PHILOSOPHICAL TRANSACTIONS B

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Research

Cite this article: Siomko SA, Greenspan SE, Barnett KM, Neely WJ, Chtarbanova S, Woodhams DC, McMahon TA, Becker CG. 2023 Selection of an anti-pathogen skin microbiome following prophylaxis treatment in an amphibian model system. *Phil. Trans. R. Soc. B* **378**: 20220126.
https://doi.org/10.1098/rstb.2022.0126

Received: 23 July 2022
Accepted: 3 November 2022

One contribution of 14 to a theme issue 'Amphibian immunity: stress, disease and ecoimmunology'.

Subject Areas:
microbiology, immunology

Keywords:
acquired resistance, *Batrachochytrium dendrobatidis*, chytridiomycosis, host microbiome, microbiome memory, wildlife prophylaxis

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.6630769.

THE ROYAL SOCIETY PUBLISHING

Selection of an anti-pathogen skin microbiome following prophylaxis treatment in an amphibian model system

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With emerging diseases on the rise, there is an urgent need to identify and understand novel mechanisms of prophylactic protection in vertebrate hosts. Inducing resistance against emerging pathogens through prophylaxis is an ideal management strategy that may impact pathogens and their host-associated microbiome. The host microbiome is recognized as a critical component of immunity, but the effects of prophylactic inoculation on the microbiome are unknown. In this study, we investigate the effects of prophylaxis on host microbiome composition, focusing on the selection of anti-pathogenic microbes contributing to host acquired immunity in a model host–fungal disease system, amphibian chytridiomycosis. We inoculated larval *Pseudacris regilla* against the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) with a *Bd* metabolite-based prophylactic. Increased prophylactic concentration and exposure duration were associated with significant increases in proportions of putatively *Bd*-inhibitory host-associated bacterial taxa, indicating a protective prophylactic-induced shift towards microbiome members that are antagonistic to *Bd*. Our findings are in accordance with the adaptive microbiome hypothesis, where exposure to a pathogen alters the microbiome to better cope with subsequent pathogen encounters. Our study advances research on the temporal dynamics of microbiome memory and the role of prophylaxis-induced shifts in microbiomes contributing to prophylaxis effectiveness.

This article is part of the theme issue 'Amphibian immunity: stress, disease and ecoimmunology'.

1. Introduction

Infectious diseases among humans and wildlife have been on the rise in the last few decades, and detrimental impacts are predicted to increase in association with globalization and climate change [1,2]. While managing wildlife diseases has proven difficult, inducing immunological resistance against emerging pathogens through prophylaxis has been successful in reducing viral and bacterial diseases in several taxa, including mammals [3–6], fish [7,8] and birds [9,10]. Prophylactics against fungal pathogens have also been developed [11], but research on the mechanisms by which wildlife species acquire immunity against invasive fungal pathogens remains in the early stages [12–14].

Exposure to a pathogen or prophylaxis can prime the immune system for future infections through upregulation of genes in the major histocompatibility complex (MHC), T-cell response, antibody production [15,16] and induced innate defences [17]. These conserved features of the complex vertebrate immune system are

retained in amphibians and provide a convenient model system to study the role of the host microbiome in disease resistance. The host microbiome is a critical component of the host defence against pathogens, capable of competing with pathogenic microbes [18], producing bioactive secondary metabolites or modifying antimicrobial skin peptides [19–22], and training the internal and external immune system of the host to respond defensively to pathogens [23,24]. Tenetts of the adaptive microbiome hypothesis include the following responses: (i) a decrease in microbial richness owing to pathogen colonization that outcompetes rare microbial taxa, followed by (ii) a reduction in microbial community stochasticity (dispersion), and (iii) an increase in competitive function of the microbiome against the pathogen as hosts recover from infection [25]. According to the adaptive microbiome hypothesis, exposure to pathogens or their metabolic products could shift the community composition of the host microbiome to include more anti-pathogenic bacteria by selecting for anti-pathogenic species and strains that contribute more effective responses to subsequent exposures, termed 'microbiome memory' [25,26]. Alternatively, microbial community shifts in response to pathogen exposure could lead to dysbiosis, i.e. imbalances in the microbiome, which can leave hosts more susceptible to subsequent pathogen infection [27].

Batrachochytrium dendrobatidis (*Bd*) is a waterborne fungal pathogen that has negatively impacted amphibian populations since its global emergence [28]. Efficacious disease control tools are urgently needed to curb *Bd*-induced biodiversity loss. There is evidence that prophylactic exposure to killed or attenuated *Bd* or the metabolites *Bd* naturally produces (i.e. non-infectious antigenic chemicals produced by *Bd*) can provide some protection against *Bd* infections [13,14,29–31]. For instance, amphibians prophylactically exposed to killed *Bd* zoospores and the water-soluble chemical metabolites they produced exhibited increased resistance when subsequently challenged with live *Bd* [13]. While we know that previous exposure yielded an upregulation of splenocytes and increased splenocyte proliferation [13], the mechanisms for this acquired resistance are not well understood, particularly for larval amphibians, and adaptive host-associated microbiome shifts may also play an important role [32,33]. As larval amphibians only acquire infection on keratinized mouthparts [34], the oral microbiome is likely important in adaptive defence [35]. Amphibian hosts carry diverse skin microbiomes [36] and may experience frequent low-dose *Bd* exposures in water bodies throughout their lives and high-load exposures during breeding periods [36–40]. Therefore, microbiome memory is a plausible immunological response as hosts mount adaptive defences against subsequent infection [15]. Prophylactic treatment may thus bolster adaptive shifts in the microbiome that can also occur from natural pathogen exposures. For example, *Bd* secondary metabolites promoted growth of anti-*Bd* bacteria, including *Janthinobacterium lividum*, *in vitro* [41]. These changes in host-associated microbial community structure may be analogous to trained innate immunity and other long-lasting non-specific effect of prophylactic treatments [42].

This study investigates the effects of prophylaxis on microbiome composition of an amphibian host, focusing on selection of anti-pathogenic microbes as a contribution to host acquired immunity. As a model system, we used *Pseudacris regilla* tadpoles immunized against the fungal pathogen *Bd* using a *Bd* metabolite prophylactic (BMP) consisting of soluble *Bd* metabolic products and void of *Bd* zoospores or zoosporangia. A concurrent study has determined the efficacy of the BMP against *Bd* infections in the same experimental trial and examined the impact of duration and concentration of exposure on degree of acquired resistance [43]. Our study addresses the prophylaxis-induced microbiome response, focusing on selection of bacterial taxa that are capable of suppressing *Bd* growth *in vitro*. We cultured and sequenced host-specific bacteria and measured *Bd* growth inhibition with bacterial secondary metabolites in bioassays to complement an existing database of 7274 bacteria that were tested for putatively *Bd*-inhibitory properties (bacteria with confirmed *Bd*-inhibitory properties hereafter called '*Bd*-inhibitory') [44]. We then determined effects of BMP concentration and duration of exposure on proportions of *Bd*-inhibitory bacterial sequences detected on tadpole mouthparts, the body region colonized by *Bd* in larval amphibians. If microbiome memory is a mechanistic explanation for the protection provided by *Bd*-metabolite prophylaxis, then we would predict a more pronounced shift in microbiome community composition towards higher proportions of *Bd*-inhibitory bacterial taxa with longer and higher concentration exposures to the BMP. This response in immunized vertebrates provides future avenues for research on the temporal dynamics of microbiome memory as a novel component of acquired resistance.

2. Methods

(a) Experimental methods

(i) Bd metabolite prophylactic preparation and trials

Initial prophylaxis trials were conducted, and determined high efficacy of a BMP on acquired *Bd* resistance in a model host, tadpoles of *P. regilla* [43]. For these trials, four egg masses of *P. regilla* were collected in Blue Oak Ranch Reserve, Santa Clara County, CA, USA, shipped overnight, and housed in a *Bd*-free facility at the University of Tampa, FL, USA. Tadpoles from all egg masses were consolidated, and when they reached Gosner stage 25 [45], tadpoles were randomized and transferred to individual experimental containers (500 ml plastic cups containing 200 ml of artificial spring water [46], ASW). Tadpoles were fed organic spinach ad libitum. The laboratory was maintained at 20°C with a 12/12 h light/dark cycle. Unconsumed spinach and waste were removed daily with a sterile pipette to maintain suitable water quality. After cleaning, ASW was added to return water volume to 200 ml, which resulted in an approximately 20% water change every 3 days.

Trials were designed to test prophylactic efficacy across a gradient of metabolite concentrations and durations of exposure. To prepare the BMP, *Bd* isolate JEL0270 (obtained from Dr Joyce Longcore, collected August 1999 in Point Reyes, CA, USA from *Rana catesbeiana*) was grown on 1% tryptone agar plates for 14 days at 18°C. Stock *Bd* zoospore solutions were obtained by flooding *Bd*-positive plates with ASW, the flooded plates were allowed to sit for 10 min, and then the supernatant from all plates was homogenized. To produce BMP, stock zoospore solutions were filtered (1.2 µm in pore size filters; GE Whatman Laboratory Products) to remove *Bd* zoospores and zoosporangia (greater than 2–5 µm in size) [47], leaving only fungal metabolic products (the BMP). A previous study characterized three metabolites in cell-free *Bd* metabolic product, two of which had strong immune-inhibitory effects at low concentrations, so we expect that BMP will trigger the immune response in a similar way. Light microscopy was used to verify the concentrated BMP was *Bd*-free, and 1 ml of this concentrated BMP was plated on three 1% tryptone plates to confirm no growth after 8 days. The concentrated

Table 1. Sample size of replicates per treatment (*N*) of *Pseudacris regilla* tadpoles exposed to different concentrations and durations of a *Batrachochytrium dendrobatidis* (*Bd*) metabolite-based prophylactic. Control individuals were exposed to artificial spring water.

	prophylaxis concentration (*Bd* zoospores ml^{-1} prior to filtration)					
no. weeks daily treatment doses	control	10^2	10^3	10^4	10^5	10^6
1	—	7	—	7	—	6
2	—	—	—	7	—	—
3	—	6	5	7	6	5
4	—	—	—	6	—	—
5	15	6	—	6	—	4

Table 2. Timeline of *Batrachochytrium dendrobatidis* (*Bd*) metabolite-based prophylaxis application on *Pseudacris regilla* tadpoles. Concentration (measured as *Bd* zoospores removed ml^{-1}) level 10^4 was tested across all durations, whereas 10^2 and 10^6 concentrations were tested over a five-, three- or one-week duration period, and 10^3 and 10^5 concentrations were only tested over a three-week duration. Artificial spring water (ASW) was used as the control.

week 1	week 2	week 3	week 4	week 5	weeks 6 and 7	end
10^2, 10^4, 10^6	10^2, 10^4, 10^6	10^2, 10^4, 10^6	10^2, 10^4, 10^6	10^2, 10^4, 10^6	*Bd* exposure	euthanasia, *Bd* infection diagnostics, microbiome
ASW	10^4	10^4	10^4	10^4		
ASW	ASW	10^2, 10^3, 10^4, 10^5, 10^6	10^2, 10^3, 10^4, 10^5, 10^6	10^2, 10^3, 10^4, 10^5, 10^6		
ASW	ASW	ASW	10^4	10^4		
ASW	ASW	ASW	ASW	10^2, 10^4, 10^6		
ASW	ASW	ASW	ASW	ASW		

BMP was then diluted to a series of treatment concentrations. We refer to prophylaxis treatments by their pre-filtration zoospore concentrations (i.e. the concentration of zoospores removed ml^{-1}) with ASW. Diluted BMP stocks were frozen and stored at −20°C, and aliquots were thawed to room temperature immediately prior to use.

The experiment included five BMP concentrations: 0 ($n = 15$), 10^2 ($n = 16$), 10^3 ($n = 5$), 10^4 ($n = 30$), 10^5 ($n = 4$) and 10^6 ($n = 6$) zoospores ml^{-1}, and five prophylaxis durations: 1 ($n = 20$), 2 ($n = 7$), 3 ($n = 29$), 4 ($n = 6$) and 5 ($n = 31$) weeks (table 1). Each prophylaxis dose was administered by adding 1 ml of the assigned BMP concentration directly to the tadpole tank water. Tadpoles assigned to exposure durations of less than five weeks were administered daily doses of 1 ml ASW during the weeks prior to the start of their exposure period. Control individuals were exposed to ASW daily for five weeks.

After the five-week prophylaxis period, all tadpoles were exposed to 1 ml of live *Bd* (1.1×10^6 zoospores ml^{-1}; JEL0270), followed by a two-week incubation period (table 2). During this time, tadpoles were held individually in 1 l plastic cups containing 700 ml ASW and fed organic spinach ad libitum. The laboratory was maintained at 18°C on a 12 h/12 h light/dark cycle. Daily water changes did not occur, and a 50% water change was conducted one week after live *Bd* exposure. After the two-week infection period, tadpoles were euthanized with an overdose of MS-222 and dissected to remove the mouthparts (table 1). Since mouthpart extraction requires host sacrifice, these samples represent the endpoint microbiome only.

Whole mouthparts were stored in a laboratory-grade freezer at −20°C. Prepman Ultra (Applied Biosystems) was used to extract DNA from each mouthpart individually, using a previously described protocol [48]. Briefly, mouthparts were agitated with 0.035 ± 0.005 g of zirconia beads in a tissue disruptor (Disruptor Genie, Scientific Industries) for a total of 2.25 min. After this they were maintained in a dry hot bath at 100°C for 10 min. These samples were again maintained at −20°C.

(b) Bacterial culturing

(i) Cultured bacteria isolation

Mouthparts from laboratory-raised *P. regilla* tadpoles hatched from wild egg masses collected in the same source wetland as our experiment (Blue Oak Ranch Reserve, CA, USA) and wild *Osteopilus septentrionalis* adults from wetlands not associated with the experiment (Tampa, FL, USA) were obtained to isolate additional bacterial taxa with potential anti-*Bd* properties. Individual tadpoles of *P. regilla* used in bacterial isolation were not associated with the original prophylaxis experiment but were sourced from the same population at a similar time of year and held in identical laboratory conditions to obtain representative microbiota, while *O. septentrionalis* adults sourced from wetlands not associated with the experiment were included to supplement data. *Osteopilus septentrionalis* adults were held in 1 l plastic cups with ASW-soaked paper towels before swabbing. Frogs were then rinsed with ASW and swabbed five times on each foot and 10 times on the underside with sterile cotton-tipped swabs. *Pseudacris regilla* tadpoles were euthanized with an overdose of MS222, and the mouthparts were removed with sterile dissecting tools. Swabs from adult frogs and dissected mouthparts from tadpoles were stored in 1 ml 20% (v/v) glycerol to suspend bacteria in solution. We plated 2 µl of glycerol from

samples on 1% R2A agar, and incubated plates at room temperature to allow bacterial growth for 2–14 days. Colonies of unique morphotypes were stored in 1 ml of 1 : 10 solution of R2A broth : 20% glycerol at −20°C.

(ii) Cultured bacteria metabolite collection

We thawed bacterial isolates at room temperature then streaked a loopful on 1% tryptone agar to ensure the presence of a pure isolate. After a sufficient growth period that produced individual colonies (2–14 days), we transferred one colony to 2 ml of 1% tryptone broth. Liquid cultures were placed on a shaker at constant moderate motion for 3 days at room temperature (approx. 21°C). After the growth period, the liquid was centrifuged at 10 000 r.p.m. (15 700*g*) for 5 min and the bacterial metabolite supernatant was removed, passed through a 0.22 µm syringe filter (Lab Safety Supply) to remove cells, and frozen at −20°C separately from the remaining dry pellet. This resulted in 85 isolates from *P. regilla* and 207 isolates from *O. septentrionalis*.

(iii) Bacterial metabolite versus *Bd* assays

To determine if the isolated bacteria could inhibit *Bd* growth, we exposed live *Bd* zoospores to the extracted metabolites from each bacterial isolate using a previously described method [49]. Two *Bd* strains (JEL0270 California; JEL0419 Panama) as used for the BMP trials were used for testing. JEL0270 is the same strain used in the original BMP preparation (collected August 1999 in Point Reyes, CA, USA from *R. catesbeiana*, obtained from the Collection of Zoosporic Eufungi at the University of Michigan, MI, USA), and JEL0419 (collected December 2004 in El Cope, Panama, obtained from the Collection of Zoosporic Eufungi at the University of Michigan, MI, USA) was used to increase sample size and will provide valuable information for concurrent experiments. We cultured *Bd* in 1% tryptone broth at 21°C for 1–3 days until zoospores were present and transferred stock to 6°C once exponential growth was established. For use in assays, *Bd* was grown on 1% tryptone agar at 21°C for 2–4 days until zoospores were visible under light microscopy, and the zoospores were resuspended in 1% tryptone broth. We filtered the resuspended zoospore solution using sterilized 20 µm gravity filters (StonyLab) to remove only zoosporangia, and diluted the zoospore solution to 2×10^6 zoospores ml^{-1} using a haemocytometer under light microscopy, staining with trypan blue [50].

Bd inhibition assays were performed in 96-well plates. We plated 16 unique bacterial cell-free supernatants containing metabolites per plate with three replicates per isolate and four controls: positive with *Bd* and 1% tryptone broth, negative with only 1% tryptone broth, heat-killed *Bd* with 1% tryptone broth, and nutrient-depleted with *Bd* in ASW. Treatment replicates contained 1 : 1 solutions of metabolite supernatants to filtered and diluted zoospore solution, resulting in a final zoospore concentration of 1×10^6 zoospores ml^{-1}. Positive controls contained a 1 : 1 solution of diluted zoospores to 1% tryptone broth to compare treatments with uninhibited zoospore growth. Negative controls contained only 1% tryptone broth at the same volume as treatment wells. Nutrient-depleted controls contained Provosoli water [51] instead of metabolite solutions along with the diluted zoospore solution. Nutrient-depleted controls still contained 1% tryptone from the preparation of the diluted zoospore solution; however, the resulting growth curves showed that this was not a sufficiently nutritive environment to support substantial growth. Heat-killed controls consisted of the diluted zoospore solution that was incubated at 75°C for 30 min for spectrophotometric comparison with live, growing zoospores [52]. We incubated assay plates at 21°C and recorded optical density of each well at 492 nm on days 0, 3, 5 and 7 using a Spectramax i3x microplate reader (Molecular Devices, San Jose, CA, USA), which was sufficient time for the growth curve to exhibit a plateau [53].

Bacterial isolates were considered to be inhibitory if their metabolites caused at least 80% inhibition of *Bd* growth during assays as determined by spectrophotometry at 600 nm. This is a conservative cutoff indicating only strongly inhibitory isolates. Inhibition was determined using the slope of the growth curves for treatments and controls. For each plate, we subtracted the average slope of the three heat-killed controls from the slope of each replicate to account for the absorbance of the initial zoospore solution before growth. We then divided this resulting value by the average slope of the three nutrient-depleted controls to determine the proportion of growth outside of the initial addition of the zoospore solution. The resulting proportion of growth was averaged across the three replicates per treatment.

(c) Molecular methods

(i) Microbiome DNA extraction and sequencing

The tadpole mouthpart microbial DNA was extracted with Prepman Ultra (Applied Biosystems) using a previously described protocol [48]. Bacterial DNA amplification and sequencing were achieved using the Earth Microbiome Project 16S Illumina Amplicon Protocol [33]. We amplified samples using Phire Hot Start II DNA Polymerase (Finnzyme, Espoo, Finland) in duplicate [54,55], including a negative control without template DNA in each 96-well plate. To confirm amplification and gel band strength, we used 1% agarose gel to visualize amplicons. Equal volumes of samples were pooled to create the amplicon library. The library was purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA), and we measured DNA concentration using the Qubit 2.0 fluorometer dsDNA Broad-Range Assay kit (ThermoFisher Scientific, San Jose, CA, USA). We sequenced the V4 region of the 16S rRNA gene with a dual index approach using universal primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVHHHTWTCTAAT-3′) from bacterial genomes to characterize sub-operational taxonomic units (sOTUs), a classification method of closely related organisms determined by molecular markers. The library was sequenced at Tufts University Core Facility (TUCF Genomics), Boston, MA, USA using an Illumina MiSeq (2 × 250 bp) sequencer.

We used Quantitative Insights into Microbial Ecology (QIIME2 v. 2020.2) for initial processing of bacterial sequences [56,57]. We used the Deblur workflow to cluster sequences into sOTUs [58]. Only forward reads were used as they are typically higher-quality and are recommended for the Deblur workflow [58], and we filtered low-quality reads using standard quality control settings. Reads were trimmed to 150 bp to maximize read quality. We aligned sequences (alignment plugin) with MAFFT [59] and constructed a phylogenetic tree (phylogeny plugin) using FastTree with QIIME default parameters [60], and then assigned taxonomy (feature-classifier plugin) with the scikit-learn naive Bayes taxonomy classifier and the Greengenes 13.8 reference sequence database [61–63]. We discarded sequences identified as chloroplasts or mitochondria, and sOTUs with fewer than 0.005% (15 reads) of the total reads (307 362) in the dataset of 98 sOTUs [64]. To standardize read counts across samples, samples were rarified to 900 reads (electronic supplementary material, figure S1), resulting in 85 of 96 samples being retained. No PCR controls were retained after filtering and rarefaction of the sample set. After all filtering steps, the final dataset contained 408 sOTUs and 100 800 sequence reads.

(ii) Bacterial isolate DNA extraction and sequencing

We sequenced the V4 region of the 16S rRNA gene with a dual index approach using universal primers 8F (5′-AGAGTTTGATCCTGG CTCAG-3′) and 907R (5′-CCGTCAATTCMTTTGAGTTT-3′) from the pellet remaining from bacteria metabolite collection. We added

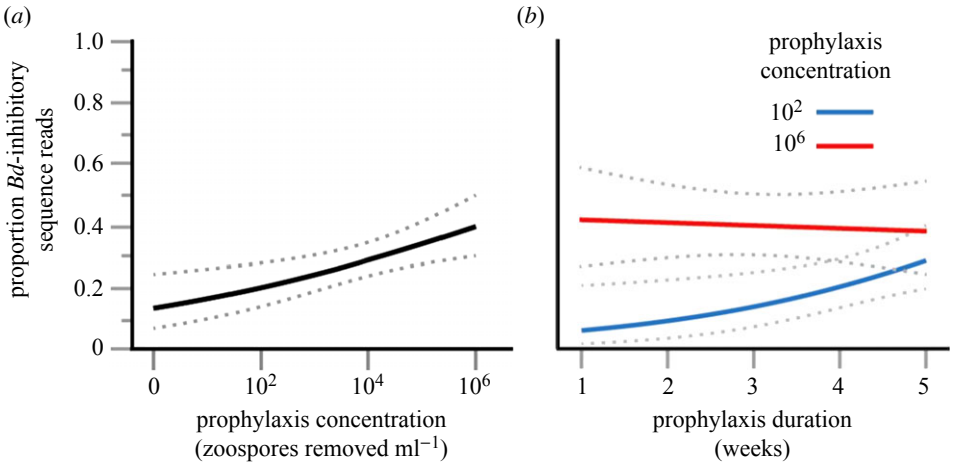

Figure 1. Predicted proportion (± 95% confidence intervals shown in grey) of *Batrachochytrium dendrobatidis* (*Bd*)-inhibitory bacterial sequence reads detected in *Pseudacris regilla* tadpole mouthparts with increasing concentration of a *Bd* metabolite prophylaxis (*a*) and increasing weeks of daily prophylaxis exposures (*b*) at the lowest (10^2; blue) and highest (10^6; red) prophylaxis concentrations tested across all durations. (Online version in colour.)

100 µl of 5% Chelex solution to each sample and incubated at 99°C for 20 min to extract DNA following the manufacturer's protocol. We diluted each sample 1 : 20 DNA : DI water to reduce PCR inhibition. We amplified samples using Phire Hot Start II DNA Polymerase (Finnzyme, Espoo, Finland) in duplicate [54,55], including a negative control without template DNA in each 96-well plate. To confirm amplification and gel band strength, we used 1% agarose gel to visualize amplicons. We measured DNA concentration using the Qubit 2.0 fluorometer dsDNA Broad-Range Assay kit (ThermoFisher Scientific, San Jose, CA, USA). The library was purified and sequenced at MCLabs, San Francisco, CA, USA using Sanger sequencing on an ABI 3730XL sequencer.

We aligned forward and reverse reads and trimmed sequences using the program Geneious (https://www.geneious.com/). Reads were clustered and sOTUs were assigned using the vsearch global matching function at 97% match (https://github.com/torognes/vsearch). We then imported sequences into QIIME2 to match inhibitory sOTUs with our microbiome data [56,57]. From the previous 33 inhibitory isolates, 19 unique sOTUs were clustered at 97% similarity. Of those 19 unique sOTUS, two sOTUs were found to be 80% inhibitory at least half of the time against both strains of *Bd* and were included in the analysis alongside the previously existing database of inhibitory sOTUs (electronic supplementary material, table S1). The two sOTUs we determined to be inhibitory still met these qualifications at 99% similarity; however, we clustered at 97% to increase the number of isolates that could be referenced when determining the inhibition qualities of each sOTU. As 16S is a relatively small gene fragment, it is possible that some matches in the microbiome may not have the same inhibitory power as isolates. However, data indicate that this will be unlikely to result in significant bias in relatively large datasets [44].

(d) Statistical methods

For bacterial alpha diversity analyses, we calculated the sOTU richness per sample, Faith's phylogenetic diversity, Pielou's evenness, Shannon's diversity index and effective number of species [65]. We identified sOTUs matching *Bd*-inhibitory isolates in our samples at 97% sequence similarity and calculated the proportion of sequence reads matching *Bd*-inhibitory isolates in each sample (proportion *Bd*-inhibitory). For bacterial beta diversity analyses, we calculated the distance between samples using unweighted and weighted UniFrac dissimilarity matrices. We additionally calculated beta dispersion using the unweighted UniFrac matrix, partitioned by treatment, which measures the relative distance from the centroid of each sample in multidimensional space (betadisper function from vegan package in program R [66]).

We tested for main and interactive effects of BMP concentration and duration on several individual response variables using linear models. Owing to the complications this design introduces, we analysed exposure duration as a continuous variable. We included all concentration treatments regardless of sample size as excluding the concentrations that were not applied across the entire experiment would greatly reduce our explanatory power. We used a generalized linear model with a binomial probability distribution and logit link function when including proportion of *Bd*-inhibitory bacterial sequence reads as the response variable. With Shannon diversity index, Faith's phylogenetic diversity, Pielou's evenness and unweighted UniFrac dispersion as individual response variables, we used general linear models with least-squares fit. For sOTU richness and effective number of species as the response, we used generalized linear models with a Poisson distribution and log link function. We used JMP Pro v.15 software (SAS Institute, 2019) for all models.

We performed permutational multivariate analysis of variance (PERMANOVA) on weighted and unweighted UniFrac distance matrices of microbiome dissimilarity only using treatments with high sample size (concentrations: control, 10^2, 10^4, 10^6; durations: 1, 3, 5), with concentration and duration separately as predictors, using the adonis function from the vegan package in program R v. 10.0.153 [67]. The chosen concentrations represent a range of *Bd* infection loads previously detected in the wild [68] as well as previous *in vitro* experiments [69]. Control exposures to ASW are included in the five-week treatment group regardless of prophylaxis concentration since all controls were exposed over a five-week period. We used the linear discriminant analysis (LDA) effect size (LEfSe) method on the Galaxy platform (https://huttenhower.sph.harvard.edu/galaxy/) with default parameters to determine differentially abundant sOTUs to determine bacterial taxa that may explain differences between control and high-concentration treatments [70].

3. Results

We found significant effects of BMP concentration and duration on the proportion of *Bd*-inhibitory bacteria in the tadpole mouthpart bacteriome (whole model test: $\chi^2 = 9.43$, d.f. = 3, $p = 0.024$). Tadpoles exposed to higher BMP concentrations carried on average higher proportions of bacteria capable of inhibiting *Bd* growth *in vitro* (concentration: $\beta = 0.219$, $\chi^2 = 8.32$, $p = 0.004$; figure 1*a*). We also found that a higher number

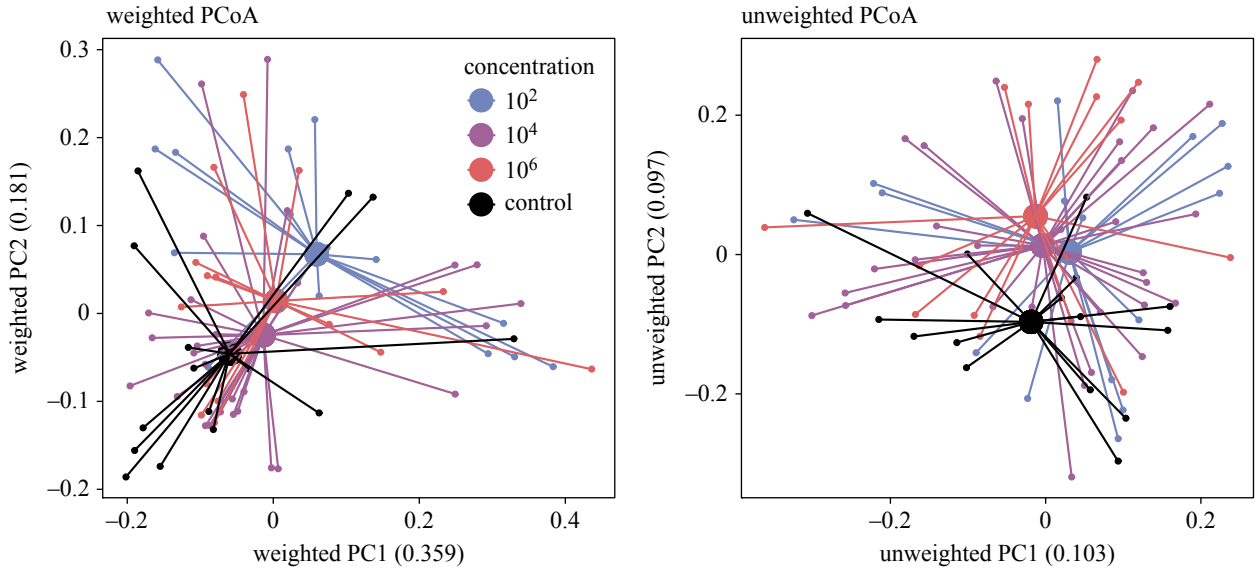

Figure 2. Principal coordinates analysis (PCoA) of effect of *Batrachochytrium dendrobatidis* (*Bd*) metabolite prophylaxis (BMP) concentration (zoospores removed ml^{-1}) on shift in microbiome structure, with treatments of low sample size removed. BMP concentration is denoted by colour. (Online version in colour.)

of consecutive BMP exposures were associated with higher proportions of potentially inhibitory bacteria (duration: $\beta = 0.193$, $\chi^2 = 4.32$, $p = 0.037$; figure 1*b*), with the exception that there was no significance at the highest BMP concentration (concentration × duration interaction: $\beta = -0.082$, $\chi^2 = 3.32$, $p = 0.068$; figure 1*b*). Using the entire microbiome dataset, we found a significant effect of BMP on the treatment group (treatment grouping all concentrations and durations versus no treatment using ASW control), predicting higher Shannon diversity index ($F_{1,83} = 4.91$, d.f. = 1, $p = 0.029$), Pielou's evenness ($F_{1,83} = 5.36$, d.f. = 1, $p = 0.023$), and effective number of species ($\chi^2 = 4.64$, d.f. = 1, $p = 0.031$), but we did not detect a significant effect of BMP on other alpha diversity metrics (sOTU richness: $\chi^2 = 0.147$, d.f. = 1, $p = 0.147$; Faith's phylogenetic diversity: $F_{1,83} = 0.715$, d.f. = 1, $p = 0.400$). However, we did not find significant effects of BMP concentration or duration on any of the metrics of microbiome alpha diversity, in either fully factorial or pruned models (Shannon diversity index: $F_{3,81} = 0.747$, d.f. = 3, $p = 0.527$; effective number of species: $\chi^2 = 0.601$, d.f. = 3, $p = 0.896$; sOTU richness: $\chi^2 = 1.26$, d.f. = 3, $p = 0.739$; Faith's phylogenetic diversity: $F_{3,81} = 0.250$, d.f. = 3, $p = 0.861$; Pielou's evenness: $F_{3,81} = 1.03$, d.f. = 3, $p = 0.385$). Additional generalized linear model results can be found in the electronic supplementary material.

BMP concentration influenced host microbiome composition when sOTU relative abundance was taken into account between treatments (PERMANOVA for weighted UniFrac: $F_{3,54} = 2.32$; $R^2 = 0.1$; $p = 0.009$; figure 2*a*) and when grouping all concentration treatments versus no treatment (PERMANOVA for weighted UniFrac: $F_{1,62} = 2.99$; $R^2 = 0.045$; $p = 0.017$), but not while using bacteria sOTU presence/absence data as a proxy for composition between treatments (PERMANOVA for unweighted UniFrac: $F_{1,54} = 0.989$; $R^2 = 0.047$; $p = 0.492$; figure 2*b*) nor when grouping all concentration treatments versus no treatment (PERMANOVA for unweighted UniFrac: $F_{1,62} = 1.20$; $R^2 = 0.019$; $p = 0.196$). BMP duration did not influence microbiome sOTU composition (PERMANOVA for weighted UniFrac: $F_{2,54} = 1.31$; $R^2 = 0.038$; $p = 0.237$; PERMANOVA for unweighted UniFrac: $F_{2,54} = 1.02$; $R^2 = 0.033$; $p = 0.405$). Additionally, we did not detect an influence of BMP concentration or duration on beta dispersion of the microbiome ($F_{3,60} = 0.35$, d.f. = 3, $p = 0.790$). Variation in concentration did not affect host microbiome composition (lowest concentration ASW control PERMANOVA for weighted UniFrac: $F = 4.053$; $R^2 = 0.123$; $p = 0.057$; Highest concentration versus lowest concentration: PERMANOVA for weighted UniFrac: $F = 4.053$; $R^2 = 0.123$; $p = 0.057$). Our

LDA effect size (LEfSe) indicated that high-concentration treatments of BMP had higher abundances of nine sOTUs, intermediate concentration treatments had higher abundances of 11 sOTUs, and low concentration treatments had higher abundances of 17 sOTUs when compared with the ASW control (electronic supplementary material, table S2). We did not detect any sOTUs that were differentially less abundant for any treatment. None of these differentially abundant sOTUs matched cultured bacteria in our *Bd*-inhibitory database nor was isolated and tested, and therefore cannot be confirmed as having *Bd*-inhibitory properties. The dominant bacteria detected belonged to the phyla Proteobacteria, Fusobacteria, Bacteroidetes, Firmicutes, Verrucomicrobia and Actinobacteria (electronic supplementary material, figure S2). Details of the effect of BMP exposure on *Bd* will be given in a separate study; however, exposure to BMP of any concentration and duration treatment concentration was highly effective at reducing *Bd* intensity when compared with the controls [43].

4. Discussion

Our study suggests that one mechanism underlying the BMP effectiveness is enrichment of antifungal microbial taxa. We found that the *Bd* metabolite prophylaxis, or BMP, boosted proportions of putatively *Bd*-inhibitory bacteria in the tadpole mouthpart microbiome of a model amphibian species, *P. regilla*. Our findings indicate that increasing BMP concentration was linked to a higher proportion of *Bd*-inhibitory bacterial sequences in the amphibian skin. Our findings suggest that duration of BMP exposure may also stimulate a change towards a Bd-inhibitory skin microbiome, but only at low BMP exposure concentrations. Understanding the mechanism

behind acquired immunity will improve our understanding of BMP application and function in ecological scenarios.

We found increased abundance of *Bd*-inhibitory bacteria in response to BMP exposure, which supports the idea that exposure to BMP is leading to strong selective pressures towards a pathogen-inhibiting microbiome. Pathogens such as *Bd* must compete for limited resources and space when colonizing and proliferating within the host. If the distribution and composition of external bacterial taxa permit initial colonization of the pathogen, it may only persist and become established if it continues to successfully compete with resident microbes armed with antimicrobial metabolites. Such antimicrobial metabolites are often capable of repelling or inhibiting the growth of pathogenic invaders, which may provide these microbes a competitive advantage [20,71]. Thus, selection for pathogen-inhibiting microbial genotypes should be initiated at the time of first pathogen contact, and importantly, the same responses may be stimulated by exposure to pathogen metabolites even in the absence of living pathogen cells. This process could occur through at least two potentially antagonistic mechanisms acting within the host microbiome. One mechanism of pathogen resistance driven by the host microbiome could be a shift towards higher bacterial diversity through selection, which blocks pathogen establishment by closing niche gaps, consistent with principles of invasion biology [72–74]. For example, more diverse bacterial biofilm communities were more likely to suppress *Bd* growth *in vitro*, a result attributed to higher overall functional performance of *Bd* growth suppression, as well as more synergistic relationships among more diverse bacterial communities [75]. By contrast, exposure to *Bd* metabolites could shift the host bacterial community towards dominance by one or a few taxa that are able to outcompete *Bd*, lowering microbiome diversity. For instance, treatment with the single bacterial species *J. lividum* prevented *Bd*-associated morbidity and mortality in endangered mountain yellow-legged frogs [18] and red-backed salamanders [21].

While priming the microbiome with single or few *Bd*-inhibitory bacterial species may be effective in suppressing subsequent pathogen infections, evidence suggests that robust bacterial community-level processes are a key component of microbiome-mediated pathogen resistance [75,76]. Importantly, our results found an increase in alpha diversity (but not richness) between control and inoculated samples; however, there were similar levels of bacterial alpha diversity with increasing prophylactic concentrations. This suggests that increasing concentrations of the BMP may have facilitated increased proportions of *Bd*-inhibitory bacteria in the microbiome without compromising optimal overall microbiome alpha diversity (i.e. Shannon diversity index, Faith's phylogenetic diversity, effective number of species and Pielou's evenness) despite initial increases in alpha diversity when exposed to the prophylactic. This could reduce the likelihood of dysbiosis, where reduced microbiome diversity associated with selection of a few *Bd*-inhibitory taxa could widen niche gaps, facilitating colonization by other pathogens or *Bd* genotypes in the wild [77]. While we detected shifts in bacterial relative abundance in response to prophylaxis concentration, those shifts did not appear to be related to the taxa that we identified as putatively *Bd*-inhibitory. Additionally, our findings showing an increase in *Bd*-inhibitory bacteria with increasing durations at low BMP concentrations but not high BMP concentrations indicate that a significant lag time may exist. Therefore, host protection increases with increasing duration of BMP exposures at low concentrations as the microbiome shifts to a *Bd*-inhibitory protective state [78].

Prophylaxis may work similarly to natural low-load exposures to *Bd* that occur in aquatic systems. The infective life stage of *Bd* is an aquatic, free-swimming zoospore that is easily transmitted among hosts in water bodies [38,39,47,79]. Thus, amphibians may be subjected to repeated but mild exposures to *Bd* at levels that do not cause morbidity or mortality, which may potentially prime the microbiome with anti-pathogenic microbes that are resistant to *Bd* infections. This mimics the concept of prophylaxis, where preemptive but tolerable exposures to the pathogen or pathogen metabolites increase host immunity and thus decrease infection risk. By contrast, previous studies have found that direct-developing amphibians that have limited contact with water bodies, and therefore *Bd* reservoirs, develop higher pathogen burdens than aquatic-breeding amphibians when exposed to *Bd* [30], mimicking a scenario where the host lacks the benefits of 'natural' prophylaxis. Much like probiotics, prophylaxis can be a form of directional microbiome manipulation that has implications in wildlife disease prevention and mitigation. However, whereas probiotics inoculate the individual with a chosen subset of bacterial taxa that could interfere with microbiome stability, prophylaxis drives microbiome shifts towards protective communities more naturally, while retaining consistent microbiome alpha diversity, in response to a pathogen trigger based on the interactions of the host and symbiotic microbes [80]. This may form a more comprehensive bacterial community composition and diversity within the microbiome and preserve functional diversity without a potentially harmful rapid shift in the microbiome towards homogeneity.

Bd-inhibitory sOTUs increased with mounting prophylaxis durations at all prophylaxis concentrations except the highest, which suggests that higher doses apply greater selection pressure equivalent to multiple low-load doses. However, high concentrations of *Bd* metabolites have been shown to cause pathogenesis in some non-amphibian hosts [40,81]. Methylthioadenosine and kynurenine, both molecules produced by *Bd*, inhibit frog T-cell survival and proliferation, with increasing concentration causing higher inhibition of splenocytes [82]. *Bd* metabolites also contain several molecules, including polyamine spermidine, that enhance the lymphocyte inhibition properties of other molecules at concentrations lower than required for the molecule to inhibit lymphocyte proliferation alone [83]. These findings suggest that prophylaxis dosing must be optimized to prevent pathogenic effects of *Bd* metabolite exposure that counteract the benefits of immunization even when the individual has been previously exposed to *Bd*. However, amphibian prophylaxis studies to date generally support minimal negative fitness effects of metabolite prophylaxis. For instance, tadpoles exposed to the BMP developed faster but the metabolites had no negative effects on tadpole growth, mortality or adult size, indicating minimal impacts of prophylaxis [84]. Interestingly, bacterial community composition of controls not exposed to BMP was statistically similar to higher-concentration BMP treatments (figure 2). Still, at the highest concentration the proportion of *Bd*-inhibitory sOTUs was equally higher across duration treatments, indicating that a few low-

abundance taxa with *Bd*-inhibitory properties might be driving resistance to *Bd* in our focal host species.

While our results suggest a clear anti-pathogen microbial response to BMP, there may be other potential mechanisms acting in concert with microbiome memory that underlie this observed acquired resistance. Microbiome memory is only one of several non-mutually exclusive mechanisms that likely underlie the effectiveness of this prophylaxis. For example, some amphibians produce lysozymes and antimicrobial peptides (AMPs) via skin mucosal secretions that serve as primary defences against invading pathogens; however, some previous studies have found that AMP abundance was not impacted by exposure to BMP [13]. These defences exist alongside the microbiome we examined in this study, and metabolites produced by bacteria may work synergistically with AMPs produced by the organism to mount a larger defence against *Bd* infections [20]. Additionally, adaptive immune responses include antibodies that are produced by the host and exist in the mucosa [15]. *Bd* may acquire mechanisms to subvert one or many host immune responses, and developing varied mechanisms of resistance, including the microbiome community composition as we explored here, may aid in preventing mortality or morbidity caused by *Bd* and slow the evolution of *Bd* immune evasion [85].

Exposure to *Bd* metabolites may cause selection within the microbiome, and enrichment of bacteria with antifungal properties may be one among several mechanisms underlying prophylaxis function. Microbiome memory could be occurring naturally in response to low-load exposure to pathogens when seasonally visiting infected environmental reservoirs (e.g. aquatic environments where *Bd* persists), and prophylaxis could therefore be a promising form of mitigation against wildlife disease. Application of a BMP on captive-breeding and wild populations may be more feasible than probiotics through widespread distribution of cell-free BMP in aquatic systems, which would not require inoculation of amphibians individually and may persist longer in the environment. Further, a previous study found that cell-free BMP was more effective at reducing *Bd* infection than killed zoospores [81], so this method not only improves the chances of reducing pathogen loads but also presents a lower risk of accidental live exposure. Similarly, future studies should also examine the cross-reactivity of this treatment as a prophylactic against *Batrachochytrium salamandrivorans* (*Bsal*) chytridiomycosis, which is a salamander-specific fungal pathogen that is projected to invade the Western Hemisphere and cause declines similar to *Bd*. A BMP would present no risk of accidental *Bsal* introduction into uncontaminated environments. Our results may be specific to the species and life stage used in this investigation, and further studies should identify if this trend is similar for other species or adult frogs. Specifically, a microbiome reorganization post-metamorphosis may disrupt the protective effects of the microbiome influenced by BMP exposure; however, carryover effects and microbial hysteresis have been previously described in amphibians [35]. The impact of BMP on metamorphosed frogs which are susceptible to mortality from chytridiomycosis will better determine the ability of this prophylactic to protect frogs at all life stages.

The acquired immunity conferred by the microbiome is a promising method for preventing further species loss by emerging pathogens. Given these results, managers at captive-breeding and reintroduction programs may consider applying a dose of the BMP of 10^4 zoospores removed ml^{-1} to amphibians one or more times as a prophylactic treatment. Further studies focusing on bacteria-specific metabolic products may parse the compounds that are providing immunity and provide further insight into the mechanisms that are responsible for the BMP function. Though little is known about the response of the microbiome to prophylactics, understanding responses of the microbiomes may help explain variation in immune response following prophylaxis in other vertebrates.

Ethics. Permits were provided by CA-DGF (#S-1935000003-20017-001) and University of Tampa Institutional Animal Care and Use Committee (TAM 2018-2). Sample sizes of the vertebrate hosts were kept low to reduce unnecessary burdens on host populations (*Pseudacris regilla* is listed as Least Concern on the IUCN Redlist).

Data accessibility. Sequence data that support the findings of this study are available from the NCBI Sequence Read Archive: https://www.ncbi.nlm.nih.gov/sra with the accession code SUB11387491.

Data are provided in the electronic supplementary material [86].

Authors' contributions. S.A.S.: conceptualization, data curation, formal analysis, investigation, methodology, project administration, writing—original draft, writing—review and editing; S.E.G.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, software, supervision, writing—original draft, writing—review and editing; K.M.B.: conceptualization, writing—review and editing; W.J.N.: data curation, formal analysis, methodology, software, writing—review and editing; S.C.: conceptualization, project administration, writing—review and editing; D.C.W.: methodology, writing—review and editing; T.A.M.: conceptualization, funding acquisition, methodology, project administration, resources, supervision, writing—original draft, writing—review and editing; C.G.B.: conceptualization, formal analysis, investigation, project administration, resources, supervision, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

Funding. This research was funded by the National Science Foundation (C.G.B.: IOS-1947681; T.A.M.: IOS-1754862, IOS-2113544; K.M.B.: GRFP DGE-193797), the National Institutes of Health (T.A.M.: 1R01GM135935-0, KK 2022), the University of Tampa (T.A.M.: Faculty Development Dana and Delo Grants) and the University of Alabama.

Acknowledgements. We thank Brandon LaBumbard, Ross Whetstone, Brandon Kim, Xiaohui Yan, Caroline Krieger, Brodie Frew, Maxfield Bell, Bridget Hilgendorff, Alexis Garcia, Britany Soompholphakdy, Vanessa Marshall and the Johnson laboratory for reading previous versions of this manuscript and for providing support in the laboratory.

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