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ANIMAL HOST-MICROBE INTERACTIONS

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Do host-associated gut microbiota mediate the effect of an herbicide on disease risk in frogs?

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Abstract

1. Environmental stressors, such as pollutants, can increase disease risk in wildlife. For example, the herbicide atrazine affects host defences (e.g. resistance and tolerance) of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), but the mechanisms for these associations are not entirely clear. Given that pollutants can alter the gut microbiota of hosts, which in turn can affect their health and immune systems, one potential mechanism by which pollutants could increase infection risk is by influencing host-associated microbiota.
2. Here, we test whether early-life exposure to the estimated environmental concentration (EEC; 200 μg/L) of atrazine affects the gut bacterial composition of Cuban tree frog (*Osteopilus septentrionalis*) tadpoles and adults and whether any atrazine-induced change in community composition might affect host defences against *Bd*. We also determine whether early-life changes in the stress hormone corticosterone affect gut microbiota by experimentally inhibiting corticosterone synthesis with metyrapone.
3. With the exception of changing the relative abundances of two bacterial genera in adulthood, atrazine did not affect gut bacterial diversity or community composition of tadpoles (in vivo or in vitro) or adults. Metyrapone did not significantly affect bacterial diversity of tadpoles, but significantly increased bacterial diversity of adults.
4. Gut bacterial diversity during *Bd* exposure did not predict host tolerance or resistance to *Bd* intensity in tadpoles or adults. However, early-life bacterial diversity negatively predicted *Bd* intensity as adult frogs. Specifically, *Bd* intensity as adults was associated negatively with the relative abundance of phylum Fusobacteria in the guts of tadpoles.
5. Our results suggest that the effect of atrazine on *Bd* infection risk is not mediated by host-associated microbiota because atrazine does not affect microbiota of tadpoles or adults. However, host-associated microbes seem important in host resistance to *Bd* because the early-life microbiota, during immune system development, predicted later-life infection risk with *Bd*. Overall, our study suggests that increasing gut bacterial diversity and relative abundances of Fusobacteria might have lasting positive effects on amphibian health.

KEYWORDS
atrazine, bacteria, *Batrachochytrium dendrobatidis*, chytrid fungus, corticosterone, Fusobacteria, *Osteopilus septentrionalis*, stress

1 | INTRODUCTION

Anthropogenic factors, such as pollutants, can dramatically affect the health of organisms (Martin, Hopkins, Mydlarz, & Rohr, 2010; Newman, 1979; Vitousek, Mooney, Lubchenco, & Melillo, 1997). Pollutants directly affect the development, reproductive output and survival of organisms and indirectly affect fitness by increasing disease risk (Figure 1, *paths a-b*) (Martin et al., 2010; Rohr et al., 2008, 2013). For example, pollutants can decrease immune function and thus decrease resistance to infection (Arkoosh et al., 1998; Koprivnikar, 2010; Rowe, Brundage, Schafer, & Barnett, 2006; Rohr et al., 2008; reviewed in Martin et al., 2010); host resistance reduces parasite damage by reducing parasite fitness (Read, Graham, & Råberg, 2008; Schmid-Hempel, 2011). Additionally, exposure to pollutants, such as the herbicide atrazine, can reduce amphibian tolerance of the fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*) (Rohr et al., 2013); host tolerance minimizes damage caused by parasites without affecting parasite fitness (Medzhitov, Schneider, & Soares, 2012; Miller, White, & Boots, 2006; Råberg, Sim, & Read, 2007; Read et al., 2008). *Bd* is an important pathogen because it has contributed, in part, to the global decline of amphibians (Wake & Vredenburg, 2008), which are the most threatened class of vertebrates in the world (Stuart et al., 2004). Therefore, it is important to understand the mechanisms by which pollutants affect diseases like *Bd* in order to mitigate the impact of these factors on hosts. Despite mounting evidence that pollutants increase infection risk, mechanisms mediating these increases remain relatively untested.

One potential mechanism for how pollutants increase disease risk is by altering the symbiotic microbiota of the host (Figure 1, e.g. *paths c-e*, *g-a-b*, *a-f-e*) (Claus, Guillou, & Ellero-Simatos, 2016). Many microbes are instrumental in breaking down pollutants in the environment (Figure 1, *path g*) (Bansal, 2012; Häggblom, 1992; Horvath, 1972; Staley, Harwood, & Rohr, 2015) and thus the contaminants can serve as a resource for microbiota, potentially increasing some of their abundances. In contrast, many contaminants can be directly toxic to microbiota (Figure 1 *path c*) (Staley et al., 2015). In hosts, exposure to pollutants, especially during formative stages of life, can induce immediate and lasting changes to gut bacterial communities (Figure 1, *path c*) (Kohl, Cary, Karasov, & Dearing, 2015; Shehata, Schrödl, Aldin, Hafez, & Krüger, 2013). Changes to the normal gut and skin microbiota of hosts just before parasite exposure have been shown to decrease host resistance (Figure 1, *path e*) (Koch & Schmid-Hempel, 2011; Schuijt et al., 2016; Schwarz, Moran, & Evans, 2016; Theriot et al., 2014; Woodhams, Bletz, Kueneman, & McKenzie, 2016). For example, the gut bacterial community can protect their host from infections through direct competition with the parasite (Figure 1, *path e*) (Costello, Stagaman, Dethlefsen, Bohannan, & Relman, 2012; Dethlefsen, McFall-Ngai, & Relman, 2007). In contrast to this direct effect, host-associated microbiota may indirectly affect infections by influencing the maintenance or development of the immune system (Hooper, Littman, & Macpherson, 2012; Macpherson & Harris, 2004; Round & Mazmanian, 2009), which in turn, can increase later-life infection risk (Figure 1, *paths d-b*) (Knutie, Wilkinson, Kohl, & Rohr, 2017).

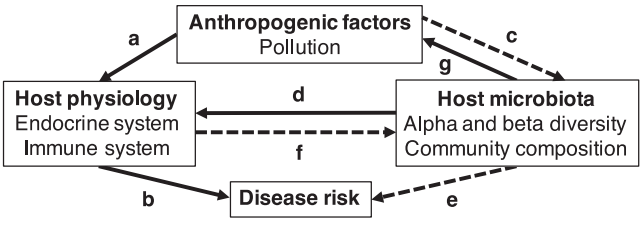

FIGURE 1 Potential interactions among anthropogenic factors, such as pollution, host-associated microbiota and physiology, and disease risk. Pathways are designated with letters and described in the main text. Dotted lines indicate the pathways which we addressed in this study

Amphibians may be at high risk of alterations to their early-life microbiota via pollutants because most amphibians spend their formative stages in water bodies that frequently receive run-off containing chemical contaminants (Schwarzenbach et al., 2006). Several studies have found that amphibian skin and gut microbiota are important in determining infection risk. For example, Woodhams et al. (2016) found that several bacterial taxa, such as *Pseudomonas* sp., *Janthinobacterium lividum* and *Rhodococcus fascians*, produce volatile antifungal compounds that directly reduce the growth of *Bd*, which suggests a direct interaction between the microbiota and infection risk. Additionally, Knutie, Wilkinson, Kohl, et al. (2017) found that an early-life disruption of the gut microbiota affects later-life resistance to a parasitic gut worm infection; these results suggest that there is an indirect interaction between the microbiota and infection risk, which is likely mediated by the immune system (Figure 1, *paths d-b*). If pollutants interact with the early-life microbiota of amphibians during immune development and disruptions in microbiota affect later-life infection risk, then the microbiota may be mediating the effect of early-life exposure to pollutants on later-life infection risk.

Host-associated microbiota may not be the only factor mediating the effect of pollutants on disease risk. Pollutants can affect host physiology, such as their endocrine and immune systems (Figure 1, *path a*), which can alter their ability to resist and tolerate infections (Figure 1, *paths a-b*) (Martin et al., 2010). For example, pollutants can cause dysregulation of the stress hormone corticosterone (Laws et al., 2009; McMahon et al., 2011) and can decrease host immunity (Bellinger, Lubahn, & Lorton, 2008; Hopkins, Mendonça, & Congdon, 1999). Interestingly, corticosterone can also interact with host-associated microbiota (Figure 1, *paths d-f*) (Clarke et al., 2014). For example, corticosterone levels in hosts have been shown to be negatively correlated with bacterial diversity (Stothart et al., 2016), which is likely mediated by the immune system (Bailey et al., 2011; O'Mahony et al., 2009).

Gabor, Knutie, Roznik and Rohr (2017) recently conducted a study related to our experiment to determine whether the effect of atrazine on *Bd* infection risk in Cuban tree frog (*Osteopilus septentrionalis*) tadpoles and post-metamorphic frogs (from here on, we refer to them as adults) was mediated by corticosterone. In this study, Gabor et al. (2017) exposed tadpoles to metyrapone, a corticosterone synthesis inhibitor, and atrazine (in a fully crossed design) and then inoculated tadpoles and adults with *Bd*. While metyrapone countered atrazine-induced corticosterone elevations, atrazine exposure reduced

Bd abundance across life stages in the absence of metyrapone but increased abundance in the presence of metyrapone. Although atrazine affected host tolerance of *Bd* (also shown in Rohr et al., 2013), Gabor et al. (2017) found that corticosterone did not mediate this relationship.

In this study, we explore whether another mechanism, that is host-associated gut microbiota, mediates the effect of atrazine on *Bd* infections (Figure 1, *paths c-e*; Figure 2). Skin bacterial taxa (e.g. *Janthinobacterium lividum*, *Pseudomonas* sp. and *Rhodococcus fascians*) of frogs can release metabolites that can inhibit *Bd* (Woodhams et al., 2014, 2015, 2016); however, we are unaware of any studies linking skin bacteria to immune development in any vertebrate. In contrast, gut bacteria, the focus of our study, has been associated with effects on parasite fitness through direct (Koch & Schmid-Hempel, 2011; Schuijt et al., 2016; Schwarz et al., 2016; Theriot et al., 2014) and indirect pathways, such as by affecting immune system development (Hooper et al., 2012; Round & Mazmanian, 2009). For example, an early-life disruption of frog gut microbiota affected later-life resistance to parasites, a result attributed to the effect of gut microbiota on amphibian immune development (Knutie, Wilkinson, Kohl, et al., 2017). Thus, this study will explore how the microbiota of the gut affects parasites on the skin (probably via the immune system), which has received little attention.

First, we test whether an early-life exposure to atrazine and metyrapone (in a fully crossed design) affects the gut bacteria of Cuban tree frog tadpoles and whether there are lasting effects on gut bacteria into adulthood (Figure 1, *path c*). We also conduct an in vitro experiment to determine the direct effects of atrazine on the gut bacteria of tadpoles (Figure 1, *path c*). Then, we sought to determine whether any changes in these bacteria affect early- and later-life resistance and tolerance of *Bd*. We hypothesize that exposure to atrazine changes gut bacterial communities of hosts because this pattern has been observed with other pollutants (Figure 1, *path c*) (Kohl et al., 2015; Shehata et al., 2013; Theriot et al., 2014) and atrazine interacts with bacteria in the environment (Newcombe & Crowley, 1999). If atrazine does alter the microbiota, we hypothesize that these changes in microbiota will be associated with changes in defences against *Bd* (Figure 1, *paths c-e*). Specifically, we determined whether the abundance of phylum Fusobacteria, which has been shown to affect infectious and non-infectious disease risk (Burns, Lynch, Starr, Knights, & Blekhman, 2015; Knutie, Wilkinson, Kohl, et al., 2017; McCoy et al., 2013; Morton et al., 2015; Scher et al., 2013), affects *Bd* infection risk. We also hypothesize that the effects of atrazine on microbiota are mediated by corticosterone (Figure 1, *paths a-f*). If so, then the effect of atrazine on microbiota should be counteracted by the corticosterone synthesis inhibitor metyrapone.

2 | MATERIALS AND METHODS

2.1 | Tadpole collection and husbandry

We collected multiple clutches of tadpoles of *Osteopilus septentrionalis* in August 2014 from the Botanical Gardens of the University of

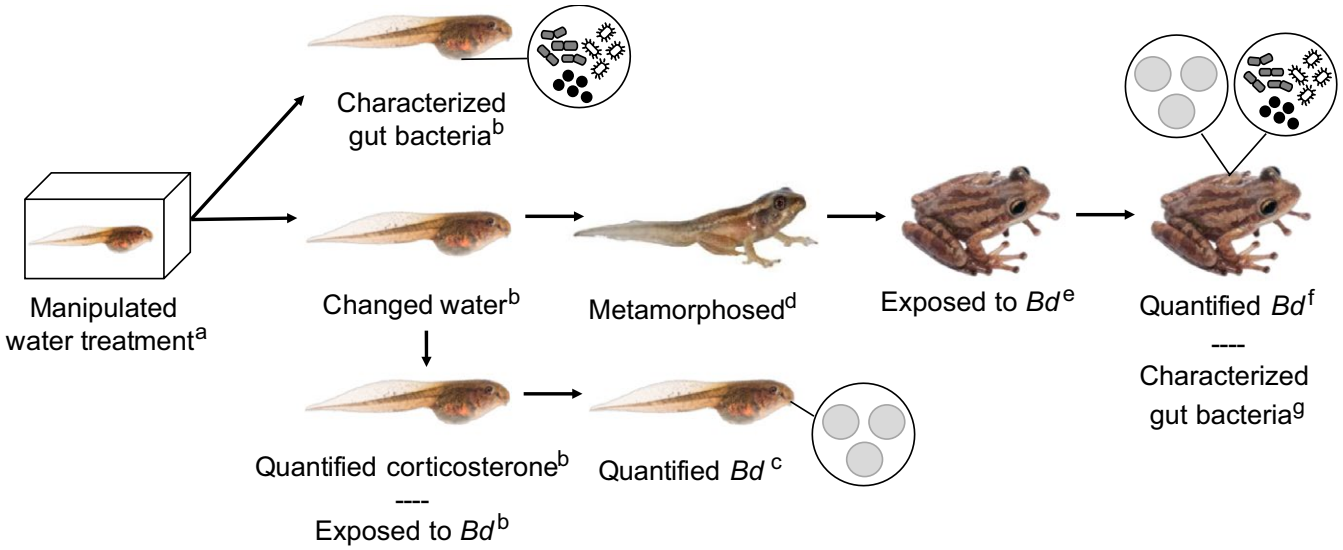

FIGURE 2 Methods to determine the relationships among atrazine exposure, corticosterone levels, host-associated gut microbiota and *Bd* infection risk in Cuban tree frogs. Superscript letters represent the timing of treatment exposure and sampling effort: [a]On day one, tadpoles were placed in tanks with water that was treated experimentally with atrazine and/or metyrapone using a 2 × 2 factorial design (eight tadpoles per tank). [b]On day six, tadpoles from each tank: (1) were euthanized and their gut bacterial community was characterized (n = 1 tadpole per tank), (2) remained in their respective tanks after the pond water was changed to remove residual chemicals from the water treatment (n = ≤3 tadpoles per tank) or (3) were used to quantify their corticosterone levels in response to water treatment and then exposed to *Bd* or a solvent control; four tadpoles from each original tank were either exposed to *Bd* (n = 2) or the solvent control (n = 2) in new tanks. [c]On day 27, *Bd* load was quantified from the skin of tadpoles that were exposed to *Bd* or the solvent control. [d]Tadpoles that remained in their respective tanks, after the experimental water treatment of atrazine and/or metyrapone, were allowed to metamorphose, which occurred, on average, on day 50. [e]On day 84, post-metamorphic (adult) frogs were exposed to *Bd* or the solvent control and then [f]*Bd* was quantified on approximately day 100 from the skin of adults (n ≤ 2 adults per treatment per tank). [g]On day 119, adult frogs were euthanized and their gut bacterial community was characterized. Photographs by Mark Yokoyama

South Florida (N 28°03.537′ W 82°25.410′). We maintained them in the laboratory for at least a week until the majority reached Gosner stage 35 (Gosner, 1960). All tadpoles were fed a mixture of fish food and spirulina suspended in agar ad libitum and were maintained at 21°C with a 12:12 h light:dark cycle. Survival was noted daily.

2.2 | Atrazine and metyrapone exposure in tadpoles

We filled forty 12-L tanks with 8 L of water from a pond at Trout Creek Park, FL (N 28°092250′, W 082°348083′), which was not exposed to agricultural run-off (i.e. no measurable level of atrazine; see below). We assigned 16 *O. septentrionalis* tadpoles haphazardly to each tank. We randomly assigned each tank to one of four exposure treatments: (1) the estimated environmental concentration (EEC) of atrazine (200 μg/L based on US Environmental Protection Agency GENEEC v2 software; Chemservice; technical grade, purity more than 98%) by dissolving atrazine in 120 μl of ethanol (n = 10), (2) 110 μM of metyrapone (Sigma Chemical Co. # M2696) dissolved in 120 μl of ethanol, (n = 10), (3) the EEC of atrazine and 110 μM of metyrapone jointly dissolved in 120 μl of ethanol (n = 11) and (4) only 120 μl of ethanol (n = 10) as a control. We used 110 μM of metyrapone because this level reduced whole-body corticosterone in tadpoles by >50% (but does not block it entirely), and exposure is non-toxic (Glennemeier & Denver, 2002). Previous work did not detect effects of ethanol on any measured trait, and thus a water control was not included (reviewed by Rohr et al., 2013). Tadpoles were exposed to these treatments for 6 days. See Figure 2 for experimental design.

Water samples were collected from each of the 40 tanks 1 hr after dosing, and atrazine was quantified in these samples using the Abraxis ELISA microtiter plate kit (Abraxis LLC). Mean ± *SE* atrazine concentration was 178.2 ± 7.8 μg/L. All atrazine values for the non-atrazine exposed tanks were below the detection limit of 0.06 μg/L (this was the level in the pond water). We redosed each tank with 110 μM of metyrapone every third day (following Hossie, Ferland-Raymond, Burness, & Murray, 2010). We did not redose with atrazine because its half-life is on the order of weeks, and Rohr et al. (2004) found no detectable breakdown of atrazine over 7 days under similar conditions.

After 6 days in the treated water, we measured the snout-vent length (SVL; mm) and body mass (to the 0.001 g) of one tadpole from each tank (41 total). We removed the digesta (guts) of each tadpole using sterile technique, and guts were frozen at −80°C until DNA extraction. We also obtained water-borne corticosterone from two tadpoles per replicate (80 total). Briefly, we placed tadpoles individually in 250-ml beakers filled with 75 ml of water for 1 hr then measured their body mass and SVL. Water samples were frozen at −20°C immediately after collection until hormone extraction.

We also tested the direct effect of atrazine on gut bacteria of tadpoles in vitro. Ten tadpoles (that were not used in the in vivo experiment) were staged and measured before they were euthanized; their whole guts were then placed in a sterile 200-ml Nalgene glass bottle containing either atrazine in an ethanol solvent (n = 5) or solvent only (n = 5). Bottles from the atrazine treatment contained 50 ml sterile deionized water and atrazine (200 μg/L) dissolved in 0.1 ml of ethanol, and bottles from the control treatment contained 50 ml sterile deionized water with only 0.1 ml of ethanol. We swabbed the solution after the guts were added to the bottles but immediately (<1 min) before the atrazine was added and 24 and 144 hr (6 days) after the atrazine was added to collect a subsample of the bacteria. Swabs were swirled in the solution for 3 s then placed in a 1.5-ml tube and immediately frozen at −80°C until the DNA extraction.

2.3 | *Bd* exposure in tadpoles

A subset of eight tadpoles from each replicate was exposed to either *Bd* (SRS812 isolate; McMahon, Romansic, & Rohr, 2013) or a solvent control. Briefly, tadpoles were removed from each tank and divided between two 6-L plastic shoeboxes with 2 L of fresh pond water (n = 4 tadpoles per tank and 80 total tanks); one of the pairs of shoeboxes received a 6-ml inoculum containing 7×10^4 *Bd* zoospores /ml in deionized (DI) water, and the other received an inoculum that was identical to the *Bd* inoculum but was free of *Bd* (i.e. we washed clean agar plates with DI water). We re-exposed all tadpoles to *Bd* (2 ml of 3×10^5 zoospores/ml) or DI water 3 days later and maintained the tadpoles in these boxes for a total of 21 days. We then euthanized tadpoles with an overdose of MS-222 and measured their mass and SVL. We used the quantitative PCR procedure described by Boyle, Boyle, Olsen, Morgan and Hyatt (2004) to quantify *Bd* samples taken from up to two tadpoles per *Bd*-exposed tank (depending on survival, n = 69 total), and a total of 10 tadpoles (each from separate tanks) that were not exposed to *Bd*.

2.4 | *Bd* exposure in adults

The remaining subset of tadpoles was reared through metamorphosis. Their water was changed after the 6-day chemical treatment, and water was subsequently changed every 2 weeks until all tadpoles metamorphosed. When frogs had all four limbs, individuals were removed from the tanks and placed in cups (6 cm high × 12 cm diameter) with moist *Sphagnum* sp. moss. The post-metamorphic frogs were maintained in the laboratory (12 hr light cycle, 22°C) and fed ad libitum vitamin- and mineral-dusted crickets twice per week. Eighty-four days after the start of the experiment and *c*. 1 month after most of the tadpoles metamorphosed, adult frogs were randomly assigned to receive an inoculum of either *Bd* (isolate SRS812) or solvent control (each tank had one to two frogs exposed to each treatment depending on survival). Adult frogs were weighed then exposed to *Bd* by pipetting 1 ml of 6×10^4 zoospores/ml onto the frog's dorsal side. Excess inoculum remained in each frog's plastic container, which contained moist sterile *Sphagnum* moss. Control frogs received the inoculum without *Bd*. Survival was monitored daily for 5 weeks. Frogs were also weighed weekly and swabbed at 2 and 3 weeks after *Bd* exposure. *Bd* from the swabs was quantified using the qPCR methods described above. Frogs were then euthanized using an overdose of Anbesol®, which was applied to the dorsal side of the frog. Frogs were then weighed, their SVL was

measured, and their guts were removed using sterile technique. The guts were then frozen at −80°C until DNA extraction.

2.5 | Hormone extraction and validation

We extracted water-borne hormones following Gabor et al., (2017). We resuspended the dried hormone residue in 260 μl enzyme immunoassay (EIA) buffer (provided by Cayman Chemicals Inc.), and we further diluted all samples to 1:2. We measured corticosterone in duplicate using a corticosterone EIA kit (Cayman Chemicals Inc.) on a spectrophotometer plate reader set to 405 nm (BioTek ELX800). We ran four plates, and based on our control samples, our intra-plate variation ranged from 0.09% to 4.01% and the interplate variation was 6.02%. We previously validated the use of water-borne corticosterone collection method from *O. septentrionalis* on EIA plates (Gabor et al., 2017).

2.6 | Bacterial DNA extraction and sequencing

We isolated total DNA from frog guts using a MoBio PowerFecal DNA Isolation Kit; DNA extracts were then sent to Argonne National Labs for sequencing. Microbial inventories were conducted by amplifying the V4 region of the 16S rRNA gene using primers 515F and 806R and paired-end sequencing on an Illumina MiSeq platform (Caporaso et al., 2012). Sequences were analysed using QIIME version 1.9.1 (Caporaso, Kuczynski, et al., 2010). We applied standard quality control settings and split sequences into libraries using default parameters in QIIME. Sequences were grouped into operational taxonomic units (OTUs) using pick_open_reference_otus.py with a minimum sequence identity of 97%. The most abundant sequences within each OTU were designated as a "representative sequence" and aligned against the Greengenes core set (DeSantis et al., 2006) using PyNAST (Caporaso, Bittinger, et al., 2010) with default parameters set by QIIME. A PH Lane mask supplied by QIIME was used to remove hypervariable regions from aligned sequences. A phylogenetic tree of representative sequences was built using FastTree (Price, Dehal, & Arkin, 2009). OTUs were classified using UCLUST (Edgar, 2010) against the Greengenes database (DeSantis et al., 2006). Singleton OTUs and sequences identified as chloroplasts or mitochondria were removed from the analysis. Additionally, any OTUs present in the "blank samples" were considered contaminants and were removed from all other samples.

Several measurements of alpha diversity were calculated. We calculated the number of observed OTUs (species richness), equitability (species evenness), the Shannon index and Faith's phylogenetic diversity (Faith, 1992), the latter of which measures the cumulative branch length on the phylogenetic tree of all representative sequences. For these measurements, we calculated the mean of 20 iterations for a random subsampling of 6,800 sequences for in vivo tadpole and adult samples and 570 sequences for in vitro samples (the minimum number of sequences returned from each sample). We calculated unweighted and weighted UniFrac distances between samples in QIIME for bacterial community composition analyses.

2.7 | Statistical analyses

We used a GLM to determine the effect of water treatments (atrazine and metyrapone) on gut bacterial diversity of tadpoles (in vivo). We used a GLMM to determine the direct effect of atrazine on gut bacterial diversity of tadpoles (in vitro) with bottle (i.e. replicate) as a random effect. We also used GLMs to determine the relationship between bacterial diversity and log *Bd* infection intensity in tadpoles and adults (measure of resistance), corticosterone release rates in tadpoles and bacterial diversity of tadpoles and adults, and bacterial diversity of tadpoles and bacterial diversity of adults. We also determined the effect of bacterial diversity on host tolerance by testing for interactions between *Bd* intensity and the treatments on mass loss using GLMs. We did not include atrazine treatment as a fixed effect in our analyses of the relationship between bacterial diversity and infection intensity because we did not find evidence that atrazine affected the gut microbiota. Tadpole and adult samples were collected from different individuals within the same replicate (tank) because tadpole sampling required destructive sampling.

We used GLMM with Gaussian errors and tank as a random effect to determine the effect of tadpole water treatment on gut bacterial diversity of adults. We only used tank as a random effect for analyses with adults because we often had more than one individual per tank, whereas for tadpoles, we only sampled one individual per tank. We present Faith's phylogenetic diversity as our measure of alpha diversity in the main text because it accounts for phylogenetic differences among taxa. Results based on other alpha diversity measurements can be found in the Supplemental Tables. Gaussian analyses without and with random effects were conducted using the glm (GLM) and lmer (GLMM) functions in the LME4 package. We generated ANOVA tables using the Anova function in the car package in RStudio (version 0.98.1062).

We determined the effect of tadpole water treatment on gut bacterial community membership (unweighted) and structure (weighted) with PERMANOVAs (with 999 permutations) using the PERMANOVA add-on to the software PRIMER. For adults, tank was included as a random effect. Unweighted scores represent bacterial community membership, which is based on the presence or absence of bacterial taxa, whereas weighted scores represent bacterial community structure, which also takes into account relative abundance of bacterial taxa.

To compare relative abundances of microbial taxa across treatments, we first removed any phyla that were present in less than 25% of samples (White, Nagarajan, & Pop, 2009). Given that the gut microbial community is largely restructured over the course of metamorphosis (Kohl, Cary, Karasov, & Dearing, 2013), we compared relative abundances of bacteria in tadpoles and adult frogs separately. We determined the effect of tadpole water treatment (atrazine and metyrapone) on relative abundances (arcsine square root transformed; Kumar, Mason, Brooker, & O'Brien, 2012; Shchipkova, Nagaraja, & Kumar, 2010) of bacterial phyla in tadpoles and adults using ANOVAs in JMP (version 12) with water treatment as an independent variable and for adults, tank as a random effect. For these analyses, *p*-values were corrected using the Benjamini–Hochberg

False Discovery Rate for multiple comparisons. See Table S1 for individual- and tank-level sample sizes for analyses on the effect of treatment on bacterial communities.

3 | RESULTS

3.1 | Effect of water treatment on microbiota of tadpoles

Tadpole water treatment did not significantly affect bacterial alpha diversity (Figure 3a; Tables S1 and S2), community structure (PERMANOVA, atrazine: $F_{1,36} = 0.79$, $p = .59$; metyrapone: $F_{1,36} = 2.00$, $p = .07$; interaction: $F_{1,36} = 0.77$, $p = .62$), or community membership in tadpoles in vivo (atrazine: $F_{1,36} = 0.90$, $p = .69$; metyrapone: $F_{1,36} = 1.10$, $p = .21$; interaction: $F_{1,36} = 0.99$, $p = .46$). Similarly, tadpole corticosterone release rates were not related significantly to bacterial diversity in tadpoles (Table S5; GLM, $\chi^2 = 0.05$, $df = 1$, $p = .82$). Metyrapone exposure significantly increased relative abundance of phyla Actinobacteria (one-way ANOVA, $F = 10.66$, $p = .04$) and Verrucomicrobia ($F = 12.08$, $p = .04$) in tadpoles. More specifically, metyrapone exposure significantly increased the relative abundance of genus *Mycobacterium* from phylum Actinobacteria ($F = 14.39$, $p = .02$) and genus *Candidatus Xiphinematobacter* from phylum Verrucomicrobia ($F = 17.17$, $p = .007$). However, atrazine and the interaction between atrazine and metyrapone did not significantly affect abundances of microbial taxa in tadpoles (all $p > .05$).

For the in vitro tadpole experiment, bacterial phylogenetic diversity decreased over time (Tables S3 and S4; GLMM, $\chi^2 = 5.91$, $df = 1$, $p = .02$). However, there was no effect of atrazine on bacterial diversity (Tables S3 and S4; $\chi^2 = 0.37$, $df = 1$, $p = .54$) or an effect of the interaction between time and treatment on diversity ($\chi^2 = 0.09$, $df = 1$, $p = .76$). Atrazine also did not affect abundances of microbial taxa (one-way ANOVA, all $p > .05$), community structure (PERMANOVA, $F_{1,8} = 1.40$, $p = .19$) or community membership ($F_{1,8} = 1.05$, $p = .36$) after 6 days of atrazine treatment.

3.2 | Effect of water treatment on microbiota of adults

Gut bacterial diversity of tadpoles did not predict bacterial diversity of adults (Table S6). Adults exposed to metyrapone as tadpoles had significantly higher bacterial diversity ($n = 9$, 69.47 ± 1.89; Figure 3b; Tables S1 and S7) compared to adults not exposed to metyrapone ($n = 15$, 58.37 ± 2.71). Interestingly, however, bacterial diversity of adults was not significantly related to corticosterone release rates in tadpoles from the same tanks (Table S5; GLM, $\chi^2 = 0.97$, $df = 1$, $p = .32$). Metyrapone exposure did not significantly affect abundances of microbial taxa (one-way ANOVA, all $p > .05$), community structure (PERMANOVA, $F_{1,49} = 0.87$, $p = .48$) or community membership in adults ($F_{1,49} = 0.97$, $p = .52$). Atrazine and the interaction between metyrapone and atrazine also did not significantly affect bacterial alpha diversity (Figure 3b; Tables S1 and S6), community structure (atrazine: $F_{1,49} = 0.81$, $p = .52$; interaction: $F_{1,49} = 0.98$, $p = .38$) or community membership (atrazine: $F_{1,49} = 0.89$, $p = .79$; interaction: $F_{1,49} = 1.16$, $p = .12$). However, exposure to atrazine as tadpoles significantly increased the relative abundance of the genus *Desulfovibrio* (one-way ANOVA, $F = 12.99$, $p = .008$) and decreased the abundance of *Delftia* ($F = 11.51$, $p = .01$) in adults.

3.3 | Host-associated microbiota and defences against *Bd*

Gut bacterial diversity of tadpoles did not significantly predict *Bd* intensity of tadpoles nor did bacterial diversity of adults significantly predict *Bd* intensity of adults (Figure 4a,c; Table S8). However, bacterial diversity of tadpoles negatively predicted *Bd* intensity in adults raised in the same tanks (Figure 4b; Table S8). More specifically, the relative abundance of phylum Fusobacteria in tadpoles negatively predicted *Bd* intensity in adults (Figure 5; Table S8). The gut bacterial diversity did not significantly affect host tolerance (measured using the reaction norm between *Bd* intensity and change in mass during

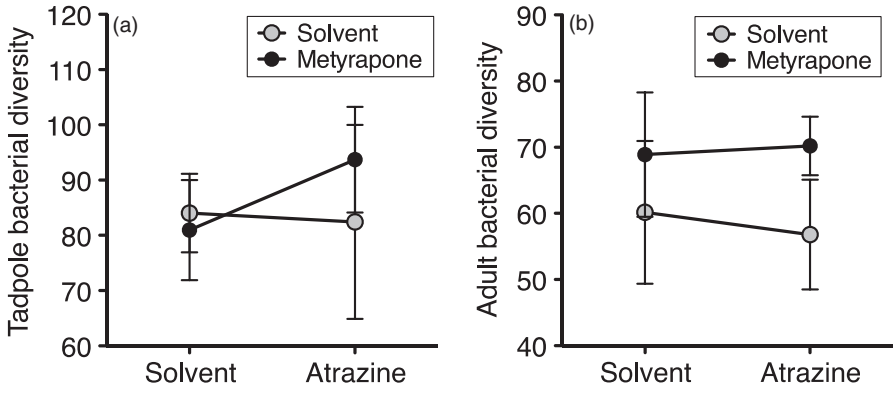

FIGURE 3 Mean (± 95% CI) alpha Faith's bacterial diversity (phylogenetic diversity metric) across water treatments for samples from the guts of (a) tadpoles and (b) adults. Water treatment did not significantly affect bacterial diversity of tadpoles (GLM, atrazine: $\chi^2 = 1.85$, $df = 1$, $p = .17$, metyrapone: $\chi^2 = 0.66$, $df = 1$, $p = .42$, interaction: $\chi^2 = 2.19$, $df = 1$, $p = .14$), but adults exposed to metyrapone as tadpoles had significantly higher bacterial diversity compared to adults that were not exposed to metyrapone (GLMM, atrazine: $\chi^2 = 0.20$, $df = 1$, $p = .65$, metyrapone: $\chi^2 = 4.56$, $df = 1$, $p = .03$, interaction: $\chi^2 = 0.54$, $df = 1$, $p = .47$)

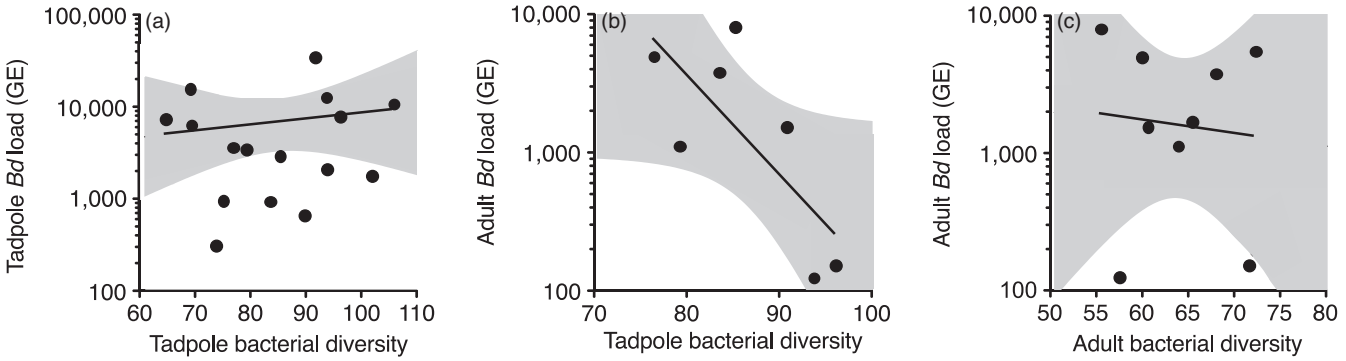

FIGURE 4 Relationship between bacterial phylogenetic diversity in the guts and *Bd* intensity (zoospore genetic equivalent [GE]). Bacterial phylogenetic diversity in tadpoles did not predict *Bd* intensity in tadpoles (n = 16 tanks; GLM, χ^2 = 0.22, df = 1, p = .64), but significantly predicted *Bd* intensity in adults (n = 7 tanks; χ^2 = 6.22, df = 1, p = .01). Bacterial diversity in adults did not predict *Bd* intensity in adults (n = 9 tanks; χ^2 = 0.05, df = 1, p = .83)

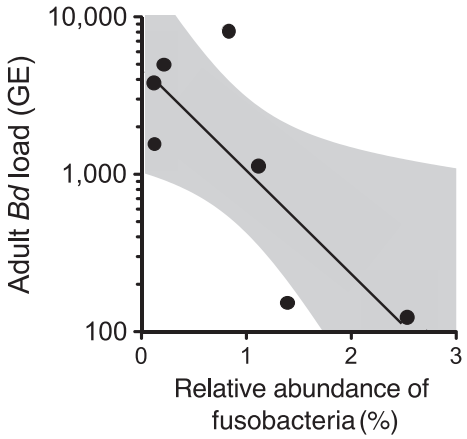

FIGURE 5 Relative abundance of phylum Fusobacteria in tadpoles significantly predicted *Bd* intensity (zoospore genetic equivalent [GE]) later in life (n = 7 tanks; GLM, χ^2 = 8.48, df = 1, p = .004)

infection) of *Bd* intensity in tadpoles (n = 18) (Table S9) or adults (n = 9 tanks) (Table S10).

4 | DISCUSSION

Our study found that early-life exposure to atrazine did not generally affect gut microbiota of tadpoles or adults (Figure 1, *path c*). Exposure to metyrapone also did not affect microbiota of tadpoles, but significantly increased gut bacterial diversity of adults, which was not driven by corticosterone release rates in tadpoles (Figure 1, *path f*). Host-associated microbiota at the time of *Bd* exposure did not affect *Bd* intensity in tadpoles or adults. However, early-life gut bacterial diversity of tadpoles negatively predicted *Bd* intensity in adult frogs (Figure 1, *path e*). Specifically, higher relative abundances of phylum Fusobacteria in tadpoles were associated with decreases in *Bd* intensity in adults. These results suggest that host-associated microbiota do not mediate the effect of atrazine and/or corticosterone on *Bd* infection risk (Figure 1, *paths c-e* and *c-d-b*), but instead, the early-life microbiota itself likely predicts later-life resistance to infection (Figure 1, *path e*).

With the exception of changing the relative abundances of two bacterial genera in adulthood, atrazine did not affect gut bacterial diversity or community composition of tadpoles (in vivo or in vitro) or adults (Figure 1, *path c*). Other studies have found an effect of pollutants, such as PCBs and antibiotics, on microbial communities (Kohl et al., 2015; Schwarz et al., 2016; Shehata et al., 2013; Theriot et al., 2014), but in several cases, the concentrations of these chemicals were quite high. There are several potential reasons why atrazine exposure did not affect the microbiota of tadpoles or adults. First, atrazine might not directly (e.g. via toxicity in soil microbes, DeLorenzo, Scott, & Ross, 2001) or indirectly (e.g. via host physiology) affect bacterial communities in hosts. Second, the concentration of atrazine that we used in our study might not have been effective at altering gut bacterial communities in the frogs. In the environment, tadpoles could be exposed to atrazine concentrations up to 1,000 μg/L (Graymore, Stagnitti, & Allinson, 2001) and perhaps the EEC concentration that we used was too low to affect bacterial communities. Alternatively, the microbiota might have a non-monotonic dose response to atrazine, where only low and high concentrations affect microbiota. Non-monotonic dose–response patterns in relation to the fitness and physiology of frogs have been observed in response to other agrochemicals (McMahon et al., 2011; Shelley, Balfry, Ross, & Kennedy, 2009; Storrs & Kiesecker, 2004), but the mechanism underlying this response remains unknown. A future study could look at the dose response of host-associated microbiota to atrazine in vivo and in vitro to determine if there is an effect of atrazine on microbiota at different concentrations. Additionally, although we did not find an effect of atrazine on bacterial diversity metrics, we did find that bacterial phylogenetic diversity decreased by *c.* 30% over the 6-day treatment period, which suggests that the in vitro experiment itself affected bacteria survival. We also did not account for the degradation of tissue in the experiment, which may have influenced bacterial communities. Future in vitro experiments should attempt to exclude the gut tissue in the experiment and determine if there are more desirable conditions for the bacteria (e.g. temperature and light conditions).

Like atrazine, metyrapone did not have a significant effect on the microbiota of tadpoles. In contrast, exposure to metyrapone as tadpoles had a lasting effect on the microbiota of adults, but this effect was not related to corticosterone release rates in tadpoles. These results suggest that metyrapone did not solely affect corticosterone production but also had non-target effects, which, in turn, had lasting effects on gut bacterial communities. One example of a non-target effect of metyrapone is that it can decrease aldosterone production (Tucci, Espiner, Jagger, & Lauler, 1967), which is a hormone responsible for the reabsorption of sodium and water reabsorption in the gut, and the regulation of extracellular potassium and blood pressure (Randall, Burggren, & French, 2002). In turn, these physiological changes may be responsible for the increase in gut bacterial diversity of adults in response to metyrapone, but this hypothesis requires further investigation.

Host-associated microbiota do not appear to have a direct effect, or mediate the effect of atrazine or corticosterone, on host tolerance against *Bd* (Figure 1, *paths c-e*, *a-f-e*). Instead, the effect of atrazine on host tolerance of *Bd* is likely caused by direct effects of atrazine, such as energy lost to atrazine detoxification or repair from damage caused by atrazine (Figure 1, *paths a-b*) (Nieves-Puigdoller, Bjornsson, & McCormick, 2007). Alternatively, these effects could be from indirect effects of atrazine on unmeasured hormones, such as thyroxine. For example, atrazine exposure in salamanders can increase their thyroxine levels, a hormone associated with amphibian condition (Larson, McDonald, Fivizzani, Newton, & Hamilton, 1998), which, in turn, could affect the ability of hosts to withstand the effects of *Bd*. These factors serve as candidate mechanisms that could mediate the effect of atrazine on host tolerance to *Bd* and should be tested in the future.

Gut bacterial diversity at the time of *Bd* exposure did not affect host resistance or tolerance in tadpoles or adult frogs (Figure 1, *path e*). In contrast, several other studies have found that the symbiotic microbiota of hosts can increase resistance to infection (Koch & Schmid-Hempel, 2011; Schuijt et al., 2016; Theriot et al., 2014). These conflicting findings may be explained by the mechanism by which microbiota affect infection risk. Previous studies mostly examined the relationship between microbiota and pathogens in the gut of the host, which suggests that the microbiota may help the host resist the pathogen by either directly competing with it (Figure 1, *path e*) or locally upregulating the immune system (Figure 1, *paths d-b*). It is possible that the gut microbiota at the time of *Bd* exposure does not affect resistance to *Bd* on the skin. Instead, during *Bd* exposure, the symbiotic microbiota on the skin, particularly *Janthinobacterium lividum*, *Pseudomonas* sp. and *Rhodococcus fascians*, likely promote resistance to *Bd* (Woodhams et al., 2016). These results suggest that skin and gut microbiota have different modes of action at different life stages to protect frogs against *Bd*.

Gut bacterial phylogenetic diversity in tadpoles was negatively correlated with *Bd* intensity in adults, which suggests that the microbiota might be priming immune system development. Interestingly, this pattern was not significant with regards to the other diversity metrics (i.e. Shannon index, species richness and species evenness) suggesting that the phylogenetic relatedness of bacterial taxa influences later-life infection risk. Similarly, Knutie, Shea, et al. (2017) found that bacterial diversity of tadpoles was positively related to later-life resistance to parasitic gut nematodes. This suggests that either (1) the microbiota of tadpoles primes multiple components of the immune system that affect resistance to a diversity of parasites in different regions of the body or (2) the microbiota primes a specific immune response that is effective against both *Bd* and parasitic worms. Previous studies support the latter hypothesis by showing that frogs produce an IgY antibody response to both parasitic worms (Knutie, Shea, et al., 2017; Knutie, Wilkinson, Wu, Ortega, & Rohr, 2017) and *Bd* (Ramsey, Reinert, Harper, Woodhams, & Rollins-Smith, 2010). Germ-free mice devoid of bacteria exhibit lower analogous IgG antibody production to pathogens when compared to conventional mice (Slack et al., 2009). Thus, the IgY antibody response could provide a candidate immune mechanism for our results that could be explored in future studies.

Particular bacterial taxa can affect the immune system of hosts (Fulde & Hornef, 2014; Kabat, Srinivasan, & Maloy, 2014; Rakoff-Nahoum et al., 2015); thus, our findings might provide insight into which bacterial taxa could have driven the long-term changes in host resistance to infections (Figure 1, *paths d-b*). In our study, the relative abundance of Fusobacteria in tadpoles was negatively correlated with *Bd* intensity in adults. In previous studies, higher relative abundance of Fusobacteria was related to lower prevalence of infectious and non-infectious diseases in both human and non-human vertebrate hosts (Burns et al., 2015; Knutie, Wilkinson, Kohl, et al., 2017; McCoy et al., 2013; Morton et al., 2015; Scher et al., 2013). This suggests that Fusobacteria are important in predicting immunity and disease risk. However, all previous studies are correlational and require experimental tests to determine the causal link between phylum Fusobacteria and the immune system, including the mechanism (e.g. butyrate production; Furusawa et al., 2013) driving the relationship between Fusobacteria and immunity.

The fungal pathogen *Bd* is responsible for the decline and extinction of many amphibians worldwide (Wake & Vredenburg, 2008). Determining which factors affect amphibian risk of *Bd* infection might help mitigate the effect of *Bd*-driven population declines. Even though we did not find that host-associated microbiota mediate the effect of atrazine on *Bd* infections, our work suggests that there are critical windows in development where the loss of microbiota can have adverse persistent effects on infection risk. Specifically, increasing the presence of Fusobacteria during formative times of development may decrease infection risk later in life. Overall, our study suggests that increasing gut bacterial diversity and relative abundance of Fusobacteria might have lasting positive effects on amphibian health.

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AUTHORS' CONTRIBUTIONS

S.A.K., C.G. and J.R.R. conceived the ideas and designed methodology; S.A.K. and C.G. collected the data; S.A.K. and K.D.K. analysed the data; S.A.K. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

The authors declare that all other relevant data supporting the findings of the study can be found on figshare https://doi.org/10.6084/m9.figshare.5417629 (Knutie, Gabor, Kohl, & Rohr, 2017).

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SUPPORTING INFORMATION

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