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Research Article

An experimental test of disease resistance function in the skin-associated bacterial communities of three tropical amphibian species

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One sentence summary: Subtle differences in the microbiome predict responses to pathogen exposure in three species of neotropical frogs.
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Abstract

Variation in the structure of host-associated microbial communities has been correlated with the occurrence and severity of disease in diverse host taxa, suggesting a key role of the microbiome in pathogen defense. However, whether these correlations are typically a cause or consequence of pathogen exposure remains an open question, and requires experimental approaches to disentangle. In amphibians, infection by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) alters the skin microbial community in some host species, whereas in other species, the skin microbial community appears to mediate infection dynamics. In this study, we completed experimental *Bd* exposures in three species of tropical frogs (*Agalychnis callidryas, Dendropsophus ebraccatus,* and *Craugastor fitzingeri*) that were sympatric with *Bd* at the time of the study. For all three species, we identified key taxa within the skin bacterial communities that were linked to *Bd* infection dynamics. We also measured higher *Bd* infection intensities in *D. ebraccatus* and *C. fitzingeri* that were associated with higher mortality in *C. fitzingeri*. Our findings indicate that microbially mediated pathogen resistance is a complex trait that can vary within and across host species, and suggest that symbiont communities that have experienced prior selection for defensive microbes may be less likely to be disturbed by pathogen exposure.

Keywords: amphibian, bacteria, *Batrachochytrium dendrobatidis*, microbiota, neotropical, pathogen

Introduction

Among individuals, populations, and species, there can be substantial variation in susceptibility to pathogenic microorganisms (Lloyd-Smith et al. 2005). This has long been recognized in domesticated plants and animals; however, in natural populations, variation in disease susceptibility can be difficult to detect and measure (Williams et al. 2002, Wobeser 2007). For example, field surveys can be used to quantify levels of infection in individuals that are encountered during a survey, but this approach may miss susceptible individuals that succumb to infection before they are sampled. Moreover, individuals and populations that do not show appreciable signs of disease or decline are often not a research priority. However, this oversight may be a missed opportunity. Undetected variation in disease susceptibility within these populations can offer insight into potential mechanisms of resistance or tolerance, and may ultimately inform the development of treatments and conservation efforts (Scheele et al. 2019).

Microbially mediated pathogen resistance is increasingly receiving attention as another form of host defense that can complement the immune system of vertebrate hosts (Myers et al. 2012, Florez et al. 2015, Chiu et al. 2017, Varga et al. 2018, McLaren and Callahan 2020). This resistance occurs *via* several mechanisms. Microbes can prevent colonization of pathogens by physically occupying space on host tissues (Buffie and Pamer 2013), or microbial symbionts may produce antimicrobial metabolites that inhibit pathogen growth (Loudon et al. 2014a, Mattoso et al. 2012, Koehler et al. 2013). These protective mechanisms can be enhanced by biofilm formation (Jones and Versalovic 2009, Berendsen et al. 2018). Microbial symbionts also act indirectly on pathogens by promoting host immune function (Shi and Mu 2017). Early in host development, microbes are involved in training of the immune system to distinguish pathogenic from commensal or beneficial microbial taxa (Hooper and Gordon 2001). In later developmental stages, microbes can stimulate the host to activate

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immune responses in reaction to immediate threats (Evans and Lopez 2004, Pieterse et al. 2014).

Accordingly, host organisms have evolved ways to control the composition of their symbiont communities by vertically transmitting beneficial symbionts (Funkhouser and Bordenstein 2013, Gerardo and Parker 2014), developing specialized environments within organs and tissues that affect microbial persistence (Douglas 2020), and modulating immune responses to hinder proliferation or promote colonization (Franzenburg et al. 2012, Mulcahy and McLoughlin 2016). However, ecological and evolutionary forces influencing the diversity and composition of microbial symbiont communities in hosts are not entirely host-driven (Foster et al. 2017). These communities experience stochasticity through the processes of dispersal, ecological drift, and diversification (Vellend 2010, Costello et al. 2012, Adair and Douglas 2017). In addition, they may be influenced by many environmental factors, resulting in both spatial and temporal variability in symbiont communities (Uhr et al. 2019, Ruuskanen et al. 2021). Ultimately, this contributes to variation in symbiont diversity among hosts—and potentially also function (Arumugam et al. 2011).

The fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*), which infects the skin of amphibians and causes the disease chytridiomycosis, is of global concern because of its continued impact on amphibian diversity (Lips 2016, Scheele et al. 2019). Bacterial symbionts are one line of defense against this pathogen (Harris et al. 2009a, b, Rebollar et al. 2020), and all amphibians studied to date appear to maintain diverse communities of bacterial symbionts on their skin (Kueneman et al. 2019). Individual frogs typically harbor multiple bacteria capable of inhibiting *Bd in vitro* (Becker et al. 2015b, Rebollar et al. 2019), indicating that redundancy for protective function likely exists in these systems. However, skin bacteria vary in their inhibitory ability (Bell et al. 2013, Woodhams et al. 2015), and the composition and structure of the bacterial symbiont community differ among individuals, populations, species, and over time (McKenzie et al. 2012, Kueneman et al. 2014, Belden et al. 2015, Rebollar et al. 2016, Hughey et al. 2017, 2019, Estrada et al. 2019). Thus, which taxa are present or sufficiently abundant may influence the effectiveness of the skin symbiont assemblage to confer protection against *Bd* (Bell et al. 2005).

We conducted experimental infections on three tropical amphibian species (*Agalychnis callidryas*, *Dendropsophus ebraccatus*, and *Craugastor fitzingeri*) to assess the impact of *Bd* exposure directly on the frogs (in terms of mass, survival, and bacterial community structure and diversity) and to determine if skin bacterial community diversity or structure influenced an individual's susceptibility to infection. *Agalychnis callidryas* and *D. ebraccatus* are treefrogs in the family Hylidae and have similar natural histories. Both species are primarily arboreal, but come down from the canopy to breed at ponds. Eggs are laid on leaves by both species, but also in the water by *D. ebraccatus* (Touchon and Warkentin 2008); upon hatching, tadpoles of these two species develop aquatically. *Craugastor fitzingeri* (family Craugastoridae) is a leaf litter inhabitant found in the forest or along the edges of streams. This species undergoes direct development: eggs are laid terrestrially, forego the tadpole stage, and hatch as froglets. All three species are considered to be relatively resistant to *Bd* infection, maintaining robust populations in areas that have experienced epizootic outbreaks of *Bd* (Puschendorf et al. 2006, Crawford et al. 2010, Rebollar et al. 2014, Belden et al. 2015). Each frog species harbors a distinct skin bacterial symbiont community, but there is substantial overlap in community composition, and all three amphibian species' communities are particularly enriched in Actinobacteria from the family Cellulomonadaceae and Proteobacteria from the family Pseudomonadaceae (Belden et al. 2015, Estrada et al. 2019, Rebollar et al. 2019). Bacteria that can inhibit the growth of *Bd in vitro* have been isolated from all three species (Rebollar et al. 2019). Given this knowledge, we predicted that (a) none of these species would develop high infection intensities when exposed to *Bd*, and (b) their mass and survival would not be negatively impacted by exposure to *Bd*. Furthermore, given that these species have evolved with this pathogen for some time (since ~2007 at site of collection; Woodhams et al. 2008), and their microbial communities could thus be selected for resistance (Rebollar et al. 2019), we predicted that (c) their skin bacterial communities would remain similar prior to and after exposure to *Bd* and (d) natural variation in the skin bacterial community among individuals of a given species would not affect their response to *Bd* exposure.

Methods

Experiments

Separate experiments were conducted for each species, but the experimental design was the same and consisted of two treatments: (1) exposed to *Bd* and (2) unexposed controls. All individuals of *A. callidryas* ($N = 24$) and *D. ebraccatus* ($N = 24$) were collected from a single pond near Gamboa, Panama, on 14 June 2013. *Craugastor fitzingeri* ($N = 22$) were collected along a drainage ditch in Gamboa on 17 July 2013. Upon returning to the laboratory, frogs were weighed, measured, and randomly assigned to treatment containers. Treatment containers were 415 ml plastic containers with 50 ml of a *Bd* solution (water plus *Bd* zoospores at a concentration of 10 000 zoospores per ml) for the exposed treatment or 50 ml of water only for controls. We used sterilized purified water (http://www.aquacristalina.com) for all aspects of the experiment. Zoospores were obtained from cultures of *Bd* strain JEL 423 (originally isolated from a treefrog, *Agalychnis lemur*, in El Cope, Panama), following standard protocol (Longcore et al. 2019). Frogs were housed at 19–20°C with a 12:12 h light cycle. Frogs remained in treatment containers for 24 h and were checked hourly to make sure they were continuously bathed in solution. After 24 h, frogs were transferred to plastic enclosures with lids (31 L × 21 W × 22 H cm) containing two paper towels on the bottom and a small blue plastic cup for cover. Treatment containers, experimental enclosures, paper towels, and plastic cups were autoclaved prior to the start of the experiment.

The experiment lasted 41 days for each frog species. Frogs were checked daily to assess their condition. Each week, enclosures were cleaned and moistened with 100 ml sterile water; after the first week, the amount of water added to each enclosure was reduced to 50 ml. Each individual also received five crickets each week. Individuals were swabbed at several time points to assess skin bacterial community diversity using sterile rayon swabs (MW113; Medical Wire Equipment & Co. Ltd., Corsham, United Kingdom) as in Walke et al. (2014). The first swab was taken on Day 0, upon collection in the field. Additional swabs were taken on Days 6, 13, 27, and 41 of the experiment. Our standardized swabbing procedure consisted of rinsing each frog with 50 ml of sterile water and then swabbing its ventral surface 20 times, each thigh five times, and each hind foot five times for a total of 40 strokes/individual. We placed swabs in sterile, empty 1.5-ml microcentrifuge tubes on ice and transferred them to a −80°C freezer until further processing. Swabbing for skin metabolite profiles was also completed as a second swab as in Belden et al. (2015) at all swabbing time points, but due to an instrument problem during sample processing, those data are not included.

At the end of the experiment, all animals were again weighed and then euthanized *via* dermal application of lidocaine. Samples of spleen, liver, and skin tissues were collected for use in other studies (Ellison et al. 2014). Carcasses were fixed in formalin, preserved in 95% ethanol, and deposited in the Collection of Herpetology at the Smithsonian Tropical Research Institute under the following accession numbers: CH-9759–CH-9782 (*A. callidryas*), CH-9783–CH-9803 (*D. ebraccatus*), and CH-9723–CH-9736 (*C. fitzingeri*).

Sample processing

Whole genomic DNA was extracted from swabs as in Rebollar et al. (2014) using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). The final elution volume was 100 μl.

We determined *Bd* infection status and intensity as in Rebollar et al. (2014) for all swabbing time points (Days 0, 6, 13, 27, and 41). This approach used the Taqman quantitative PCR assay developed by Boyle et al. (2004). We tested for PCR inhibition and false-negatives by applying internal positive controls (Applied Biosystems Exogenous Internal Positive Controls, Life Technologies, Carlsbad, CA) to a subset of randomized samples; no PCR inhibition was detected. We used *Bd* strain JEL 423, the same strain used in the experiment, to prepare serial dilutions of 0.1–10 000 zoospore genome equivalents (hereafter, zoospore equivalents) for our DNA standards. We considered values ≥ 0.045 zoospore equivalents positive for *Bd*, based on the ITS copy number of the JEL 423 strain. Initially, we ran three qPCR reactions for each sample. If one or more of the initial triplicate samples yielded a value between 0.045 and 1 zoospore equivalents, the qPCR was repeated in triplicate for a total of six reactions. We estimated infection intensity in terms of zoospore equivalents by taking the average of all reactions for each sample.

We assessed bacterial community diversity as described in Belden et al. (2015) using the primers 515F and barcoded 806R targeting the V4 region of the 16S rRNA gene (Caporaso et al. 2012). PCR amplicons were quantified using Quantifluor (Promega, Madison, WI). Composite samples for sequencing were created by combining equimolar ratios of amplicons from the individual samples, followed by cleaning with the QIAquick PCR clean up kit (Qiagen). Barcoded composite PCR products were sequenced on an Illumina MiSeq at the Dana Farber Cancer Institute of Harvard University using a 300 bp paired-end strategy; however, only forward reads were used for analyses because the quality of reverse reads was poor. We processed and sequenced Day 0 swabs to characterize bacterial community diversity in nature, and swabs from Day 13—by which time *Bd* infection prevalence in the exposed treatment had reached its highest levels in all three species—to assess effects of treatment and captivity.

Raw forward sequences from each of the three runs were imported into Qiime2 (Bolyen et al. 2019) and demultiplexed. Resulting reads were then filtered and denoized with DADA2 (Callahan et al. 2016) which also removed chimeric reads. In DADA2, we used the recommended 'big data' workflow, and truncated reads with a phred quality score < 11. We trimmed the reads to 250 bp and used 10 000 reads to build the DADA2 error model, which we have found adequate for our high-quality sequence data. The three resulting ASV tables were then merged into a single table for continued processing, as were the three resulting representative sequence files. To remove rare taxa, we filtered the ASV table to remove any ASVs that represented less than 0.01% of the total reads. This reduced the number of ASVs from 12 707 to 289. We then assigned taxonomy to the remaining 289 ASVs using a scikit-learn naive Bayes classifier (Pedregosa et al. 2011) with the SILVA132 database (Quast et al. 2012). Based on that taxonomy, we removed two ASVs that were assigned as chloroplast sequences. All the remaining 287 ASVs were assigned as bacteria. We then rarefied the entire dataset to 20 000 reads, which resulted in the loss of two samples. The final dataset contained 130 samples and 287 ASVs (46 from *A. callidryas*, 46 from *D. ebraccatus*, and 38 from *C. fitzingeri*). These sample sizes partially reflect a loss of individuals that escaped or died during the course of the experiment (N = 0 *A. callidryas*, 3 *D. ebraccatus*, and 8 *C. fitzingeri*; Table S1, Supporting Information). A phylogeny was produced for the final set of representative sequences to be used in phylogenetics-based analyses. We first aligned the sequences and masked the bases that were not useful for tree construction. Then we produced a maximum likelihood tree with RAxML (100 bootstrapped replicates) using the GTRCAT substitution model and added a midpoint root. Raw 16S rRNA amplicon sequences were deposited in the NCBI Sequence Read Archive under accession number PRJNA504463 and the mapping file in Dryad (DOI: 10.5061/dryad.8sf7m0cq6).

Statistical analysis

Statistical analyses were conducted in R v 3.3.0 using the base (Team 2016) and vegan (Oksanen et al. 2019) packages unless specified otherwise. We conducted separate analyses for each host species. In all three species, 1/3–1/2 of individuals across both control and exposed treatments came into the study infected with *Bd*. However, in all of these cases, *Bd* infection levels were extremely low (≤ 2 zoospore equivalents). When possible (i.e. unless too few individuals were infected for statistical comparison), we tested for correlations between initial infection levels and infection levels at later time points in both the control and exposed groups (each group tested separately). We found no significant relationships (Pearson correlations, all $P > .05$), which suggests that low levels of initial infection probably had little impact on the outcome of experimental treatment. Therefore, in all analyses, we focused on effects of *Bd* exposure treatment regardless of initial infection status.

To assess host outcomes following *Bd* exposure, we tested for changes in *Bd* infection prevalence and intensity between treatments and over time using linear mixed models (package lme4) (Bates et al. 2015), including 'Individual' as a random effect in models to account for repeat sampling of individuals over time. Differences in prevalence (proportion infected/total) were modeled using an underlying binomial distribution and logit function, and differences in infection intensity (measured as counts of zoospore equivalents) were modeled using an underlying Poisson distribution and log function. We tested for differences in mass and survival based on treatment. For mass, we calculated the proportional change in mass from Days 0 to 41 for each individual (= (mass Day 4−mass Day 0)/mass Day 0), and used linear models to test for differences between treatments. For survival, we used one-sided Fisher's exact tests.

We determined if exposure to *Bd* caused changes in the skin bacterial communities by testing for differences in skin bacterial diversity or community structure between treatments, swab days (i.e. pre/postexposure) and their interaction. Differences in diversity, measured as the effective number of species (i.e. exp(Shannon Index); Jost 2006) and Faith's Phylogenetic Diversity (package picante; Faith 1992, Kembel et al. 2010), were assessed using generalized linear mixed models, including 'Individual' as a random effect to account for repeat sampling of individuals over time. Differences in community structure, measured as weighted and unweighted UniFrac distances (Lozupone and Knight 2005,

Lozupone et al. 2007), were assessed using permutational analysis of variance (Anderson 2001) and Anderson's (Anderson et al. 2006) distance-based test of homogeneity of multivariate dispersions.

Finally, we asked if natural variation in skin bacterial diversity or community structure could explain responses to experimental *Bd* exposure. We first tested if a host's natural (Day 0) skin bacterial community diversity (measured as the effective number of species and phylogenetic diversity) predicted infection intensity on Day 13 using generalized linear models with an underlying negative binomial distribution. Second, to determine if variation in bacterial community structure explained responses to *Bd* exposure, we used Deterministic Grouped Covariate Regression (dGCR) (Loftus 2015). dGCR pares down a dataset, while retaining as much information as possible to explain a response, until the number of variables in the model is less than the number of observations. It does this by grouping the covariates in a dataset into clusters and identifying important groups of covariates, while accounting for the possibility of mis-assigning covariates to clusters. This process occurs iteratively until the number of covariates in the selected clusters is less than the sample size, resulting in a model that includes a small subset of ASVs that predict *Bd* infection intensity without having the model be overfit. We included only individuals that were experimentally exposed to *Bd* in these analyses to focus on the outcomes of acute exposure to the pathogen.

Results

What were levels of *Bd* infection in nature?

Swabs taken in the field indicated that, depending on the species, just under 1/3–1/2 of all individuals were infected with *Bd* prior to the start of the experiment (prevalence of *Bd* infection based on Day 0 samples: 29, 27, and 50% for *A. callidryas*, *D. ebraccatus*, and *C. fitzingeri*, respectively). However, all but a few animals from the control treatment tested positive for *Bd* at some point during the experiment ($N = 7/12$ *A. callidryas* controls, 10/12 *D. ebbracatus* controls, and 8/11 *C. fitzingeri* controls), indicating that most frogs in the experiment carried very low loads of *Bd* when we collected them. Detecting the presence of *Bd* at very low levels is a known challenge (Bletz et al. 2015, Byrne et al. 2018). In 13 controls ($N = 5$ *A. callidryas*, 3 *D. ebraccatus*, and 5 *C. fitzingeri*), *Bd* infection went undetected for two weeks or more into the experiment.

Detectable *Bd* infection levels at Day 0 were extremely low for all infected individuals—not exceeding 2 zoospore equivalents—and most controls that were infected had low infection intensities throughout the experiment. For *A. callidryas,* infection intensity in controls never exceeded 5 zoospore equivalents for any individual at any time point. Similar to *A. callidryas*, infection intensity in *C. fitzingeri* controls was low, never exceeding 16 zoospore equivalents. *Dendropsophus ebraccatus* was unique in that two *D. ebraccatus* controls developed relatively severe infections, with infection intensities peaking at 39 117 zoospore equivalents for one individual and 616 733 zoospore equivalents for the other. The latter individual died 2 days after this infection intensity was reached.

How did experimental exposures to *Bd* affect infection intensity and host health?

Bd infection dynamics in the exposed treatments were different from those of controls, and outcomes following exposure differed by species (Table 1, Fig. 1A–F). For *A. callidryas*, prevalence of infection was always higher in the exposed treatment relative to the control, and individual infection intensities increased over time in the exposed treatment, although not significantly. Still, infection intensities were relatively low, not exceeding 409 zoospore equivalents in any exposed individuals, with the exception of one individual whose infection increased throughout the experiment, peaking at 77 141 zoospore equivalents on Day 41. For *D. ebraccatus*, all exposed individuals were positive for *Bd* by Day 6 and infection levels remained elevated relative to the initial time point for the duration of the experiment. Interestingly, for many individuals of *D. ebraccatus*, infection intensity began to subside after Day 27. *Craugastor fitzingeri* exposed to *Bd* also experienced an increase in infection prevalence and sustained the highest infection intensities of all three species (max infection intensity: 3 767 749 zoospore equivalents). The four individuals of *C. fitzingeri* with the highest infection intensities on Day 27 (= 445 800, 720 658, 950 389, and 3 767 749 zoospore equivalents) died before the end of the experiment.

Table 1. Results of generalized linear mixed models testing for effects of treatment and time on *Bd* infection prevalence and intensity during a 41-day *Bd* exposure experiment. Separate experiments were conducted for each frog species (*A. callidryas, D. ebraccatus,* and *C. fitzingeri*).

	Prevalence		Intensity	
A. callidryas				
	χ^2	*P*	χ^2	*P*
Treatment	5.5457	**.019**	8.8961	**.003**
Time	1.0954	.295	12.8533	**.0003**
D. ebraccatus				
	χ^2	*P*	χ^2	*P*
Treatment	17.5379	**< .001**	28.874	**< .001**
Time	7.1086	**.008**	88.01	**< .001**
C. fitzingeri				
	χ^2	*P*	χ^2	*P*
Treatment	13.645	**.0002**	72.726	**< .001**
Time	17.173	**< .001**	134.714	**< .001**

Experimental exposure to *Bd* did not alter mass or survival in *A. callidryas* or *D. ebraccatus* (proportional change in mass, mean ± SD, *A. callidryas* controls: 0.04 ± 0.12; exposed: −0.007 ± 0.11; *D. ebraccatus* controls: 0.22 ± 0.42; exposed: 0.14 ± 0.16; mass (ANOVA): *A. callidryas* $F_{1,22} = 0.96$, $P = .34$; *D. ebraccatus* $F_{1,19} = 0.37$, $P = .55$; survival (Fisher's exact test): *A. callidryas* NA—all individuals survived; *D. ebraccatus* $P = .26$). Mass of *C. fitzingeri* was unaffected by treatment (proportional change in mass, mean ± SD, controls: −0.07 ± 0.07; exposed: −0.10 ± 0.14; $F_{1,11} = 0.24$, $P = .64$); however, *C. fitzingeri* exposed to *Bd* did show a trend toward increased mortality relative to controls (Fisher's exact test $P = .07$).

Did *Bd* exposure alter the skin bacterial community?

We identified 223 ASVs associated with frogs sampled in the wild (ASVs per species, *A. callidryas*: 142 (range per individual: 34–68), *D. ebraccatus*: 151 (range: 41–76), and *C. fitzingeri*: 194 (range: 42–101)). Most of these ASVs could be found on more than one species, with 106 shared among all three species.

Exposure to *Bd* did not alter the diversity nor community structure of the skin bacterial communities of any of the three species (i.e. no interaction; Tables 2 and 3). However, the skin communities did change over the course of the experiment (i.e. significant swab day effect; Tables 2 and 3). The skin bacterial communities of all three species increased in the effective number of species, and those of *D. ebraccatus* increased slightly in phylogenetic diversity (Fig. 2A–F). Community structure shifted, and the variation among

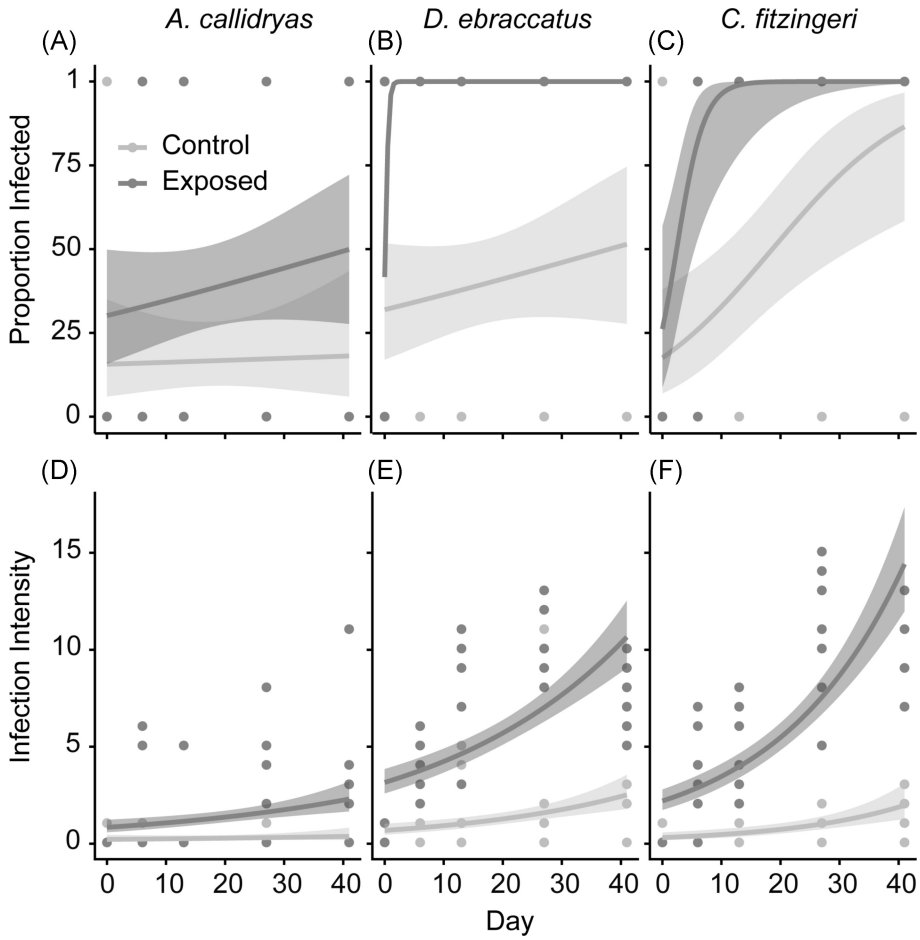

Figure 1. Changes in infection prevalence **(A)–(C)** and intensity **(D)–(F)** in an experimental test of exposure to the fungal pathogen, *Bd*. 'Exposed' frogs were exposed to zoospores of *Bd* for 24 h at the start of the experiment; 'Control' frogs were exposed to water during the same time period. Experiments for each of the three species of tropical frogs (*A. callidryas, D. ebraccatus*, and *C. fitzingeri*) were conducted separately and lasted 41 days. Individuals were sampled on Days 0 (prior to the start of the experiment), 6, 13, 27, and 41 and infection was assessed using quantitative PCR. Prevalence was calculated as the proportion of infected individuals out of the total for each treatment at each time point. Infection intensity is presented as log(number of zoospore genome equivalents) per individual, averaged across three or six replicate PCR reactions (see Methods for details).

individuals increased in captivity in all three species, regardless of treatment (Fig. 2G–K, Table 4). Of the three amphibian species, the bacterial communities of *C. fitzingeri* displayed the greatest consistency in structure across time (Fig. 2I and L).

Shifts in community diversity and composition were influenced in part by the turnover of ASVs from Days 0 to 13. A total of five ASVs were only detected in wild (D0) samples and 50 ASVs were only detected in captive (Day 13) samples.

Did Day 0 bacterial community diversity or structure predict Day 13 infection?

Diversity of skin bacterial communities at the time of *Bd* exposure did not predict how infected an individual would be two weeks later (GLM, all $P > .05$; Table S2, Supporting Information). However, dGCR identified one, three, and two ASVs for *A. callidryas*, *D. ebraccatus*, and *C. fitzingeri*, respectively, that best predicted individual host's responses to *Bd* infection (i.e. Day 13 *Bd* infection intensity; dGCR probabilities of significance: *A. callidryas* Cluster1 > 0.9999, *D. ebraccatus* Cluster1 = 0.9999, Cluster2 = 0.9927, *C. fitzingeri* Cluster1 = 0.9833, and Cluster2 = 0.9120). Phylogenetically, these ASVs were diverse, spanning three phyla, and including the genera *Sphingobacterium, Kaistia, Pseudomonas, Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium, Acinetobacter,* and *Paenarthrobacter* (Figs 3A–F; Table S3, Supporting Information). In all three frog species, individuals that displayed high levels of infection on Day 13 of the experiment were deficient in dGCR-selected ASVs compared to individuals whose infections remained low. The one exception was a *Kaistia* associated with *C. fitzingeri*. Individuals with high *Bd* infection loads two weeks post exposure supported higher relative abundances of this ASV (Fig. 3E).

Discussion

Experimental infections can greatly inform our understanding of disease susceptibility and offer critical insight into the mechanisms by which differences in microbiome diversity or structure relate to disease in the host organism. For example, invasion theory adapted for microbial systems has focused on how the diversity of a community should affect its ability to resist potential invaders: i.e. more diverse symbiont communities can more effectively monopolize available resources, leaving little way for pathogens to gain a foothold (Eisenhauer et al. 2012, Mallon et al. 2015, Kinnunen et al. 2016, Vila et al. 2019). Some hosts, however, may be better served by harboring just a few key symbionts in sufficient abundance (Rebollar et al. 2016). The differences in

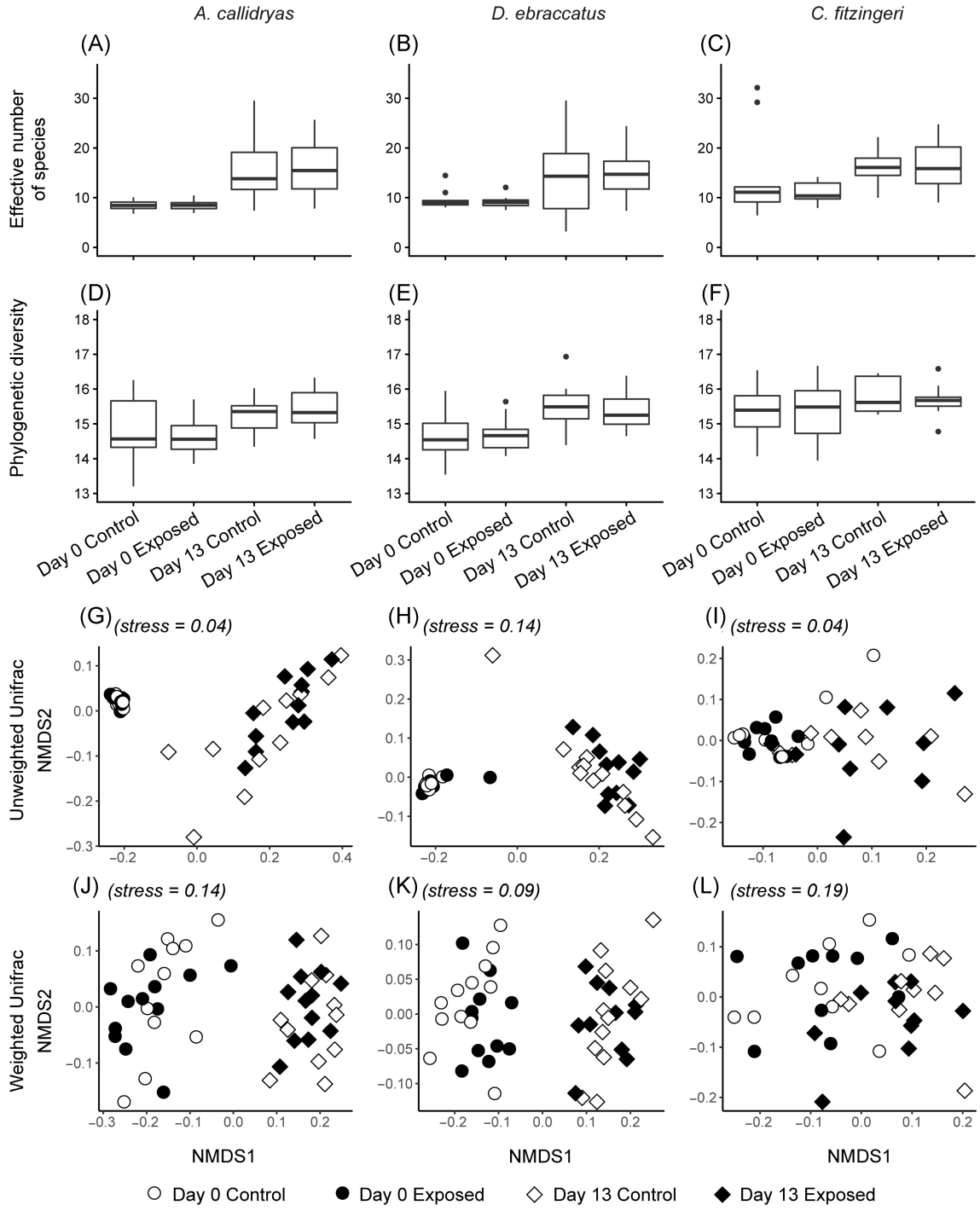

Figure 2. Patterns in alpha **(A)–(F)** and beta **(G)–(L)** diversity of skin bacterial communities in *Bd*-exposure experiments involving three species of frogs (*A. callidryas*, *D. ebraccatus*, and *C. fitzingeri*; left, center, and right columns, respectively). Each species was tested separately. Bacterial communities were sampled prior to the start of the experiment (Day 0) and again two weeks later (Day 13). Ordinations (G)–(L) were constructed using non-metric multidimensional scaling; stress values are provided in parentheses at the top of each ordination. Swab day is denoted by symbol type (Day 0 = circle or Day 13 = diamond) and experimental treatment by color (Exposed to *Bd* = black or Control = white).

community composition observed between individuals that do or do not develop disease can be dramatic (Turnbaugh et al. 2006, Duvallet et al. 2017). Our findings, however, suggest that even subtle differences in the relative abundance of bacterial taxa on the skin may substantially alter responses to disease (Mockler et al. 2018). In addition, we found that the bacterial taxa that may drive disease outcomes varied by host species.

In nature, *A. callidryas*, *D. ebraccatus*, and *C. fitzingeri* harbored extremely low levels of *Bd* infection, complementing previous findings that these three species are relatively resistant to or tolerant of *Bd* (Puschendorf et al. 2006, Goldberg et al. 2009, Crawford et al. 2010, Garcia-Roa et al. 2014, Belden et al. 2015, Rebollar et al. 2016). In the laboratory, however, *D. ebraccatus* and *C. fitzingeri* showed unexpected vulnerability to *Bd*. By contrast, most

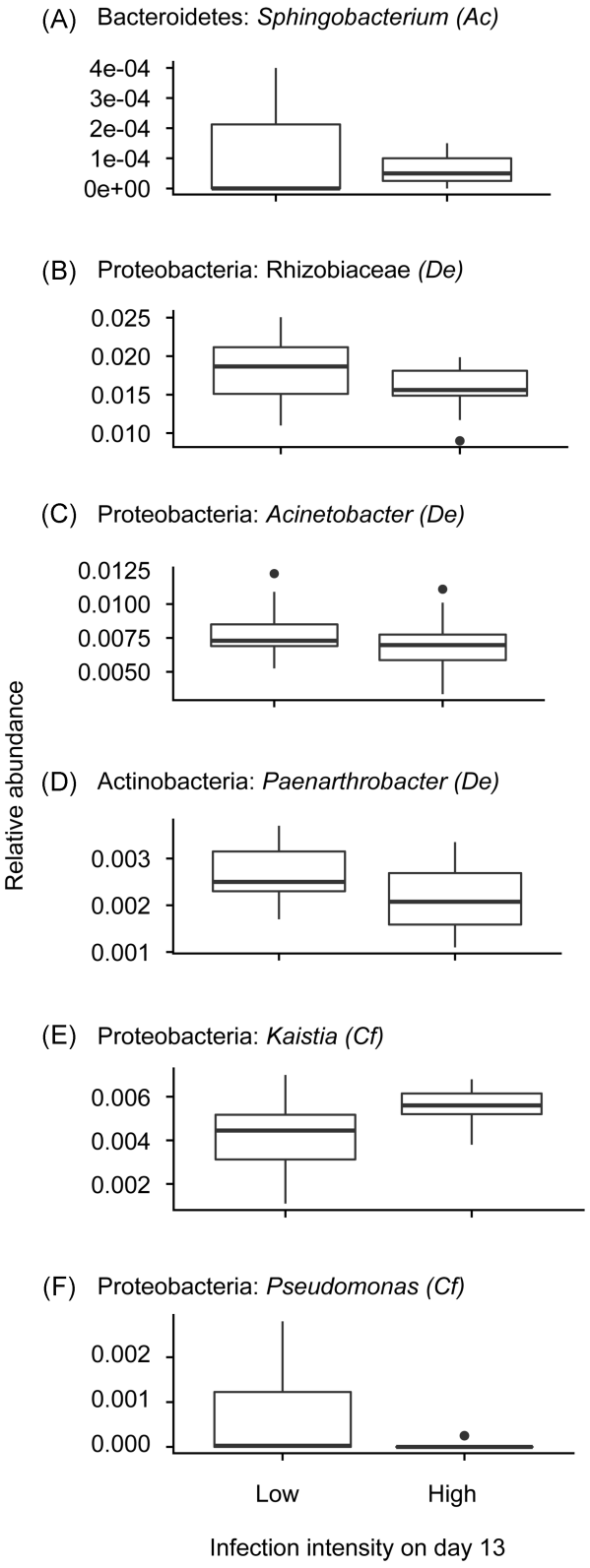

Figure 3. Relative abundance of ASVs predicting host responses at 2 weeks postexposure to *Bd* in a dGCR analysis. Separate analyses were conducted for each of the three frog species. Bacterial phylum and genus (if assigned) are provided for each ASV, along with the frog species the ASV was associated with (*A. callidryas*—Ac; *D. ebraccatus*—*De*; and *C. fitzingeri*—*Cf*). Full taxonomic information for each ASV is provided in Table S3 (Supporting Information). X-axis is infection intensity measured on Day 13 of the experiment. Y-axis is ASV relative abundance on Day 0. Relative abundances are grouped according to low (0–60 zoospore equivalents) and high (133–65 044 zoospore equivalents) infection intensities to ease interpretation of results.

Table 2. Results of generalized linear mixed-effects models testing for effects of treatment, swab day, and their interaction on bacterial community diversity two weeks postexposure during a 41-day *Bd* exposure experiment. Separate experiments were conducted for each of three frog species (*A. callidryas*, *D. ebraccatus*, and *C. fitzingeri*). Alpha diversity was calculated as the effective number of species (Jost 2006) and Faith's Phylogenetic Diversity (Faith 1992).

	Effective number of species		Faiths' phylogenetic diversity	
A. callidryas				
	χ^2	*P*	χ^2	*P*
Treatment	0.0019	.9652	0.0002	.988518
Swab day	47.9252	**< .001**	9.0426	**.002638**
Treatment: swab day	0.0063	.9365	1.3192	.250742
D. ebraccatus				
	χ^2	*P*	χ^2	*P*
Treatment	0.0392	.8430	0.0271	.8693
Swab day	28.3295	**.0002**	17.4238	**< .001**
Treatment: swab day	0.0074	.9314	0.0539	.8165
C. fitzingeri				
	χ^2	*P*	χ^2	*P*
Treatment	0.5813	.445814	0.0066	.9352
Swab day	8.5157	**.003521**	2.6740	.1020
Treatment: swab day	2.6602	.102891	0.2795	.5970

individuals of *A. callidryas* did not become infected following *Bd* exposure; just a few individuals developed mild infections postexposure. For all three species, individuals that developed infections tended to harbor lower relative abundances of specific bacterial taxa relative to those that did not, suggesting that accommodating higher abundances of certain skin symbionts may be advantageous with regard to pathogens. Indeed, three ASVs identified by dGCR matched isolates classified as inhibitory based on *Bd* growth *in vitro* inhibition assays from the Woodhams et al. (2015) antifungal isolates database at 100% sequence similarity: the *Sphingobacterium* from *A. callidryas*, the *Acinetobacter* from *D. ebraccatus*, and the *Pseudomonas* from *C. fitzingeri* (Fig. 3A, C, and F, respectively; Table S3, Supporting Information). These findings, however, should be interpreted with caution, as the 16S rRNA gene may not be a reliable indicator of inhibitory function (Becker et al. 2015b).

While several experimental studies have demonstrated that hosts with specific microbial community assemblages are better at resisting pathogen invasion (Becker et al. 2015a, Walke et al. 2015, Longo and Zamudio 2017, Rosshart et al. 2017, Harrison et al. 2019, Jones et al. 2021b, Rosales et al. 2019, Vanderwolf et al. 2021), others have determined that differences in community assemblages in relation to pathogen infection are the result of pathogen invasion disturbing the symbiont community (Jani and Briggs 2014, Zaneveld et al. 2017, Muletz-Wolz et al. 2019, Jani et al. 2021). We found no evidence that *Bd* exposure altered skin bacterial community diversity or structure in *A. callidryas*, *D. ebraccatus*, or *C. fitzingeri*. One possibility for why we did not see changes after infection is that all three of these species were collected from a site where *Bd* is endemic, so selection may have acted on their symbiont communities before we brought them into the laboratory. A second possibility is that the sterile conditions under which the animals were maintained altered the structure of their skin microbial communities and may have masked treatment

Table 3. Results of permutational analysis of variance testing for effects of treatment, swab day, and their interaction on bacterial community structure two weeks post-exposure during a 41-day Bd exposure experiment. Separate experiments were conducted for each of three frog species (*A. callidryas*, *D. ebraccatus*, and *C. fitzingeri*). Beta diversity was calculated based on weighted and unweighted UniFrac distances (Luzopone *et al.* 2005, 2007).

	Weighted Unifrac			Unweighted Unifrac		
A. callidryas						
	pseudoF	R^2	P	pseudoF	R^2	P
Treatment	1.728	0.01085	.191	0.753	0.01008	.561
Swab day	113.717	0.71417	**.001**	31.2447	0.41784	**.001**
Treatment:Swab day	1.785	0.01121	.174	0.7788	0.01041	.528
D. ebraccatus						
	pseudoF	R^2	P	pseudoF	R^2	P
Treatment	1.419	0.00794	.242	1.143	0.01450	.309
Swab day	134.512	0.75299	**.001**	34.725	0.44033	**.001**
Treatment:Swab day	0.706	0.00395	.409	0.993	0.01259	.364
C. fitzingeri						
	pseudoF	R^2	P	pseudoF	R^2	P
Treatment	0.4006	0.00813	.816	0.7425	0.01793	.678
Swab day	14.1102	0.28640	**.001**	5.8445	0.14113	**.001**
Treatment:Swab day	0.7566	0.01536	.506	0.8252	0.01993	.623

Table 4. Results of distance-based test of homogeneity of multivariate dispersions for effects of swab day on bacterial community structure two weeks postexposure during a 41-day Bd exposure experiment. Separate experiments were conducted for each of three frog species (*A. callidryas*, *D. ebraccatus*, and *C. fitzingeri*). Tests were conducted for both weighted and unweighted UniFrac distances (Luzopone *et al.* 2005, 2007).

	Weighted UniFrac			Unweighted UniFrac		
A. callidryas						
	Df	F	P	Df	F	P
Swab day	1	99.39	**< .001**	1	1.0129	.3197
Residuals	44			44		
D. ebraccatus						
	Df	F	P	Df	F	P
Swab day	1	36.738	**< .001**	1	0.462	.5002
Residuals	44			44		
C. fitzingeri						
	Df	F	P	Df	F	P
Swab day	1	11.494	**.001707**	1	1.3986	.2447
Residuals	36			36		

effects, particularly if they were subtle (Wuerthner et al. 2019). Environmental reservoirs—which we did not include in our experiment to maintain sterility—appear to be an important source of microbial symbionts for amphibians (Loudon et al. 2014b, Fitzpatrick and Allison 2014, Rebollar et al. 2016, Bates et al. 2019, Hughey et al. 2019, Jones et al. 2021a), and future studies incorporating dispersal from environmental source pools may provide additional insight into host–pathogen–microbiome interactions.

Our experimental exposures resulted in higher pathogen loads than are typically observed in the field for these host species (Rebollar et al. 2014, Belden et al. 2015). This may be in part because we conducted our experiments at a temperature that was ideal for *Bd* growth (Piotrowski et al. 2004, Stevenson et al. 2013). This temperature was lower than what the populations of frogs used in this study experience naturally (19–20°C in the lab vs. 25–26°C in the field); however, it was similar to what all three species experience at higher elevations within their geographic range. Importantly, our experiment revealed that even for amphibian species that persist with *Bd* in the wild, there may be individuals that are susceptible to this pathogen, especially *D. ebraccatus* and *C. fitzingeri*. These susceptible individuals may be missed by field surveys if they die quickly after reaching high infection levels.

These three frog species persist in high elevation regions with climates that favor the growth of *Bd* (Crawford et al. 2010). There are at least three possible explanations for their continued coexistence with this pathogen. First, these species' bacterial communities may show local adaptation in terms of *Bd* resistance. Elevational gradients in taxonomic and functional variation in the microbiome, potentially in relation to *Bd*, have been observed in other species of amphibians (Bresciano et al. 2015, Hughey et al. 2017, Muletz-Wolz et al. 2017, Medina et al. 2019). The microbiome of *C. fitzingeri* generally shows signatures of selection for *Bd* resistance in *Bd*-endemic sites relative to *Bd*-naïve sites in terms of both taxonomic and functional diversity (Rebollar et al. 2016, 2018). Second, there are other ways that these species defend themselves against *Bd* infection, such as mounting acquired immune responses to pathogen infection (Ellison et al. 2014) and producing antimicrobial skin peptides (Davis et al. 2016). Lastly, the ecological niches these species occupy may put them at lower risk of developing severe infections (Lips 1998), even if they are vulnerable under experimental settings (Becker et al. 2019, Mesquita et al. 2017). In the Neotropics, increased association with aquatic habitats is one of the best predictors of susceptibility to *Bd* (Lips et al. 2003), and all three species that we studied spend at least part of their lives away from water. *Agalychnis callidryas* and *D. ebraccatus* are arboreal as adults and typically lay their eggs out of water, and *C. fitzingeri* has a fully terrestrial life cycle, although juveniles and adults can be found along stream margins (Hughey and Ibáñez, personal communication).

Resisting disease caused by potentially lethal microbial pathogens is critical to survival, and leads to the selection of defenses to protect against pathogens, including the recruitment of commensal and beneficial symbionts (McLaren and Callahan 2020). Previous work in a diverse array of animal systems, ranging

from corals to humans, has established that both the diversity and the composition of the symbiont community can influence disease resistance functions (van Rensburg et al. 2015, Rosales et al. 2019). However, disease resistance afforded by microbial symbiont communities may be a more nuanced property than has been previously appreciated and may be dependent on the attributes of hosts or pathogens, environmental context, as well as how long the pathogen and bacterial communities have co-existed.

Supplementary data

Supplementary data are available at *FEMSEC* online.

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