

Research



Cite this article: Becker CG *et al.* 2019

Low-load pathogen spillover predicts shifts in skin microbiome and survival of a terrestrial-breeding amphibian. *Proc. R. Soc. B* **286**: 20191114.

<http://dx.doi.org/10.1098/rspb.2019.1114>

Received: 14 May 2019

Accepted: 23 July 2019

Subject Category:

Ecology

Subject Areas:

ecology, health and disease and epidemiology

Keywords:

Batrachochytrium dendrobatidis, chytridiomycosis, community composition, host microbiome, direct development

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Electronic supplementary material is available online at <https://dx.doi.org/10.6084/m9.figshare.c.4593827>.

Low-load pathogen spillover predicts shifts in skin microbiome and survival of a terrestrial-breeding amphibian

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Wildlife disease dynamics are strongly influenced by the structure of host communities and their symbiotic microbiota. Conspicuous amphibian declines associated with the waterborne fungal pathogen *Batrachochytrium dendrobatidis* (Bd) have been observed in aquatic-breeding frogs globally. However, less attention has been given to cryptic terrestrial-breeding amphibians that have also been declining in tropical regions. By experimentally manipulating multiple tropical amphibian assemblages harbouring natural microbial communities, we tested whether Bd spillover from naturally infected aquatic-breeding frogs could lead to Bd amplification and mortality in our focal terrestrial-breeding host: the pumpkin toadlet *Brachycephalus pitanga*. We also tested whether the strength of spillover could vary depending on skin bacterial transmission within host assemblages. Terrestrial-breeding toadlets acquired lethal spillover infections from neighbouring aquatic hosts and experienced dramatic but generally non-protective shifts in skin bacterial composition primarily attributable to their Bd infections. By contrast, aquatic-breeding amphibians maintained mild Bd infections and higher survival, with shifts in bacterial microbiomes that were unrelated to Bd infections. Our results indicate that Bd spillover from even mildly infected aquatic-breeding hosts may lead to dysbiosis and mortality in terrestrial-breeding species, underscoring the need to further investigate recent population declines of terrestrial-breeding amphibians in the tropics.

1. Introduction

Most emerging pathogens of wildlife, livestock, plants and humans infect multiple host species [1–3]. In multi-host pathogen systems, patterns of pathogen transmission are influenced not only by levels of pathogen resistance and tolerance within individual host species but also by community-wide interactions among host species [4]. Thus, particular community assemblages of hosts may experience different patterns of pathogen transmission, depending on proportions of tolerant and resistant species as well as aspects of host life history that may

influence infection risk and interspecies pathogen spillover, including reproductive behaviour and habitat use [5–8].

On a smaller scale of community assembly, animals also host complex communities of microorganisms (the microbiome) that may interact with invading pathogens [9]. Host microbiome structure is shaped by both host and environmental factors such that co-occurring host species may carry distinctive bacterial communities influenced by the micro-environment of host surfaces, microhabitat use, previous pathogen exposure and horizontal bacterial transmission [10–13]. Host-associated bacterial communities may influence host health by inhibiting or outcompeting pathogens, and at the same time, pathogenic infections may lead to imbalances in the microbiome [14,15]. Thus, disentangling multi-host pathogen dynamics requires understanding not only patterns of pathogen spillover, but also how the distinctive microbiomes of host species interact with invading pathogens and whether transmission of symbiotic microbes among host species may influence disease outcomes.

In response to several high-visibility population declines and extinctions of aquatic-breeding amphibians globally, epidemiological studies of the waterborne chytrid fungus *Batrachochytrium dendrobatidis* (Bd) have primarily focused on amphibian species undergoing aquatic larval development (hereafter aquatic-breeding). However, recent studies in South America, Africa and the Caribbean reveal that terrestrial-breeding frogs (a diverse group of tropical frogs that breed terrestrially through direct larval development) occasionally carry extremely high Bd infection intensities [16]. It is now clear that studies of Bd exposure and transmission must encompass effects of cross-species infection spillover on population stability in these previously overlooked terrestrial-breeding species [17–19] and that aquatic and terrestrial-breeding species must be jointly considered in community-based studies of Bd.

We focused on frog assemblages from Brazil's Atlantic forest, a biodiversity hotspot highly impacted by Bd [17], and took into account that the cutaneous microbiomes of amphibians can play a role in their defenses against the disease chytridiomycosis [20–22]. We selected four aquatic-breeding amphibian species with varying life histories (stream and pond breeders; tree frogs and frogs), and one focal terrestrial-breeding species: the pumpkin toadlet *Brachycephalus pitanga*. Terrestrial-breeding amphibians tend to be less exposed to waterborne Bd throughout their ontogeny [23] and thus show lower acquired immunity and resistance to the pathogen than aquatic-breeding species [24]. To maximize ecological realism, we conducted an experiment using wild-collected hosts harbouring natural symbiotic microbiomes and Bd infection loads. We housed amphibians in the laboratory under single-species (four conspecific individuals) and multi-species treatments (four heterospecific individuals), and quantified changes in their Bd infection loads, skin bacterial communities and mortality rates during the experiment. By experimentally manipulating multiple tropical amphibian assemblages harbouring natural microbial communities, we tested (1) whether Bd spillover from naturally infected aquatic-breeding frogs could lead to disease and mortality in our focal terrestrial-breeding species and (2) whether the strength of spillover could vary depending on skin bacterial transmission among different assemblages of hosts. Our results reveal detailed mechanisms of pathogen spillover and shifts in microbial communities with important implications for the field of disease ecology and the conservation of tropical amphibians.

2. Material and methods

(a) Host species

We captured adult amphibians of five locally abundant species in January 2016 from Parque Estadual da Serra do Mar–Núcleo Santa Virginia and contiguous forested areas outside the park boundaries in São Luiz do Paraitinga, in Brazil's Atlantic forest (−23.32° S, −45.15° W). We captured three pond-breeding amphibian species with aquatic larval development (*Aplastodiscus leucopygius* (LEU), *Dendropsophus microps* (MIC) and *Scinax hayii* (HAY); Hylidae), one stream-breeding species (*Hylodes phyllodes* (PHY); Hylodidae), and one exclusively terrestrial, direct-developing species that occupies the forest leaf litter (*Brachycephalus pitanga* (PIT); Brachycephalidae). These five species represent an ecologically relevant subset of the original community in terms of life histories, habitat associations and relative abundances. All five species are found foraging in relatively high abundance within the same leaf-litter and understory environments, both near and away from water bodies [25]. We photographed unique colour patterns to identify each individual. Animals were individually housed and brought to the laboratory 1 day prior to the onset of the laboratory experiment.

(b) Experimental design

We randomly assigned host individuals to treatments and experimental units. Each experimental unit consisted of a rectangular terrarium (42 × 34 × 13.5 cm) with terrestrial habitat at one end of the container (autoclaved moist *Sphagnum* sp. moss) and aquatic habitat at the other end (300 ml of distilled water). To control for host density, each experimental unit within both treatments (single-species or multi-species) contained four amphibians. We replicated single-species treatments four times for each of the five host species, totalling 20 experimental units. For the multi-species treatment, we assigned four unreplicated host species (species richness = 4) to each experimental unit. We replicated the five possible combinations of four host species three times, totalling 15 multi-species experimental units, which led to a balanced experimental design with every host species having the same representation in both single-species ($n = 12$ individuals per species) and multi-species treatments ($n = 16$ individuals per species).

(c) Experimental procedures

We collected skin swabs from frogs at the time of capture in the field (day 0) and when we noted a significant increase in mortality on day 24 of the experiment [26]. To avoid confounding effects of mortality-associated shifts in host density on bacterial exchange and Bd spillover among individuals, we housed amphibians individually in similar containers from day 24 to 42. To remove unwanted environmental bacteria from the host skin prior to swabbing, we rinsed each individual with distilled water. We monitored amphibians daily and fed them pinhead crickets (*Gryllus* cf. *assimilis*) ad libitum. Given the short period of this experiment and the small biomass of our experimental animals, we did not perform terrarium cleaning during the experiment. Laboratory temperatures were $18.96^{\circ}\text{C} \pm 0.51$ s.d. and we used a 12 L:12 D cycle.

(d) Sequence and statistical analyses

Detailed descriptions of DNA extraction, qPCRs, sequencing and processing are available as electronic supplementary material. For statistical analysis, we first investigated host Bd infections and survival among treatments. To test whether Bd infection loads (logged zoospore genomic equivalents (g.e.)) varied between single-species and multi-species treatments and among species, we used a generalized linear mixed model (GLMM). We included the following explanatory categorical

variables in the model as fixed effects: number of host species (single species versus multi-species), host species (PIT, PHY, LEU, HAY and MIC) and the interaction between these two variables. Additionally, we included the experimental unit as a random effect and both Bd infection loads and number of bacterial operational taxonomic units (OTUs) from field swabs (day 0) as continuous fixed effect controls.

We compared survival curves between single- and multi-species treatments for each host species using independent Wilcoxon statistics. We performed a parametric survival analysis testing for effects of Bd load (field swabs) of aquatic-breeding host species on the survival of terrestrial-breeding pumpkin toadlets in multi-species treatments. We employed a GLM with binomial distribution and *logit* link to test for differences in Bd prevalence (proportion of individuals with Bd load > 1 g.e.) between pumpkin toadlets and aquatic-breeding species at the time of capture in the wild.

Second, we investigated the effects of host community structure on skin bacterial communities. We calculated bacterial community dissimilarity (Bray–Curtis) between single-species and multi-species treatments independently for each host species. We compared Bray–Curtis distance values among species using a non-parametric FDR test, corrected for multiple comparisons. We also calculated weighted UniFrac distances among samples and quantified variation in bacterial community structure among host species from the field samples and after applying experimental treatments by performing permutational multivariate analyses of variance (PERMANOVAs) with host species as the main fixed factor [27]. We also performed pairwise PERMANOVAs to further explore significant main effects. Pairwise distance matrices between species at field sampling, within single-species treatments and multi-species treatments, were calculated in QIIME v. 1.9.1 with `make_distance_comparison_plots.py`.

To characterize structural differences in host microbiomes from single-species and multi-species treatments, we used analyses derived from network theory. We plotted the following four unipartite networks as described by Araújo *et al.* [28]: day 0 (field)/multi-species, day 0/single-species, day 24 (experiment)/multi-species and day 24/single-species. We compared the global efficiency of unipartite networks following Costa *et al.* [29]. Global efficiency describes how distant experiment/field individuals are in terms of the main components of species composition (see electronic supplementary material). We also used bipartite networks to visualize differentially abundant bacterial taxa (genus level) among host species in multi-species treatments (see electronic supplementary material).

To quantify bacterial richness and diversity, we calculated OTU richness, Chao1, Shannon diversity and Faith's phylogenetic diversity. We used the same GLMM approach described above to test whether OTU richness varied between single-species and multi-species treatments and among host species. We repeated this analysis, in turn, with Chao1, Shannon and Faith's phylogenetic diversity as response variables.

Third, we investigated interactions between host community structure, host bacterial communities and disease outcomes (Bd infection loads and survival). We used a parametric survival model to test for effects of the initial number of OTUs (day 0) on the survival of pumpkin toadlets from multi-species treatments. We also ran parametric survival models testing for associations between toadlet survival and relative abundance of each of the five bacterial genera that were differentially abundant in pumpkin toadlets from multi-species treatments. These analyses were performed independently for each bacterial genus due to high cross-correlation among OTU relative abundance, which would lead to multi-collinearity if all were included in a single model.

To further explore the relationship between host skin microbiomes and Bd independently for each species, we performed distance-based linear modelling (DISTLM) in Primer7, which

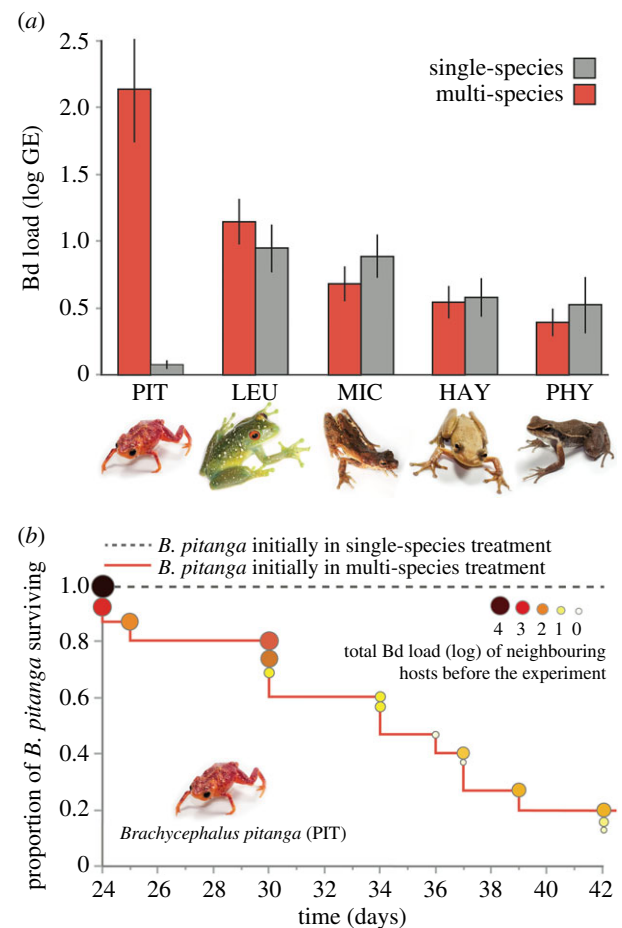


Figure 1. (a) Average Bd infection load across multi-species and single-species treatments (least-square mean \pm s.e.). Host species abbreviations: *Brachycephalus pitanga*, PIT; *Aplastodiscus leucopygius*, LEU; *Dendropsophus microps*, MIC; *Scinax hayii*, HAY; *Hylodes phyllodes*, PHY. (b) Proportion of *B. pitanga* surviving in both single-species and multi-species treatments after day 24. Coloured circles represent total infection loads of aquatic-breeding frogs in multi-species treatments at the time of capture in the wild. (Online version in colour.)

determines the proportion of variation in microbial community structure explained by individual Bd loads. In addition, we applied a piecewise path analysis to test for the relative strength of direct and indirect effects of aquatic-breeding frog species on Bd infection loads (day 24), OTU richness (day 24) and survival of pumpkin toadlets. In the model, we allowed the presence of aquatic-breeding frogs to influence Bd loads, bacterial diversity and survival of toadlets.

3. Results

(a) Effects of host community structure on Bd loads and host survival

We found that terrestrial-breeding pumpkin toadlets (PIT) carried higher average Bd infection loads in multi-species compared to single-species treatments (figure 1a; electronic supplementary material, table S1). The four aquatic-breeding species showed similar Bd loads in single-species and multi-species treatments (figure 1a). All toadlets from the single-species treatment survived to the end of the experiment, while toadlets in multi-species treatments showed significantly lower survival (Wilcoxon $\chi^2 = 20.032$, d.f. = 2, $p < 0.0001$; figure 1b). Survival of toadlets in multi-species

treatments was explained by Bd infection loads of neighbouring aquatic-breeding host species. Specifically, toadlets experienced lower survival rates in multi-species assemblages that included highly infected aquatic-breeding frogs (parametric survival test: $\chi^2 = 7.047$, d.f. = 1, $p = 0.008$; figure 1b). In contrast to pumpkin toadlets, aquatic-breeding species showed similarly high survival rates in both single- and multi-species treatments (LEU: Wilcoxon $\chi^2 = 3.200$, d.f. = 2, $p = 0.2019$; PHY: Wilcoxon $\chi^2 = 4.121$, d.f. = 2, $p = 0.1274$; MIC and HAY showed 100% survival in both treatments). Pumpkin toadlets showed lower Bd prevalence (PIT = 7.14%) than aquatic-breeding species (LEU = 89.28%, PHY = 57.14%, MIC = 50%, HAY = 46.42%) at the time of capture in the wild ($\chi^2 = 29.589$, d.f. = 1, $p < 0.0001$).

(b) Effects of host community structure on skin bacterial communities

We detected differences in bacterial community composition among host species at capture in the field (day 0: PERMANOVA pseudo- $F = 5.752$, $p = 0.001$) and in single-species treatments (day 24: PERMANOVA pseudo- $F = 4.964$, $p = 0.001$). Bacterial community composition after frogs were housed in multi-species assemblages, however, did not differ significantly among host species (PERMANOVA pseudo- $F = 1.032$, $p = 0.401$). Detailed pairwise distances among host species obtained from samples prior to and during the experiment (day 24) are available in electronic supplementary material table S2 and figure S1. Our unipartite network representation of host-microbiome composition at the time of capture in the field also showed a clear clustering by host species (electronic supplementary material, figure S2), reinforcing our PERMANOVA results. The proportion of microbiota similarity between two individual frogs (i.e. higher network connectance) increased during the experiment for single-host and multi-host treatments. However, the overall microbiota similarity was higher (i.e. the network was denser) and the patterns of microbiota similarity across individuals were more intricate in the multi-host treatment, indicating microbial homogenization (electronic supplementary material, figure S2). The global network efficiency analyses corroborated this pattern, as the mean network efficiency from field samples to day 24 samples did not change significantly in the single-species treatment (field mean = 0.636; day 24 mean = 0.585; $p > 0.05$). Conversely, we detected a significant increase in mean network efficiency for multi-species treatments (field mean = 0.552; day 24 mean = 0.811, $p < 0.05$), quantitatively describing the increase in the overall level of microbiota similarity across individuals. Our FDR analysis indicated that terrestrial-breeding pumpkin toadlets (PIT) showed the highest shifts in bacterial composition (Bray–Curtis dissimilarity), followed by LEU and MIC, from single-species to multi-species treatments (figure 2). We also found higher average skin bacterial diversity in multi-species than in single-species treatments for one species (*Aplastodiscus leucopygius* (LEU)), while the other four amphibian species maintained similar bacterial diversity (e.g. richness, Chao1, Shannon and Faith's phylogenetic diversity of OTUs; electronic supplementary material, table S3).

Our bipartite networks indicated that pumpkin toadlets experienced the largest shifts in bacterial relative abundance while in contact with other host species (figure 3). Toadlets showed increases in relative abundance of *Staphylococcus*, *Desulfovibrio*, an unclassified genus of *Proteobacteria*, *Bacteroides*

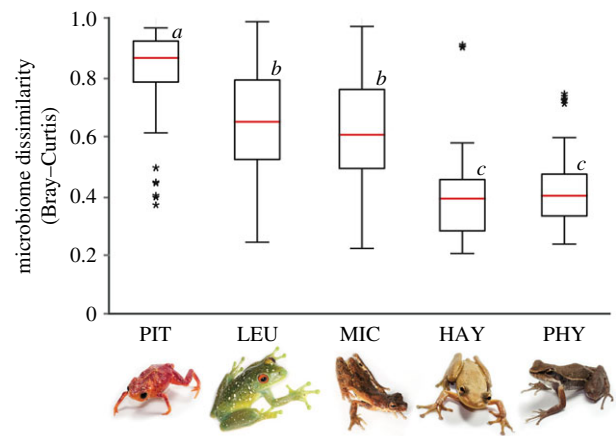


Figure 2. Median skin bacterial community dissimilarity between single-species and multi-species treatments calculated independently for each host species. Different letters indicate significant differences between groups based on non-parametric FDR test. Host species abbreviations: *Brachycephalus pitanga*, PIT; *Aplastodiscus leucopygius*, LEU; *Dendropsophus microps*, MIC; *Scinax hayii*, HAY; *Hylodes phyllodes*, PHY. (Online version in colour.)

and *Exiguobacterium* in multi-species treatments, while an unclassified genus of *Bacteroidetes* and *Elizabethkingia* became less abundant. The bacterial taxa of toadlets showing increased relative abundance were highly abundant in multiple amphibian species in single host treatments, except for *Staphylococcus*, which was only differentially abundant in *Dendropsophus microps* (MIC).

(c) Interactions between Bd, host skin bacteria, and host survival

We found that toadlet survival in multi-species treatments was not explained by their initial number of bacterial OTUs (parametric survival test: $\chi^2 = 0.001$, d.f. = 1, $\beta = -0.234$, $p = 0.974$). However, one of the five differentially abundant bacteria in pumpkin toadlets from multi-species treatments (figure 3) was correlated with toadlet survival times. Specifically, our parametric survival analyses found significantly positive associations between the relative abundance of the unclassified genus of *Proteobacteria* ($\chi^2 = 8.860$, $\beta = 39.692$, d.f. = 1, $p = 0.003$) and toadlet survival. Three other bacteria showed similar positive trends: *Bacteroides* ($\chi^2 = 3.078$, $\beta = 26.937$, d.f. = 1, $p = 0.079$), *Exiguobacterium* ($\chi^2 = 2.541$, $\beta = 6.037$, d.f. = 1, $p = 0.111$), and *Desulfovibrio* ($\chi^2 = 1.965$, $\beta = 8.416$, d.f. = 1, $p = 0.161$). *Staphylococcus* relative abundance showed a negative non-significant trend with toadlet survival ($\chi^2 = 1.033$, $\beta = -0.346$, d.f. = 1, $p = 0.309$).

Our distance-based linear modelling (DISTLM) indicated that Bd infection loads and bacterial community structure were not correlated in aquatic-breeding frog species, but Bd loads were tightly linked to bacterial community structure in the pumpkin toadlets (table 1), reinforcing results from our bipartite network analysis (figure 3). Our path analysis detected a negative effect of aquatic-breeding species on toadlet survival through Bd spillover (figure 4). In this path model, the presence of aquatic-breeding species had a strong positive effect and a weak, indirect negative effect on OTU richness in toadlets, but shift in microbial diversity was not a significant predictor of toadlet survival (figure 4).

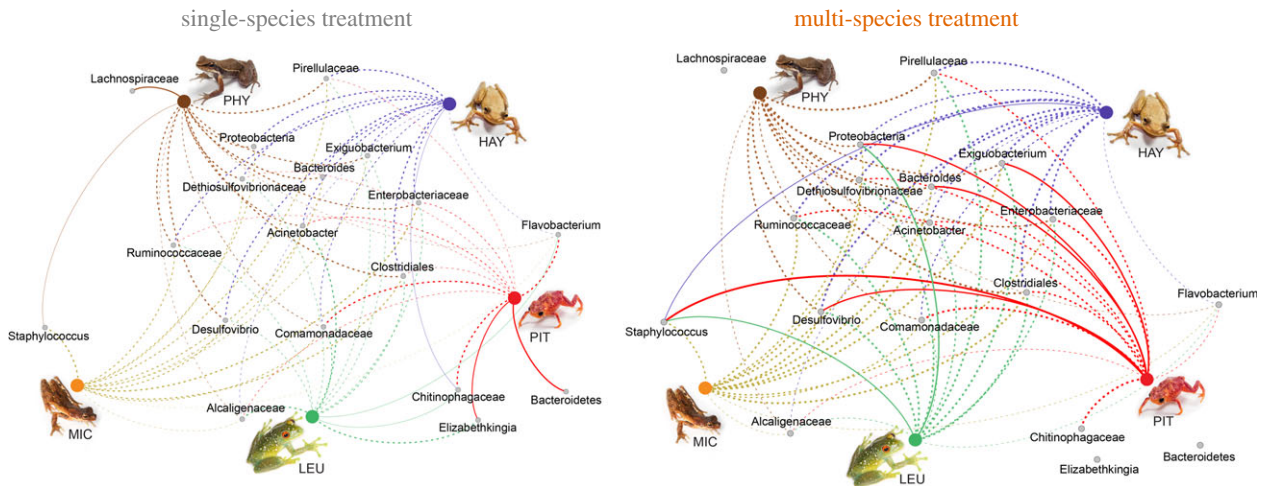


Figure 3. Bipartite networks for common bacterial taxa in single-species and multi-species treatments (day 24). Dashed lines represent constant host–bacterial associations in single-species and multi-species treatments. Solid lines represent host–bacterial associations showing higher differential abundance in single-species or multi-species treatments. Line thickness represents OTU relative abundance. Host species abbreviations: *Brachycephalus pitanga*, PIT; *Aplastodiscus leucopygius*, LEU; *Dendropsophus microps*, MIC; *Scinax hayii*, HAY; *Hylodes phyllodes*, PHY. When a Phylum or Family level name is indicated this denotes an unclassified genus within this group. (Online version in colour.)

Table 1. Distance-based linear modelling (DISTLM) determining the proportion of variation in microbial community structure explained by individual Bd infection loads. Bold type indicates statistical significance ($p < 0.05$).

species	% of variation explained	pseudo F	p -value
<i>Brachycephalus pitanga</i> (PIT)	0.331	4.442	0.012^a
<i>Dendropsophus microps</i> (MIC)	0.040	0.719	0.651
<i>Aplastodiscus leucopygius</i> (LEU)	0.030	0.589	0.638
<i>Scinax hayii</i> (HAY)	0.035	0.859	0.374
<i>Hylodes phyllodes</i> (PHY)	0.034	0.627	0.675

^aMantel test for PIT: $\rho = 0.655$; $p = 0.017$.

4. Discussion

Wildlife disease dynamics are strongly influenced by patterns of host and microbial community assembly [7,30,31]. In our study, the bacterial skin microbiomes of terrestrial-breeding pumpkin toadlets shifted in multi-species assemblages as a result of Bd spillover infections, leading to significant mortality. However, we found little support for (i) host species diversity and composition influencing disease in aquatic-breeding species and (ii) initial bacterial diversity and composition shaping anti-pathogen responses. Notably, all host species harboured naturally low Bd infection loads at the beginning of the experiment and pumpkin toadlets also exhibited remarkably low infection prevalence. In addition, we did not supplement initial infections with experimental inoculations that could build infections to unrealistic levels. Thus, our study suggests that pathogen spillover even from mildly infected reservoir hosts may be sufficient to induce mortality and population instability among terrestrial-breeding amphibian species. These results are consistent with the finding that low-load Bd

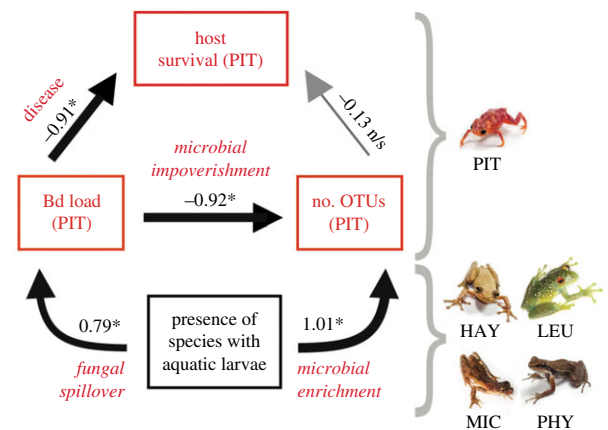


Figure 4. Path analysis showing direct and indirect effects of host species with aquatic larvae (*Scinax hayii*, HAY; *Hylodes phyllodes*, PHY; *Dendropsophus microps*, MIC; *Aplastodiscus leucopygius*, LEU) on survival of *Brachycephalus pitanga* (PIT) through changes in both Bd loads (log g.e.) and number of bacterial OTUs. In this model, Bd loads were also allowed to influence number of bacterial OTUs. Numbers are standardized path coefficients ($*p < 0.05$). The thickness of the arrows represents the relative strength of each relationship. Non-significant path shown in grey. (Online version in colour.)

transmission from Bd-resistant reservoir hosts led to mortality in heterospecifics sharing a body of water [32], but our study is novel in reporting lethal spillover from aquatic-breeding to terrestrial-breeding frogs.

Our data revealed strong links between Bd spillover and host–microbiome composition in pumpkin toadlets. Specifically, pumpkin toadlets were the only species in which Bd infection loads predicted skin bacterial community structure, suggesting that Bd infection altered the skin microbiome [33]. We also found some correlational support for the defensive activity of the toadlet microbiome. For instance, the relative abundance of one of the differentially abundant OTUs was linked to enhanced host survival in pumpkin toadlets, consistent with recent findings that amphibian hosts are able to recruit beneficial bacteria [34]. While these findings indicate that host community composition could have an indirect effect on host survival through shifts in microbiome

composition, we found evidence that survival of toadlets responded more strongly to the infection loads of neighbouring aquatic-breeding species. Results from our path model also supported a scenario in which the presence of aquatic-breeding species influenced toadlet mortality through Bd spillover. Our path model indicated that bacterial transmission from neighbouring species was offset by pathogen-mediated bacterial losses and that overall shifts in bacterial richness did not predict toadlet survival.

Our experimental results are compatible with recent field-based studies linking Bd emergence, pathogen spillover and population declines of direct-developing amphibian species, such as our focal pumpkin toadlet [17,35]. Epizootics and long-term endemicity of Bd on several continents [17,36–38] allowed significant evolution of host tolerance and resistance among amphibian species undergoing aquatic larval development [24]. In Brazil's Atlantic forest, for instance, Bd prevalence in amphibians with aquatic larvae could reach up to approximately 80% in natural forest habitats without causing apparent die-offs in tolerant species [35]. The present study provides additional support that aquatic-breeding frogs could act as Bd reservoirs and sources of spillover [7] for susceptible terrestrial-breeding amphibians [24,39]. Field studies (and our results) support higher Bd prevalence among aquatic-breeding frogs, although terrestrial-breeding species often carry much higher average infection loads in the wild [16,40]. The likelihood of capturing an infected terrestrial-breeding frog in nature is also lower on average, because chytridiomycosis might quickly lead to host mortality in these species [40]. A rapid disease progression in a small fraction of the population thus precludes terrestrial-breeding amphibians from evolving acquired resistance and/or tolerance to Bd [24]. For instance, a previous study carried out at our field site showed that some individuals of terrestrial-breeding *Ischnocnema guentheri* carried very high natural infection loads (greater than 10 000 zoospores) [16]. Furthermore, the cryptic terrestrial-breeding frog *Holoaden bradei*, endemic to a pristine National Park in Brazil, was last seen in the wild right before a period of Bd upsurge in that area [17]. Examples of Bd negatively affecting direct-developing frogs are not exclusive to Neotropical frogs. The direct-developing species in the genus *Arthroleptis* were among the most impacted by the emergence of Bd in Cameroon and a retrospective study detected high average infection loads in species of *Arthroleptis* at the onset of population declines [18]. The notion that droughts increase host exposure to Bd and facilitate density-dependent transmission near environmental reservoirs [41] coupled with the fact that terrestrial-breeding frogs might congregate near water bodies during dry periods (C.F.B.H. & C.G.B. 2016, personal observation) suggest that accelerated climate shifts might bring together host species with varying historical adaptations to waterborne chytrids. These recent findings, combined with our results, highlight that alteration in host species interactions could cause Bd spillover and trigger epizootics among species that are not frequently exposed to Bd in the natural environment [24].

In addition to the imminent threats posed by accelerated habitat loss, narrow geographical range distributions, such as those observed in most direct-developing amphibians [42], are also linked to lower host adaptive immunity to pathogens [43]. A potential mechanism behind this prediction is that animals with narrow ranges tend to be less exposed to selective pressures from multiple pathogens, which may in turn

decrease host adaptive defences. Additionally, species occupying smaller geographical areas are also exposed to a narrower array of symbiotic microbes [44] that might reduce the protective capacity of their cutaneous microbiome. *Brachycephalus* occupies a more homogeneous environment (leaf-litter) than any of our aquatic-breeding species, which could also limit exposure to a larger pool of environmental microbes. Narrow geographical range distribution is also linked to higher local population densities in tropical frogs [45], which could facilitate direct transmission among terrestrial-breeding species in the natural environment. For instance, pumpkin toadlets are only known from Santa Virginia park and the immediate surrounding landscape, yet they occur at very high population densities. Although there is no evidence of ongoing direct transmission in nature for pumpkin toadlets (low Bd prevalence in the wild according to our field sampling), Bd could potentially threaten this species in the event of direct transmission, spillover and/or increased exposure to Bd in the natural environment. Our study also highlights that exposure to low zoospore concentrations of local Bd genotypes is capable of causing disease and quickly driving local terrestrial-breeding frogs to mortality, underscoring the fragility of the few populations of pumpkin toadlets in the wild.

In conclusion, our study provides evidence that host community structure can impact the composition of host-associated microbiota and host–pathogen dynamics. Additionally, we experimentally describe a mechanism of microbial spillover and pathogen amplification with important theoretical inferences for the study of wildlife diseases and critical applications to the global amphibian crisis. Pathogen amplification in diverse host communities is considered rare, because it strongly depends on transmission from tolerant host to susceptible species [5]. In amphibians, however, many susceptible terrestrial-breeding frog species might be experiencing pathogen spillover and silent population declines and extinctions in the wild.

Ethics. Research permits were provided by Instituto Chico Mendes—Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis/Brazil—ICMBIO (29964-9 and 57098-1), Instituto Florestal do Estado de São Paulo (260108-007.600/2015) and the local Animal Care and Use Committee—CEUA/UNESP.

Data accessibility. Data available from the Dryad Digital Repository at <https://doi.org/10.5061/dryad.18dv32q> [46].

Authors' contributions. C.G.B. designed research, carried out fieldwork, set up laboratory experiment, performed molecular laboratory work and statistical analyses, and wrote the paper with important contributions from all co-authors. M.C.B. carried out molecular laboratory work and participated in data analysis. L.F.T. and C.L. carried out fieldwork. A.P.A.A., P.R.G. and M.C.B. performed network analysis. M.V., D.R., R.G., and M.J. carried out molecular laboratory work. All authors gave final approval for publication.

Competing interests. We declare we have no competing interests.

Funding. Our work was funded by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq (400177/2014-5 and 300896/2016-6), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—CAPES (88881.062205/2014-01), Deutsche Forschungsgemeinschaft—DFG (VE247/9-1), Fundação de Amparo a Pesquisa do Estado de São Paulo—FAPESP (2013/50741-7 and 2016/25358-3) and the Department of Biological Sciences at the University of Alabama.

Acknowledgements. We thank R. Martins, M. A. Passos, C. H. Nunes-de-Almeida, T. Carvalho, A. Belassen, I. Haga and F. Andrade for field support.

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