Title: Skin defenses and host-environment microbiome interactions in spotted salamanders

Running title: Skin defenses in spotted salamanders

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Abstract

Emerging infectious diseases have been of particular interest as a major threat to global biodiversity. In amphibians, two fungal sister taxa, *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans* (Bsal) along with the viral pathogen ranavirus have affected global populations. Factors such as host traits, abiotic and biotic environmental conditions, and pathogen prevalence contribute to species specific disease susceptibility. The eastern United States is home to the Appalachian Mountain system, known as a “hotspot” for salamander biodiversity. Bd and ranavirus are present throughout the Appalachians, and a Bsal emergence could be imminent. Also throughout the Appalachians are the spotted salamanders, *Ambystoma maculatum*, a mostly terrestrial salamander that participates in mass breeding migration in the late spring. Previous experimental studies have shown that spotted salamanders appear to be resistant to Bd and Bsal infection, but the mechanism of this defense remains unknown. Spotted salamanders emerging from their overwintering habitats were hypothesized to have potent anti-Bd function expressed in their mucus and in their skin microbiomes, as a countermeasure to pathogen presence. We used non-invasive sampling at two pools during the spotted salamander annual breeding event to (I) determine pathogen prevalence, (II) quantify the antifungal potential of salamander skin mucus, and (III) characterize the diversity and composition of the salamander skin microbiome and contrast it to that of the corresponding environmental microbiome. We did not detect any Bd, Bsal, or ranavirus in the salamanders. The salamander mucus did not inhibit Bd growth in-vitro and few anti-Bd bacteria at low relative abundance were present in the microbiome. The salamander microbiome sourced a proportion of bacteria from the environment and appears to select rare taxa from their respective pools, however their functional relevance in pathogen defense is unclear. Our results suggest that the spotted salamander mucosal secretions or skin microbiome do not appear to be the mechanisms of defense against Bd and Bsal during post-winter emergence. Instead, we suggest that a synergistic mechanism within the salamander mucosal environment (e.g., AMPs-microbiome synergies) or skin immune cells may confer resistance. This study contributes to the understanding of salamander intra- and interspecies variation in disease susceptibility.

Introduction

Global biodiversity faces threats from a variety of sources, which include habitat loss (Cushman 2006), climate change (Blaustein et al. 2010), overexploitation (Stuart et al. 2004) and emerging infectious diseases (Fisher et al. 2020). Emerging infectious diseases have been of particular interest as a major causal factor in amphibian declines and extinctions (Luedtke et al. 2023). The role of amphibian immune and microbial defenses in observed differential species susceptibility during epidemics have been of increasing interest (Madison et al. 2017; Scheele et al. 2019; Grogan et al. 2023). Understanding the origin of host defenses against emerging infectious diseases and the mechanisms that promote them can reveal key processes in host-microbial ecology and evolution, which may be critical to preserving amphibian biodiversity in the event of disease outbreaks (Smith et al. 2018a; Ribas et al. 2022; Longo et al. 2019).

In the eastern United States, the Appalachian Mountain system is home to the most speciose group of salamanders in the world (Petranka and Smith 2005). In the Appalachians, three pathogens have been of concern to salamander biodiversity including two fungal sister taxa, *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans (*Bsal) (Martel et al. 2014; Scheele et al. 2019; Lötters et al. 2024) and double-stranded DNA viral pathogens in the genus *Ranavirus* (Sutton et al. 2015; Bartlett et al. 2021). The spread of the amphibian chytrids, Bd and Bsal, have exacerbated global declines in at least 500 amphibian species (Scheele et al. 2019). Bd is now enzootic in the US and examining host-pathogen trade-offs is of interest in disease ecology (reviewed in Van Rooij et al. 2015; Grogan et al. 2023; Rollins-Smith 2024). Fortunately, Bsal has not yet been detected in the US (Waddle et al. 2020), but a Bsal introduction could alter extinction-speciation trends in Appalachian salamanders (Martel et al. 2014, DiRenzo et al. 2022, Gray et al. 2023). *Ranavirus* is widespread in the US and their prevalence is likely underestimated in the Appalachians (Mosher et al. 2018; Bartlett et al. 2021; Millikin et al. 2023). Previous die-offs of Appalachian amphibians have been attributed to *Ranavirus* (Green et al. 2002) and species susceptibility is higher in vernal pool breeding amphibians, particularly in larvae (Hoverman et al. 2011), with limited sampling in adults.

Amphibians have a variety of host immune characteristics to combat pathogen infections (Grogan et al. 2018, 2023). Many salamander species are considered tolerant to Bd infection such as the eastern newt

(*Notophthalamus viridescens*) or resistant to infection, such as the red-backed salamander (*Plethodon cinereus*) (Muletz Wolz et al. 2018; Jiménez et al. 2022). The amphibian skin mucus layer and its innate defenses are the first physical barrier to pathogens. Peptides, secondary bacterial metabolites, and symbiotic microbes make up each species' unique mucus composition and are predicted to be a key factor in innate resistance mechanisms (Smith et al. 2018a; Jiménez et al. 2022; Rosa et al. 2022; Rollins-Smith 2023). Additionally, host-associated microbial communities are known to contribute to pathogen defense and maintain host health. However, the mechanisms of interaction between the host microbiome and invading pathogens are not clear. Previous *in vitro* studies have shown the Bd-inhibitory potential of bacteria present on amphibian skin (e.g. *Janthinobacterium lividum*, *Serratia marcescens, Stenotrophomonas sp.* and *Chryseobacterium sp.*), yet few of these putative anti-Bd bacteria consistently inhibit chytrid *in vivo* (Brucker et al. 2008; Park et al. 2014; Madison et al. 2019; Muletz-Wolz et al. 2019a). Specific interactions between bacterial taxa, the host, and the environment are likely altering chytrid susceptibility. Examining the host and environmental microbiome in parallel can help reveal the mechanisms of host microbe filtering and mucosal defense.

To examine pathogen occurrence and mucosal defenses our study focused on the spotted salamander, *Ambystoma maculatum*, a primarily terrestrial salamander with a distribution throughout the Appalachian Mountains. Spotted salamanders can be found infected with ranavirus as larvae in the wild (Millikin et al. 2023) and as adults can become infected with Bsal and Bd (Patillo 2019), but generally considered resistant based on experimental trials (Martel et al. 2014; Barnhart et al. 2020; Gray et al. 2023). Spotted salamanders participate in mass breeding events from their overwinter habitats to vernal pools and ponds in late winter and early spring. This annual event could provide suitable conditions for pathogen transmission and impact host-microbiome interactions. We sampled adult spotted salamanders as they migrated from their overwintering habitat into two breeding sites in Maryland with three objectives (I) determine the prevalence of Bd, Bsal, and ranavirus as well as individual's infection intensity, if infected (II) quantify the inhibitory potential of salamander skin mucus against Bd, and (III) characterize the diversity and composition of the spotted salamander skin microbiome and contrast it to that of their pool environmental microbiome. The salamanders emerging from their overwintering habitats were hypothesized to have potent anti-Bd function expressed in their mucus and in their skin microbiomes, as

a countermeasure to pathogen presence. Our research aims to uncover microbial mechanisms related to *Batrachochytrium* susceptibility and contribute to the conservation of wild amphibian species in Maryland and the Appalachian region.

Methods

Sample collection

We sampled a total of 30 spotted salamanders (visibly sexed to 26 males and 4 females) at one vernal pool and one semi-permanent pond in the Frederick City Municipal Forest in Frederick County, Maryland (Figure S4, 39.563769, -77.478283) on March 6th and 9th, 2024 (Table S1). The vernal pool is referred to as the small pool, while the pond is referred to as the big pool. The small pool is a natural ephemeral pool and is filled by a nearby pond and rainfall in early spring. The big pool is of unknown origin but is likely man-made similar to the surrounding stocked ponds. We had permits from Maryland DNR (#58925) and approval from Smithsonian IACUC (#SI-24006) for sampling.

Adult spotted salamanders were captured by hand or dip net. Then, each salamander was placed briefly in a deli cup (rinsed with pond water) for transport to sampling location, rinsed with sterile MilliQ water and then placed in a sterile Whirl-Pak bag. All salamanders were handled with high-density polyethylene gloves (food safe deli gloves) as opposed to powder free nitrile gloves to reduce the likelihood of powder free nitrile gloves inhibiting Bd or Bsal growth (Thomas et al. 2020). Then, each salamander was swabbed five times on one forelimb, one hindlimb, their ventrum, dorsum, and tail resulting in 25 streaks per salamander. Swabs were placed in 1.5 mL tubes containing a 70/30 silicon bead mix (70% 0.1mm and 30% 0.5 mm by volume) and immediately placed on dry ice. After swabbing, we measured each salamander's mass and snout-vent length (SVL) and determined their sex. Males were smaller with a very pronounced vent, and this was the key feature in sexing the salamanders. Females were larger with an enlarged midsection. Then, each salamander received a 20-minute water bath in sterile MilliQ water to obtain a mucus solution. The volume of water used in each salamander's water bath was based on their mass (Table S2). Following the water bath, each salamander was returned to its initial capture site. The mucus solution was transferred to sterile falcon tubes and immediately placed on dry ice, and then lyophilized in the lab and stored in a -80°C freezer until assays were conducted. For environmental

samples, swabs were placed in unique locations at each sampling site at the interface between the water and the soil surrounding the pools and swished through below surface pool debris for 15 seconds (n = 12; Table S1). Negative controls were twirled through the air for 15 seconds at each sampling site (n = 4). All swabs and mucus samples were transferred and stored at -20°C within 24 hours of collection.

Mucosome-Bd challenge assays

Bd strain GPL1-JEL404 was cultured on 1% tryptone agar plates at 20°C for 7 days to stimulate zoospore production. The Bd isolate is from the eastern United States (Maine) and part of the Global Panzootic Lineage that occurs throughout Appalachia (James et al. 2009). Following incubation, the plates were flooded with 1% tryptone broth, allowed to sit for 20 mins, and then filtered through a 20-micron mesh filter to exclude zoosporangia. Zoospore concentration was counted with a hemocytometer and diluted with 1% tryptone broth to 1 x 10^6 zoospores ml^{-1}. To assess the inhibitory potential of the salamander mucus, 50 µL of re-constituted mucus samples were cultured in quadruplicate with 50 µL of our zoospore solution on 96-well tissue-culture treated polystyrene plates. Quadruplicate control wells consisted of a (i) heat-killed zoospore solution, (ii) a nutrient depleted positive control (NDPC) with 50 µL of zoospore solution and 50 µL sterile water (iii) a positive control with 50 µL of our zoospore solution and 50 µL 1% tryptone and (iv) a sterile water negative control. The positive control wells were used as the threshold for determining Bd augmentation in our experimental wells. 96-well plates were incubated at 20°C and Bd growth was assessed via optical density (OD) at 480 nm on days 0, 4, 5, 7, 11 and 12. Bd inhibition score calculations were performed using OD readings from 0 – 7 days (showing exponential growth) according to Muletz-Wolz et al. (2017) using the NDPC to determine Bd inhibition. Briefly, we visually inspected OD readings and excluded unusually high densities (indicating contamination or error). We corrected for baseline zoospore OD by subtracting the average Bd heat-killed from the experimental wells then log transformed the corrected OD readings. We fit linear regression models to the transformed OD readings over time in each well and extracted the average slope of Bd growth in the NDPC on each plate. We calculated the Bd inhibition score using this equation: [Inhibition score = 1 – (slope sample well / average slope NDPC].

DNA Extraction and sequence processing

DNA was extracted from the swabs using a DNeasy PowerSoil HTP 96 kit (Qiagen) with the bead-beating step consisting of 90 seconds on a Biospec 96 machine. We used qPCR for the quantification of Bd, Bsal, and ranavirus infection using synthesized gene fragments (gBlocks) as in Standish et al. (2018). All swabs were tested in duplicate. We used a one-step PCR library preparation and dual-indexed paired-end sequencing to sequence the microbiome of each salamander skin swab sample and all controls. The V3–V5 region of the 16S rRNA gene (~380 bp) was amplified from samples and controls (field, extraction, and PCR controls) using the universal primers 515F-Y and 939R as fully detailed in Bornbusch et al. (2024) and sequenced the library on one MiSeq (Illumina) run (2 x 300 V3 kit) at the Center for Conservation Genomics at the National Zoo & Conservation Biology Institute, Washington, DC.

16S rRNA Sequencing

Demultiplexed reads were downloaded from Basespace (Illumina) and processed in R version 4.3.2. The package “dada2” (Callahan et al. 2016) was used to perform quality filtering (maxEE = 2), collapsed high quality reads into amplicon sequence variants (ASV) and removed chimeras. Bacterial taxonomy was assigned using Silva (version 138.2). The package “phyloseq” was used to import and merge the final ASV table, taxonomy table, and metadata for downstream analysis. Mitochondria, Cyanobacteria, chloroplasts, and singletons were filtered out. We used “decontam” to remove contaminants (method = combined, threshold = 0.25) as well as ASVs that occurred in 2 or more negative samples. We used BLAST to search against an Antifungal Isolates Database (Update 2020 strict database [>80% inhibition and any facilitating isolate matches removed] received from M. Bletz) and ASVs with 100% identity to anti-Bd bacteria were considered to have Bd-inhibitory activity. We also matched our ASVs to the 2023 Database including all antifungal taxa to compare to estimates of anti-Bd relative abundance of spotted salamanders in Barnhart-McCarty et al. (2024). Sequence counts ranged from 1483 to 14527 sequences per sample. We analyzed alpha and beta diversity metrics with both non-rarefied and rarefied (to 1483 sequences per sample) and found that the statistical inference was the same; we report the results based on the non-rarefied dataset analyses.

Microbiome Analysis

In alpha and beta diversity analyses, we examined if these microbiome measures were influenced by sample type (salamander or environment), sample location (small pool or big pool) or their interaction. For alpha diversity, we examined ASV richness and anti-Bd richness using two-way ANOVA or the non-parametric Scheirer-Ray-Hare (SRH) test, respectively. Anti-Bd relative abundance was calculated using the equation: [anti-Bd ASV count in sample X / count of ASVs in sample X] and we used a SRH test for comparison. For beta diversity, we examined Bray-Curtis dissimilarity and Jaccard distances using PERMANOVAs from the package “vegan” (Oksanen et al. 2024). Then, we used FEAST, a tool for fast expectation-maximization microbial source tracking (Shenhav et al. 2019), to represent the scaled proportions of each salamander microbiome (sinks) that can be attributed to the environmental microbiome (source) and to an ‘Unknown source’ (i.e. all source proportions that cannot be attributed to the environmental microbiome). Shared and unique ASVs were determined with R by finding ASVs that intersect between the adult salamanders and the sample location. To determine if the salamanders were selecting for rare or functionally relevant taxa, we used the R package “ALDEx2”(Gloor et al. 2024) (128 Monte-Carlo simulations, gamma = 0.5) on centered-log ratio transformed data to identify differentially abundant ASVs that are shared between the environmental and salamander microbiomes. We used an effect size threshold greater than 1 or less than -1 and Benjamini-Hochberg corrected p-value from Wilcoxon-test less than 0.05 to determine if an ASV was significantly differentially abundant. Additionally, we performed Kendal's tau ranked correlations for the relative abundance of ASVs between each salamander and its corresponding environment as in Rebollar et al. (2016). Correlations were focused on (i) all ASVs and (ii) ASVs with relative abundance of 0.1% or higher, determined by calculating total relative abundance values of both sample types.

Results

The average salamander weight was 20.97g (SD = 5.47) and snout-vent-length was 92.45 mm (SD = 5.58) (Table S1). There was no Bd, Bsal, or ranavirus detected in any of the salamander swabs. In mucus-Bd assays, we found that spotted salamander skin secretions generally did not inhibit Bd growth. Bd was slightly inhibited in only one sample (WSBP16, $\mu = 4.70\%$) (Figure 1).

The environmental microbiome at the pond/terrestrial interface had higher number of overall bacterial ASVs and anti-Bd bacterial ASVs than salamander skin microbiomes (Figure 2; overall ASVs ANOVA, $F_{1,33} = 12.96$, $p < 0.01$; Anti-Bd ASVs SRH test, $H_{1,33} = 12.72$, $p < 0.001$). Interestingly, while the number of anti-Bd bacterial ASVs were higher in the environment, the relative abundance of that community, the anti-Bd bacteria, were similar between the environment and the salamanders (SRH test, $H_{1,33} = 1.70$, $p = 0.19$), albeit at a relatively low relative abundance (μ = 3.2% and 3.0% respectively). The environmental and salamander overall richness, anti-Bd bacterial richness and anti-Bd bacterial relative abundance were similar between the two pools (Overall ASVs ANOVA, location $p = 0.18$; Anti-Bd ASV SRH test, location $p = 0.87$; Anti-Bd relative abundance ANOVA, location $p = 0.56$). In all three measures, the interaction between sample type and location was not significant. The bacterial community composition differed between salamanders and their environment (Figure 3; Bray-Curtis: $F = 2.61$ $R^2 = 19.2\%$, $p < 0.01$ | Jaccard: $F = 1.75$ $R^2 = 13.7\%$, $p < 0.01$).

The salamander microbiome selected less bacteria from the environment than expected (Figure 4A; 555 shared bacterial ASVs in the big pool and 228 in the small pool). Similarly, our source tracking predicted around 1/3 of the salamander microbiome to be sourced from the environment (Figure S2; FEAST prediction, μ = 29% source, μ = 71% unknown). We found 30 putatively anti-Bd bacterial ASVs that match the strict anti-Bd isolates database (>80% inhibition, strains with matches to facilitating strains removed), however, none were present across all salamander samples. Some anti-Bd ASVs were shared with the environment (Figure 4B; 18 anti-Bd ASVs in the big pool and 10 in the small pool). We found 33 putatively anti-Bd bacterial ASVs that match the non-strict 2023 anti-Bd isolates database (all strains with >0% inhibition) with average relative abundance of 2.8% in the small pool and 3.4% in the big pool. We prefer to use a conservative approach in estimating anti-Bd relative abundance and focus our results on the strict database estimates. Of all the ASVs that were shared between sample types, none were significantly differentially abundant per our threshold (corrected $p < 0.05$ and effect size >1 or < -1). ASV34 (family *Acetobacteraceae*) was the only ASV to meet the effect size threshold with higher abundance in the environment (effect size < -1, $p > 0.05$). In the Kendall's correlation of all ASVs and higher relative abundance ASVs (0.1% or higher) the ASVs on the salamander and their respective environment were significantly negatively correlated, showing that ASVs in higher abundance on

salamander skin were in lower abundance in the environment (Figure S3, all ASVs: small pool: τ = -0.20, $p < 0.001$, big pool: τ = -0.31, $p < 0.00$; >0.1% ASVs: small pool: τ = -0.40, $p < 0.001$, big pool: τ = -0.23, $p < 0.001$).

Figure 1. Spotted salamander skin mucus (mucosome) and Bd (GPL JEL404) challenge assays. Grey dashed line at 0 indicates no inhibition of Bd growth. Red dashed line at -45.9 is the average score of the positive control, which reflects a normal Bd growth pattern.

Figure 2. Bacterial ASV richness and putative Bd-inhibitory(anti-Bd) relative abundance by sampling location. (A) ASV richness and (B) richness of putative anti-Bd ASVs (C) relative abundance of anti-Bd bacterial ASVs. Yellow boxes represent salamander samples, darker blue boxes represent environmental samples.

Figure 3. Bacterial community composition differed between spotted salamanders and their environment similarly at two sampling locations. Bray-Curtis principal coordinates shown. Jaccard principal coordinates showed a similar structure (Figure S4).

Figure 4. Shared and unique bacterial ASVs and anti-Bd bacterial ASVs by sample type and location. (A) Presence/absence count of ASVs in the environment and salamander samples by location (B) Presence/absence count of putative anti-Bd ASVs. Yellow bars are ASVs found only on the salamander skin, while blue bars are ASVs found only in the environment. Grey bars are ASVs shared between salamander and the environment.

Discussion

We sought to determine pathogen prevalence and innate immune defenses in adult spotted salamanders during their annual mass breeding event. We hypothesized that the environmental effects around the time of the breeding event in early-March (cooler temperature, frequent rains, frost melting) would coincide with higher Bd prevalence (Woodhams et al. 2008; Le Sage et al. 2021; Basanta et al. 2023) and to counteract Bd exposures, the immune capabilities of the spotted salamander would be heightened (e.g. increased AMP expression, dominant antifungal microbes). However, Bd was not detected and surprisingly, wild mucus samples showed little to no ability to inhibit the growth of Bd *in vitro* (GPL1-

JEL404). We found that all but one salamander hosted a number of anti-Bd bacterial taxa on their skin. However, their relative abundance was lower compared to estimates found in lab reared spotted salamanders in a Massachusetts, USA population (~50% relative abundance using full anti-Bd 2023 database) (Barnhardt-McCarty et al. 2024), and in other terrestrial Appalachian salamanders (i.e., *Plethodon* species: ~15% relative abundance using strict anti-Bd 2020 database) (Osborne et al. 2024). It is likely that Bd and *Ranavirus* occur at our sampling sites, at least seasonally, as they host various amphibian species known to become infected regularly with Bd (e.g., eastern newts: Jiménez et al. 2022) and *Ranavirus* (e.g., wood frog larvae: Mosher et al. 2018). Perhaps other immune mechanisms or environmental conditions limit susceptibility or exposure. *Ambystoma* salamanders, including spotted salamanders, appear to be resistant to both Bd and Bsal (Patillo 2019; Barnhart et al. 2020; Basanta et al. 2023; Gray et al. 2023; Barnhart-McCarty et al. 2024) with our evidence suggesting that their mucosal defenses are limited against Bd. Therefore, they may serve as useful models to understand the contribution of innate and adaptive immune cells in amphibian chytrid resistance (e.g., Barnhart et al. 2020; Hauser et al. 2024).

Amphibian skin mucus consists of host-defense antimicrobial peptides (AMPs; Pereira and Woodley 2021) larger antimicrobial proteins (Smith et al. 2018b), and bacterial metabolites (Woodhams et al. 2014) that can work synergistically to kill Bd (Myers et al. 2012). Here we tested crude skin mucus samples to examine the naturally available anti-Bd content of the salamander mucus and observed limited inhibition of Bd. Le Sage et al. (2021), similarly found skin mucus from frogs in a cooler environment (4°C compared to 21°C) were less effective at inhibiting Bd (Le Sage et al. 2021). The two nights of collection were recorded at approximately 10°C and 6°C respectively, with average daily temperature ranging from 5°C – 10°C (data via Open-Meteo.com, Zippenfenig 2024). Others have observed seasonal patterns to Bd prevalence, with a general pattern of low Bd prevalence in late-winter, which comes to a peak in spring (Wilber et al. 2022; Saenz et al. 2024)**.** Perhaps Bd prevalence is low, and environmental conditions are less favorable for Bd such that the pressure on the salamanders to upregulate AMP genes and storage following an overwintering event is limited. AMP expression patterns are variable among amphibians, and our results could possibly resemble the wood frog, with no detectable AMP expression until they are acclimated to a warmer temperature (approximately 30°C) (Matutte et al. 2000). However,

we have also observed AMPs gene expression at high levels in Bd-infected red-backed salamanders at cool temperatures (13°C) (Ellison et al. 2020) showcasing variability among amphibian species in AMPs expression that warrants future study.

Salamander microbial communities clustered with each other and were distinct from the environmental community regardless of location. We hypothesize that species-specific host traits and environmental factors that did not differ between sampling locations (Kueneman et al. 2014; Muletz Wolz et al. 2018) explain this observation. The number of overall bacterial richness and anti-Bd richness was higher in the environmental samples than the salamanders, implying a larger pool of micro-organisms for which the salamander microbiome selects a subset (Walke et al. 2014). Interestingly, we also previously observed that pond-associated salamander species (including *Ambystoma jeffersonianum* metamorphs) had lower bacterial richness than their environment, but not forest- or stream-associated species (Osborne et al. 2024), suggesting that some host factor associated with pond life history traits leads to fewer bacteria taxa living on pond-associated salamander skin. Additionally, the salamanders shared less than 50% of their ASVs with their environment suggesting ecological filtering of rare environmental bacteria as they migrate. We found a significant negative correlation between relative abundance of ASVs in the salamander and environment suggesting the salamander skin microbiome is enriched with rare environmental bacteria likely due to the unique structure of the skin (mucus pH, moisture) which favors the growth of specific bacteria, as seen in Rebollar et al. (2016). Overall, the anti-Bd microbes present were lower than we expected suggesting that the selective pressure of Bd, or possibly other fungi in the environment, on the composition of the spotted salamander skin microbiome is limited. Cooler temperatures as experienced during their migration may also explain this lower anti-Bd richness, as some species display a reduction in microbiome diversity in cold climates (Kueneman et al. 2019; Muletz-Wolz et al. 2019b). Anti-Bd microbes and host-defense AMPs can act synergistically to kill Bd (Myers et al. 2012), and it is plausible that a similar interaction is occurring in the spotted salamanders for which we were not able to capture. However, we hypothesize that innate and adaptive immune cells of *Ambystoma* salamanders may have a stronger contribution to their generally observed Bd- and Bsal- resistance.

Our results contribute to our understanding of amphibian mucosal defenses. The factors that stimulate mucus production, pathogen inhibitory potential, and microbiome structure and function are multifactorial. Future studies on identifying salamander skin innate and adaptive immune defenses and their interactions with AMPs, the skin microbiome and immune cells will help uncover possible immune or synergistic effects that confer resistance (e.g. Hauser et al. 2024). Continued research efforts on Appalachian salamanders are important to our understanding of vertebrate disease ecology and salamander conservation. Appalachian salamanders have complex, yet fascinating immune components and a better understanding of the underlying mechanisms will better equip researchers to combat the threat of Bd in a changing climate and a possible spread of Bsal to the United States.

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Data Availability

We deposited demultiplexed sequence data in the National Center for Biotechnology Information Sequence (NCBI) under BioProject ID: PRJNA1220672. All code used for data analysis is available at https://github.com/JulianU-C/SpottedSalamander2024.

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