the host. To investigate the importance of external reinfection on pathogen burden, we infected captive-bred individuals of the highly susceptible Panamanian Golden Frog, *Atelopus glyphus*, and wild-caught glass frogs, *Espadarana prosoblepon*, with *Bd*. We housed the animals in one of three treatments: individually, in heterospecific pairs, and in conspecific pairs. For 8 weeks, we measured the *Bd* load and shedding rate of all frogs. We found that *Atelopus* had high rates of increase in both *Bd* load and shedding rate, but pathogen growth rates did not differ among treatments. The infection intensity of *Espadarana* co-housed with *Atelopus* was indistinguishable from those housed singly and those in conspecific pairs, despite being exposed to a large external source of *Bd* zoospores. Our results indicate that *Bd* load in both species is driven by pathogen replication within an individual, with reinfection from outside the host contributing little to the amplification of host fungal load.

**Keywords:** Amphibians, Abiotic reservoir, Community, Disease, Epizootic, Chytridiomycosis, Panama, Multi-species, Tropical, Transmission

## INTRODUCTION

The fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*), and its associated disease, chytridiomycosis, have devastated amphibian populations worldwide (Berger et al. 1998; Lips et al. 2006; Skerratt et al. 2007; Wake and Vredenburg 2008). This emergent infectious disease, first described in poison dart frogs (Berger et al. 1998; Daszak et al. 2000),

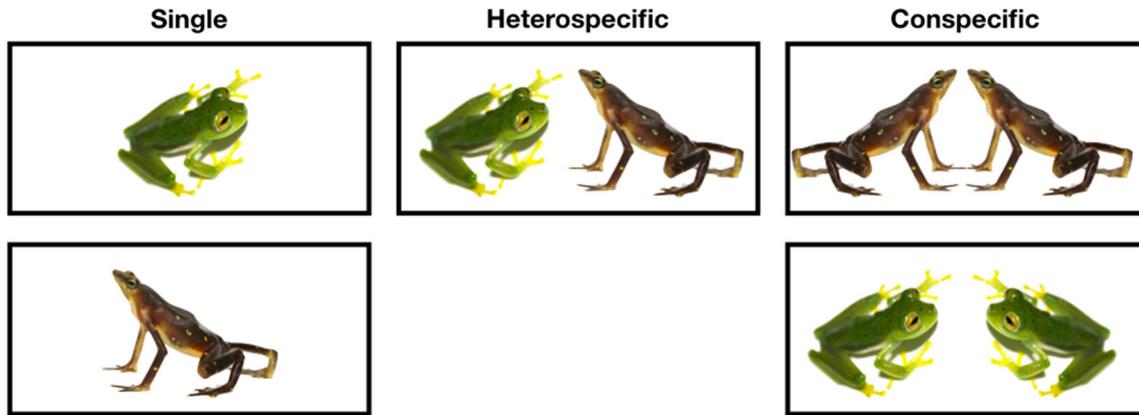
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Graziella V. DiRenzo and Tate S. Tunstall have been contributed equally to this manuscript.

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**Figure 1.** Experimental design of single, heterospecific, and conspecific pairings of *Atelopus* and *Espadarana*. The rectangles represent tanks. The green anurans are *Espadarana prosoblepon*, and the brownish anurans are *Atelopus glyphus*. There were a total of five groups.

**Table 1.** Summary of the Mean *Bd* Load, Standard Error, *Bd* Prevalence, Sample Size, and the Minimum to Maximum *Bd* Load Range per Week for Each Genus Over the Course of the Experiment.

Species	Week	Mean <i>Bd</i> load	Standard error	<i>Bd</i> prevalence	Sample size	Min–max <i>Bd</i> load
<i>Atelopus glyphus</i>	1	6.14	1.07	0.61	33	0–90.85
	2	40.46	7.04	0.58	33	0–794.8
	3	162.7	28.76	0.75	32	0–3128.56
	4	1923.22	339.98	0.88	32	0–36,638.34
	5	2689.56	475.45	0.84	32	0–84,188.22
	6	4748.58	881.79	0.93	29	0–48,258.71
	7	614.75	131.06	0.91	22	0–3370.03
<i>Espadarana prosoblepon</i>	0	0.58	0.12	0.67	24	0–3.27
	1	0.96	0.19	0.8	25	0–14.13
	2	1.67	0.31	0.4	30	0–40.3
	3	29.87	5.45	0.33	30	0–798.93
	4	121.07	22.1	0.17	30	0–3446.18
	5	156.11	28.5	0.47	30	0–4590.38
	6	9.97	1.82	0.57	30	0–233.94
7	0.77	0.14	0.73	30	0–2.12	

Week 0 refers to a swabbing event prior to the experimental inoculations. The sample size of *Espadarana* on week 0 and 1 are less than 30 because swabs were misplaced. For all other weeks, we report summary stats of all 30 *Espadarana* individuals.

has caused mortality in numerous amphibian species (Wake and Vredenburg 2008; Cheng et al. 2011) and driven several species extirpations (e.g., Crawford et al. 2010). *Bd* is highly pathogenic in many species, and it often produces high pathogen loads, prevalence, and host mortality when it is introduced into naïve populations (e.g., Lips et al. 2006; Vredenburg et al. 2010). Where *Bd* is enzootic, species often maintain low levels of infection with little to no mortality, and may serve as pathogen reservoirs (Weldon et al. 2004; Schloegel et al. 2010). *Bd* has been detected on the

skin of over 700 of amphibian species worldwide (<https://microreact.org/project/GlobalBd>). Yet, transmission between species remains poorly understood, and the exact method of transmission, whether through direct contact between individuals or mediated through the environment, remains unknown (although see Rachowicz and Vredenburg 2004; Rachowicz and Briggs 2007).

Pathogens are often broadly characterized as either microparasites or macroparasites, depending on their mode of reproduction (Anderson and May 1978, 1981).

Microparasites are characterized by reproduction within a host, whereas macroparasites are characterized by reproduction outside of the host. *Bd* is intriguing in that it exhibits properties of both micro- and macroparasites, as host infection can increase through two pathways: (1) reinfection within the host by zoospores produced by an existing infection or (2) reinfection by zoospores produced outside the host (i.e., from environmental reservoirs, or other infected hosts) (Berger et al. 2005; Briggs et al. 2005; Louca et al. 2014). The relative contribution of these pathways to infection load is unknown, even though it is essential to understanding the disease dynamics of *Bd*. External reinfection could be a particularly important driver of outbreaks in systems with multiple species, where multiple hosts with varying susceptibility could contribute to the environmental zoospore pool. For example, the shedding rate of the harlequin frog, *Atelopus*, has been estimated to be as high as 6 million zoospores per day (DiRenzo et al. 2014). If *Bd* fungal load dynamics are primarily determined by external reinfection, supershedder species (i.e., an infected host that contributes a disproportionate number of infectious agents for transmission for a short period of time), such as *Atelopus*, could have disproportionate influence on epizootics, rapidly spreading the pathogen and amplifying the pathogen burden on other species.

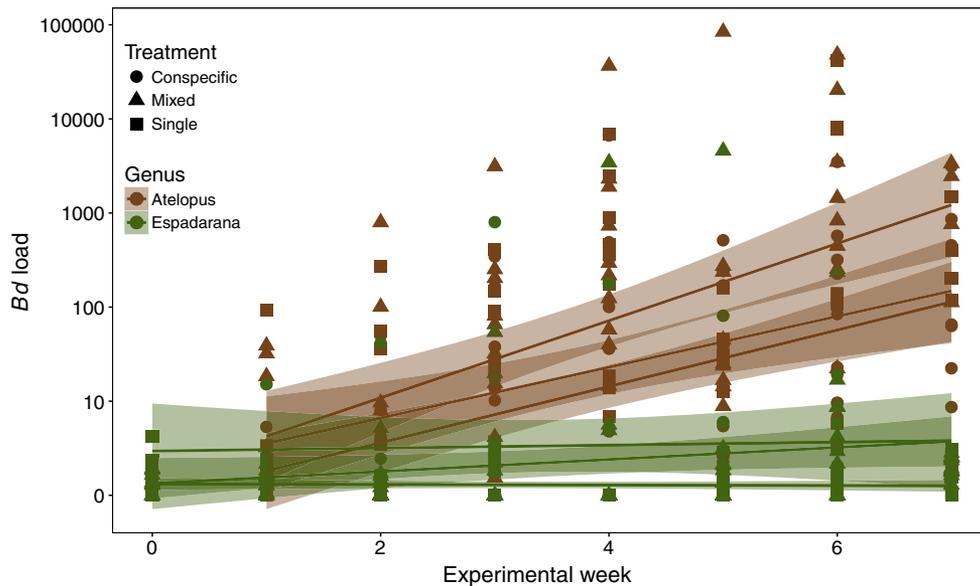
Amphibian communities with *Atelopus* species present could be at higher risks of epizootics or *Bd* invasions.

To determine whether *Atelopus* can increase infection intensity in co-occurring species, we conducted an experiment involving *Atelopus glyphus* and the emerald glass frog, *Espadarana prosoblepon*. Our experiment tests whether the presence of infected *Atelopus* is likely to increase *Bd* loads in conspecific or heterospecific hosts within a community and attempts to determine whether infection load is driven primarily from *Bd* growth on the skin of the host, or from reinfection from other infected individuals.

## METHODS

### Animal Husbandry

We received 33 uninfected adult *Atelopus glyphus* from the Panama Amphibian Rescue and Conservation project’s breeding facility in Summit Municipal Park, Panama. These animals had been raised in captivity from two clutches of eggs laid by adults caught from the field in Darien National Park, on Serranía de Pirre above Cana, Darién Province Panama. We also collected 30 adult *Espadarana prosoblepon* from the General de División Omar Torrijos Herrera National Park in El Copé, Coclé Province Panama, during the



**Figure 2.** Line predictions from the mixed effect model showing *Bd* zoospore load over time for both genera and all treatments, with individuals indicated by points. The y-axis is *Bd* zoospores load on the log 10 scale and was measured using qPCR of skin swabs. The orange lines (mean model prediction) and shading (model standard error) represent *Espadarana*; blue lines (mean model prediction) and shading (model standard error) represent *Atelopus*. The shapes represent different treatment groups.

**Table 2.** Summary Table of Post Hoc Analysis of the Mixed Effect Model Comparing the Slopes of Swab Data from Each Genus and Treatment, Including Slope Mean, Standard Error (SE), and the 95% Confidence Interval.

Genus	Treatment	Slope mean	SE	95% CI		Group
<i>Espadarana</i>	Single	0.00	0.04	− 0.07	0.07	1
	Conspecific	0.02	0.06	− 0.10	0.13	1
	Heterospecific	0.07	0.04	0.00	0.14	1
<i>Atelopus</i>	Single	0.29	0.05	0.19	0.38	2
	Conspecific	0.32	0.05	0.23	0.41	2
	Heterospecific	0.44	0.05	0.35	0.54	2

Group represents the significant difference between treatments and genera using a Tukey-HSD test with an adjusted *p* value for multiple comparisons.

**Table 3.** Summary Table of Post Hoc Analysis of the Mixed Effect Model for Soak Data Comparing the Slopes of Each Genera and Treatment, Including Slope Mean, Standard Error (SE), and the 95% Confidence Interval.

Genus	Treatment	Mean	SE	95% CI		Group
<i>Espadarana</i>	Single	− 0.06	0.09	− 0.24	0.11	1
	Conspecific	0.03	0.13	− 0.22	0.27	12
	Heterospecific	0.15	0.08	0.00	0.31	12
<i>Atelopus</i>	Single	0.32	0.08	0.17	0.48	23
	Conspecific	0.48	0.07	0.34	0.62	3
	Heterospecific	0.52	0.08	0.36	0.68	3

Group represents the significant difference between treatments and genera using a Tukey-HSD test with an adjusted *p* value for multiple comparisons.

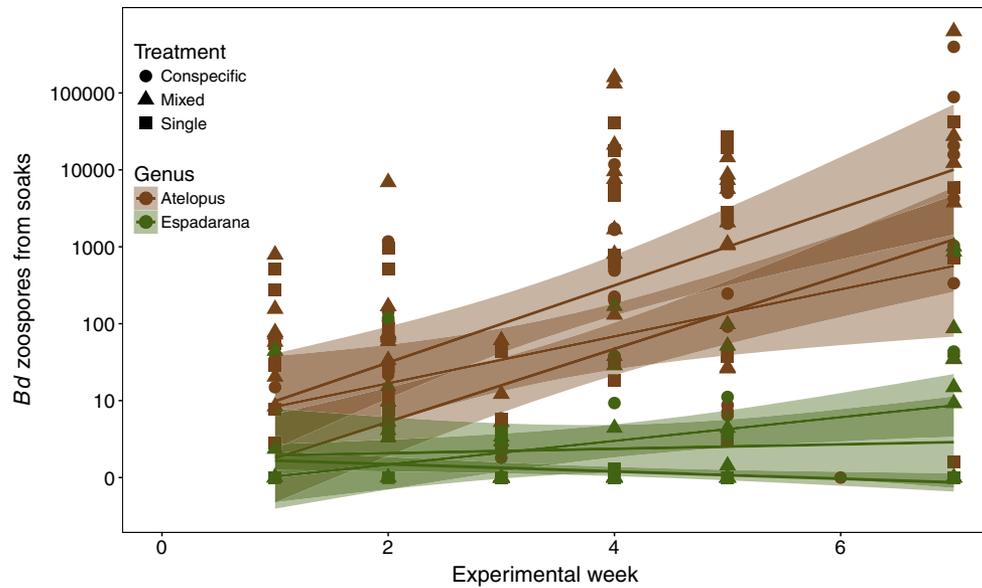
wet season in June, 2013. We selected *Espadarana* because it has persisted at the site following a chytridiomycosis epidemic in 2004 (Lips et al. 2006; Crawford et al. 2010). We transported all animals to the Smithsonian Tropical Research Institute (STRI) Center for Tropical Paleocology and Archeology (CTPA) facility in Panama City, Panama, and we housed them in 2 Liter plastic containers, with 250 mL of water, 3 paper towels, and a small perch. We sprayed each cage daily with approximately 10 mL of water to maintain humidity in the cage. We fed animals ad libitum once a week with crickets and wingless *Drosophila*. We kept animals in a laboratory maintained at 19–24°C with a 12:12 h light: dark photoperiod and were given 7–14 days to acclimate to the laboratory before the experiment began.

### Bd Inoculation Design and Data Collection

We swabbed all animals and tested for *Bd* prior to the start of the experiment. We inoculated each animal individually in a 30 mL solution containing  $3 \times 10^5$  *Bd* zoospores (isolate JEL423) for 10 h (Langhammer et al. 2013), and

then, we randomly assigned each individual to one of three treatments (Fig. 1). There were three treatments (i.e., singly housed, heterospecific housing, conspecific housing) comprising five groups. One treatment consisted of animals housed individually to measure *Bd* growth rate based on self-reinfection (i.e., singly housed individuals for each species). The second treatment consisted of a conspecific pair of *Atelopus* and *Espadarana* housed in the same container to measure the combined effects of self-reinfection and reinfection from another species. The third treatment consisted of a conspecific pair of either two *Atelopus* or two *Espadarana* housed in the same container to measure the combined effects of self-reinfection and reinfection from a conspecific animal.

We swabbed animals weekly for eight weeks by rubbing a sterile swab over the ventral surfaces 30 times to measure *Bd* infection intensity. Immediately post-swabbing, we soaked each frog in 50 mL of distilled water for 15 min to measure the shedding rate of zoospores. We filtered the solution using a 60-mL sterile syringe and 0.45- $\mu$ m filter for each sample and added 10  $\mu$ L of BSA to prevent zoospores



**Figure 3.** Line predictions from the mixed effect model showing *Bd* zoospore released during soaks over time for both genera and all treatments, with individuals indicated by points. The y-axis is *Bd* zoospores released during soaks on the log 10 scale and was measured using qPCR of filtered water. The orange lines (mean model prediction) and shading (model standard error) represent *Espadarana*, blue lines (mean model prediction) and shading (model standard error) represent *Atelopos*. The shapes represent different treatment groups.

from sticking to the plastic container. We plugged filters with syringe caps and stored them in a 4°C refrigerator until we processed them in the laboratory. Swabbing individuals before soaking could reduce the number of *Bd* zoospores estimated from the soak; thus our estimates from the soaks are a more conservative estimate of minimum zoospore output. In addition, swabs are meant to pick up residual zoospores on amphibian skin, whereas the soaks are meant to induce the zoosporangia in the skin of the amphibian to discharge the zoospores, providing an estimate of the capacity of the number of zoospores a single individual produces (Longcore et al. 1999). Thus, the swabs and the soaks give two metrics of estimating an individual's contribution to *Bd* transmission.

We euthanized animals when they lost righting reflex and used a fresh pair of latex powder-free gloves when handling individuals (Hyatt et al. 2007).

### Molecular Analysis

We tested skin swabs for *Bd* with qPCR (Hyatt et al. 2007; Vredenburg et al. 2010) using PrepMan Ultra<sup>®</sup>. We tested samples in singlicate using Taqman qPCR (Boyle et al. 2004; Hyatt et al. 2007) and ran each plate with JEL 423 standards to determine *Bd* presence and infection intensity. We categorized individuals as *Bd* positive when infection

intensity was greater than or equal to one zoospore genomic equivalent (ZGE; Kriger and Hero 2006). All infection intensity (i.e., *Bd* load estimates) corresponds to the number of ZGE on a swab. There is error in the number of zoospores quantified by qPCR (Miller et al. 2012) and swabbing (DiRenzo et al. 2018), but correcting ZGE estimates for imperfect *Bd* detection is a lesser concern for experiments than for field studies because individuals are repeatedly swabbed over time during an experiment and infection history is well recorded. The largest concern for field studies is to accurately and precisely estimate the relationships between covariates because nothing is known about prevalence or infection intensity in the population of interest. In contrast, the main concern for experimental studies is only to precisely estimate the relationship between covariates and not the accuracy because of the controlled environment; therefore, it is likely that estimates are accurate but maybe imprecise.

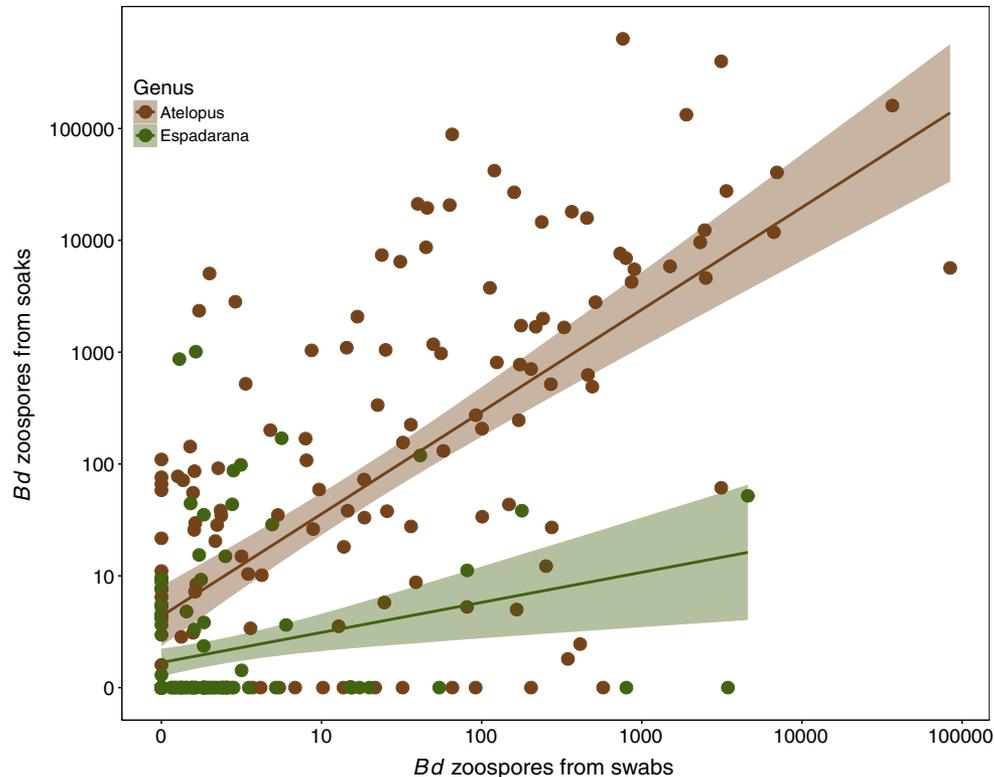
### Statistical Analysis

We analyzed all data in R, version 3.1.1 (R Core Development Team 2014). For all models, we used the package *lme4* (Bates et al. 2015) and the function `lmer()`. Then, we used the package *lsmeans* (Lenth 2016) to determine the significance between slopes using the functions `lsmeans()`

**Table 4.** Summary Table of Post Hoc Analysis of the Mixed Effect Model for Soak Data Compared to Swab Data, Including Slope Mean, Standard Error (SE), and the 95% Confidence Interval.

Genus	Slope mean	SE	95% CI		Group
<i>Espadarana</i>	0.00011714	6.13E-05	- 2.98E-06	0.00023727	1
<i>Atelopus</i>	0.00039799	2.90E-05	0.00034106	0.00045492	2

Group represents the significant difference between treatments and genera using a Tukey-HSD test with an adjusted  $p$  value for multiple comparisons.



**Figure 4.** Relationships between *Bd* load swab data and *Bd* soak data for each genus. The orange lines (mean model prediction) and shading (model standard error) represent *Espadarana*, blue lines (mean model prediction) and shading (model standard error) represent *Atelopus*. The shapes represent different treatment groups.

and `cld()`. Our experiments aim to answer the following questions:

- (1) Does *Bd* growth rate differ between treatments (single, heterospecific, conspecific) and genera (*Atelopus* vs. *Espadarana*)?

We estimated the rate of zoospores increase on each frog, defined as “*Bd* growth rate,” using a mixed linear effects model with week, species, treatment, each two-way interaction, and the three-way interaction as fixed effects, and individual as a random effect.

- (2) Does the number of *Bd* zoospores released in soaks differ between treatments and genera?

We used a mixed linear effects model with the number of *Bd* zoospores released in soaks and with week, species, treatment, each two-way interaction, and the three-way interaction as fixed effects, and individual as a random effect.

- (3) Does the number of *Bd* zoospores differ from swabs and soaks between genera?

We used a mixed linear effects model with the number of *Bd* zoospores released in soaks and with species, *Bd* load from swabs, and the two-way interaction as fixed effects, and individual as a random effect.

(4) Can the shedding rate of *Bd* zoospores of *Atelopus* predict the number of *Bd* zoospores on *Espadarana* over time?

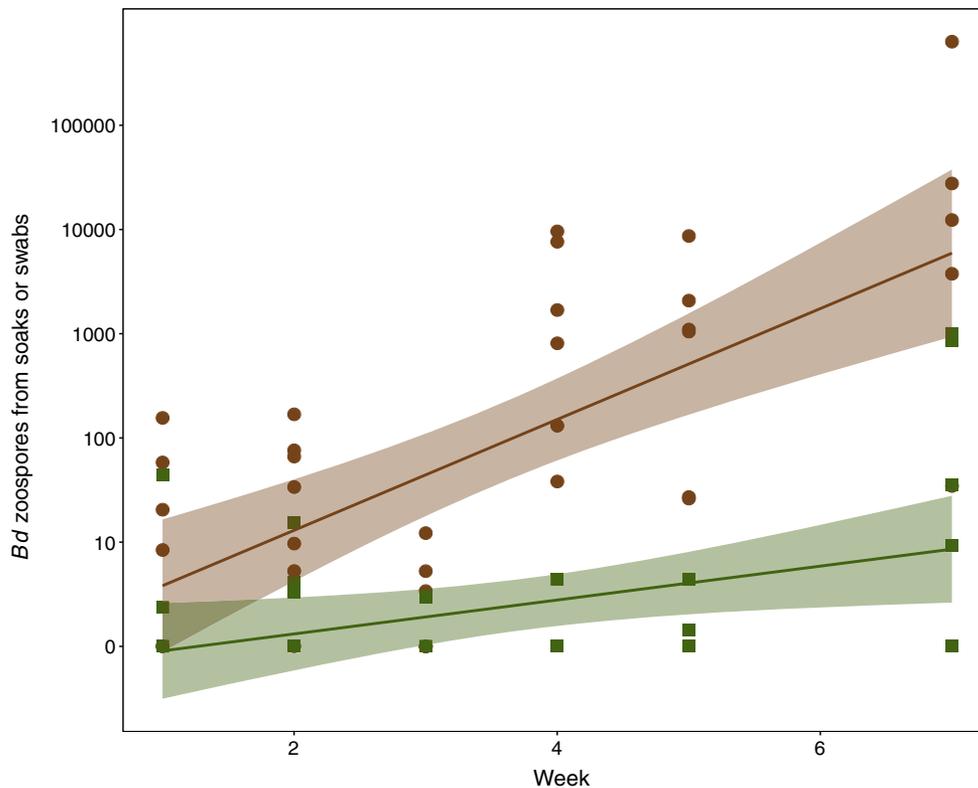
To determine whether the number of *Bd* zoospores released in soaks by *Atelopus* increased the number of *Bd* zoospores on swabs found on *Espadarana*, we used a mixed effects model with the number of *Bd* zoospores released in soaks by *Atelopus* as the response variable and of the number of *Bd* zoospores on swabs *Espadarana*, week, and their interaction as explanatory variable. We also included individual as a random effect.

(5) Does frog survival vary among treatments and genera? We compared survival rates of *Atelopus* and *Espadarana* and the effect of treatment on mortality using a Cox proportional hazard survival model using package *survival* (Therneau 2015).

## RESULTS

At the start of the experiment, all *Atelopus* tested negative for *Bd*, where 66% of *Espadarana* tested *Bd* positive (mean = 0.57 ZGE, range 0–3.27 ZGE; Table 1). All infected *Atelopus* maintained their infection for the duration of the experiment, and either died of chytridiomycosis or were euthanized. Mean survival did not vary among treatment group for *Atelopus* (all  $P > 0.05$ ; Figs. S1 & S2), and all *Espadarana* survived until the end of the experiment at 65 days.

The estimated growth rate of *Bd* in *Atelopus* was higher than that of *Espadarana* across all treatment groups (Fig. 2, Table 1), and appeared to grow exponentially on infected individuals until the death of the host. *Bd* load at death was highly variable (mean =  $7533 \pm 1375$  ZGE, Table S1). However, we found no differences in *Bd* loads among



**Figure 5.** Linear regression of zoospores for co-housed *Atelopus* and *Espadarana*. The solid blue line is the model fit for the number of zoospores released in soaks by *Atelopus* co-housed with *Espadarana*, while the solid orange line is the model fit for the number of zoospores on swabs collected on *Espadarana* co-housed with *Atelopus*. Brown circles represent individual samples for *Atelopus*, and green squares are *Espadarana*. Shaded area represents the standard error around the mean line.

treatment groups in both *Atelopus* and *Espadarana*, indicating that the growth rate of zoospores in conspecific pairs, heterospecific pairs, or animals housed singly were indistinguishable (Fig. 2, Table 1). Several individuals had infections over 10,000 ZGE (Table 2). Shedding rate of *Atelopus* and *Espadarana* increased with time (Table 3) and was linear on the log scale (Fig. 3). We also found that swab and soak data were correlated within genera even though the slopes were significantly different between genera (Table 4; Fig. 4).

*Espadarana* showed little to no *Bd* growth after initial inoculation and maintained low levels of *Bd* throughout the experiment across all treatments (Figs. 2, 3, 4). The infection loads were very low; thus, we do not know if animals were clearing infections or if infections were sub-clinical. Growth rates of *Bd* in *Espadarana* were indistinguishable from zero across all three treatment groups.

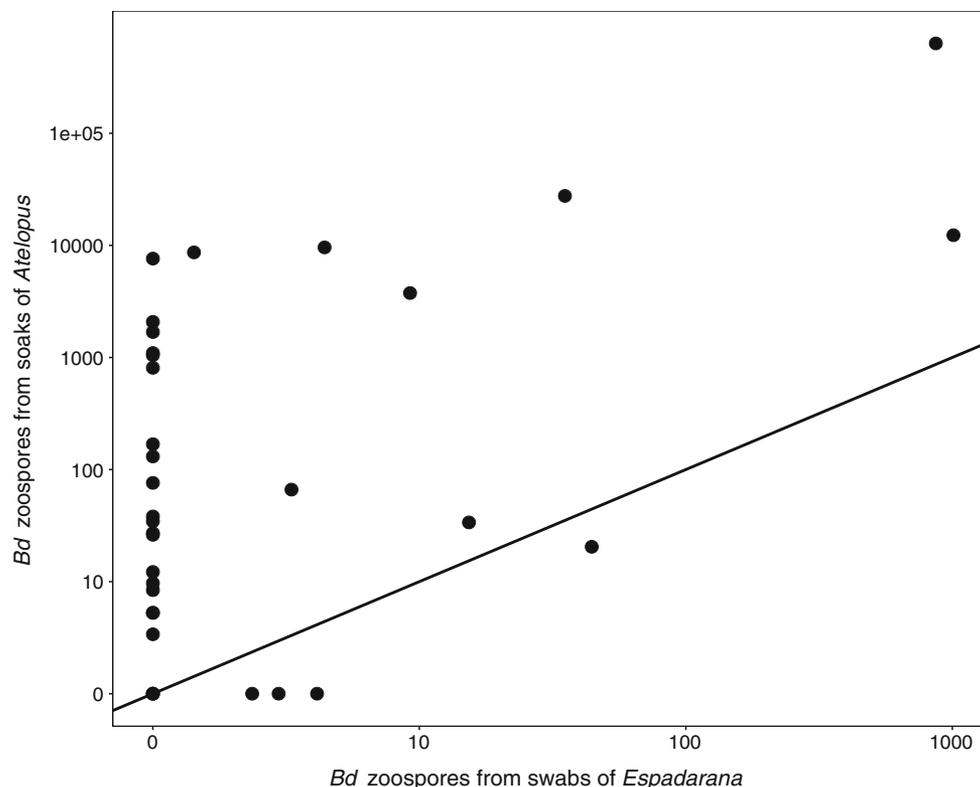
The *Atelopus* housed with *Espadarana* shed high levels of *Bd* throughout the experiment, but this did not result in any increase in *Bd* load on the skin of the *Espadarana* (Figs. 5, 6). There was a significant trend for the increase in *Bd* load over time on *Atelopus* ( $P < 0.001$ ), but there was no relationship between the number of zoospores shed by

*Atelopus* and the number of zoospores on swabs of *Espadarana*.

## DISCUSSION

Despite the fact that *Atelopus* shed increasingly high levels of zoospores throughout the course of the infection and released thousands of zoospores into the environment over this 65-day experiment, the additional zoospores did not amplify the fungal load of other susceptible hosts, be it either their conspecifics or *Espadarana*. These data indicate that increases in *Bd* infection intensity are driven primarily by the growth and reinfection of *Bd* on the individual and not from reinfection from outside the host, as neither *Atelopus* conspecifics, *Espadarana* conspecifics, nor mixed *Atelopus/Espadarana* pairs increase in *Bd* load when exposed to additional zoospores.

Models of *Bd*-amphibian disease dynamics, such as those described in Briggs et al. (2010) and Louca et al. (2014), typically account for pathogen reproduction on both the skin of the host, as well as reinfection from outside the host. These models predict that when shedding and



**Figure 6.** Relationship between the number of zoospores released in soaks by *Atelopus* and the number of zoospores on swabs collected on *Espadarana* that were co-housed. The solid black line represents a 1:1 relationship.

reinfection rates are high, frogs housed in groups should have higher loads than those housed individually, as hosts take up additional zoospores shed into the environment. However, our results indicate that once a host is infected, reinfection from additional zoospores from outside the host is less important than zoospore reproduction on the skin of the host.

In contrast to *Atelopus*, the lack of increase in infection in *Espadarana* indicates that individuals of this species are able to limit the growth of *Bd* on their skin, likely by limiting zoospore colonization and reproduction. Antimicrobial peptides secreted by amphibians are known to limit the growth of *Bd* (Woodhams et al. 2006), and *E. prosoblepon* from El Copé in particular can produce high levels of effective peptides (Woodhams et al. 2006). However, *E. prosoblepon* populations are susceptible to chytridiomycosis, and several populations have experienced large declines following the epizootics in Central America (Crawford et al. 2010; Lips et al. 2006; Lips et al. 2003; Smith et al. 2009; McCaffery and Lips 2013; Angeli et al. 2015). It is possible that the *E. prosoblepon* used in our experiment have survived in El Copé, Panama, because they have developed resistance and/or tolerance to *Bd* (Ellison et al. 2015; Voyles et al. 2018) and are now less susceptible to infection. *Bd* resistant/tolerant species demonstrate increased expression in genes associated with skin integrity and reduced skin inflammatory response (Ellison et al. 2015), which could lead to a decrease in *Bd* growth and mortality associated with infection. Therefore, the interspecific survival outcomes may be due to the fact that the amphibians were either naïve (*Atelopus*, from a breeding program) or not-naïve (*Espadarana*, wild caught), producing the results we observed.

Another possible explanation for the lack of *Bd* growth on *Espadarana* is that *Bd* transmission requires direct or close contact between hosts. Even in our small cages, the two species rarely came into direct contact with one another. We know from DiRenzo et al. (2014) as well as from the shedding rate data collected in this study that both *A. zeteki* and *A. glyphus* release large numbers of potentially infectious zoospores (~ 80% infectious zoospores released; Maguire et al. 2016). Furthermore, it is known that zoospores can at least temporarily remain infectious outside the host and that a large proportion of the zoospores shed by *Atelopus* are viable (Maguire et al. 2016). However, it is unclear if an acute supershedder is more detrimental to disease dynamics than a chronic shedder (i.e., an infected host that contributes a constant number of infectious

agents for transmission for the duration of infection). During our experiment, the increased number of shed zoospores did not intensify pathogen loads in nearby frogs, as paired frogs had similar *Bd* growth rates than frogs individually housed. Little is known about the behavior of *Bd* outside the amphibian host, but *Espadarana* could be escaping contact with infectious zoospores either because zoospores do not disperse far from the original host, or because they are too short lived outside of the host to accumulate in the environment. Direct contact was observed in our conspecific *Atelopus* treatment, making direct transmission of zoospores much more likely. If reinfection from zoospores outside of the host plays an important role in *Bd* growth, one would have expected a higher *Bd* growth rate in our conspecific paired *Atelopus* treatment. None of the growth rates estimated for our *Atelopus* treatments differed significantly, and in fact the *Bd* growth rate on *Atelopus* housed in conspecific pairs was the lowest of all three treatments, despite these conspecific pairs being exposed to tens of thousands of additional zoospores than those housed singly.

Our results demonstrate that *Bd* growth on an individual host is similar to a classic microparasite, where pathogen growth is driven primarily through pathogen replication within an individual. Assuming that *Bd* reproduction is similar to that of a typical microparasite, this finding could lead to the construction of simpler models for the invasion and spread of *Bd*. Current modeling approaches that include reinfection from outside sources require that one monitor the number of zoospores on each individual in the entire population (Briggs et al. 2010, Louca et al. 2014), which typically involves large systems of ordinary differential equations with many parameters that are difficult to estimate from field data (Briggs et al. 2010). If growth rates are similar within hosts, our results suggest that relatively simpler models could be used, where infection load may be determined primarily by time since infection, as opposed to the number of contacts with other infected hosts or zoospores.

Our results also have important conservation implications, especially given that reintroduction programs are being considered for *Atelopus* (Gagliardo et al. 2008, Poole 2008) and localized release trials for *Atelopus* have begun. If *Bd* load is strongly dependent on outside reinfection, from either the environment or other amphibian species, then introducing a highly susceptible species, such as *Atelopus zeteki*, which is capable of producing large numbers of zoospores, could put other species at risk by increasing the

mean infection intensity to lethal levels. Instead, our results show that increasing exogenous sources of zoospores does not lead to increased individual fungal loads but indicated that reintroducing *Bd* susceptible species, such as *Atelopus zeteki*, would not lead to an increase in the mean *Bd* load in an ecosystem. However, these results might differ in a field setting, or with other species combinations, and we recommend additional laboratory experiments and conducting limited field trials before *Atelopus* are profusely released into the wild. We need to understand the role of *Atelopus* in a *Bd* outbreak: can the presence of *Atelopus* amplify the amphibian mass mortality during an epizootic compared to a community without *Atelopus*? Similarly, we need to quantify its role in transmission: does *Atelopus* increase the rates of transmission within and among species? In this case, we would be focusing on the rate of becoming infected with *Bd* rather than what influences the growth rate of *Bd* after infection, which was examined in this experiment. Our experimental design did not allow us to address whether species with high shedding rates might affect other aspects of transmission, such as infectivity (i.e., the probability that a susceptible animal becomes infected given a contact with an infectious host). Given that all individuals were inoculated with *Bd* in our experiment, we could not measure how *Bd* load affects how quickly uninfected individuals become infected. Introducing a highly susceptible, supershedding species such as *A. zeteki* could result in more infectious contacts with other uninfected species, which will promote a higher overall prevalence in the community while not affecting the average infection intensity.

Little is known about the susceptibility of *Atelopus glyphus*; therefore, our experiment provides additional reasoning for concern that the entire genus *Atelopus* are supershedders (DiRenzo et al. 2014). Our experiment also adds evidence that several species of *Atelopus* are (1) highly susceptible to *Bd* (Lips et al. 2008) and (2) that the genus *Atelopus* produces a disproportionate number of zoospores compared to other species (DiRenzo et al. 2014). In the wild, the geographic distribution of *Atelopus glyphus* ranges from the eastern side of Panama into Northern Colombia. However, few amphibian surveys have been conducted in this region because many areas are difficult to access and security concerns, providing scant information about the status of these populations in the wild.

Although our experiment did not exactly represent how transmission occurs in these two species in the wild, we maximized the chance of *Bd* survival, growth, and

transmission by housing pairs in a small space under cool moist conditions conducive to *Bd* growth. These species co-occur in the same streams in the wild: *Atelopus glyphus* are diurnal and terrestrial and sleep on low vegetation at night, while *Espadarana prosoblepon* are nocturnal and arboreal and are found on higher perches than those used by *Atelopus*. We observed these behaviors in the experiment, where *Atelopus* would stay at the bottom of the tank and *Espadarana* would either be on the side or the lid of the container. We did not observe *Espadarana* sleeping on the floor of container or *Atelopus* climbing on the walls during the day. However, it is possible that *Espadarana* came into contact with the floor of the tank at night or *Atelopus* slept on the walls of the container. Given the life history traits of these two species, it is unlikely that adult stages of both species would interact as closely in the wild as the conditions simulated in our experiment, and yet even under these ideal conditions for *Bd* transmission, we were not able to induce an increased *Bd* growth rate in either *Espadarana* or in conspecific *Atelopus*.

Whether infection load is primarily determined by within host–pathogen reproduction or external reinfection has important implications for the disease dynamics of infected communities. Infections maintained primarily through reinfection from outside the individual host can lead to a stable endemic disease state, as long as there is a constant reservoir of zoospores (i.e., tadpoles) that provide a source for reinfection (Briggs et al. 2010). We found that once an individual *A. glyphus* is infected, the infection grew exponentially, regardless of external sources for reinfection, suggesting that— unless there is some mechanism limiting within host zoospore growth, such as an immune response— stable endemic disease states are unlikely. Our results also indicate that the primary driver of *Bd* growth may be within host reproduction, similar to a classic microparasite and that reinfection from outside the host does not significantly contribute to *Bd* growth rate within the host, even when ideal conditions for outside reinfection are created. As *Bd* continues to spread to naive populations, understanding what drives *Bd* growth will be essential to modeling new epizootics and managing populations afflicted by *Bd*.

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## AUTHORS' CONTRIBUTION

GVD, KRL, TST conceived of and designed the study. TST and MSdV conducted the experiment. GVD executed data analysis, modeling, and co-wrote the first draft of the manuscript. TST conducted the experiments in RI's laboratory, executed data analysis, modeling, and co-wrote the first draft of the manuscript. KRL was involved with experimental design, manuscript preparation and collected the original dataset. AVL performed the laboratory work in the KRZ laboratory. All authors edited the manuscript. The work was funded by KRL and KRZ.

## COMPLIANCE WITH ETHICAL STANDARDS

**CONFLICT OF INTEREST** The authors declare that they have no conflict of interest.

**DATA AVAILABILITY** The datasets and R code used in the current study are available through GitHub: <https://github.com/tatet2/AtelopusBdTransmission>.

**STATEMENT OF ANIMAL ETHICS** All applicable institutional and/or national guidelines for the care and use of animals were followed.

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