

**Of Hosts and Habitats: The Ecological and Evolutionary Patterns of the
Amphibian Skin Microbiome**

**A Dissertation Presented for the
Doctor of Philosophy
Degree
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**Benjamin Houston Holt
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DEDICATION

To my parents, whose unwavering support and encouragement over the years and to John Conner, who together, instilled in me what it means to appreciate, understand, and love nature.

To my siblings, Abby and Andrew, who struggle to fathom my obsession with salamanders but grant me unconditional support, nonetheless.

To my nephew, Hudson, and nieces Nora, Lily, and Caroline- may you never stop exploring the curiosities of the natural world.

Finally, to the wondrous, enigmatic salamanders of Southern Appalachia, who have given me the joy of a lifetime through the blessing of experience.

“Places and happenings, people and animals, winds at sea and on mountains, and all that I remember, it was no unpleasant path I trod when I followed this ‘questing beast’”

– Emmett Reid Dunn, 1926

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ABSTRACT

The skin microbiome of amphibian hosts can inhibit growth of pathogenic fungi, contribute to anti-predator compounds in newts, and is linked with sex-specific scents in frogs. However, despite growing evidence of symbiont importance to amphibians, how symbionts are acquired and maintained on hosts remains poorly resolved. Microbiomes exist on a dynamic spectrum from casual assemblages to intricate systems, and community members vary in fidelity and association to hosts. The establishment of these communities involves complex interactions between symbionts, host traits, and source communities. I seek to enhance our understanding by assessing the spatial-temporal patterns of the salamander skin microbiome relative to their cover objects which are likely an important, but rarely investigated source of microbes for colonization onto host skin. In Chapter 1, I investigate small-scale similarity and differentiation between host species and cover objects along an aquatic and terrestrial interface to determine the effects of host species, habitat, and microhabitat. I find that bacterial communities associated with salamanders display differentiation according to host species, which is matched in cover object communities suggesting microhabitat rather than host traits determine bacterial presence and community composition in heavily divergent communities. To understand patterns of dissimilarity, in Chapter 2, I test whether heterogeneity between salamander hosts, other salamander hosts, and their cover objects can be explained by priority effects, host selection, or stochastic dispersal in aquatic or terrestrial habitats and find differences between salamander-associated communities are consistent with stochastic colonization and extinction of environmental microbes. In Chapter 3, I use the phenology of *E. cf. wilderae* to understand the temporal variability by sampling hosts seasonally for one year as hosts move between aquatic and terrestrial habitats. Host-associated communities show turnover seasonally that is exacerbated with habitat changes. However, a small subset shows evidence of non-random associations, some of which can produce antifungal metabolites, potentially providing a defensive function for hosts. These results suggest stochastic processes dominate the amphibian skin microbiome with little evidence for deterministic structuring. Despite this high heterogeneity between hosts, we identify a few microbial taxa as candidates for investigating mechanisms that promote persistence on amphibian skin.

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INTRODUCTION

Symbiosis

The term symbiosis, originally coined by Albert Bernhard Frank in 1877, refers to ‘the living together of unlike named organisms’ (Sapp, 1994). Anton de Bary extended this definition in 1879 to describe associations between unlike species living together in physical contact (Sapp, 1994). Though initially intended specifically for outcome-independent associations, confusion over terminology has since blurred the definition, with symbiosis often conflated with mutualisms (outcome-dependent positive benefits for both parties) (A. E. Douglas, 2021; Saffo, 1992). Some have argued an outcome-independent definition of symbiosis is ‘too broad to be useful’ (D. C. Smith, 1992) since defining criteria for ‘intimacy’ is sufficiently vague and rarely agreed upon by biologists. However, the broad definition, as originally intended, offers the benefit of flexibility given evolutionary consequences are often unknown, and the associations are often dynamic and difficult to distinguish. Empirical evidence over the last 150 years has confirmed the ubiquity of symbiotic associations in ecology and evolutionary biology, making symbiosis a pertinent concept for understanding inter-specific associations contributing to physiology, reproduction, ecosystem function, and natural selection (Margulis & Fester, 1991; Peacock, 2011).

Host-associated symbiotic associations are often studied in the context of multicellular hosts and prokaryotic, fungal, or unicellular symbionts that exist on a dynamic spectrum from pathogenic to mutualistic. Transmission of these symbionts can be either vertical (inherited from a parent) or horizontal, with horizontal transmission being either abiotic (from other hosts) or environmental (acquired from external reservoirs). Endosymbionts have been particularly valuable in understanding the evolutionary consequences and mechanisms of symbiosis, with vertically transmitted endocellular mutualists engaging in reciprocal, coordinated changes to facilitate cooperation for long periods of time (e.g. *Buchnera* and the pea aphid, 160 million years) (Hansen & Moran, 2011; Moran & Degnan, 2006). The evolutionary ramifications of cooperation can yield co-dependence and co-evolution, as evolutionary fates of hosts and mutualists become intricately linked and display congruence along host and symbiont phylogenies (Moran & Sloan, 2015). Vertically transmitted endocellular parasites, such as the

‘master manipulator’ *Wolbachia*, can remarkably affect host behavior and fitness, even shaping the evolutionary trajectories of species through an array of processes (Yeap et al., 2018). Environmentally acquired symbionts are more puzzling from an evolutionary perspective since the dynamics of partner fidelity (prolonged correlated associations) are not immediately clear. Environmentally acquired symbionts can engage positive feedback cycles for mutualistic benefits, whether it be through pleiotropic interactions, partner choice, or utilizing toxic host by-products (Shapiro & Turner, 2014). The maintenance of these symbiotic interactions might be explained by the theory of biological markets and game theory, in which commodities are exchanged between hosts and symbionts through cooperation and conflict (Hammerstein & Noë, 2016; Wilkinson & Sherratt, 2001). These scenarios could promote specialization and increase the fitness of hosts and symbionts without relying on vertical transmission to align the reproductive success of hosts and symbionts. This has been shown to occur through housing organs (e.g. *Euprymna scolopes* and *Vibrio fischeri* (Visick et al., 2021)), capture and ‘farming’ (e.g. Corals and *Symbiodinium* (Wooldridge, 2010)), or symbiont harboring through selective gut physiology (e.g. Coreoidae and *Burkholderia* (Takeshita & Kikuchi, 2020)). Alternatively, hosts and symbionts can interact through diffuse associations where benefits of either party can be obtained without specialization.

Recently, advancements in high throughput sequencing have launched the investigation of extracellular symbiotic prokaryotic and fungal communities associated with a host. Symbiotic communities associated with animal hosts (referred to as a microbiome) can influence host behavior, reproduction, digestion, ecological interactions, and even cognitive function through individual associations or symbiotic consortiums more broadly (Antwis et al., 2020; Kohl et al., 2016). This importance has led to proposed paradigm shifts in evolutionary theory, departing from genome and environmental interactions to theories encompassing the assemblage of prokaryote genes associated with hosts, referred to as a ‘holobiont’ or ‘extended genotype’ (Rosenberg & Zilber-Rosenberg, 2011). Central to this proposition is that hosts and microbial traits act as the fundamental unit of natural selection, which can be problematic for complex, environmentally derived microbiomes with diverse associations and fidelity (O’Brien et al., 2019). Animal hosts exhibit vast range of symbiotic relationships, ranging from inconsequential communities lacking residency (Hammer et al., 2017) to those pertinent for dietary capabilities

and niche expansion (Kohl et al., 2016). Similarly, while metabolically active, most microbes appear to have neutral associations with host, implying complex microbiomes likely exhibit weak, if any, partner fidelity (Sieber et al., 2019). In these scenarios, host or bacterial benefits could be gained through diffuse associations rather than specialization (A. E. Douglas & Werren, 2016). As a result, a more testable framework for complex communities is to view host-associated assemblages (microbiomes) as ecological communities influenced by the relative strengths of host traits, microbial interactions, and environmental colonization. This does not downplay the potential role of symbiont contribution to host behavior, immunity, reproduction, and physiology, but acknowledges the complex ecological and evolutionary dynamics within and between host-associated communities.

Amphibians are a compelling model for studying external symbiotic communities. Ecological and physiologically resembling the earliest colonizers of land, amphibian porous skin is in constant contact with their environment. Copious secretory glands cover the integument, interacting with residing and colonizing microbes. Additionally, the skin microbiome, in tandem with host secretory profiles, acts as the first line of immune defense and can inhibit the growth of fungal pathogens *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in laboratory assays. More recent discoveries have found symbionts are capable of toxin production in unpalatable newts and sex-specific methoxypyrazines in frogs, revealing potential evolutionary ties and links to host fitness (Brunetti et al., 2019, 2022; Vaelli et al., 2020). However, high environmental colonization is presumed to be the dominant way in which microbes are acquired for hosts, making host-specificity and physiological interdependency of the skin microbiome unclear in the context of host traits and ecological dynamics. This dissertation takes a high-resolution investigation of microbes on salamander skin across aquatic and terrestrial habitats, using paired sampling of host skin and cover object surfaces, to investigate the ecology of microbiomes that exist at the interface between host and environment. I describe similarity and differentiation between host species before investigating the patterns of dissimilarity in terms of community assembly processes, and finally explores temporal dynamics and the core microbiome concept for a migratory salamander over the course of one year. Below, I outline the amphibians as models for symbiotic investigations before introducing the specific aims of each chapter, which are explicated in subsequent chapter introductions.

The amphibian skin as a stage for symbiosis

The amphibian skin, functioning as an interface between an organism and its environment, possesses two distinct layers: the epidermis and the dermis. The epidermis is a permeable layer of squamosal epithelium divided into three sections: *stratum corneum* (outermost), *stratum spinosum*, and the basal *stratum germinativum* (Duellman & Trueb, 1994; Varga et al., 2019; Wells, 2010). The *stratum corneum* is keratinized and is routinely sloughed, and can be ingested, potentially acting as an inoculum for the gut microbiome or clearing chytridiomycosis (Meyer et al., 2012; Ohmer et al., 2015). Sloughed skin is then synchronously replaced by a keratinized layer of the *stratum spinosum*. Cell morphology is most diverse in the *stratum germinativum*, containing an array of immune and dendritic cells (Colombo et al., 2015; Haslam et al., 2014; Woodhams et al., 2023a). Beneath the epidermis lies the dermis, divided into an upper layer (*stratum spongiosum*) and a lower layer (*stratum compactum*), connected via collagenous columns. The *stratum spongiosum* contains an array of glands, chromatophores, and lymphocytes while the compact layer contains mast cells associated with anti-parasitic responses (Marshall & Bienenstock, 1994; Varga et al., 2019).

Perhaps the most well-known attribute of the amphibian skin is the presence of mucosal, granular, and pheromone glands, responsible for a wide range of physiological, immunological, and behavioral processes. Nestled within the *stratum spongiosum*, mucosal glands are often smaller than granular glands and more uniformly distributed. These glands function to mediate physiological homeostasis including, but not limited to, oxygen diffusion, ion transport, water balance, and thermoregulation (Duellman & Trueb, 1994; Lewis et al., 2018; Sever & Siegel, 2015; Wells, 2010). While not extensively studied in caudates (salamanders), research in anurans (frogs and toads) and humans indicates the mucus component from these glands consists of around 95% water and contains complex networks of gel-forming mucins (Dubaisi et al., 2018). These large mucins create a medium approximately 6µm thick (in *Xenopus* tadpoles) and serve as the site of O-glycosylation through regions enriched in proline, threonine, and serine (PTS) residues (Dubaisi et al., 2018), though N-glycosylation has also been found in other Anurans (Barbosa et al., 2022). The attached sugars could provide a nutrient source for residing microbes or create a barrier for colonization. Additionally, the gel-forming mucin networks trap other

molecules (lipids, peptides, metabolites) from the host and residing microbes, creating a landscape of interaction and signaling between hosts and symbionts (Van Rooij et al., 2015).

Granular glands have received more attention due to their bioactive relevance in predator deterrent, pathogen prevention, and potential medical uses for humans. These glands are larger and more sporadically distributed than mucosal glands, but are capable of secreting a cocktail of bioactive compounds such as amines, peptides, proteins, and sequestered alkaloids (Chen et al., 2003; Pukala et al., 2006). These compounds are multifunctional, aiding in defense through neuroactivity against animals and/or antimicrobial activity against fungal or bacterial pathogens (Brandon & Huheey, 1981; Conlon, 2011; Roelants et al., 2013). Peptides with antimicrobial capabilities exhibit α -helical or β -sheet conformations and exert effects via toroidal pore or carpet mechanisms and can act synergistically to elicit effects (Huan et al., 2020; Rollins-Smith, 2009). The evolution of these genes arose through duplication events, followed by modifications for skin deployment of short, complex sequences bioactive in both mature and cleaved states (Pukala et al., 2006; Roelants et al., 2013; X. Xu & Lai, 2015). While the signal portion of AMPs are highly conserved for storage and transport in the cell, bioactive portions are hypervariable and can differ within species and populations of the same species (Tennessen et al., 2009). Nearly 2,000 peptides have been cataloged from over 20 genera of anurans and can be broadly classified into 100 families according to amino acid sequences and predicted/observed function (X. Xu & Lai, 2015). In caudates, less research has been conducted, but evidence suggests most species possess the capacity to generate cytolytic compounds from granular glands (Fredericks & Dankert, 2000; Jiménez et al., 2022; Meng et al., 2013; Pereira et al., 2023; Sheafor et al., 2008; H. K. Smith et al., 2018; von Byern et al., 2017). Caudal courtship and mental glands also occur in plethodontid salamanders. These glands are associated with mating and vary seasonally regarding size and content and likely have limited relevance to the assembly of the skin microbiome outside of breeding season (Rupp & Sever, 2018; Sever & Siegel, 2015). Taken together, the intricate processes of mucosal, granular, and courtship glands within amphibian skin not only aid in physiological, immunological, and behavioral processes, but also present dynamic chemical and nutritional environments for symbiotic interactions between amphibian hosts and microbes residing on their skin.

Plethodontid salamanders as a system for intra and inter-specific differences in the skin microbiome

Southern Appalachia harbors at least 41 species of lungless salamanders belonging to the family Plethodontidae, with recent evidence suggesting the potential number to be even higher due to cryptic species (Pyron et al., 2022, 2023). Eastward migration into Southern Appalachia during the Paleocene and Eocene was followed by rapid Miocene species accumulation within genera leading to high diversity maintained by niche conservatism in a favorable climate (Kozak, 2017; Kozak & Wiens, 2010). Plethodontid salamanders are unique among amphibians in that all 350+ species lack lungs despite occupying a diversity of habitats and exhibiting a variety of life history strategies. Lung loss is not exclusive to Plethodontid salamanders, evolving three other times in tetrapods, once in another salamander genus (*Onychodactylus*), once in Gymnophiona (*Typhlonectes eiselti*), and once in anurans (*Barbourula kalimantanensis*) (Bickford et al., 2008; Lewis et al., 2018; Nussbaum & Wilkinson, 1995). The proposed adaptive significance is attributed to buoyancy reduction for larval salamanders occupying, high-gradient streams early in plethodontid evolution (Beachy & Bruce, 1992). In the absence of lungs, extrapulmonary respiration is thought to be facilitated by a paralogue of a protein associated with pulmonary gas exchange in tetrapods that decreases mucus thickness (Lewis et al., 2018) and presents the potential for unique associations with symbionts.

In Southern Appalachia, low-order streams and riparian zones offer a fine-grained system for studying the ecological and evolutionary drivers of host-associated microflora. Co-occurring species maintain high richness through interspecific competition and intraguild predation along aquatic-terrestrial gradients (Bruce, 2008; Crawford & Semlitsch, 2007; Hairston, 1986; Southerland, 1986). Thus, a single aquatic/terrestrial interface can contain as many as eight species of salamanders in close proximity. These organisms are the dominant vertebrate in headwater streams and can exist in abundances as high as 14.7 larvae+adults per m² (Davic, 1983) and an estimated 1.8 adults/m² in riparian zones (Petranka & Murray, 2001). The biomass contribution of salamanders to aquatic and terrestrial ecosystems is oftentimes the highest among vertebrate predators and effectively links biomass transfer between aquatic and terrestrial ecosystems through biphasic lifestyles and trophic cascades (Davic & Welsh, 2004). Salamanders occupying these areas serve as bioindicators of ecosystem health and also represent

a unique system to study how symbionts are acquired and maintained on hosts, both similarly and differently, between host species at small geographical scales.

Fungal pathogens *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* and defensive symbionts

Global amphibian declines are occurring at an unprecedented scale, with 41% of species experiencing declines, over 100 species on the verge of extinction, and 9 species extinct since 1980 (Luedtke et al., 2023; Stuart et al., 2004). These declines are over 200 times historical background rates and can be attributed to an array of factors including land use change, commercial use, environmental contaminants, climate change, invasive species, infectious diseases, or combinations of these (Collins, 2010; Lips et al., 2008; Roelants et al., 2007; Skerratt et al., 2007; Stuart et al., 2004). Of particular concern are the rise and spread of two fungal pathogens, *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans* (Bsal), which cause chytridiomycosis in amphibians. These fungi are characterized by motile flagellated spores (zoospores) and inhabit freshwater and moist soils (Rollins-Smith & Le Sage, 2021). These zoospores have the propensity to spread quickly through amphibian communities since many amphibians congregate in wetlands for reproduction. When colonizing amphibian skin, zoospores encyst by absorbing the flagellum and developing a cell wall before germ tube develops and the mature sporangium discharges motile zoospores that penetrate the *stratum corneum* and *stratum granulosum* (Van Rooij et al., 2015). Protease and lipase secretions of Bd degrade skin integrity, disrupt physiological homeostasis, and decrease the expression of host genes for skin maintenance, oftentimes resulting in death (Abramyan & Stajich, 2012).

While Bd has been highly lethal to anurans, declines in North American plethodontid salamanders attributed to Bd have been less severe. Documented declines of plethodontid salamanders in Southern Appalachia were observed since the 1980s (Highton, 2005), but these declines seem unattributed to fungal pathogens, despite Bd being present in North America during this time (Muletz et al., 2014). Field surveys for Bd on *Plethodon* salamanders have only found 19 positive cases in over 2,000 individuals sampled spanning 12 species (Muletz et al., 2014). Of 976 samples of non-*Plethodon* plethodontid species occurring in Southern Appalachia, only 49 individuals sampled in the field tested positive for Bd (*Desmognathus*: 17/664, *Eurycea*:

25/232, *Aneides*: 6/41, *Pseudotriton*: 1/9, *Gyrinophilus*: 0/30) (Augustine & Neff, 2016; Blackburn et al., 2015; Chinnadurai et al., 2009; Gratwicke et al., 2011; Hossack et al., 2010; Jongsma et al., 2019; Spaulding et al., 2018). Susceptibility assays in the lab have shown Plethodontids are still susceptible to Bd and resulting chytridiomycosis, but susceptibility is lower than other amphibian clades (Chinnadurai et al., 2009; Vazquez et al., 2009). Rare detection in the wild and decreased susceptibility could be a result of plethodontid ecology, however, the skin microbiome of plethodontid salamanders also consistently contains microbes associated with anti-fungal capabilities (Harris et al., 2006; Loudon et al., 2014; Muletz et al., 2012a).

Bsal is currently restricted to Asia and Europe, but appears more lethal to salamanders and habitat modeling indicates southern Appalachia to be a high-risk area due to proximity to trade ports, abiotic characteristics, and amphibian species richness (Martel et al., 2014; H. K. Smith et al., 2018; G. Stegen et al., 2017; Yap et al., 2017). Early studies assessing the susceptibility of North American salamanders to Bsal found Eastern Newts (*Notophthalmus viridescens*) to be highly susceptible, similar to European salamandrids (Martel et al., 2014; H. K. Smith et al., 2018), whereas ambystomatids appear resistant to Bsal through potent skin activity (Barnhart et al., 2020; Pereira et al., 2023). Plethodontids show variation in their response, with Spelerpinae being more susceptible than Plethodontinae, with the exception of *Gyrinophilus* (Carter et al., 2020; DiRenzo et al., 2021; Jiménez et al., 2022; Martel et al., 2014). That said, the mechanisms involving susceptibility and resistance are not clear. Mucosome function is hypothesized to play a major role in Bd/Bsal susceptibility and resistance (Jiménez et al., 2022), but skin secretions from *Desmognathus ocoee* and *Plethodon shermani* facilitated the growth of Bsal *in vitro*, despite other members of these genera showing tolerance or resistance to Bsal infection (DiRenzo et al., 2021; Martel et al., 2014). Instead, resistance to chytridiomycosis from Bsal appears to be a result of complex interactions stemming from the synergistic associations of host secretory products and residing microflora. This could explain observed variation within genera and species regarding the susceptibility and lethality of Bsal (Carter et al., 2020).

Soil communities harbor high bacterial and fungal containing expansive interspecies interactions ranging from competition and predation to cooperation and mutualisms (Deveau et

al., 2018). The evolutionary arms race of competing bacterial/fungal species has led to complex arsenals of secondary metabolites that have been transformative in medicine and agriculture (Dror et al., 2020; Vij et al., 2021). As a result, the antifungal capabilities are likely not restricted to amphibian-associated symbionts. Defensive symbionts associated with amphibian hosts show functional and phylogenetic redundancy in inhibiting the growth of Bd in laboratory assays. For example, Becker et al. (2015) noted approximately 75% of bacteria associated with Panamanian frogs were capable of inhibiting Bd during challenge assays. Similarly, Barnes et al. (2020) found Bd inhibition in bacteria isolated from salamander skin was represented across multiple bacteria phyla and showed little evidence of trait-associated phylogenetic relatedness, with high intra-genus variation in suppressing the growth of Bd in challenge assays. Together, this implies the anti-fungal capabilities of symbionts associated with amphibian skin are likely widespread in environmental reservoirs and could show affinity for host conditions but are not necessarily engaging in persistent mutualisms.

Chapter overviews

A growing body of evidence suggests hosts receive benefits from interacting with individual and collective symbionts, and thus a key step in understanding the associations and maintenance of symbionts is assessing the relative strengths of host traits and environmental colonization in structuring host-associated communities. Several authors have additionally noted the consistency of evolutionary history in explaining microbial community dissimilarity, implying a link between host traits and microbial assemblages (Lim & Bordenstein, 2020; Mazel et al., 2018). Host traits, such as genes of the major histocompatibility complex, antimicrobial peptides, and mucins can act as selective filters for microbes leading to non-random colonization and persistence (Belley et al., 1999; Davies et al., 2022; Falk et al., 1998). Communities associated with amphibians are hypothesized to be more influenced by habitat (Allison, 2012), but others have suggested community specificity pertaining to host genera and species (A. Hill et al., 2022; Osborne et al., 2024; Ramírez-Barahona et al., 2023). In **Chapter 1**, I assess the similarity and differentiation of host-associated communities for five species of salamanders and their immediate cover objects in an aquatic-terrestrial interface to determine the role habitat and host phylogeny play in shaping the amphibian skin microbiome.

Host-associated communities are comprised of varying strengths of deterministic and stochastic processes (Hubbell, 2005; Nemergut et al., 2013; Vellend, 2010). Results from Chapter 1 discovered amphibian-associated communities were statistically differentiated from cover objects and also displayed high inter-host dissimilarity, showing only subtle similarities in the form of indicator taxa. This refutes strong deterministic processes at the community level and suggests salamander-associated communities are environmentally acquired and weakly structured by host traits and microbial interactions. Ecological null models are a powerful tool for assessing such dissimilarities by comparing observed dissimilarity to expectations from probabilistically assembled communities while maintaining richness differences (Ning et al., 2020; J. C. Stegen et al., 2013; Zhou & Ning, 2017). To understand the assembly patterns of salamander-associated communities, in **Chapter 2** I use a taxonomic and phylogenetic null ecological model as well as correlative relationships at varying taxonomic levels to evaluate signatures of stochastic and deterministic influences on salamander skin microbiomes from aquatically and terrestrially captured salamanders and their immediate cover objects.

Community stability describes the ability of a community to minimize variability or return to its original state after perturbation. As such, community stability is inherently related to disturbance and can be broken down into three linked facets: constancy, resistance, and recovery (de Bello et al., 2021). Disruption of host-associated communities can drive communities to a state of dysbiosis, which can have detrimental effects to hosts (Jiménez & Sommer, 2016). However, the extent of dysbiosis is dependent on the degree of the disturbance and the reliance of hosts to symbionts. In this regard, interpreting how a community might respond to disturbance events is defining expectations of ‘normal operating range’ for host-associated communities (Shade et al., 2012). According to my previous results, differences between salamander hosts appear largely random, consistent with stochastic colonization and extinction of environmentally acquired microbes. This implies most microbes found on salamanders are likely to be transient, making it unclear how and if microbes persist on hosts over the course of the year. In **Chapter 3**, I assess the temporal variation of the amphibian skin microbial and test four definitions of a ‘core’ microbiome to uncover candidates for future investigation that display non-random associations with salamander skin.

**CHAPTER I:
SIMILARITY AND DIFFERENTIATION OF AMPHIBIAN SKIN
MICROBIOMES BY HABITAT, MICROHABITAT, AND HOST SPECIES**

Abstract

Host-associated communities exist on a spectrum ranging from casual assemblages to highly co-evolved systems. Casual, or transient, assemblages rely heavily on source pools and are thus expected to mirror environmental communities and vary with host habitats. Highly coevolved systems are dependent on host traits and thus remain relatively constant over time and space. However, diffuse mutualisms are likely to exhibit convergence when similar host species have similar interactions with microbes. To assess intrinsic (host traits) and environmental influences on amphibian-associated bacterial communities, we utilized the high diversity of Plethodontid salamanders in Great Smoky Mountains National Park. Salamanders and cover objects (their local environments) were sampled along an aquatic-terrestrial gradient and bacterial diversity was assessed using 16S rRNA meta-amplicon sequencing. Cover objects show strong ties to habitat (aquatic/terrestrial) with some evidence of microhabitat partitioning. Salamander-associated communities show high individual variation with little relationship to habitat, and only slight differences among salamander species. A subtle pattern of convergence among host species was suggested by the identification of 21 sequence variants with strong associations to salamanders, providing evidence for diffuse mutualisms, or microbes well suited for salamander skin. However, our results find little other evidence for salamander or species specificity, meaning ecological assembly patterns and environmental reservoirs largely drive symbiotic communities on salamander skin.

Introduction

Host-microbe interactions have become a focal point in ecology and evolution, as host associated microbes can have profound consequences for the evolution, behavior, and physiology of the host (Bordenstein & Theis, 2015; Huitzil et al., 2018; O'Hara et al., 2020). Microbes residing on the host can be thought to exist on a dynamic spectrum ranging from mutualistic to pathogenic (Steinert et al., 2000). In amphibians, the skin microbiome has garnered attention due to its ability to inhibit the growth of two chytrid fungi, *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans* (Bsal) through the production of antifungal metabolites in laboratory experiments (Lauer et al., 2007; Muletz et al., 2012a; Woodhams et al., 2014). These two chytrid fungi, associated with global amphibian declines,

colonize the skin and disrupt physiological homeostasis by damaging host tissues, disrupting immunological response, and afflicting metabolic dysregulation (Fites et al., 2013; Grogan et al., 2018; Van Rooij et al., 2015). As a result, understanding how microbes are acquired and sustained on the host has important implications for understanding host-microbe relations and the conservation of amphibians.

To what extent amphibian hosts shape their cutaneous microbiome has been explored previously, with studies coming to predominantly three conclusions. First, across all studies, the environment was the largest influence in the cutaneous microbiome, acting as a reservoir through which continuous horizontal colonization can occur. This can be concluded from strong correlations between amphibians and their immediate cover objects and higher similarities among co-occurring species than between a single species from different geographical sites (Bird et al., 2018a; Fitzpatrick & Allison, 2014; Wolz et al., 2018). Differences in environmental pools are reflected in skin microbial communities, with elevation, pH, and temperature being the dominant drivers of free-living assemblages (Jiménez & Sommer, 2016; Kueneman et al., 2019).

Secondly, all studies also found that host-associated samples differ from environmental samples in relative abundance and tend to be slightly less diverse. These patterns suggest that while the environment offers a pool of microbes for colonization, amphibians may have filtering mechanisms such as skin sloughing (Meyer et al., 2012), secretory products (Myers et al., 2012; Rollins-Smith, 2009), ontogeny (Kueneman et al., 2014; Longo et al., 2015), diet (Antwis et al., 2014), and habitat selection (Bletz, Archer, et al., 2017). It is also plausible that ecological processes, such as priority effects, can facilitate or inhibit the colonization of immigrants through the metabolic activities of residing community members (Toju et al., 2018). A less often discussed possibility is that animal skin is a sink, an inhospitable habitat that free-living microbes fall upon by accident and gradually emigrate or die. In this case, differences between host samples and environmental samples would arise from differences in tolerance or mobility of microbial taxa. The observation of bacterial cell division on salamander skin via scanning electron microscopy shows that growth and reproduction can happen on the host (Lauer et al., 2007), but does not confirm that host-associated populations are self-sustaining.

Finally, some studies have emphasized the consistency of microbiomes within host species, and differences among host species (McKenzie et al., 2012; Prado-Irwin et al., 2017;

Walke et al., 2014). Such patterns might be consistent with stabilizing selection on microbiome composition and have been interpreted as suggesting a defensive mutualism in which amphibians favor bacteria with anti-Bd metabolites (Loudon et al., 2016; Prado-Irwin et al., 2017). However, these interpretations remain speculative, and evidence for host-specificity is limited. Higher taxonomic comparisons have revealed significant differences between Anurans and Caudates, but perhaps blurred by microhabitat differences (Bletz, Perl, & Vences, 2017; Kueneman et al., 2014). McKenzie et al. (2012) showed statistical differences between frog and salamander larvae within the same ponds, but differences between frog species were subtle or absent. In terrestrial salamanders, the similarity of cutaneous communities was associated with co-occurrence rather than host species (Allison, 2012). Thus, any patterns of phyllosymbiosis, where more closely related hosts have more similar microbial communities, is likely attributed more to differences in environmental source communities associated with different microhabitats rather than trait-based filtering, particularly of closely related hosts. However, recent analyses including salamanders in Southern Appalachia suggest some evidence otherwise (A. Hill et al., 2022; Osborne et al., 2024), concluding host species have higher explanatory power than environmental reservoirs.

Amphibian skin systems are porous, i.e., there is extensive opportunity for the movement of microbes between the skin and the external environment. This leads to a high likelihood that relationships are diffuse, i.e., many microbial species with similar effects on many host species. Mutualism in such diffuse and porous systems may promote specialization or generalism. Generalism is expected to result in community convergence, where different host species harbor similar assemblages of microbes that are non-random (filtered) draws from the environmental pool. Specialization is expected to result in distinctive microbial assemblages associated with each host species. Here, we attempt to evaluate convergence vs. differentiation by comparing microbial samples from the skin of several co-occurring salamander species across a terrestrial-aquatic interface.

The Southern Appalachians are a biodiversity hotspot for salamanders in the family Plethodontidae, making it an ideal region to study the effects of habitat and host species on the cutaneous microbiome. In this study, we sought to quantify differences in microbial communities of five salamander species spanning three genera within Great Smoky Mountain National Park. Our goal was to test the relative importance habitat (terrestrial vs. aquatic), microhabitat (cover

objects), and host species in the formulation of the skin microbiome. Three of the sampled species (*Desmognathus gvnigeusgwotli*, *Desmognathus monticola*, and *Desmognathus conanti*), occupy different positions along an aquatic to semi-aquatic gradient, while the other two species (*Plethodon teyahalee* and *Eurycea* cf. *wilderae*) occupy neighboring terrestrial habitats in the summer (Petranka, 2010).

Materials and Methods

Field sampling

We sampled plots across three sites within Great Smoky Mountain National Park (GSMNP). All sites were low-order streams under 800m in elevation. Upon arriving at each site, a 10mx10m plot was set up evenly encompassing aquatic and terrestrial habitats. Up to ten salamanders of each species were captured with a gloved hand or net. Only salamanders with a snout-vent length (SVL) over 25mm were collected for swabbing due to difficulty in consistently swabbing organisms below this size class. Upon capture, salamanders were placed in a new Ziploc bag and transported to a central location for swabbing. At each salamander capture location, a flag was dropped to ensure organisms were returned to the exact location. Prior to swabbing, salamanders were rinsed with 50ml of sterile spring water to remove transient bacteria not considered part of the host microbiome (Lauer et al., 2007; Walker & Lawrence, 2015). After rinsing, salamanders were placed in a new Ziploc bag and swabbed 30 times (10 times on the dorsum, 10 times on the venter, and five times on each lateral side) with a sterile MW-113 Rayon-tipped swab (Medical Wire and Equipment, UK). Gloves were changed between the handling of each salamander. Swabs were stored in either 1.5ml or 2.0ml sterile centrifuge tubes and placed on dry ice. A swab of the cover object under which the salamander was found was also taken and stored in the same manner. At the end of each site, salamanders were released where collected. Swabs were stored at -80°C until extraction. For all sites, we had at least five biological replicates of at least three species occurring in aquatic or terrestrial habitats.

16S library preparation

DNA was extracted from the swabs using DNEasy PowerSoil Pro Kit (Qiagen), following the manufacturer's protocol, with the addition of a five-minute incubation in solution

C6 prior to the final spin column elution. The V3-V4 region of the 16S Ribosomal RNA gene was amplified with 341F-785R primers in triplicate. Polymerase chain reactions (PCR) reactions consisted of 6.25µl of KAPA HiFi HotStart ReadyMix, 2.5µl of forward primer, 2.5µl of reverse primer, and 2.5µl of template DNA. Cycles for PCR were as follows: 95 °C for 3 minutes, followed by 35 cycles of 95 °C (30s), 55 °C (30s), and 72 °C (30s), followed by a five-minute elongation step at 72 °C. Amplification was verified on a 1% agarose gel. Amplified triplicates were pooled and cleaned using AMPure beads (Beckman Coulter) and Nexterra XT sequencing primers containing distinct barcodes were added during a second PCR with following the same conditions, only with eight cycles. Purified products were quantified via nanodrop, pooled to equimolar concentrations, and sequenced PE 2x275 using a V3 600 flow cell on an Illumina Miseq with a 20% PhiX spike at the University of Tennessee Genomics Core.

Bioinformatics

Following sequencing, primers were removed from demultiplexed samples using Cutadapt and samples entered the DADA2 pipeline (Bolyen et al., 2018; Callahan et al., 2016; Martin, 2011). Reads were trimmed to a length 250 (forward) and 225 (reverse) and quality filtered with a maxEE score of three for both forward and reverse reads. MaxEE is considered to be a more accurate filtering scheme than the traditional Q score threshold, as accuracy is assessed for every base along a sequence, and rejection criteria are based on expected errors along each read (Edgar & Flyvbjerg, 2015). Reads were then merged, and chimeras removed. Sequences were then clustered using to 97% similarity using Vsearch and a SEPP fragment insertion tree was created using the Silva 128 database in qiime2 for both ASV and 97% clustering (Janssen et al., 2018). The phylogeny was then time-calibrated using TreePL (S. A. Smith & O'Meara, 2012) and taxonomy was assigned to the sequences via the IdTaxa algorithm, using the Silva SSU 132 database as a reference in R (Murali et al., 2018; Quast et al., 2013). Contamination from control samples (sterile water, extraction, and PCR blanks) were removed using the 'decontam' package (Davis et al., 2018). This resulted in the removal of 205 ASVs from our dataset. Following contamination removal, control samples and any sample with reads below the highest control library size were removed from the dataset. If this resulted in samples no longer being paired, the matching sample was also removed. Prior to analyses, reads assigned

to Chloroplast and Mitochondria were removed as well as singletons, resulting in 3,124,159 reads mapped to 21,057 OTUs (97% clustering) and 62,065 ASVs in 165 samples.

Rarefaction and diversity

Estimating diversity for microbial assemblages from a single sample poses challenges for researchers, since each sample is only a momentary snapshot of a larger picture and thus true diversity is likely underestimated. Not only does sampling result in rare species going undetected, but also tends to inflate the unevenness of species abundances. These biases can be further exacerbated by PCR, and sequencing (Gohl et al., 2016; Pollock et al., 2018; Willis, 2019). Post sequencing, differences in library sizes can drastically affect diversity estimates, thus transformations are necessary to adequately compare differences within and between samples in a biologically and statistically meaningful manner. Scaling, rarefying, and log-transforming are three commonly used techniques to post-hoc standardize raw abundance data, but only rarefaction removes the effects of unequal library sizes (Weiss et al., 2017). Despite comparable raw library sizes between salamanders and cover objects, we observed differences in richness and a positive relationship between ASV richness and library size (**Appendix Fig. A1**). To ensure equal sampling for diversity estimations, we performed multiple rarefactions of all samples to the lowest sample library (2,785 reads) 500 times and estimated alpha diversity (a) and taxonomic pairwise dissimilarities (b diversity) for each rarefaction using the R packages *vegan* and *vegetarian* (Charney & Record, 2012; Oksanen et al., 2019).

Beyond biases associated with sequencing, a large proportion of rare sequences can distort the interpretation of alpha diversity. One remedy to this issue is estimating diversity using Hill numbers and assessing differences across orders of q (Chiu & Chao, 2014; M. O. Hill, 1973). This approach applies monotonic transformations to a standard formula, establishing an intuitive and comparable scale: effective number of species (Jost, 2007). Hill numbers also follow the replication principle, which states that diversity in N completely distinct communities of equal sizes, when pooled, must be N times the diversity of a single assemblage (Chiu & Chao, 2014). Hill Numbers have gained recent traction for estimating α diversity since their formulation in the 1970's and have been extended to evaluate β diversity using a multiplicative

β , allowing researchers to conveniently compare differences within and between samples in a unified framework (Jost, 2007).

Beta diversity

Our sampling scheme allows us to examine the relationship between salamanders and their immediate environment reservoir. We used multivariate ordination and variance partitioning to test whether bacterial assemblages were consistently different among salamander species and between aquatic and terrestrial habitats. Ordinations use pairwise dissimilarities, which we estimated corresponding to different orders of Hill Numbers. Sørensen dissimilarity uses the presence-absence of taxa in pairwise communities to represent the fraction of species in one community not shared with the other, corresponding to a $q=0$ β -diversity metric. The 1-Horn index of overlap ($q=1$) considers the relative abundance of each species using Shannon information, while the Morisita-Horn index for equally weighted communities, is a $q=2$ pairwise β -diversity metric reflecting the probability of identity (Hurlbert, 1971). The Horn contribution to the Morisita index keeps the pairwise dissimilarity values constrained between zero and one and all dissimilarities are transformations of multiplicative β (Horn, 1966; Jost, 2006). Dissimilarities were estimated for each rarefaction using the R packages *vegan* and *vegetarian*, and the mean dissimilarity across all rarefactions was used for analyses (Charney & Record, 2012; Oksanen et al., 2019). A phylogenetic-based dissimilarity incorporating relative abundance (Weighted Unifrac) was also estimated for 100 rarefactions on an ultrametric tree, with a mean standard deviation for pairwise comparisons of 0.0004 across all rarefactions (Lozupone et al., 2007).

We used variance partitioning and distance-based redundancy analyses (dbRDA) in *vegan* to assess clustering patterns and model performance. Variance partitioning assesses the explanatory power of each variable independently and tandemly via coefficients of determination (adjusted r^2), which can then be modeled and tested via constrained dbRDA and permutation tests. Differences between salamanders and cover object samples was assessed first before breaking up the dataset to only salamanders or only cover object samples to test the effects of habitat and species (or microhabitat) within each dataset. Hypothesis testing was conducted using an *anova.cca*, with 1,000 permutations one level below the tested variable.

Alpha diversity

To test the prediction that host-associated assemblages are less diverse than free-living microbial communities, we compared Hill numbers at three orders of q . At $q=0$, the effective number of species is the estimated richness of a sample (given a rarefaction sample size). At $q=1$, the Hill number is the exponential of Shannon entropy (a diversity measure accounting for relative abundance), while the inverse of Simpson's concentration index is the Hill number at $q=2$ (a scaled probability of "interspecific encounter"). The changes in diversity between each additional q can be used to evaluate the evenness of species abundances within a given sample. We estimated alpha diversity at $q=0$, $q=1$, and $q=2$ for each rarefaction and compared group (salamander, aquatic/terrestrial environment) alpha diversity estimations using a Dunn's test in the *rstatix* package (Kassambara, 2020).

Indicator species analysis and community composition

We used an indicator species analysis to identify sequence variants (ASVs) with strong association with a group (salamanders, aquatic environments, and terrestrial environments) (De Cáceres et al., 2010). ASVs were used to provide a finer resolution in identifying microbes associated with each salamander species or habitat. To do this, we rarefied our dataset 1,000 times and evaluated the distribution of mean point-biserial coefficients for each sequence. We then identified top candidates whose average point-biserial correlation coefficient for salamanders was equal or higher than 0.2. To assess species specificity and salamander specificity across sites, we used the 'multipatt' function, which allows for individual and multiple group attributions of indicators identified using the point biserial correlation coefficient. Finally, to investigate taxonomic distributions in our samples, we constructed composition plots for the top ten phyla as well as the top 20 most abundant families. All visualizations were made in R v4.2.0 using the MicEco, ggpubr, and phyloseq packages (Kassambara, Alboukadel, 2020; McMurdie, Paul J. & Holmes, 2013; R Core Team, 2022; Russel, 2020).

Results

Heterogeneity is high for salamander hosts

Using 97% clustered OTUs, we assessed dissimilarities at three different levels pertaining

to our research questions. The first assessed differences between salamanders and their environment (cover objects), while the other two were conducted on split datasets to analyze habitat and species on only cover objects and only salamanders. For each level tested, we visualized data dispersion using unconstrained ordinations (**Fig. 1** for Horn Dissimilarity). We then used variance partitioning to assess the explanatory power of each variable (**Fig. 2**). Site was important in explaining variation in our dataset for each dissimilarity (**Table A1**), and due to low biological replicates of Site, we conditioned our model to partial out these effects. This did not change the significance of any variable tested. To isolate and test only the effect of species, we additionally partialled out the effects of habitat as well. Our models explained anywhere from 5-36% of total inertia in our dataset, evaluated by the adjusted r^2 from variance partitioning. Salamander swabs were significantly different from cover object swabs and aquatic cover objects were significantly different from terrestrial cover objects, but aquatic and terrestrial salamanders were not significantly different. When comparing salamanders to cover objects, both Weighted Unifrac and all ecological-based dissimilarities were statistically significant (**Table 1**). Unconstrained ordinations illustrate the near absence of overlap between aquatic and terrestrial cover samples on the first two PC axes. Variance partitioning confirmed that aquatic vs terrestrial habitat accounts for the largest fraction of the variation overall and for cover objects, but for very little of the variation among salamanders (**Fig. 2**).

Swabs from different salamanders were not very similar. Pairwise turnover (**Fig. 3**) indicates that the signal of differentiation might be largely driven by consistency among cover object swabs from the same habitat rather than similarity among salamander samples. Salamander swabs were as different from each other or more, on average, as they were from most cover object swabs (**Fig. 3**). Salamanders, in general, exhibit more heterogeneity than cover objects but show subtle evidence of increased similarity to their immediate cover object and cover objects of the same species compared to those of differing species or habitat (**Fig. 3**). Further evidence of high dissimilarity between salamanders is evident in the average gamma diversity across rarefactions, which was 9,461 OTUs for all cover objects and 8,334 for all salamanders.

Swabs from different salamander species were somewhat differentiated. After partialling out the effects of habitat and site, both salamander species and the cover objects associated with

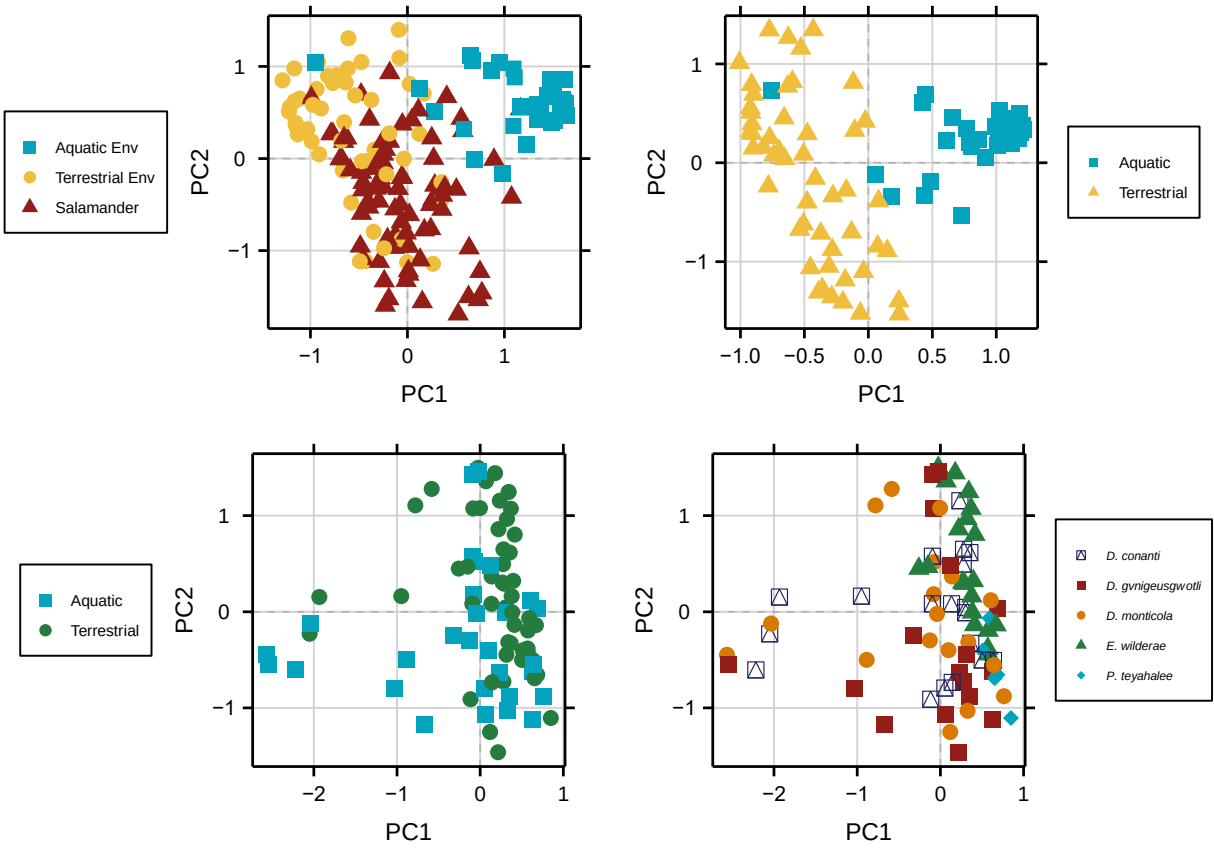


Figure 1: Unconstrained ordinations of the Horn dissimilarity for all samples (top left), only cover objects (top right) and salamanders colored by habitat (lower left) and species (lower right)

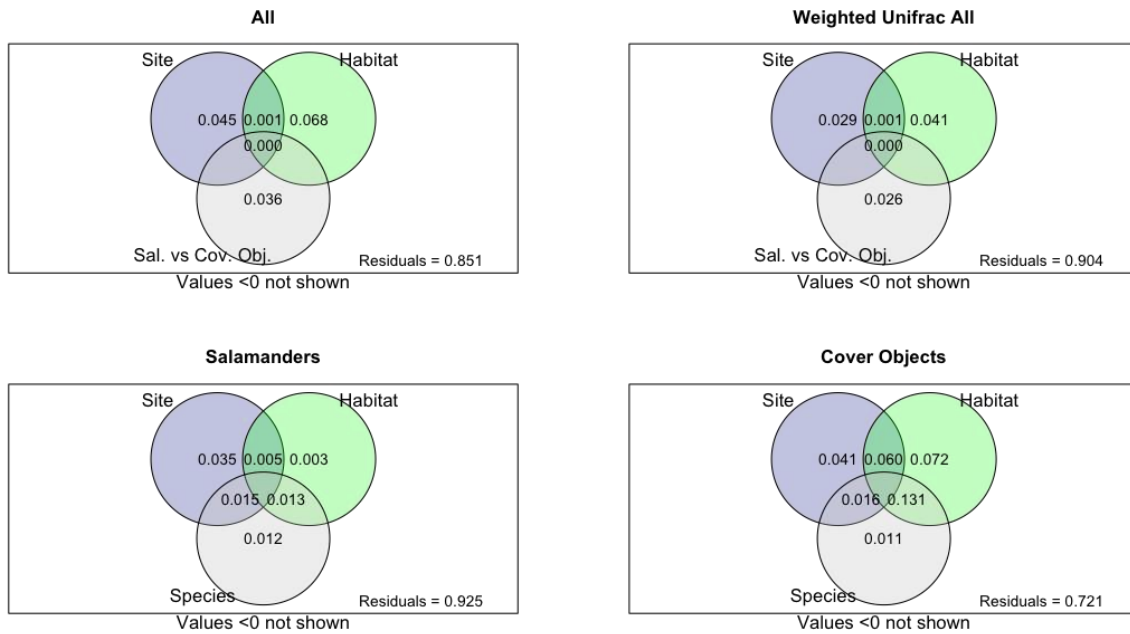


Figure 2: Variance partitioning of variables. Values denote adjust r^2 . All diagrams show partitioning of the Horn dissimilarity, except where denoted.

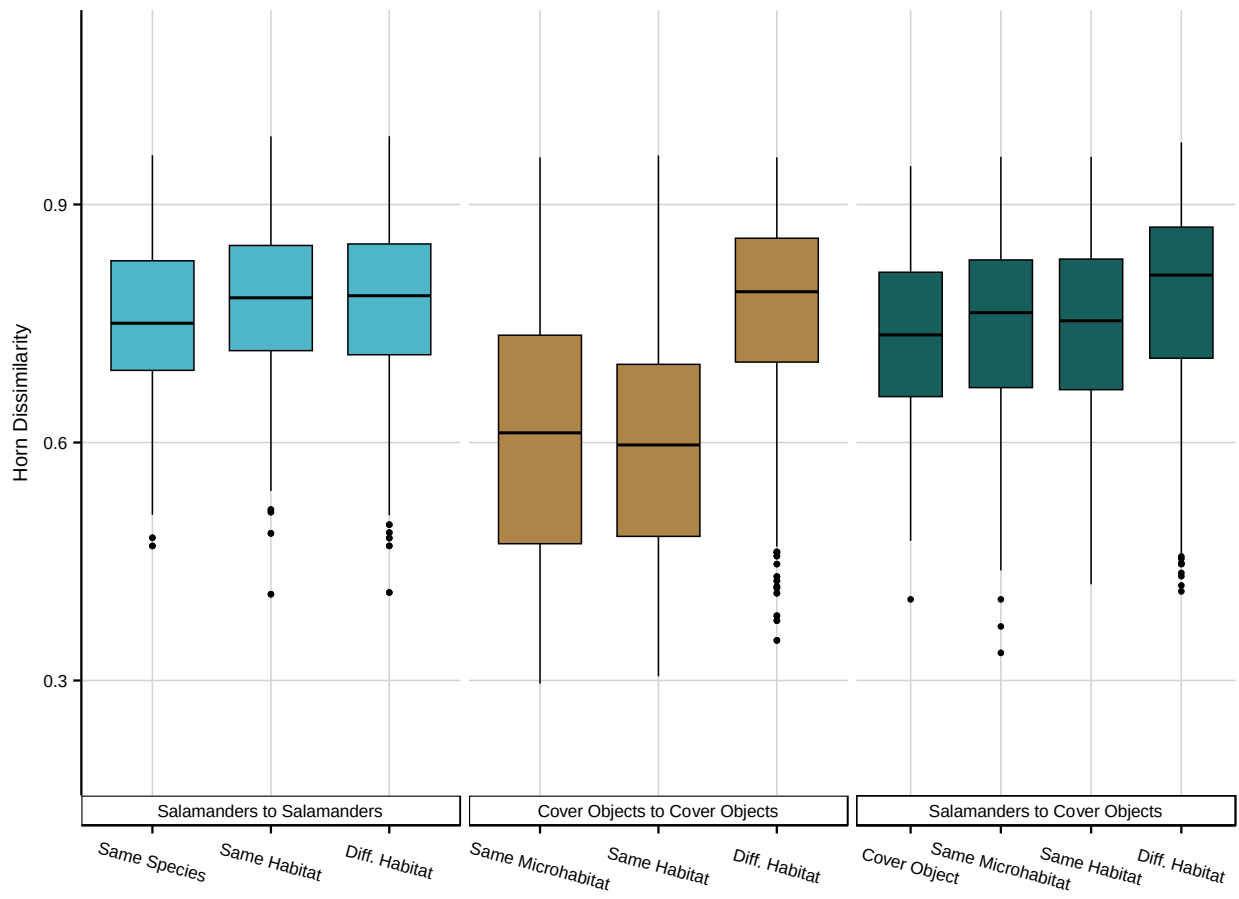


Figure 3: Boxplots of OTUs of pairwise Horn dissimilarity. "Same Microhabitat" means same salamander species. All comparisons are within a site.

Table 1: Results from dbRDA and anova.cca. Columns correspond to the tested hypothesis and rows indicate the dissimilarity metric.

Dissimilarity	Comparisons				
	Salamanders to Cover Objects	Cover Objects: Aquatic vs Terrestrial	Salamanders: Aquatic vs Terrestrial	Cover Objects of Different Salamander Species	Salamanders of Different Species
Sørensen	<0.001	<0.001	0.1096	0.015	0.0222
Horn	<0.001	<0.001	0.1378	0.0358	0.0338
Morisita-Horn	<0.001	<0.001	0.1858	0.07899	0.1188
Weighted Unifrac	0.001	<0.001	0.5305	0.1628	0.1792

salamander species had significant differences only in Sørensen and Horn dissimilarity (**Table 1**), indicating the effects of species/microhabitats on patterns of differentiation stem largely from rare or lesser abundant OTUs. Hierarchical clustering of the Horn dissimilarity shows low agreement with the host species phylogeny (**Fig. 4**) and instead reflects widespread differentiation, with some resemblance to niche partitioning of salamander species along aquatic-terrestrial gradients within a site, particularly for *E. cf. wilderae* and *D. gvnigeusgwotli*. While the number of salamander species is far too small to support a statistical test of phylogenetic signal (Boettiger et al., 2012), the dissimilarity between species was only slightly greater, on average, than dissimilarity between individuals of the same species.

Hosts less diverse than cover objects

Host-associated samples were generally less diverse than samples from cover objects. Alpha diversity was assessed at three orders of diversity. Average richness across all salamanders was 362 OTUs, while the average richness on aquatic and terrestrial cover objects was 596 and 461, respectively. Diversity estimates at $q=1$ were 199 for salamanders, 248 for terrestrial cover objects, and 320 for aquatic cover objects. At every order of q , salamanders were less diverse than aquatic cover objects (**Fig. 5**, all $p<0.05$). Additionally, aquatic cover objects were more diverse, on average, than terrestrial cover objects at $q=0$ and $q=1$. Salamanders were significantly less diverse than terrestrial cover objects at $q=0$ and $q=1$, but not $q=2$.

Effective diversity decreased as the order of q increased for all salamanders, aquatic, and terrestrial samples as expected for assemblages without perfectly even abundances. However, salamanders exhibited the least decline of all groups, indicating a more even distribution of abundances on salamanders. When focusing only on salamanders, there were no statistically significant differences in alpha diversity between host species or host habitat (**Fig. A2**).

Indicator species analysis and community composition

The density distribution of point biserial correlation coefficients in our dataset pertaining to salamanders showed a bimodal distribution (**Fig. 6**). Of the over 62k sequences, only 21 possessed an average point biserial correlation coefficient greater than 0.2 across rarefactions (**Fig. A3, Table 2**). These ASVs amounted to a mean relative abundance of 0.02 on salamander samples and 0.0008 on cover objects. The top indicator, an unnamed sequence variant in the

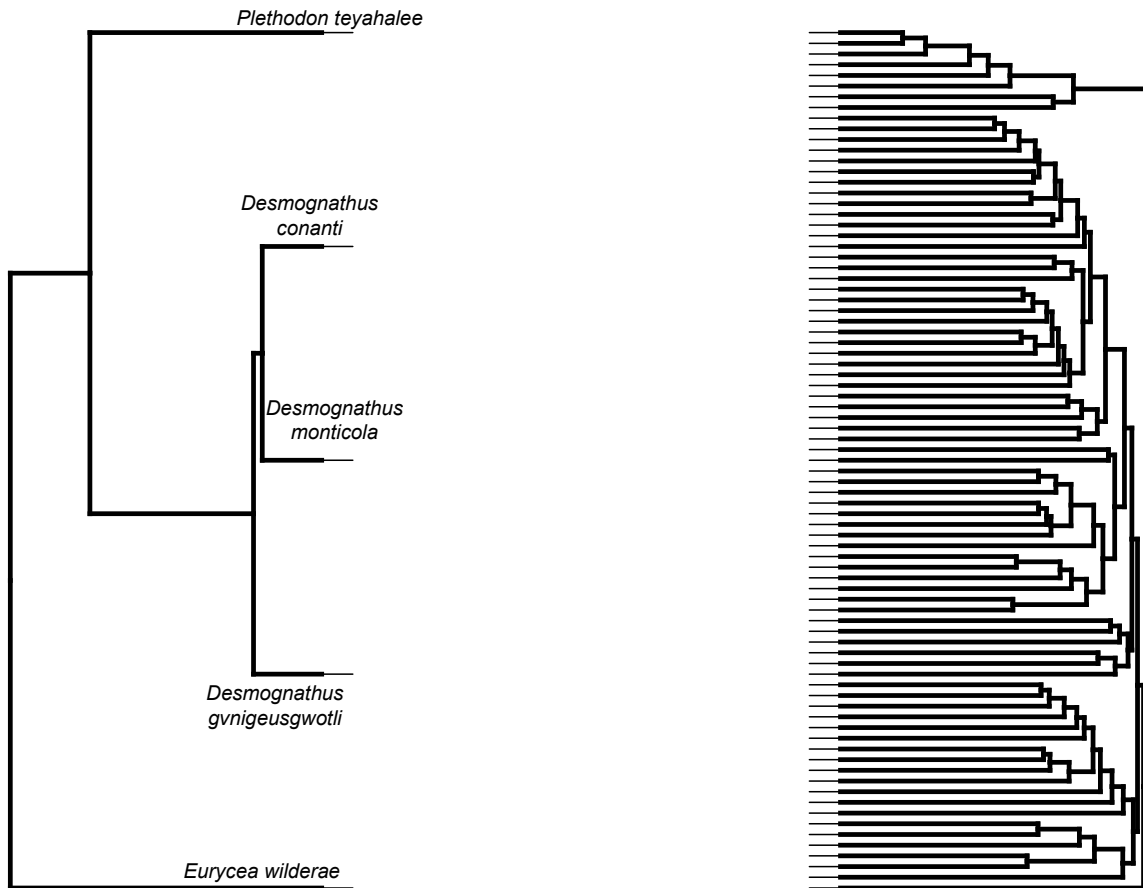


Figure 4: Host phylogeny (left) and hierarchical clustering of the Horn dissimilarity (right).

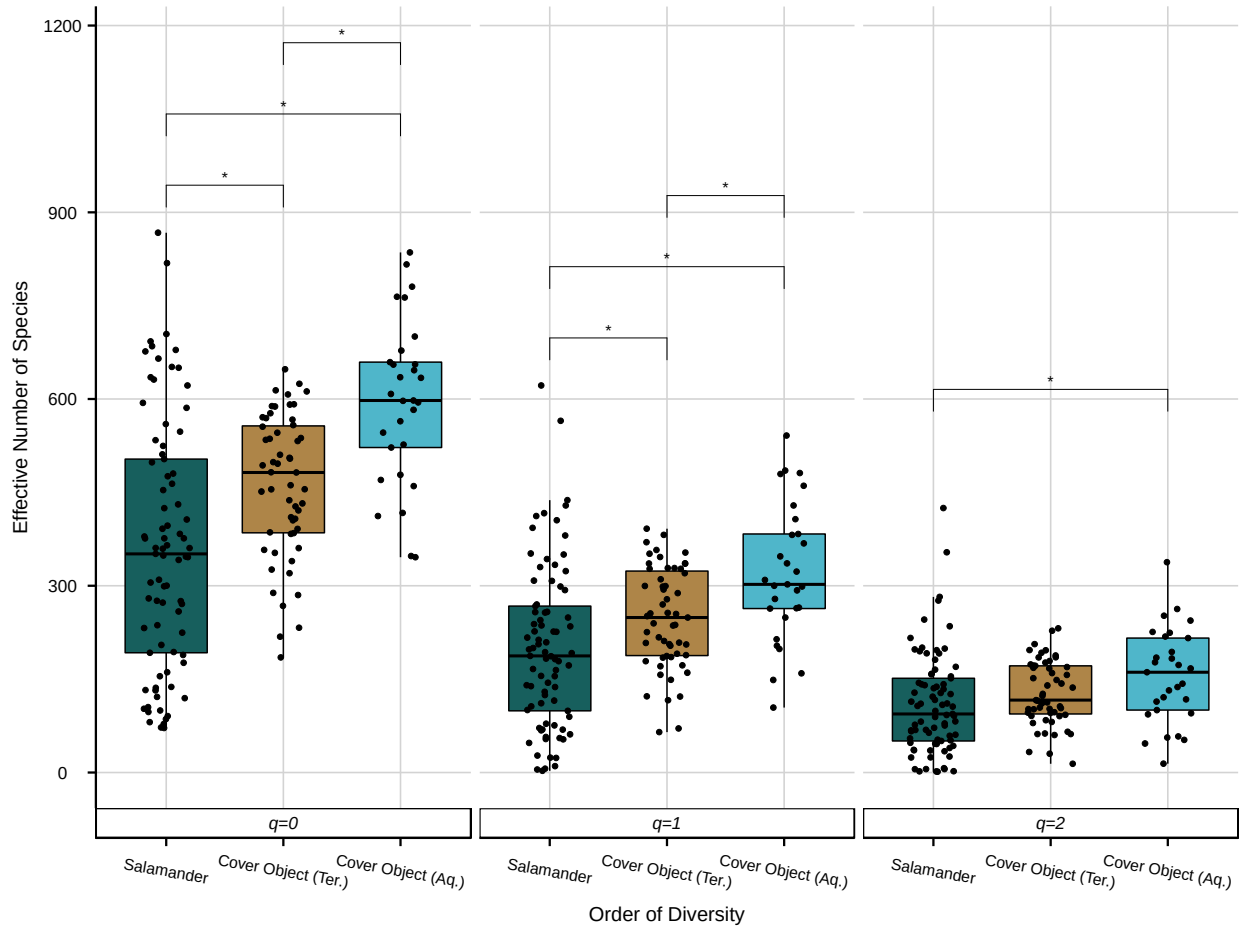


Figure 5: Alpha Diversity estimates for communities on salamanders, terrestrial cover objects, and aquatic cover objects at three orders of diversity.

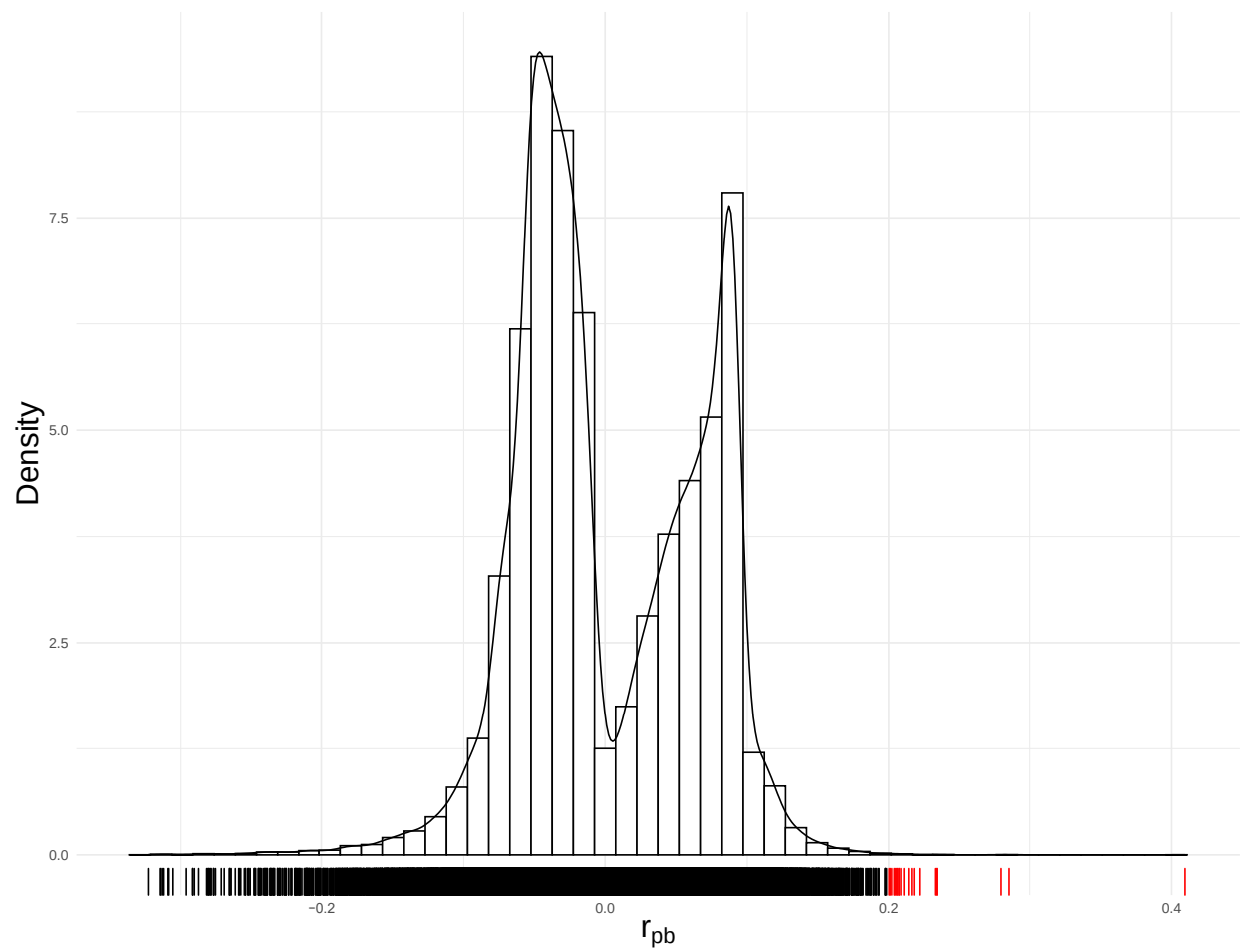


Figure 6: Distribution of point biserial correlation coefficients describing strength of association of ASVs and salamander hosts.

Table 2: Taxonomic information of indicator species and the strength of association

Order	Family	Genus	Mean r_{pb}
Xanthomonadales	Xanthomonadaceae	<i>Stenotrophomonas</i>	0.40905203
Propionibacteriales	Propionibacteriaceae	<i>Cutibacterium</i>	0.28534226
Cytophagales	Spirosomaceae	<i>Flectobacillus</i>	0.28048033
Solirubrobacterales	Solirubrobacteraceae	<i>Patulibacter</i>	0.23431044
Burkholderiales	Comamonadaceae	<i>Variovorax</i>	0.235148
Sphingomonadales	Sphingomonadaceae	<i>Sphingomonas</i>	0.23188597
Rhizobiales	Beijerinckiaceae	1174-901-12	0.22252176
Sphingomonadales	Sphingomonadaceae	<i>Novosphingobium</i>	0.2166361
Pseudomonadales	Moraxellaceae	<i>Moraxella</i>	0.21541869
Pseudomonadales	Moraxellaceae	<i>Acinetobacter</i>	0.21371188
Corynebacteriales	Mycobacteriaceae	<i>Mycobacterium</i>	0.20907266
Rhizobiales	Beijerinckiaceae	NA	0.20719538
Rhizobiales	Xanthobacteraceae	<i>Tardiphaga</i>	0.20672952
Corynebacteriales	Mycobacteriaceae	<i>Mycobacterium</i>	0.20563616
Verrucomicrobiales	Rubritaleaceae	NA	0.20614392
Micromonosporales	Micromonosporaceae	<i>Dactylosporangium</i>	0.20569715
Burkholderiales	Comamonadaceae	<i>Ramlibacter</i>	0.2049049
Burkholderiales	Comamonadaceae	NA	0.20517004
Sphingomonadales	Sphingomonadaceae	<i>Sphingobium</i>	0.2016855
Enterobacterales	Erwiniaceae	<i>Pantoea</i>	0.20240385
Rhizobiales	Beijerinckiaceae	<i>Methylobacterium</i> - <i>Methylorubrum</i>	0.20135468

genus *Stenotrophomonas* and family Xanthomonadaceae was detected on nearly 75% of salamander samples, despite appearing in only four environmental samples (mean relative abundance 0.0076). This sequence consistently had a r_{pb} of greater than 0.4, and is a member of a genus known to produce anti-fungal metabolites (Barnes et al., 2020) and a family (Xanthomonadaceae) known for poor dispersal capabilities and tight relationships with hosts (Nemergut et al., 2013). This family was also found to have genetic differentiation and positive associations with other terrestrial salamander (*Plethodon jordani*) hosts (Fitzpatrick & Allison, 2014). Three sequences with high indicator values could not be identified at the genus level, but only one genus, *Mycobacterium*, had multiple sequences identified as indicators (2). At the family level, three members each of Comamonadaceae, Sphingomonadaceae, and Beijerinckiaceae had positive correlations. Taxa in Beijerinckiaceae have been shown to undergo strong seasonal variation in the frog cutaneous microbiome, with the highest abundance in summer (A. J. Douglas et al., 2020), while members of Comamonadaceae have been associated with potential anti-Bd capabilities in laboratory assays (Kruger, 2020). Sphingomonadaceae is a family commonly found on amphibian skin, ranging from North America (Hernández-Gómez, Byrne, et al., 2020a), Europe (Sanchez et al., 2017), the tropics (Hughey et al., 2017), and Asia (L. Xu et al., 2020).

To assess whether these taxa are associated with a salamander species or the salamander skin characteristics more broadly, we conducted a multi-level pattern indicator species analysis using ‘multipatt’ in the R package indicpecies (De Cáceres et al., 2010). This identifies ASVs associated with specific groups but can also identify ASVs associated with combinations of groups, making it useful for understanding whether salamander hosts contain species-specific ASVs, or whether the small set of indicators described above is a result of convergence (i.e., recruitment by hosts of multiple species). Because *P. teyahalee* was poorly represented in sampling (5 samples from a single site), we removed these samples and their associated cover objects, leaving us with four species/microhabitat groupings across three sites. Results indicated each host salamander species possessed between 29-68 ASVs significantly associated with a single host species (**Table A1**). However, this number was dwarfed by the number of sequence

identified using the point-biserial correlation coefficient. These were the sequence variants belonging to the genera *Stenotrophomonas*, *Dactylosporangium*, *Cutibacterium*, and *Methylobacterium-Methylobacterium*. The results of both indicator species broadly show salamander skin microbiomes are enriched for a subset of microbes relatively uncommon in environmental swabs, but specificity is driven predominately by local effects, and evidence for ‘salamander-specific’ microbes is limited to a tiny subset of microbes.

The top 10 phyla in our samples encompassed 70% of all taxa in our dataset while capturing 95.6% of relative abundance in pooled aquatic cover object samples, 97.5% of relative abundance in pooled terrestrial cover object samples, 95.6 % of relative abundance in pooled aquatic salamander samples, and 95.5% of relative abundance in pooled terrestrial salamander samples. The dominant phyla in our samples, regardless of sample type, were Proteobacteria followed by Acidobacteriota, Actinobacteriota, Bacteroidota, and Verrucomicrobiota (**Fig. A4**). Plotting the top 20 most abundant families accounted for just over 26% of total taxa and resulted in 54.1%, 60.3%, 51.5%, and 55.5% of relative abundance in each pooled sample types, following the same order as above. While families share common trends across sample types, differences can be observed in the relative contribution of each family. Aquatic environmental samples contained a higher relative abundance of Comamonadaceae, Sphingomonadaceae, and Pedosphaeraceae, while terrestrial cover objects contained a higher relative abundance of Xanthobacteraceae, Acidobacteriaceae (**Fig. A5**). Both aquatic and terrestrial salamanders possessed a higher relative abundance of Pseudomonadaceae and Beijerinckiaceae than cover objects. Sequence variants in these families were identified to have strong associations with salamanders and are common symbionts found on amphibian skin. Pseudomonadaceae has been linked to TTX production in newts and sex-specific compounds in frogs, with certain members also possessing anti-Bd capabilities (Brunetti et al., 2019; Vaelli et al., 2020; Woodhams et al., 2015).

Discussion

With the growing awareness that host-associated microbiomes can affect host fitness, researchers have begun to examine how host ecology and evolution contribute to associated assemblages. Amphibian-associated communities have received attention due to the cutaneous

microbiome's contribution to host resistance to chytrid. Our results support the existing body of research describing amphibian microbiome as a complex ecological community heavily dependent on the environmental reservoir (Fitzpatrick & Allison, 2014; Loudon et al., 2014; Wolz et al., 2018). That is, host-associated and free-living bacterial samples are composed of largely the same taxa, and host-associated assemblages likely result from continuous horizontal colonization onto the host from the environment. Host-associated samples showed high heterogeneity among individual salamanders regardless of species and habitat utilization. We find only weak evidence of host species specificity, which is confounded by differences in host-species microhabitat. Convergence among hosts (across species and habitat) is suggested by the presence of a small number of indicator taxa, while the majority of the skin microbiome might reflect idiosyncrasies of stochastic colonization-extinction and priority effects.

Host species and site were more effective in explaining variance than habitat (aquatic vs terrestrial) in our analyses evaluating salamander-associated communities. Site characteristics such as geological and geographical factors (climate, elevation, latitude, slope) shape reservoirs at a site that is reflected on salamander communities. Site alone consistently explained between 2-5% of variation in salamander analyses and salamanders show subtle evidence of site-specificity in turnover analyses. Hernández-Gómez et al. (2017) found similar results with skin microflora of Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) across latitude and elevation. Local effects were also found in attempts to explain global variation among amphibian cutaneous microbiomes (Kueneman et al., 2019). However, for cover objects, habitat designation (aquatic or terrestrial) explained more inertia than site in each dissimilarity tested, meaning the difference between aquatic and terrestrial environments was more important than geographic differences between sites for both the detection of taxa and the composition of communities of cover objects (i.e., aquatic cover objects were more similar to an aquatic cover object at a different site than a terrestrial cover object at the same site).

Host species explained a significant proportion of variation in our dataset for salamanders only at binary and $q=1$ ecological β diversity metrics, but not phylogenetic or Morisita-Horn dissimilarities. Phylosymbiosis, the notion that more closely related hosts have more similar microbial communities, can arise through deterministic or stochastic processes (Kohl, 2020). Deterministic phylosymbiosis refers to the concordance of host phylogeny and community

dissimilarity stemming from the direct mediation of host traits on community structure (Lim & Bordenstein, 2020). Stochastic phylosymbiosis occurs when concordance exists as a byproduct rather than direct mediation from host traits. For example, closely related hosts occupying similar habitats could show strong resemblance because of exposure to similar microbial reservoirs and not selection of microbes due to host traits. While we lack adequate statistical power to test this hypothesis, our results suggest host species lack sufficient differentiation to support deterministic structuring at the community level, and only slight evidence in terms of ASV sequence specificity, which could be an artifact of sampling. In other words, the significant statistical pattern of species-specificity does not necessarily imply strong intrinsic differences between host species especially since cover objects associated with different species show similar trends. This suggests differences in microhabitats could drive differences between hosts and perhaps blur aspects of host-species specificity. Either way, these differences were subtle relative to the overall pattern of heterogeneity among individual salamanders in comparison to environmental samples from aquatic and terrestrial habitats, meaning salamander skin does not exert a coherent selection pressure at the community level.

Significant differences between salamander-associated assemblages and assemblages living on their own cover objects could be evidence that salamander skin is an alternative niche for certain microbes, or that salamander skin is simply better at inhibiting the growth of some microbes more than others. The skin of salamanders is an important organ for host physiological homeostasis. Histological studies in *Desmognathus*, *Plethodon*, and *Eurycea*, confirm the presence of mucosal and granular glands embedded within the dermal layer of the integument (Rupp & Sever, 2018; Sever & Siegel, 2015). Uniformly distributed mucus glands maintain physiological homeostasis through secretions of glycoproteins and mucopolysaccharides, which likely act as a nutrient source for host-associated microbes and pathogens (Austin, 2000; Elkan, 1968; Garbe & Collin, 2012). More notably, amphibian skin also possesses more sparsely distributed granular glands, which are responsible for a cocktail of neuroactive and cytolytic peptides. Some of these bioactive peptides are capable of inhibiting Bd and Bsal but can also facilitate the growth of resident microbes (Flechas et al., 2019). Thus, the amphibian skin might facilitate colonization or growth of "desirable" microbes, leading to dissimilarity between host-

associated and free-living microbial assemblages. Such dissimilarity might also result from differential inhibition colonization or growth (these possibilities are not mutually exclusive).

Beyond the skin, differences between salamanders and their environment could be attributed to ecological processes such as diversity-area relationships and priority effects. The significant difference in alpha diversity could drive differences in beta diversity (Chase et al., 2011). In particular, if salamanders were all colonized by all species in the environmental pool, then independent random extinctions would make their assemblages more different from each other, while non-random extinctions would make them more similar to each other. The high observed turnover rates between individual salamander samples, despite clustering in ordinations, indicate that random extinction and/or priority effects could be important in amphibian-associated communities. For example, Toju et al. (2018) found priority effects to have lasting impacts on community composition in plants and Sprockett et al. (2018) identified priority effects to be key in the establishment of gut microbiome communities. In this sense, metabolic activities of members already residing on the host engage in cooperation, competition, and niche partitioning, and thus can facilitate or prohibit the colonization of new taxa (Burke et al., 2011; Coyte & Rakoff-Nahoum, 2019). Given the significant differences between microhabitats and the moderate turnover rate even within a microhabitat, heterogeneity of environmental communities and differential colonization success on hosts due to priority effects, in tandem with host physiology could explain the high heterogeneity of salamander-associated communities despite similar organismal filtering processes.

Finally, we identified 21 sequence variants with positive associations with salamanders, evidence of subtle convergence in the salamander skin microbiome. Many of these taxa were commonly found on amphibians in other studies and it is plausible these variants could play a role in host immunity, physiology, and/or establishment of other members if neutral to the host (Estrada et al., 2019; Sabino Pinto et al., 2017; Walke et al., 2020). From this standpoint, understanding the temporal constancy of these indicator taxa on hosts could prove valuable for understanding both the evolutionary and ecological processes dictating the community structure and potential function on hosts.

CHAPTER II:
SIGNATURES OF STOCHASTIC COLONIZATION AND EXTINCTION
DOMINATE SALAMANDER SKIN MICROBIOMES

Abstract

Symbiotic communities associated with animal hosts are widespread across the animal kingdom. The ramifications of these associations can affect host physiology and fitness but understanding the processes underlying community structure remains elusive for large, complex communities in the context of host ecology and physiology. In this study, we use the amphibian skin microbiome as a system for understanding the processes leading to differentiation between individual hosts, other host species, and their environmental reservoirs. Null ecological models were utilized to understand the relative strengths of host filtering, priority effects, environmental transmission, and random differences between individual salamanders, salamander species, and free-living assemblages in aquatic and terrestrial habitats. Results indicate community disparity among individual salamanders was greater than among environmental samples, suggesting widespread differentiation stemming from idiosyncratic, as opposed to salamander-specific, conditions. Apart from a small core community, the salamander skin microbiome is a product of weak and random ecological processes, with differentiation from cover objects stemming primarily from stochastic dispersal or random local extinction at the lowest taxonomic levels.

Introduction

The patterns and processes leading to the establishment of vertebrate-associated microbial communities are commonly understood through the lens of island biogeography and community assembly (McFall-Ngai et al., 2013; Nemergut et al., 2013; Sprockett et al., 2018). In particular, host-associated communities can be broken down into three linked facets resulting from stochastic and deterministic processes. First, a source community is needed to establish on or in the host, whether it be through vertical transmission from a parent or horizontally from other animals or the environment (Bright & Bulgheresi, 2010; Kouete et al., 2023; Nemergut et al., 2013). Environmental transmission is reminiscent of the mainland-island model of community ecology where dispersal makes local communities similar to regional communities while local extinctions make them more dissimilar (Wilson & MacArthur, 1967). Second, host-mediated effects can act as non-random barriers to colonization or causes of extinction (Lim & Bordenstein, 2020; Mazel et al., 2018). These effects may be direct (arising from host traits such as secretions) or indirect (arising from things like host habitat utilization or diet). These factors

can make host-associated communities appear more similar to those of closely related hosts than expected by chance alone (Kohl, 2020). Alternatively, host-associated communities could be largely dissimilar from each other stemming from neutral processes such as drift. Finally, residing microbes competing for resources can shift the relative abundances of local communities through cooperation, interference, or exploitation. These ecological interactions can have varied effects on community composition between hosts depending on the complexity of interaction networks, resource availability, and niche space.

A less-often discussed structuring factor in host-associated communities is historical contingency, chiefly priority effects. Priority effects can shape a community through two mechanisms: niche preemption and niche modification. Niche preemption occurs when nutrient depletion from an early colonizer prevents colonization of microbes occupying the same niche (exploitative competition), thus the inhibitory relationship is dependent on the order of colonization if the two microbes are competitively similar (Debray et al., 2022). Alternatively, niche modification can be facilitative or inhibitory when early colonizers alter niche availability for later colonizers (Fukami, 2015). This type of priority effect is more likely for large, diverse communities with high dispersal rates from environmental reservoirs (Fukami, 2015; Kohl, 2020), and is expected to decrease similarity between hosts in the absence of strong selection or structuring hub taxa. Priority effects have lasting impacts on the function and taxonomic composition of the microbiome by mediating selection and dispersal as microbes on the host engage in competition or cooperation with potential colonizers (Furman et al., 2020; Jackrel et al., 2020; Martínez et al., 2018; Sprockett et al., 2018; Toju et al., 2018).

External microbiomes are in constant contact with environmental reservoirs and thus present an appealing system for studying assembly patterns. Host-associated communities could range from isolated islands inhospitable to dispersing microbes to entirely nested communities differing only in the number of species. The amphibian skin possesses a thin layer of gel-forming mucins hypothesized to act as both a nutrient source for residing microbes and a barrier for colonization and can result in the subsistence of microbes even in the absence of a reservoir (Loudon et al., 2016). More distantly distributed granular glands secrete a cocktail of bioactive molecules with varied effects on residing and dispersing microbes (Flechas et al., 2019; Pereira et al., 2023). However, amphibian skin microbiomes are generally heavily reliant on

environmental reservoirs, with studies typically find hosts to be statistically ‘different’ from reservoirs in community dissimilarity analyses, implying potential alternative assembly processes (Bird et al., 2018b; Fitzpatrick & Allison, 2014; Kueneman et al., 2019; Prado-Irwin et al., 2017). Dissimilarity between amphibian hosts and environmental reservoirs could be a result of taxonomic replacement via host filtering mechanisms or community processes (priority effects, species interactions, or drift) (Whittaker, 1972). Alternatively, amphibian-associated communities are also typically less diverse than environmental reservoirs (Jiménez & Sommer, 2016). Because maximum similarity between two communities is limited by the less diverse sample (Chase et al., 2011), richness differences contributing to β diversity might lead to communities appearing ‘different’, but are in fact largely nested versions of a regional pool (Podani & Schmera, 2011). While this does not exclude potential fitness implications of host-associated microbes, it does limit the potential evolutionary interactions between host and symbionts.

Previously, we sampled plethodontid-associated communities along an aquatic-terrestrial interface in Southern Appalachian streams and found host-associated communities displayed subtle convergence for a small number of indicator taxa in otherwise divergent communities. While terrestrial and aquatic cover object communities showed strong ties to habitat, salamander-associated communities were more influenced by sampling location and host species. Importantly, statistically significant differentiation of host species was matched in communities found on cover objects pertaining to host species, indicating species specificity could be blurred by host microhabitat utilization. Furthermore, salamander-cover object comparisons were highly dissimilar, indicating a need to better understand the patterns of dissimilarity between hosts and environmental reservoirs as well as hosts and other hosts in the context of horizontal transmission, host filtering, and microbial interactions. In this study, we sought to address the relative strengths of random processes, environmental transmission, host filtering, and priority effects on the amphibian skin microbiome. For observational data alone, we can make the following predictions: a) Lower diversity on hosts, in tandem with strong host filtering should produce more similarities between host-associated communities than their respective cover objects (**Fig. 7**, panel 1); b) Priority effects, in combination with random dispersal events and the absence of strong host filtering, should produce more differences

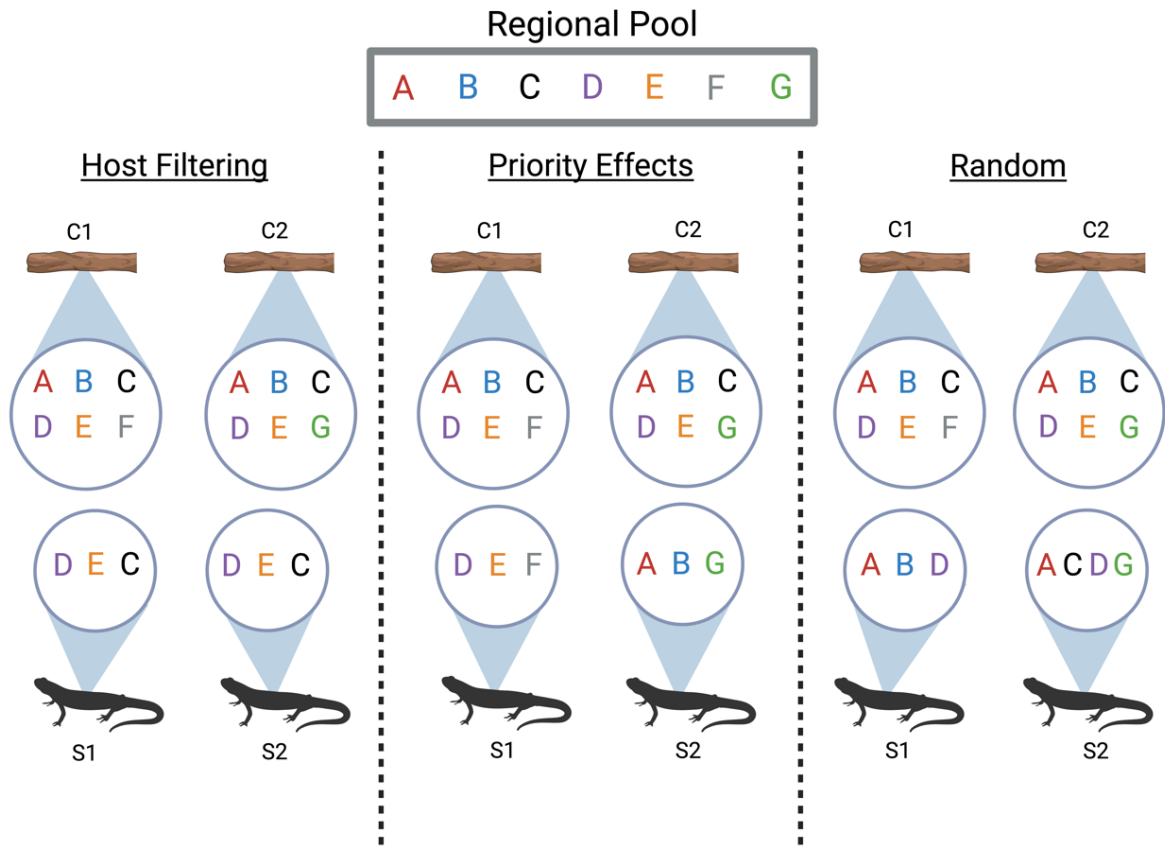


Figure 7: Possible scenarios driving community (dis)similarity between two salamanders (S1 and S2) and their respective cover objects (C1 and C2).

between hosts than their immediate cover object (**Fig. 7**, panel 2); c) Random chance should produce communities with incoherent patterns of similarity and differentiation, consistent with neutral or random assembly (**Fig. 7**, panel 3). The host species focused on for this study were sympatric plethodontid species *Eurycea* cf. *wilderae*, *Desmognathus monticola*, *Desmognathus conanti*, and *Desmognathus gvnigeusgwotli*, which partake in niche partitioning along aquatic and riparian habitats during summer months in low-order streams and are thus exposed to slightly different microbial species pools in a small area.

Materials and Methods

Sample collection

Four sites were sampled in Great Smoky Mountain National Park in low-order streams below 800m during the Summer of 2019. We focused on differences within a single habitat, thus sampling was done focusing on either aquatic or terrestrial habitats, with two replicates of each. Between five and eight salamanders of two species were captured wearing new nitrile gloves and placed in clean Ziplock bags. Salamanders were handled and swabbed as described previously. The cover object under which the salamander was found was also swabbed in the same manner. All salamanders were released where captured. Samples were stored in sterile 1.5ml centrifuge tubes in the field and kept on dry ice before storage at -80°C at the University of Tennessee, Knoxville. **Table 3** lists the species, numbers, and habitat distribution at each Site used for analyses.

Library preparation, sequencing, and bioinformatics

DNA was extracted from the swabs using Qiagen DNEasy PowerSoil Kit, following the manufacturer's protocol, with the addition of a five-minute incubation step before spin column elution. The V3-V4 region of the 16S Ribosomal RNA gene was amplified using 341F-785R primers in triplicate. Polymerase chain reactions (PCR) consisted of 6.25µl of KapaTaq Mastermix, 2.5µl of forward primer (10µM), 2.5µl of reverse primer (10µM), and 2.5µl of template DNA. PCR cycles were as follows: 95 °C for 3 minutes, followed by 35 cycles of 95 °C. (30s), 55 °C (30s), and 72 °C (30s), with a final five-minute elongation at 72 °C. Amplification was verified on a 1% agarose gel and amplified triplicates were pooled and

Table 3: Species, habitat, and sample numbers used in Chapter 2.

Site	Species	Habitat	Number of Salamander/Cover Object Pairs
1	<i>Desmognathus conanti</i>	Terrestrial	4
1	<i>Desmognathus monticola</i>	Terrestrial	6
2	<i>Desmognathus monticola</i>	Terrestrial	6
2	<i>Eurycea cf. wilderae</i>	Terrestrial	8
3	<i>Desmognathus conanti</i>	Aquatic	4
3	<i>Desmognathus monticola</i>	Aquatic	6
4	<i>Desmognathus monticola</i>	Aquatic	6
4	<i>Desmognathus gvnigeusgwotli</i>	Aquatic	6

purified using AMPure beads (Beckman Coulter). Unique sample barcodes and sequencing primers (Nexterra XT) were added during a second PCR with conditions the same conditions described previously, only with eight cycles. Purified products were quantified via nanodrop, pooled to equimolar concentrations, and sequenced on an Illumina MiSeq at the University of Tennessee Genomics Core. Paired-end 2 X 275 sequencing was carried out using a V3 600 flow cell with a 20% PhiX spike.

After removing primers, sequences were trimmed, quality filtered and merged in the same manner as previously described. Taxonomy was assigned via the Silva 132 database via the IdTaxa algorithm in R v2.4.0 (R Core Team, 2022) and a fragment insertion phylogeny was created using the Silva 128 reference database in qiime2 (Bolyen et al., 2019; Janssen et al., 2018) and time-calibrated using TreePL (S. A. Smith & O'Meara, 2012). We also clustered sequences at a 97% sequence similarity using VSEARCH (Rognes et al., 2016) with the same taxonomic classification and phylogenetic methods as described above. After removing chimeras, singletons, and contaminants associated with sterile water and laboratory blanks as well as any sample with lower reads than the highest control, the final dataset resulted in 1,731,197 reads mapped to 38,836 sequence variants in 46 environmental samples and 46 salamanders. Clustering to 97% similarity yielded 12,796 OTUs. OTUs were used for diversity analyses, while ASVs were used for our indicator species analysis, and both ASVs and OTUs were used for assessing the strength of relationship between relative abundance on cover objects and salamanders.

Diversity within and between samples

To evaluate whether microbiomes are less diverse than environmental reservoirs, we assessed alpha diversity both taxonomically and phylogenetically using three orders of Hill numbers, amounting to the richness ($q=0$), exponentiated Shannon index (as the limit approaches $q=1$), and inverse Simpson's index ($q=2$) for taxonomic Hill Numbers (M. O. Hill, 1973; Jost, 2007). Phylogenetic diversity is associated with the diversity of traits in a microbial community. Despite horizontal gene transfer and rapid gene loss, bacteria show trait conservatism along their phylogeny, especially when a particular trait is associated with functional complexity (A. C. Martiny et al., 2013; J. B. H. Martiny et al., 2015). Thus, phylogenetic diversity can be

informative for understanding the distribution of potential traits in the context of community resiliency and assembly. Phylogenetic alpha diversity using Hill Numbers represents the effective number of equally distinct lineages (Chao et al., 2016). At $q=0$, this is the commonly used Faith's PD, while as the limit approaches $q=1$, it becomes an extension of Allen's phylogenetic entropy and $q=2$ corresponds to Rao's quadratic entropy (Chao et al., 2016). To estimate alpha diversity, we rarefied each sample to the lowest read count (2,354 reads) and obtained Hill Number estimates ($q=0$, $q=1$, and $q=2$) for each of 100 rarefactions and used the mean for statistical comparisons. To compare alpha diversity between paired samples, we used a paired Wilcoxon test in the *rstatix* package (Kassambara, 2020).

Null model for community assembly

To distinguish diversifying or homogenizing processes from random assembly and drift, we used the approach of Stegen et al. (2013), which incorporates phylogenetic relatedness and taxonomic dissimilarity in a null model context. This has been used previously to infer community assembly processes, but we adopted this framework to understand patterns of differentiation within and between groups and thus do not strictly adhere to the common verbiage associated with this analysis. Continuous random immigration should result in microbiomes that are statistically indistinguishable from environmental samples. Random colonization and extinction should result in microbiomes that are less diverse, but still a random subset of the environmental species pool. Strong host filtering should result in significant differences between host-associated and environmental samples and strong similarities among hosts. Priority effects should result in idiosyncratic differences among host-associated communities.

The approach uses null models to assess whether pairwise communities differ more or less than expected from a null distribution generated through probabilistically assembled communities- first phylogenetically, and then taxonomically (J. C. Stegen et al., 2013). The mean nearest taxon index (β NTI) for pairwise comparisons is evaluated by calculating the mean nearest taxon distance (β MNTD) before randomizing communities using tip shuffling and recalculating 999 times to produce a null distribution (J. C. Stegen et al., 2013; Webb et al., 2008). The deviation from the null distribution can then be used to identify pairwise comparisons

with strong phylogenetic (dis)similarity. Values of $|\beta\text{NTI}| > 2$ (two deviations from null distribution) imply strong phylogenetic ties, with $\beta\text{NTI} > 2$ indicating phylogenetically different, and $\beta\text{NTI} < -2$ indicate strong phylogenetic similarity. Alternatively, values of $|\beta\text{NTI}| < 2$ fall within the expected range of the null distribution, and differences can be assessed taxonomically.

The RC_{Bray} is a relative-abundance-based extension of the Raup-Crick (β_{RC}) index of pairwise β dissimilarity, which uses randomizations of a ‘species pool’ keeping α diversity consistent in each pairwise comparison of samples (Chase et al., 2011; J. C. Stegen et al., 2013). The resulting value can be interpreted as the difference between two samples not explained by differences in α diversity. In the RC_{Bray} format, alpha diversity for each randomized sample is drawn from a community pool, where the probability of drawing a given taxon is dependent on the number of samples containing that taxon. Once the random taxon list has been obtained, relative abundance is assembled, with the relative abundance being the relative abundance in the metacommunity of selected taxa. The number of iterated dissimilarities greater than observed plus $\frac{1}{2}$ those which are equal are divided by the total number of iterations to produce a value, which can then be standardized between -1 and 1. Pairwise comparisons with a value less than -0.95 are considered to be more similar than expected under a null distribution, while comparisons > 0.95 are considered to be more dissimilar than expected under a null distribution. Values $< |0.95|$ fall into the category of undetermined, or not any more taxonomically similar/different than a probabilistically assembled community.

Both BNTI and β_{RC} were calculated using the iCAMP package in R 4.2.0 (Ning, 2022; R Core Team, 2022). We modified existing functions in iCAMP to produce a RC_{Horn} as opposed to RC_{Bray} , with the former representing the Horn ($q=1$) dissimilarity using relative abundance of pairwise communities. For our data, we considered the species pool to be any taxa observed within a given site, and estimated BNTI and RC_{Horn} on each of 50 rarefactions. Community pools (total number of available species) averaged from 3,867 to 4,554 OTUs. We then assessed each pairwise comparison of groups (salamanders to cover objects, salamanders to other salamanders, and cover object samples to other cover object samples) by calculating the proportion of comparisons falling into five categories: phylogenetically similar, taxonomically similar, phylogenetically different, taxonomically different, or random. Pairwise comparisons between

cover objects were also included to provide context relative to salamander comparisons with the same sample size.

Correlation of shared sequences and identification of indicator species

If environmental transmission is the dominant driver of salamander skin communities, a strong correlation is expected between relative abundance of taxa on cover objects and relative abundance on salamanders (Fitzpatrick & Allison, 2014). However, if many differences exist, a weak correlation coefficient would be indicative of alternative symbiont acquisition and maintenance on hosts. We assessed the strength of the relationship between relative abundance on salamanders and on cover objects using Kendall's tau correlation coefficient at four hierarchical levels: ASVs, 97% clustering, genus, and family level 'glomming'. We divided our dataset to assess trends between aquatic and terrestrial samples. Sequences not assigned at the respective taxonomic level were assigned the taxonomy one level above to prevent spurious glomming resulting from classification failure.

Even if the dominant pattern is one of high correlation and no significant community dissimilarity between salamander skin and environmental samples, there might be a small number of salamander specialists (or microbes systematically excluded from salamander skin). Indicator species analysis is designed to identify such taxa (De Cáceres et al., 2010). We used the point biserial correlation coefficient in the R package 'indicspecies' following the methods described previously to screen for ASVs statistically associated with salamanders or cover objects. To ensure point biserial estimates were not a result of subsampling (rarefaction), we rarefied our dataset 1000 times and computed the point biserial correlation coefficient for each rarefaction. We then extracted the taxa with an average correlation coefficient greater than 0.25 for comparisons within a habitat and 0.20 for indicators of all salamanders (Varma, 2006).

Results

Hosts harbor less species than cover objects

Skin swabs from both terrestrial and aquatically captured salamanders had lower bacterial richness than their corresponding cover objects (**Table 4**, $p < 0.05$, $p < 0.05$). However, only aquatically captured salamanders had lower diversity than aquatic cover objects at $q=1$ and $q=2$

Table 4: Hill Number taxonomic and phylogenetic alpha diversity estimates for aquatic/terrestrial salamanders and cover objects. Superscripts denote statistical significance ($p < 0.05$).

Order of Diversity	Group	Effective Species (Taxonomic)	Effective Species (Phylogenetic)	Samples
$q=0$	Env T	559.79 ^A	361.39 ^A	24
$q=0$	Env W	623.24 ^B	445.59 ^B	22
$q=0$	Sal T	390.30 ^A	301.77 ^A	24
$q=0$	Sal W	260.19 ^B	247.31 ^B	22
$q=1$	Env T	304.49	40.43	24
$q=1$	Env W	344.34 ^C	47.44 ^C	22
$q=1$	Sal T	237.71	41.69	24
$q=1$	Sal W	124.18 ^C	36.21 ^C	22
$q=2$	Env T	145.10	19.38 ^D	24
$q=2$	Env W	170.99 ^D	20.87 ^E	22
$q=2$	Sal T	136.65	20.12 ^D	24
$q=2$	Sal W	59.61 ^D	18.89 ^E	22

($p < 0.05$, $p < 0.05$). At higher orders of diversity, terrestrial captured salamanders were not significantly different from terrestrial cover objects ($p = 0.128$, $p = 0.63$ for $q = 1$ and $q = 2$, respectively), meaning terrestrial cover objects contain comparable bacterial diversity to terrestrial salamander skin when diversity metrics take into account the abundances of taxa.

Phylogenetic alpha diversity showed similar trends to taxonomic alpha diversity regarding richness. Both terrestrial and aquatically captured salamanders had significantly fewer effective lineages at $q = 0$ ($p < 0.05$, $p < 0.05$, **Table 4**). Assemblages on salamanders captured in water also had lower phylogenetic diversity than aquatic cover objects at $q = 1$, while terrestrial salamander bacterial communities had slightly higher phylogenetic diversity than terrestrial cover objects (41.69 vs 40.43), but not significantly so. Interestingly, at $q = 2$, corresponding to Rao's quadratic entropy, terrestrial salamander samples had a significantly higher effective number of lineages than terrestrial cover objects, while aquatically captured salamanders had significantly fewer than their counterparts, indicating increased phylogenetic breadth on terrestrial captured salamanders. While there were no significant differences pertaining to sampling sites or between species of salamanders, salamanders captured in aquatic habitats had significantly less taxonomic diversity at all orders of q , but only significantly less phylogenetic diversity at $q = 1$ ($p = 0.039$), meaning comparable lineage representation despite taxonomic diversity differences.

Stochasticity dominates host communities

We assessed pairwise phylogenetic and taxonomic dissimilarity through βNTI and RC_{Hom} to disentangle abundance-based community dissimilarity. We focused on three comparisons for 97% clustered sequences and broke up samples into aquatic or terrestrial origin. The first compared each single cover object to other cover objects within the site. The second and third compared each salamander to their immediate cover object and to other salamanders captured within the site, respectively. Aquatic cover object comparisons found only 3/111 (2.7%) were more phylogenetically similar than expected under a null distribution, while 13/111 (11.7%) were phylogenetically more different (**Fig. 8, facet 1, left**). The remaining 95 comparisons were neither more phylogenetically similar nor different than expected and had to be parsed taxonomically. Of these, 22 (19.8%) were taxonomically similar while 16 were taxonomically

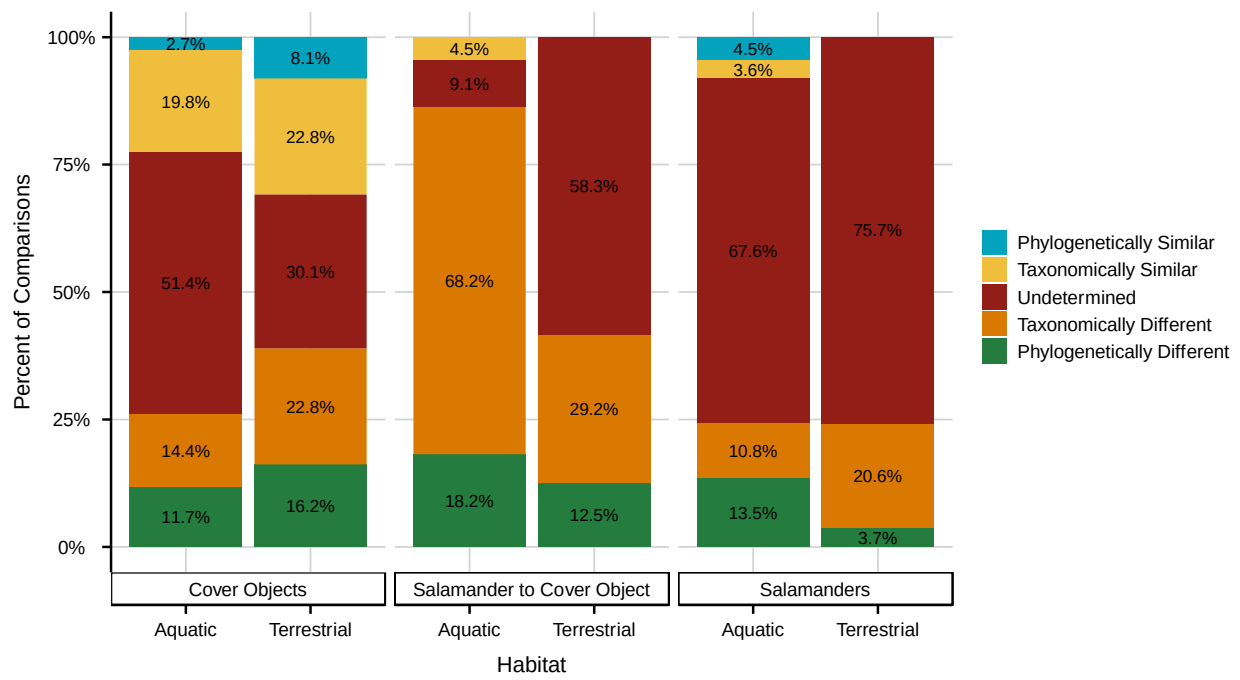


Figure 8: Similarity and Differences between Sample groups. Colors represent average Phylogenetic (BNTI) and Taxonomic (RC_{Hom}) values for each sample.

different (14.4%). The remaining 57 (51.4%) of comparisons were ‘Undetermined’ (not significantly different from their null distributions). Comparing terrestrial cover objects to other terrestrial cover objects within a site (136 pairwise comparisons) yielded similar results, with only 11/136 (8.1%) of samples being more phylogenetically similar, compared to 22/136 (16.2%) being phylogenetically different (**Fig. 8, facet 1, right**). The remaining 103 comparisons needed to be parsed taxonomically. Nearly twenty-three percent of these (31/136) were either taxonomically similar or taxonomically different and 30.1% (41/136) were undetermined, meaning they resembled similarity expected from a probabilistically assembled community. Thus, comparing cover objects to other cover objects reveals some heterogeneity associated with microhabitat across a small space, whether it be taxonomic or phylogenetically driven, and a substantial contribution of randomness.

Horizontal colonization from cover objects to salamanders is presumed to be the predominant way salamanders acquire microbes. However, salamander skin and microbial interactions have been hypothesized to result in differences between salamanders and cover objects. Aquatically captured salamanders had no ‘Phylogenetically Similar’ samples and only 4/22 (18.2%) being phylogenetically different (**Fig. 8, facet 2, left**). Fifteen of 22 comparisons (68.2%) were taxonomically different, while only one comparison was taxonomically similar and two comparisons were undetermined, respectively. Terrestrially captured salamanders showed slightly different trends. Of the 24 terrestrial salamander-cover object comparisons, none showed phylogenetic similarity, while only 3 (12.5%) were ‘Phylogenetically Different’ (**Fig. 8, facet 2, right**). When parsing the remaining (dis)similarities taxonomically, 7 (29.2%) were taxonomically different, while none were taxonomically more similar than expected under a null distribution. The majority of terrestrial salamander-cover object comparisons (14/24) were undetermined. Comparing salamanders to any other cover object within a site showed consistent patterns, with ‘Undetermined’ and ‘Taxonomically Different’ comprising the majority of group comparisons. Thus, salamanders are comprised of microbes that are phylogenetically similar to their cover objects, with differences arising at lower taxonomic levels. Differences between aquatically captured salamanders and their cover objects consistently showed taxonomic differences, while terrestrial comparisons are more stochastic. Finally, if salamanders exert strong selection for associated microbial communities, we expect convergent host-filtering

mechanisms to produce phylogenetically and/or taxonomically similar communities. For pairwise comparisons among aquatically captured salamanders, 13.5% (15/111) were phylogenetically different while only 5 (4.5%) were phylogenetically similar (**Fig. 8, facet 3, left**). Twelve comparisons (10.8%) were taxonomically different compared to just 4 (3.6%) being taxonomically similar. The remaining comparisons (75, 67.6%) were undetermined. In 136 terrestrial salamander comparisons, 5 (3.7%) were differentiated phylogenetically, with all being phylogenetically different (**Fig. 8, facet 3, right**). Taxonomic (dis)similarities revealed 28/136 (20.6%) were taxonomically more different than expected under a null distribution, while none were taxonomically similar. The remaining 103 (75.7%) of comparisons between terrestrial salamanders were undetermined, meaning they are as different taxonomically as probabilistically assembled communities. Together, this analysis finds little evidence for strong host-filtering mechanisms capable of producing community-level similarities in salamander-associated assemblages. Instead, random processes tend to be more common in salamander-salamander comparisons.

Shared relative abundance is strongly correlated

High environmental transmission is expected to produce strong correlations between the relative abundance in reservoirs and the relative abundance on hosts. Subtle differences between hosts and cover objects could arise via strain-level filtering, in which particular genotypes have greater success colonizing hosts. Prevalence of this would be evident by assessing the strength of association of hosts to their cover objects. The strength of relationships between relative abundance on salamanders and relative abundance of cover objects was assessed through Kendall's tau at four levels. Using ASVs, we had 3,332 (14%) shared terrestrial sequence variants and 2,027 (11%) shared aquatic sequences out of 24,612 total terrestrial sequence variants and 18,409 aquatic. The strength of relationship between shared relative abundance was 0.19 (aquatic) and 0.26 (terrestrial) (**Fig. 9**). Clustering sequences at a 97% similarity yielded 33% and 26% of OTUs shared between terrestrial and aquatic paired samples, with correlation coefficients of 0.41 (terrestrial) and 0.24 (aquatic). Glomming at the genus taxonomic level produced tau coefficients of 0.62 and 0.46, with 72% and 69% of genera shared for terrestrial and aquatic comparisons, respectively. Finally, glomming at the family taxonomic level showed

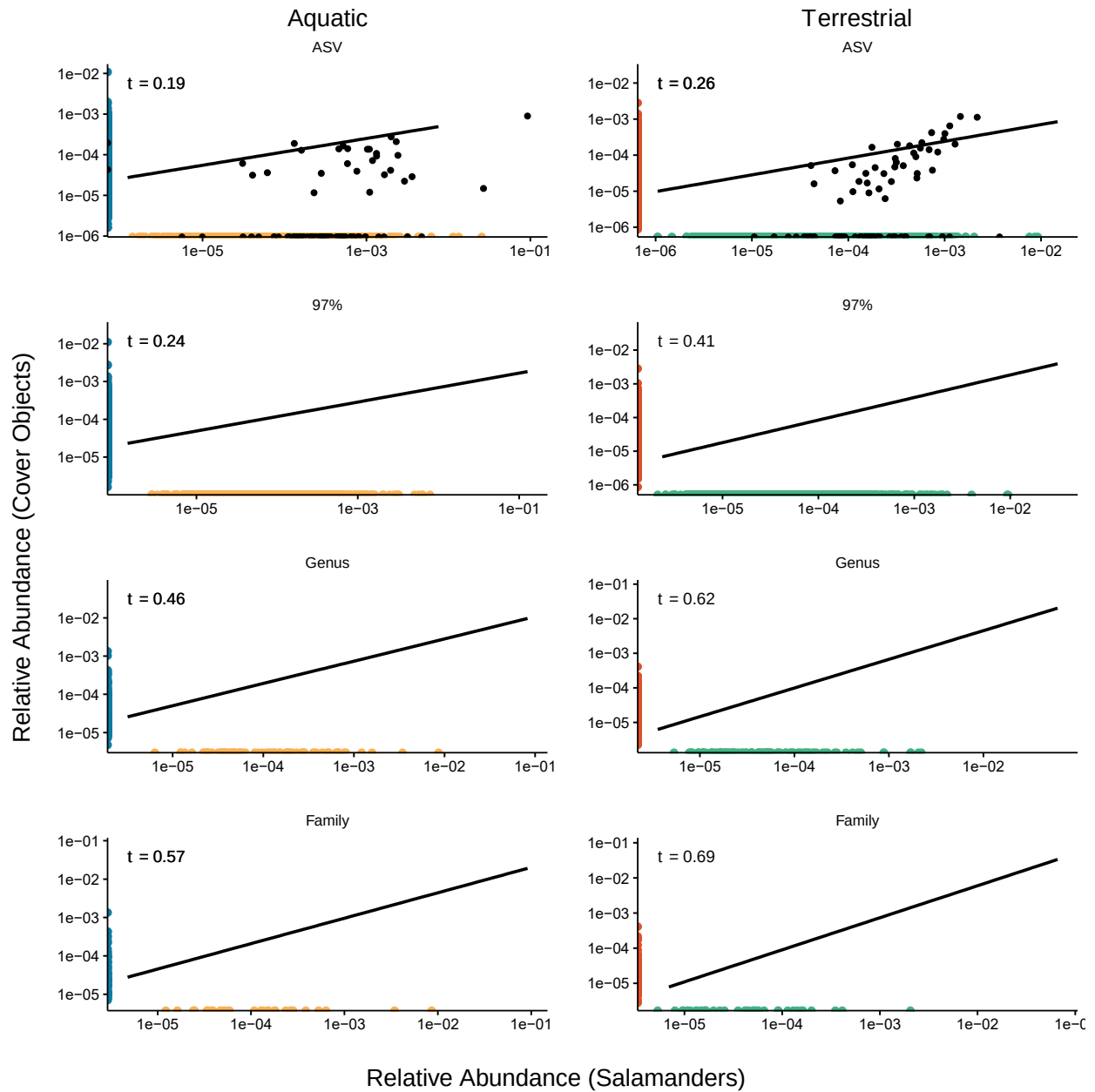


Figure 9: Correlation between relative abundance on salamanders (x-axis) and relative abundance on cover objects (y-axis). Gray points denote taxa detected in both salamander and cover object samples. Note the log-transformed x and y axes.

a strong correlation between relative abundance on salamanders and cover objects (0.69, 0.57), with 79% and 75% of respective terrestrial and aquatic families shared. Taxonomic differences are widespread at the sequence level and the relationship between the relative abundance on salamanders and the relative abundance on cover objects increases with more inclusive clustering. Thus, the most abundant bacterial taxa on salamander skin tended to be the most abundant taxa in the environmental pool, however, many taxa observed on salamanders were not detected in environmental samples and the differences are most prevalent at the sequence level.

Affinity for hosts restricted to a small subset of bacteria

We used an indicator species analysis to identify sequence variants with positive correlation coefficients with salamanders compared to their cover objects for both aquatic and terrestrial samples. While beta diversity analyses found salamander communities were largely stochastically assembled, sequence variants showing positive associations with salamander hosts could be salamander specialists. To identify sequence variants associated with salamanders found both within a habitat and across all habitats, we first identified indicator taxa associated with salamanders from a particular habitat and then associated with all salamanders. Forty-five indicator species were identified for terrestrially captured salamanders, all having an average r_{pb} greater than 0.25, with values ranging from 0.25 to 0.37 (**Fig. 10**). Average cumulative relative abundance of all indicators in terrestrial salamander samples (45 sequence variants) was 0.02, compared to 0.004 average cumulative relative abundance in cover objects. Of the 45 sequence variants, five belonged to the family Xanthobacteraceae, while three each belonged to Oxalobacteraceae and Pseudonocardiaceae (**Table A3**). Aquatically captured samples had 75 taxa identified as indicators, with correlations ranging from 0.25 to 0.44 and an average cumulative relative abundance of 0.18 in salamanders and 0.002 in cover objects. Beijerinckiaceae was the most represented family, with 12 sequence variants, followed by Comamonadaceae and Microbacteriaceae with 6 and 4 sequence variants respectively. Of these 120 unique sequence variants, 9 displayed 97% sequence similarities to isolates capable of inhibiting Bd in laboratory assays, with Pseudomonadaceae and Xanthomonadaceae, and Comamonadaceae containing two sequences variants each (Woodhams et al., 2015). The other three belonged to Moraxellaceae, Microbacteriaceae, and an unidentified sequence in

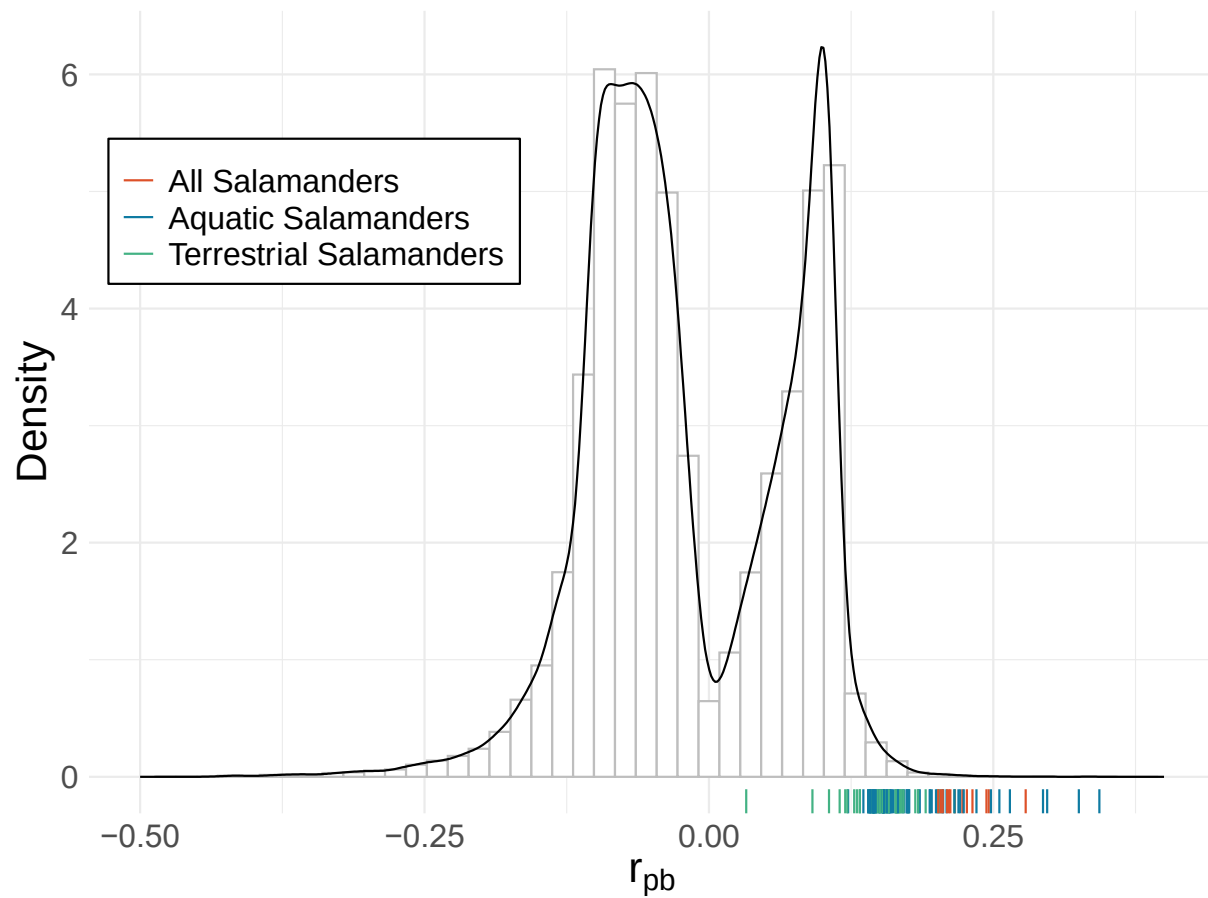


Figure 10: Distribution of point biserial correlation coefficients (r_{pb}) of sequence variants for salamanders and cover objects. Rug lines denote group membership

Enterobacterales. Eight of these came from aquatically captured salamanders, while only one from terrestrially captured salamanders. Thirty-two sequence variants were identified as indicators for all salamanders. Of 16 were previously identified with aquatic salamanders and three with terrestrial salamanders, leaving 13 sequence variants associated with all salamanders regardless of habitat. Three of these belonged to Microbacteriaceae while two belonged to the families, Acidobacteriaceae and Nocardiodaceae. Mean cumulative relative abundance of these indicators (13 sequence variants) was 0.005 for salamanders and 0.0008 for cover objects. Of the 32 sequences identified as having strong associations to all salamanders, four were matched isolates known to inhibit Bd, belonging to the genera *Chryseobacterium*, *Comamonas*, *Stenotrophomonas*, and *Pseudomonas*, with the latter two also included in the preceding paragraph.

Discussion

Untangling complex influences of host-associated communities is paramount to understanding both the ecological and evolutionary processes underlying a host's microbiome. The amphibian skin microbiome is particularly important due to its potential to inhibit the growth of fungal pathogens associated with global amphibian declines (Barnes et al., 2020; Becker et al., 2015; Harris et al., 2009). As such, understanding the patterns of assembly and sources of differentiation between hosts and environmental reservoirs is important for developing conservation strategies and uncovering the contributions of microbes to host fitness. The amphibian skin microbiome appears both 'heavily reliant' yet 'different' from their environmental reservoirs, but the mechanisms driving these differences are poorly understood, as is the relationship between intra and interspecific differences observed on hosts (Belden et al., 2015; Bird et al., 2018b; Kueneman et al., 2014; Prado-Irwin et al., 2017). In this study, we assessed the patterns of differentiation between salamanders, other salamanders, and their cover objects in the context of small-scale microhabitat differences. We implemented null models classically used in community assembly to understand the degree to which stochastic and deterministic processes might explain differentiation of host microbiomes from each other and surrounding reservoirs. While salamander skin microbiomes are less diverse than their environmental reservoirs, diversity is not the sole reason for differentiation. Furthermore,

differences between salamanders suggest idiosyncratic patterns of ecological assembly inconsistent with strong host filtering and more in line with random assembly stemming from random dispersal, weak host filtering, and weak priority effects.

Lower diversity within host-associated communities compared to environmental reservoirs with similar sampling efforts remains a consistent pattern in microbiome research. The underlying mechanisms driving this pattern for amphibian skin are likely linked to energy availability, surface area, and symbiont colonization. Energy availability is likely to affect the number of individuals that can survive at a site, and therefore the number of species that can maintain viable populations (More Individuals Hypothesis), or the promotion of specialists utilizing ‘rare’ resources (Niche Specialization Hypothesis) (Di Cecco et al., 2022). Diversity-area and diversity-energy relationships are well established in ecology, and have recently been shown in gut microbiomes (Godon et al., 2016), but are rarely applied to host-associated vs environment communities. External microbiomes (such as those on skin) are perhaps expected to be less diverse than free-living assemblages with open dispersal and equal sampling effort. Distribution of free-living assemblages is influenced by pH, moisture, elevation, latitude, and nutrient availability (Fierer & Jackson, 2006; Serna-Chavez et al., 2013). The nutrient-rich, acidic soil of Southern Appalachia boasts healthy microbial diversity, especially under cover objects where microbes aggregate in biofilm on rocks or crevices in logs and facilitate nutrient recycling and decomposition (McTammany et al., 2008; Osburn et al., 2021). Nutrient availability on salamander skin is likely more limited, as microbes have been documented clustering around mucus glands (Lauer et al., 2007). This smaller area with more limited resources would inevitably exacerbate competition and thus promote local extinctions through competitive exclusion. Aquatically captured salamanders had the least diversity, while aquatic cover objects had the highest number of effective species. Biofilm aggregation on aquatic cover objects could be resilient to water flow and potentially exposed to a higher diversity of microbes flowing from upstream sources. The low diversity on aquatically captured salamanders could also reflect failed colonization since flowing water would be more likely to wash microbes off the surface of the skin. Alternatively, less diversity recovered could be a product of sampling error, as our previous study found no significant difference between aquatically and terrestrially

captured salamanders from the same site despite significant differences in cover object communities.

Despite having lower richness, terrestrial captured salamander skin samples were not significantly less diverse than terrestrial cover object samples at $q=1$ and $q=2$, and were, in fact, more phylogenetically diverse at $q=2$, indicating that a larger proportion of rare lineages was detected in terrestrial cover objects. Aquatically captured salamander samples were less taxonomically diverse than cover objects and all samples showed similar declines in effective species at increasing orders of diversity, indicating both salamanders and cover objects have an uneven distribution of abundances among microbes. 16S rRNA sequencing does not enable us to assess the percentage of dormant bacteria, however, other studies assessing dormancy have concluded a large proportion, sometimes up to 80% of taxa in soil communities can be dormant (Lennon & Jones, 2011; Sorensen & Shade, 2020). High temporal turnover of the amphibian skin microbiome and local extinction observed in laboratory experiments indicate colonization in wild amphibians is a continuous process (Loudon et al., 2016). Dormant bacteria, as well as taxa with poor dispersing capabilities, would be less likely to colonize the host, but would still be detected in cover object samples, which could explain the high richness in cover objects despite lower effective species at higher orders of diversity. Regardless, given the significant differences in richness between salamanders and cover objects, future research should be conscientious of the impacts of α diversity on inferences about β diversity, especially when using binary dissimilarity analyses (Jaccard or Sørensen).

To account for within sample diversity differences in our samples, we utilized a null model (RC_{Hom}) to assess the dissimilarity between samples not attributed to differences in diversity. Our results are consistent with previous inferences that salamanders acquire microbes predominantly from the environment via environmental transmission. While the differences are subtle, salamander skin assemblages are not merely passive samples from their immediate environment. In the extreme, we might expect unrestricted dispersal to result in statistical similarity between each salamander and the cover object it was captured under. This was not the case, as only a small proportion of such comparisons were taxonomically more similar than expected. Most aquatic salamander-cover object pairs were taxonomically differentiated, and terrestrial salamander cover-object pairs were often no more or less different than expected by

chance. In addition to the pairwise comparisons, positive correlations of mean relative abundance at increasing taxonomic levels indicate most differences between salamanders and cover objects occur predominately, but not exclusively, at the lowest taxonomic levels. Only a small portion of comparisons (18% and 12%) were differentiated phylogenetically, similar to pairwise comparisons among cover objects. In other words, salamander-associated communities are differentiated from cover objects in the detection and relative abundance of OTUs along predominately shared phylogenies.

While salamanders are statistically ‘different’ from cover objects, salamanders show little similarity to other salamander hosts, as evident by high pairwise turnover rates and patterns of differentiation consistent with random sampling. Host secretory products act as the first line of immune defense against pathogens and form an ecological landscape for microbe colonization. Adaptation or tolerance to these secretory products appears to be common for functionally important symbionts (Brunetti et al., 2022; Loudon et al., 2016). While these stress-induced bioactive peptides are likely not constantly released on the skin, mucosal gland secretions responsible for regulating physiological homeostasis and preventing evaporative water loss likely play a more consistent role in shaping community interactions on amphibian skin. Recent investigations indicate these mucin-rich secretions contain an abundance of N- and O- linked oligosaccharides and can vary between anuran species (Barbosa et al., 2022). The gel-forming nature of this mucus presents a stage for symbiotic interactions between hosts, residing microbes, and potential colonizers, which has been hypothesized to result in ‘distinct’ amphibian communities attributed to host filtering. In the present study, we find little evidence for strong host filtering mechanisms. Our results suggest salamander skin could act as a selective medium promoting differentiation from cover objects at the lowest taxonomic levels but does not result in a predictable salamander-specific assemblage.

Previous studies have sometimes emphasized the detection of statistically significant differences between groups without evaluating heterogeneity (diversity turnover) within groups. Thus, it can be easy to misinterpret a statistically significant difference as proof that each group has a “distinct”, “predictable”, or “specific” microbial assemblage. In many cases, this is an unwarranted inferential leap. More detailed analyses of turnover and signatures of community

assembly processes are needed to understand patterns of both differentiation and similarity among complex microbiomes composed of thousands of variants (J. C. Stegen et al., 2013).

Our results indicate differences between salamanders are largely due to neutral or weak processes. Colonization of microbes onto salamander skin is dependent on the dispersal ability of the microbe, tolerance for salamander conditions, and relationships with microbes already present. The diffuse relationships between host secretions and colonizers are further exacerbated by the diffuse interactions between residing microbes and new colonizers, meaning the appearance of random differences could be a product of weak priority effects and weak community interactions in tandem with weak host filtering and stochastic dispersal.

Using null models, the size of the regional pool can influence the deviation from randomized communities. A small regional pool can result in low deviation from a null community, while a large pool would drive RC_{Horn} values further from the null (J. C. Stegen et al., 2013). Our paired sampling design defined the regional pool as any microbe detected on a rarefied salamander or cover object sample at any given site. Moreover, pairwise comparisons among cover objects provide a valuable frame of reference for the interpretation of comparisons involving hosts. Given that cover objects possessed drastically fewer ‘Undetermined’ comparisons and even exhibited partitioning according to microhabitat utilization, our analyses were not underpowered for assessing the differences between salamanders and other salamanders. However, this analysis did reveal a subtle difference in the patterns of dissimilarity between salamanders and their associated cover object that requires contextualization. Aquatic salamander to cover object comparisons were predominately “Taxonomically Different”, while terrestrial salamander to cover object comparisons were “Undetermined”. Aquatic cover objects had higher effective diversity than terrestrial cover objects, while aquatically captured salamanders were, on average, less diverse than terrestrially captured salamanders. Average differences (Horn dissimilarity) in sites comparing aquatically captured salamanders to aquatic cover objects were higher (means: 0.84, 0.87) than in terrestrial sites (means: 0.73, 0.70). This variability, in conjunction with less diversity, and the mechanisms described in preceding paragraphs, could have driven null model estimates towards ‘Taxonomically Different’ as opposed to ‘Undetermined’. This also explains the weaker relationship between relative abundance between aquatic and terrestrial salamanders and their cover objects. While the null

model controls for differences in alpha diversity, the increased dissimilarity would result in a larger community pool, which would inevitably affect the assembling of probabilistic communities. In this sense, aquatically captured salamanders show more differences to their cover objects, which is both an artifact of the analysis and a representation of our sampling. Regardless, the notion that salamander-associated communities are nested communities of cover objects, or display convergence through host filtering, is refuted at the community level.

While salamander skin shows little evidence of community-level convergence, we were able to identify 32 sequence variants strongly associated with salamander hosts, indicating that there may be salamander specialists or genotypes favored by conditions on salamander skin. This result might support the concept of a core community (Risely, 2020), but members of this group generally account for a small proportion (2-20%) of the bacteria in samples from salamander skin. Temporal studies have also found a detectible core microbiome across seasons and habitats, with members displaying temporal constancy often associated with functional capabilities on hosts (Le Sage et al., 2021; Walke et al., 2021). Correlations between shifts in bacterial communities and bacterial community metabolites post-disturbance have also been documented (Walke et al., 2015, 2021), indicating three-way interactions between the host secretory products, residing microbial metabolites, and new colonizers. This core community could be important in mediating priority effects by acting as hub taxa and facilitating or inhibiting the colonization of new microbes via niche modification. While network analyses are perhaps speculative for large communities, lab experiments manipulating introduction order in tandem with host secretions could be fruitful in understanding these interactions. Nonetheless, these indicator taxa present the most compelling candidates for future investigation of symbiotic interactions in amphibians.

Our results provide three pertinent conclusions regarding the assembly of the plethodontid skin microbiome. First, horizontal colonization from environmental reservoirs is the dominant way in which hosts acquire microbes. Relative abundance on hosts and cover objects is strongly correlated, especially at the higher taxonomic levels, underscoring the observation that the most abundant bacteria in the environment are often most abundant on salamander skin. Secondly, despite being less diverse than cover objects, salamander hosts are also comprised of statistically ‘different’ OTUs from cover objects stemming from an array of weak ecological assembly processes that result in communities being dissimilar to both cover objects and other

salamanders. Finally, salamander skin lacks sufficient selective structuring ability to produce distinct community similarities between individual salamanders and salamander species. Instead, residing microbes, host secretions, and high environmental transmission produce largely divergent communities with only a small potential core community shared.

**CHAPTER III: TEMPORAL TURNOVER AND THE CORE
MICROBIOME OF *EURYCEA* CF. *WILDERAE***

Abstract

External microbiomes, such as the amphibian skin, are in constant contact with environmental reservoirs and experience high rates of immigration. Predicting the natural range of variation for such microbiomes is important for understanding how communities might respond to disturbance in addition to parsing transient associations from candidates linked to host benefits. In this study, we use the phenology of a migratory salamander (*E. cf. wilderae*) to understand seasonal changes in skin assemblages as hosts move between aquatic and terrestrial habitats. We additionally test the utility of a core microbiome concept using two commonly applied and two modified definitions. Seasonal heterogeneity was high between salamander hosts, but turnover was exacerbated when hosts changed habitats, consistent with immigration of microbes from a new regional pool. Potentially defensive microbes capable of inhibiting the growth of chytrid fungi were most abundant when hosts occupied terrestrial habitats but some showed persistence on hosts year-round despite low abundance in aquatic habitats. These results indicate the amphibian skin microbiome lacks temporal constancy but contains a few microbes with affinity for salamander conditions that also produce anti-fungal compounds. These non-random associations provide opportunities for deeper investigation into the mechanisms underlying persistence on hosts and the potential for reciprocal benefits in an external microbiome.

Introduction

Complex host-associated microbiomes, such as the skin and gut, consist of many individuals with diverse relationships to each other and the host. The assembly of these ecological communities is dependent on varying strengths of environmental colonization, host traits, and microbe-microbe interactions. Internal microbiomes are generally more resistant to immigrants due to an often-inhospitable gut environment and tight ecological networks (sometimes referred to as ‘colonization resistance’) (Shade et al., 2012; Sylvain et al., 2020). External microbiomes tend to be more porous and experience high rates of immigration from surrounding reservoirs, often resulting in heterogeneous communities that reflect local conditions. This reliance on external reservoirs makes parsing relationships to the host difficult for external microbiomes. Laboratory assays have found examples of hosts benefiting from individual and collective symbionts (Brunetti et al., 2019; Jani et al., 2021; Vaelli et al., 2020).

However, these isolated interactions, while informative for how symbionts could impact host fitness, offer little insight into the dynamics of host-associated complex communities in nature. Salamander-associated communities show assembly patterns consistent with stochastic dispersal and extinction, with host communities heavily reliant on environmental reservoirs. As such, heterogeneous communities- even between co-occurring hosts- make it difficult to imagine when, if ever, a stable state is reached and what the ramifications might be for hosts after short-term (pulse) or long-term (press) disturbances.

Community stability describes the ability of a community to minimize variability or return to its original state after disturbance and can be broken down into three linked facets: constancy, resistance, and recovery (de Bello et al., 2021). For host-associated communities, disturbances are diverse in their severity and modes of action but can broadly fall into three non-mutually exclusive categories- those altering the sources (changing the immigrant pool), those directly altering the species interactions (immigrants or pathogens), and disturbances to the host that evoke physiological/immunological changes to the symbiotic landscape (host injury, stress, pathogens). Community responses to these disturbances can range from a return to a stable state (original composition), alternative stable states (different, but stable composition) (Ravel et al., 2011), or a regime shift (large change between alternative stable states) (Gonze et al., 2017). In amphibian-associated skin communities, disturbances have been evaluated in the context of disruption to the immigrant pool (Hernández-Gómez, Byrne, et al., 2020b; Jiménez et al., 2020), exposure to skin pathogens (Jani & Briggs, 2014; Weeks et al., 2020), or host harm (Hernández-Gómez, Wuerthner, et al., 2020), with host-associated communities typically showing increased group dispersion, rapid compositional changes, and loss of taxa linked with anti-fungal capabilities, indicating low resistance to all types of disturbances. Similarly, community recovery post-disturbance often fails to result in a return to the original state for amphibian-associated communities, but some evidence suggests alternative stable states, although potentially confounded by captive conditions (Jani et al., 2021). Given the global decline in amphibians stemming from land-use change, climate change, and fungal pathogens, understanding the dynamics predicting how communities respond to disturbances can aid in estimating the potential effects of emerging disturbances on hosts and external symbionts.

Of particular importance for interpreting community responses to disturbance events (pathogen, climate change) is defining expectations of ‘natural variation’ or ‘normal operating range’ for host-associated communities. Temporal community turnover of bacteria on hosts is generally high for amphibians, yet there is some evidence for ‘structural stability’ in the retention of taxa temporally, with ‘rare’ taxa being the most transient, while moderately abundant taxa fluctuate over time (Ellison et al., 2021; Walke et al., 2021). For studies focusing on temporal variation in a single habitat, high turnover is likely attributed to the continuous stochastic dispersal and extinction, with weaker effects of host physiology and microbial interactions.

The biphasic lifestyle of amphibians exposes them to aquatic and terrestrial reservoirs with significantly different compositions that are reflected in skin-associated microbial communities at small scales (A. J. Douglas et al., 2020). Habitat changes often coincide with phenology, particularly courtship, mating, metamorphosis, or overwintering, which are also linked to changes in skin physiology (e.g., ion regulation or desiccation resistance) and secretory products on the skin (Duellman & Trueb, 1994; Le Sage et al., 2021; Wells, 2010). The amphibian skin serves as a landscape for symbiotic interactions, meaning subtle changes in skin chemistry could alter nutrient availability on hosts (Hernández-Gómez & Hua, 2023; Krynak et al., 2016; Varga et al., 2019; Woodhams et al., 2023b). As such, biphasic transitions likely result in disturbance events for host-associated communities by changing both the immigrant pool and nutrient availability supplied by hosts (Bletz, Perl, Bobowski, et al., 2017; L. Xu et al., 2020). Microbial turnover during phenological habitat shifts, in tandem with host physiological changes, is predicted to be greater than fluctuations within a single habitat over time. Here we describe seasonal changes in salamander skin microbiomes as the hosts move between aquatic and terrestrial habitats to evaluate the effect of the environment on skin assemblages. We test whether a non-random component of the microbiome remains constant across the environmental transition, with particular attention to symbionts with potential defensive function against fungal pathogens.

Attention towards a ‘core’ microbiome has increased since 2007, when the launch of the Human Microbiome Project included a hunt for a ‘core’ microbiome- a group of microbial taxa/genes shared by most humans (Turnbaugh et al., 2007). Ecologists readily adopted this concept, seeking to identify groups of microbes associated with host species. Implicit in the

concept is the notion that commonly detected, or more abundant taxa have important functional relationships with hosts. In some cases, this has been true, however, rare taxa can have profound consequences as well (Antwis et al., 2019). Furthermore, microbes do not have to be a part of a ‘core’ microbiome (whatever the definition may be) for the host to receive benefits from either individual symbionts or the microbial consortium more broadly. And finally, commonly detected or abundant taxa might simply be those that are abundant in the external environment. In this sense, commonly detected taxa on hosts could reflect environmental reservoirs and thus do not necessarily represent positive, or tight associations with hosts. Thus, it is critical to evaluate skin-associated microbiomes in comparison to relevant free-living assemblages (Fitzpatrick & Allison, 2014). In this study, we evaluate four definitions of the ‘core’ microbiome for salamander-associated communities by identifying common OTUs found within a given sampling period (common core), OTUs consistently detected within a site over the course of four sampling periods (temporal core), OTUs associated with anti-fungal capabilities (defense functional core), and OTUs showing high affinity for the host condition relative to environmental reservoirs (host-associated core).

The most researched contribution of amphibian-associated symbionts is the ability to inhibit the growth of fungal pathogens *Batrachochytrium dendrobatidis* (Bd) and *Batrachochytrium salamandrivorans* (Bsal) through secondary metabolites. These pathogens can have major impacts on amphibian survival, and the potential benefits of defensive symbiosis with antifungal bacteria could be enormous for the hosts (Harris et al., 2006, 2009). Anti-fungal metabolites are not exclusive to amphibian symbionts but instead likely reflect an evolutionary arms race between competing bacteria and fungi in soils. As such, anti-chytrid capabilities are phylogenetically widespread and could be functionally redundant in amphibian-associated communities (Barnes et al., 2020). The acquisition of these symbionts is presumed to be environmental colonization followed by persistence on hosts, though there has been some evidence for vertical transmission (Kouete et al., 2023; Walke et al., 2011). Importantly, certain defensive symbionts show persistence on amphibians in captivity and are resistant to host anti-microbial peptides, meaning that while defensive benefits could be widespread in nature, hosts might form reciprocal associations with a few species that can tolerate and supplement their own defensive arsenals (Flechas et al., 2019; Loudon et al., 2016).

Eurycea cf. *wilderae* is a brook salamander occupying higher elevation, high-gradient streams and is a paraphyletic member of the *Eurycea bislineata* species complex. While we refer to this species as *Eurycea* cf. *wilderae*, the populations in our study area are part of a well-delimited but undescribed species in the Blue Ridge (Pierson et al., 2021, 2023). Salamanders in this complex utilize streams for overwintering and nesting while occupying terrestrial uplands and riparian zones from late spring until late fall. Individuals are observed in October/November moving from terrestrial areas into aquatic habitats, with courtship and mating occurring along the way. While this genus has shown low susceptibility to Bd (25 positive samples of 232 field swabs), *Eurycea* is among the more susceptible Plethodontid genera to Bsal in lab experiments (Augustine & Neff, 2016; Bauer et al., 2018; Carter et al., 2020; DiRenzo et al., 2021; Jongsma et al., 2019; Muletz et al., 2014). Infection by these pathogenic fungi is most likely to occur when the animals are in their aquatic phases, therefore changes in the representation of skin symbionts with antifungal properties during and after transitions between water and land might be critical for host susceptibility. The goals of this study were to a) assess the temporal variability of skin bacteria in relation to host phenology (aquatic and terrestrial phases) and b) evaluate four definitions of the core microbiome to characterize host-microbe associations.

Methods

Sample collection

To assess the temporal constancy and core microbiome of *E. cf. wilderae*, we selected nine low-order streams within Great Smoky Mountain National Park and sampled each site seasonally for the course of one year in 2018-2019. We analyzed one salamander per site per season. Salamanders were captured, rinsed, and swabbed as described previously. The cover object where the salamander was found was also swabbed. Swabs were broken off into sterile 1.5ml centrifuge tubes and stored on dry ice in the field, before storage at -80°C in the lab. Across our nine sites sampled, six followed the anticipated seasonal habitat utilization of *E. cf. wilderae*, while two sites had salamanders on land in the Spring, and in water in the Fall during sampling. Additionally, one site had terrestrial captured salamanders for three seasons. Finally, we were unable to find salamanders during the Spring sampling at site N, and during the winter

sampling of site K, resulting in seven sites with four samples (one in each season) and two sites with only three seasons represented (**Table 5**).

Sample processing

DNA was extracted using the Qiagen DNEasy Powersoil Kit following the manufacturer's protocol with the exception of including a 5-minute incubation step at room temperature before spin column elution and a 50µl elution volume to compensate for expected low recoveries. The V3-V4 region of 16S rDNA was amplified using the 341F 785R primers in 27.5µl reactions containing 12.5µl KAPA HiFi HotStart PCR ReadyMix, 5µl forward primer (1µM), 5µl reverse primer (1µM), and 5µl template. Conditions for the PCR were as followed: 95°C for 3 minutes, followed by 30 cycles of 95°C for 30s, 55°C for 30s, and 72°C for 30s. The final elongation step was 72°C for five minutes and reactions were then held at 4°C. Amplified products were verified on a 1.5% agarose gel and purified using AMPure Beads (Beckman Coulter). Purified samples were assigned unique Nexterra barcodes in a multiplexing PCR using the same conditions described above, only with 8 cycles. Products were then purified again, quantified using a Nanodrop spectrophotometer (Thermo Fisher), and pooled to equimolar concentrations. Quality and amplicon size were confirmed and sequenced at the University of Tennessee Genomics Core on an Illumina Miseq PE250 flow cell with a 20% PhiX spike.

Bioinformatics

Demultiplexed sequences were processed using Qiime2 (Bolyen et al., 2019). Primers were removed with Cutadapt v3.5 and sequences then entered the DADA2 pipeline (Callahan et al., 2016; Martin, 2011). Forward reads were trimmed to 225 bases and reverse reads were trimmed to 215 base pairs. Quality filtering was carried out with a maxEE threshold of c(2,3) and after correcting errors, paired sequences were merged, dereplicated, and clustered at a 97% sequence similarity before formulating an OTU table and removing chimeras. A sepp-fragment insertion tree was then constructed using the Silva 128 reference database (Janssen et al., 2018), and the phylogeny was time-calibrated using TreePL (S. A. Smith & O'Meara, 2012). The OTU table was exported, and taxonomy was assigned using the IDTaxa algorithm in R v4.2.0 in the DECIPHER package (Murali et al., 2018; Quast et al., 2013). Following taxonomic assignment, reads assigned to Chloroplast and Mitochondria were removed, as were singletons and any

Table 5: Salamanders sampled in each season and their habitat(s)

Season	Habitat	
	Terrestrial	Aquatic
Spring	1	7
Summer	9	0
Fall	8	1
Winter	1	7

sequence not between 400 and 428bp long. We then used sterile water from the field and extraction/PCR blanks to assess contamination, which resulted in the removal of 23 OTUs using the decontam package (Davis et al., 2018). Additionally, any sample containing fewer reads than a ‘blank’ was discarded. The final dataset used for analyses contained 2,261,552 reads mapped to 17,667 OTUs in 67 samples, 33 of which were salamander cover object pairs in aquatic or terrestrial habitats seasonally.

Diversity within and between samples

Taxonomic and phylogenetic alpha diversity were used to assess the seasonal changes in within-sample diversity of salamander-associated communities and communities found on cover objects. Host-associated communities can vary seasonally from lower body temperature and host activity but could also reflect dormancy of environmental assemblages and decreased immigration (Le Sage et al., 2021; Shade et al., 2012; Tong et al., 2020). For taxonomic alpha diversity, we used three orders of diversity (Hill Numbers) representing the richness ($q=0$), exponentiated Shannon Index ($q=1$), and scaled probability of interspecific encounter ($q=2$) (M. O. Hill, 1973; Jost, 2006). For phylogenetic diversity, these orders correspond to Faith’s phylogenetic diversity ($q=0$), Allen’s entropy ($q=1$), and Rao’s quadratic entropy ($q=2$) and represent the effective lineages over time (Chiu & Chao, 2014). We compared differences in α diversity for three hypotheses: salamander-associated communities would be less diverse than their environmental reservoirs, alpha diversity would be lower on aquatically captured salamanders than terrestrially captured salamanders, and alpha diversity would be lowest in the winter for salamanders. Alpha diversity hypotheses were tested using either a paired t-test in the rstatix package for comparing salamanders to cover objects or an ANOVA with a Tukey Honest Significant Difference post-hoc test for parsing differences across seasons (Kassambara, 2020). Due to sampling biases arising from sampling/sequencing effort, we estimated diversity for each of 500 rarefactions and used the mean diversity estimate for each sample using the *vegan* package in R (Oksanen et al., 2019). Our rarefaction level was 13,407 reads, with rarefaction curves becoming asymptotic around 9,500 reads.

The effective number of species is a reflection of the number of species (richness) and the equitability of abundances (evenness) (Tuomisto, 2012). When richness and/or abundances vary,

Chao et al. (Chao & Ricotta, 2019) proposed two requirements for a measure of evenness: a fixed interval with minimum and maximum (unrelatedness criterion), and evenness measures that perform consistently for absolute or relative species counts (scale invariance). A proposed measure fitting these criteria is to use the normalized absolute slope of Hill-number profiles, meaning evenness (between 0 and 1) represents the normalized ratio of higher-order diversities to richness. In this study, host habitat changes expose hosts to a different immigrant pool, potentially increasing evenness through local declines of resident taxa and colonization of new microbes filling niche space. To evaluate seasonal patterns of evenness for salamander and cover object-associated communities, we estimated the taxonomic evenness of a Hill number profile from $q=0$, $q=1$, and $q=2$ across 500 rarefactions.

For assessing beta diversity pertaining to our study design, we used variance partitioning of the Horn and PhyloHorn dissimilarities ($q=1$), partitioning by Site, Season, and Habitat (as well as interaction terms). The adjusted r^2 represents the inertia explained constrained models, which was then statistically assessed using dbRDA followed by an `anova.cca` in the package *vegan*. Due to the phenology of *E. cf. wilderae*, habitat and seasons are necessarily confounded, and we thus conditioned the model to remove variance associated with habitat while testing for variance seasonally. While post-hoc analyses for crossed-sampling designs requiring conditioning are currently unavailable, Alekseyenko (2016) developed a multivariate Welch t-test for distances (Alekseyenko, 2016). This method is robust to sample size differences and was used to statistically compare consecutive seasons in the context of habitat shifts, correcting for multiple comparisons with sequential Bonferroni. To apply a more nuanced approach for visualizing temporal turnover at the community level, we assessed turnover between salamanders and cover objects, salamanders and other salamanders, and cover objects and other cover objects.

Core microbiome

We implemented four definitions of the core microbiome (common, temporal, host-associated, and defensive functional) to identify relationships of OTUs to salamanders in relation to cover objects. We defined the common core (one for each sampling season) as OTUs detected on 80% of individuals within a given sampling season (across 9 sites). The temporal core was identified as OTUs detected on all salamanders within a given site across three or more sampling

seasons. The defensive functional core was identified as any OTUs matching strains known to have antifungal properties *in vitro* (Woodhams et al., 2015) at a threshold of 99% similarity. Finally, our host-associated core identified OTUs with statistical associations with amphibian hosts relative to cover objects, which was assessed using an indicator species analysis using the *stratassoc* function (De Cáceres et al., 2010). To test whether the distribution of indicator values could be attributed to random community assembly, we simulated a ‘null’ distribution of expected associations, we probabilistically assembled to preserve the rarefied richness in each sample and shuffled group membership. In brief, a pool of OTUs was created and random taxa were selected, with the probability of each species dependent on the frequency of samples it was detected in. Relative abundance of taxa for each sample was then drawn from a probability distribution, with the probability being the relative abundance of the selected taxa in the metacommunity. This was done across 500 rarefactions and the distribution of correlation coefficients was plotted along with observed patterns. For each of the four ‘core’ definitions, we assessed the number of OTUs passing our threshold and plotted their relative abundance on amphibians and cover objects over the course of a year. All analyses were run in R v4.2.0 and graphics were created using the ggpubr, ggplot2, ggpubfigs packages (Kassambara, Alboukadel, 2020; Steenwyk & Rokas, 2021; Wickham, 2016).

Results

Alpha diversity is consistent on hosts seasonally

Studies analyzing the effects of seasonality on amphibian skin communities have often found that diversities on hosts decreased in cooler months, attributed to ambient temperatures, decreased host metabolisms, or higher dormancy in free-living assemblages. We first asked if salamanders had lower diversity than their cover objects in a particular habitat (aquatic or terrestrial), and then focused on seasonal trends on salamanders. Aquatic cover objects had significantly higher effective diversity than terrestrial cover objects at $q=1$ and $q=2$ ($p<0.05$), but microbial diversity in aquatic salamander communities was not significantly different from diversity in terrestrial salamander communities in any taxonomic order of diversity (**Table 6**) ($q=1$: 448.8 vs 432.5, $p=0.67$). Similarly, we found no significant difference in the effective number of species on salamanders seasonally, though salamanders sampled in the fall had the

Table 6: Taxonomic and Phylogenetic diversity estimates by habitat. Superscripts denote statistical significance of paired comparisons.

Group	Taxonomic			Phylogenetic		
	$q=0$	$q=1$	$q=2$	$q=0$	$q=1$	$q=2$
Aquatic Cover Objects	1206.9	548.8 ^A	262.4 ^B	428 ^C	29.6 ^D	16.9 ^E
Aquatic Salamanders	1028.3	448.8	194.2	400.4 ^F	29.3	16.9
Terrestrial Cover Objects	942	370.8 ^A	158.1 ^B	305.7 ^C	25.3 ^D	15.6 ^E
Terrestrial Salamanders	956.9	432.5	184.5	333.1 ^F	27.6	16.6

fewest effective species, while salamanders sampled in the Spring had the highest ($p=0.085$, **Table 7**).

Phylogenetic alpha diversity showed trends consistent with taxonomic assessments. While there were significantly higher effective lineages on aquatic cover objects compared to terrestrial cover objects in all phylogenetic diversity metrics (all p values <0.05), there were no significant differences between diversity in communities on salamanders and diversity in communities on cover objects for either habitat. Salamanders-associated communities tended to have slightly higher phylogenetic diversity than their cover objects (**Table 6**). Aquatic salamanders had significantly higher effective lineages at $q=0$ than terrestrial salamanders (400.4 vs 333.1, $p=0.029$), but not at $q=1$ or $q=2$ ($p=0.18$, $p=0.92$). Evaluating the effects of season on alpha diversity of salamander-associated communities found communities sampled in the Spring had significantly higher phylogenetic richness than salamanders sampled in the Fall ($p=0.029$), but this trend was not supported at higher orders of diversity, and no other seasonal comparisons were statistically significant.

Evenness increases with host habitat shifts

Standardized evenness metrics take on a maximum value of 1 (completely even) and a minimum value of 0. At $q=0$ (richness), evenness is defined as 1. Declines in the effective number of species were observed at increasing orders of diversity, implying inequity of abundances found on salamander and cover object samples. Both salamanders and cover objects show similar patterns of evenness from $q=0$ to $q=2$, with a mean evenness value of 0.45 for salamanders and 0.41 for cover objects at $q=1$, and 0.20 for salamanders and 0.18 for cover objects at $q=2$. For 7/9 sampling sites, salamanders underwent habitat shifts before Summer and Winter sampling. Evenness trends of these samples revealed salamander communities were more even in the Summer and Winter (means= 0.51, 0.49) than in Spring and Fall (means= 0.42, 0.40) at $q=1$ (**Fig. 11**), while cover objects showed no patterns in evenness seasonally.

Turnover exacerbated by host habitat shifts

Previously, we found that while salamander-associated communities were heterogenous even within a site, microhabitat utilization contributed to fine-scale differences between salamander species and aquatic and terrestrial habitats were highly differentiated. Given that our

Table 7: Seasonal taxonomic and phylogenetic diversity for salamander-associated assemblages. Superscripts denote statistical significance of paired comparisons.

Salamanders	Taxonomic			Phylogenetic		
	$q=0$	$q=1$	$q=2$	$q=0$	$q=1$	$q=2$
Spring	1218.9	520	220.9	426.23 ^A	27.6	16.5
Summer	983.1	486.1	214.6	334.5	28	16.7
Fall	823.5	318.6	120.2	318.5 ^A	28	16.9
Winter	949.6	443.4	204.8	381.1	29.8	17.1

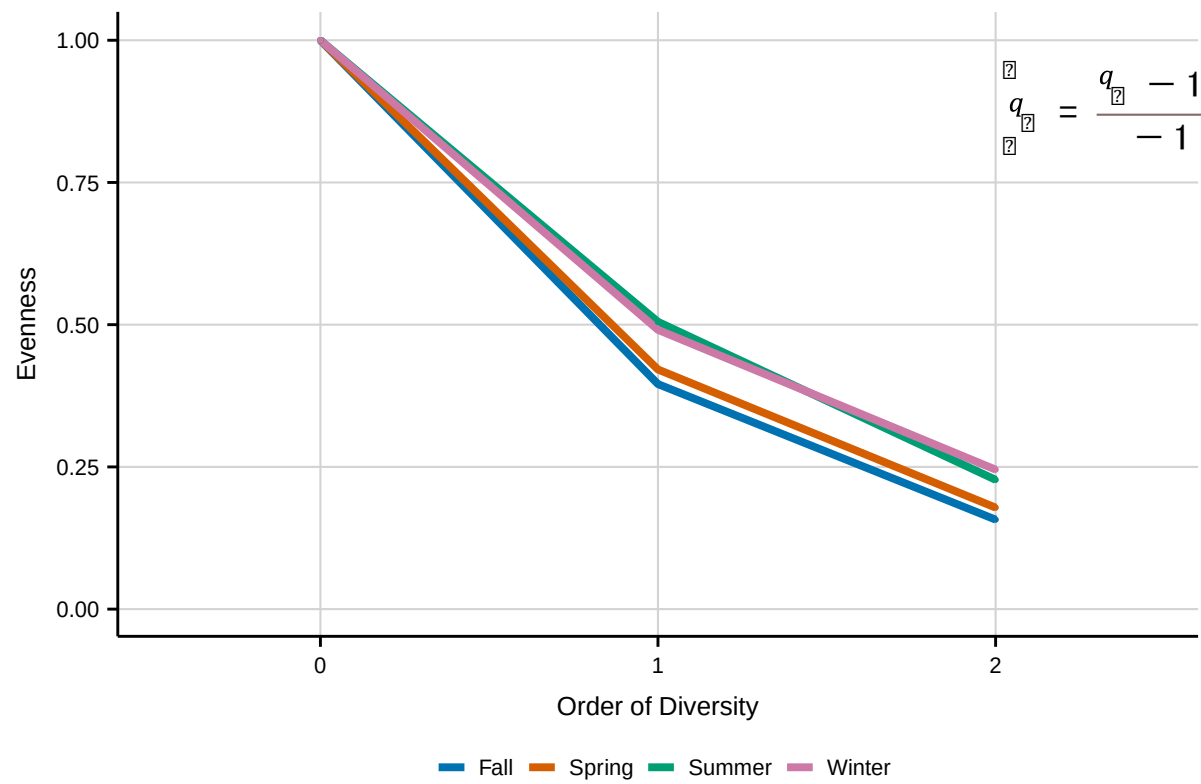


Figure 11: Evenness profile of communities on salamanders seasonally.

study sampled nine sites seasonally, we first addressed variance partitioning of the horn dissimilarity and tested variables with a dbRDA and anova.cca and then compared seasonal turnover with a multivariate Welch t-test with a Bonferroni correction.

Unconstrained ordination of the horn dissimilarity of salamander communities revealed clustering associated with habitat, with some evidence of Site and Season (**Fig. 12, Fig. A6**). Variance partitioning (adjusted r^2) found variables explained 27% of inertia, with Site explaining 6%, Habitat explaining 5% and Season explaining 4%. The bulk of inertia explained came through the interaction term of Habitat and Season (12%), as expected given our sampling scheme and research questions (**Fig. A7**). Phylogenetic turnover was considerably less than taxonomic turnover for salamanders. Our variables (Site, Habitat, and Season) explained 44% of inertia, with Site explaining 9%, Habitat 7%, and Season 8%. Similar to taxonomic turnover, the interaction between Habitat and Season explained 20% of inertia (**Fig. A8**). For both taxonomic and phylogenetic β diversity, Habitat, Season, and Site explained a significant amount of inertia, even when partialling out significant interaction terms in our dbRDA and anova.cca (all $p < 0.05$). The average taxonomic turnover between salamanders sampled in the same season (across sites) was 0.645, while the average turnover between salamanders sampled from the same site (across seasons) was 0.684. While subtle, these results indicate that seasonality within a site contributes more variation to host-associated communities at a small geographical scale than characteristics between sites, consistent with unconstrained ordination and variance partitioning.

To provide higher resolution hypothesis testing of turnover patterns seasonally, we used a Welch t-test for distances and corrected for multiple comparisons using a sequential Bonferroni. While all explanatory variables were statistically significant in dbRDA, the evenness and beta analyses suggested communities might stabilize after initial disturbance, meaning turnover within a habitat (but different seasons) would be lower than turnover between habitats (and between seasons). Multivariate Welch t-test found seasonal turnover of consecutive seasons was significantly higher when hosts either entered water (Fall to Winter, $T_w^2: 2.80, p=0.001$) or entered land (Spring to Summer: $T_w^2=3.88, p=0.001$) than when they remained on land (Summer to Fall: $T_w^2=1.24, p=0.132$) or in water (Winter to Spring: $T_w^2=1.35, p=0.18$). These results confirmed patterns of turnover within a site across seasons and habitats (**Fig. 13**) and confirmed

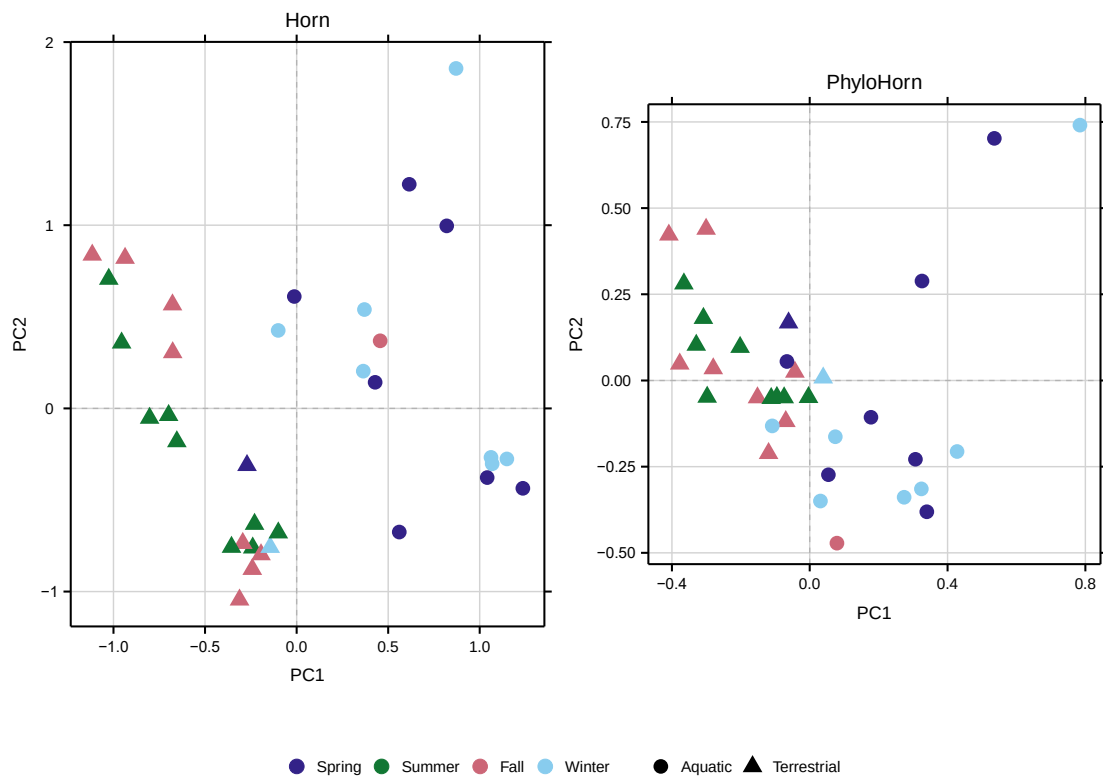


Figure 12: Unconstrained ordination of Horn and PhyloHorn dissimilarities of salamander-associated communities.

patterns of turnover within a site across seasons and habitats (**Fig. 13**) and phylogenetic dissimilarity showed identical trends (Spring to Summer: $T_w^2=6.47$, $p=0.01$, Fall to Winter: $T_w^2=3.85$, $p=0.01$, Summer to Fall: $T_w^2=1.26$, $p=0.36$, Winter to Spring: $T_w^2=1.57$ $p=0.36$).

Cover objects showed similar patterns as salamanders in terms of variance partitioning and unconstrained ordination. Site explained just 3% of inertia (adjusted r^2) for cover objects, while Habitat and Season explained 15% and 6%, respectively. Similar to salamander samples, the interaction between Habitat and Season explained 37% of the variance. While Habitat and Season predicted a significant amount of variance in our dataset ($p<0.01$, $p<0.01$), Site did not ($p=0.97$), and our unconstrained ordination showed strong clustering associated with Habitat. Evaluating the pairwise turnover described above for assessing turnover within a site, cover objects had the lowest turnover (highest similarity) to cover objects in the same habitat (0.53) and showed the highest average dissimilarity to cover objects in a different habitat (0.825).

Common core reflects environmental reservoirs

The common core describes OTUs consistently detected on hosts in each sampling period. Our common core definition identified OTUs detected on 80% of salamander hosts in each sampling season (Spring, Summer, Fall, Winter). For the common core, we identified 260 OTUs (164 unique), with spring samples having 104 taxa, 43 in winter, 44 in fall, and 69 in summer. In spring, the 104 core taxa consisted of 50 taxonomic families and averaged 0.25 relative abundance on salamander samples and 0.23 in cover objects (**Fig. 14**). Across all sites, 67 of these 104 OTUs were in the top 100 most abundant OTUs on hosts in our Spring sampling period. The most abundant taxonomic families on salamander hosts- Chitinophagaceae, Pedosphaeraceae, Xanthobacteraceae, and Sphingomonadaceae – contained core OTUs (5, 5, 2, and 3), although the relative abundance of these families was not exclusively limited to core members. Sixty-nine OTUs were identified as core members for our summer sampling, spanning 34 taxonomically identified families. These taxa comprised an average cumulative relative abundance of 0.24 on salamanders and 0.25 on cover objects, and 54/69 OTUs were in the top 100 most abundant OTUs on salamanders during the summer sampling period. The most abundant families on salamanders were again heavily comprised of core OTUs, with Chitinophagaceae (6), Comamonadaceae (4), Xanthobacteraceae (4), and Sphingomonadaceae

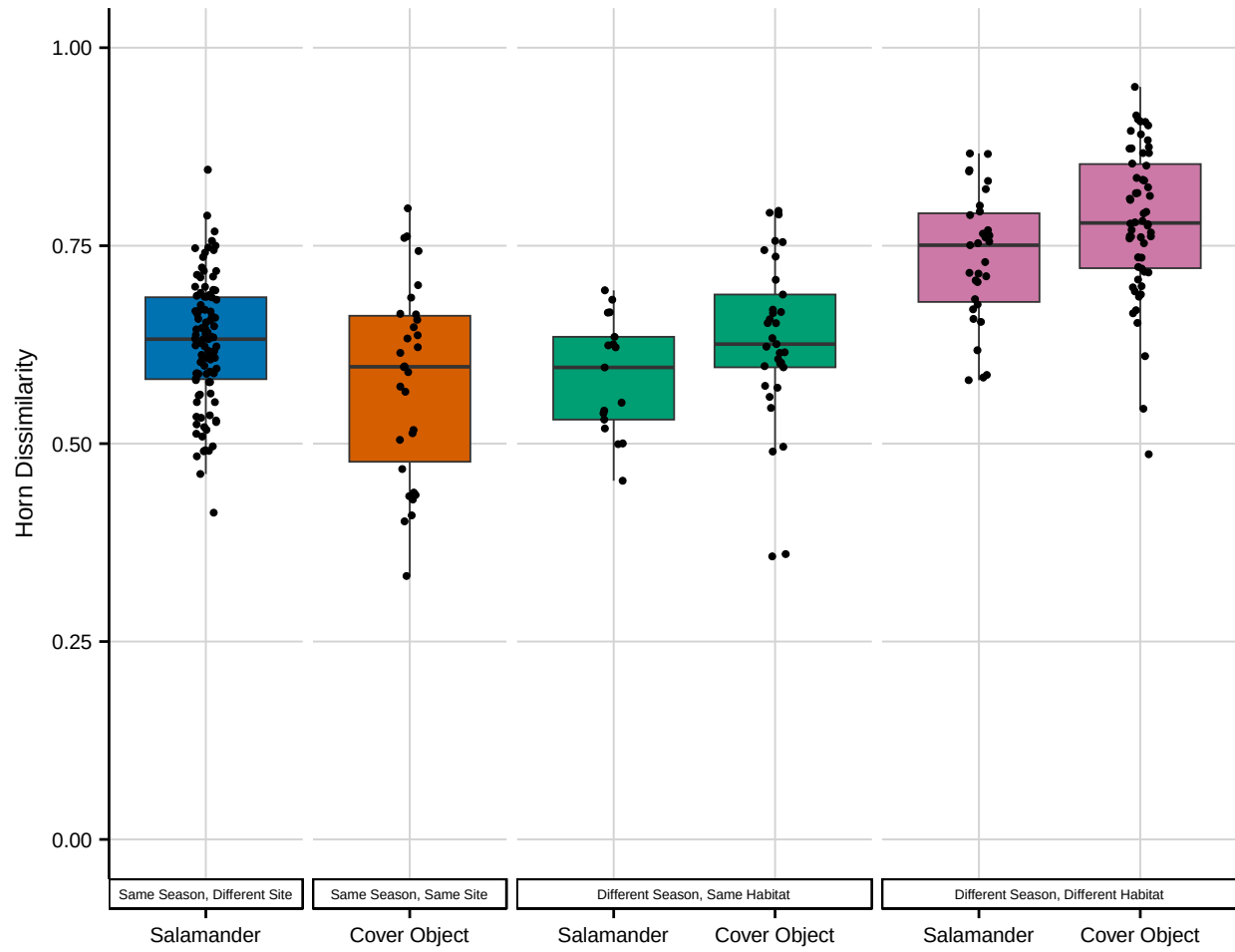


Figure 13: Pairwise turnover of salamander samples to other samples.

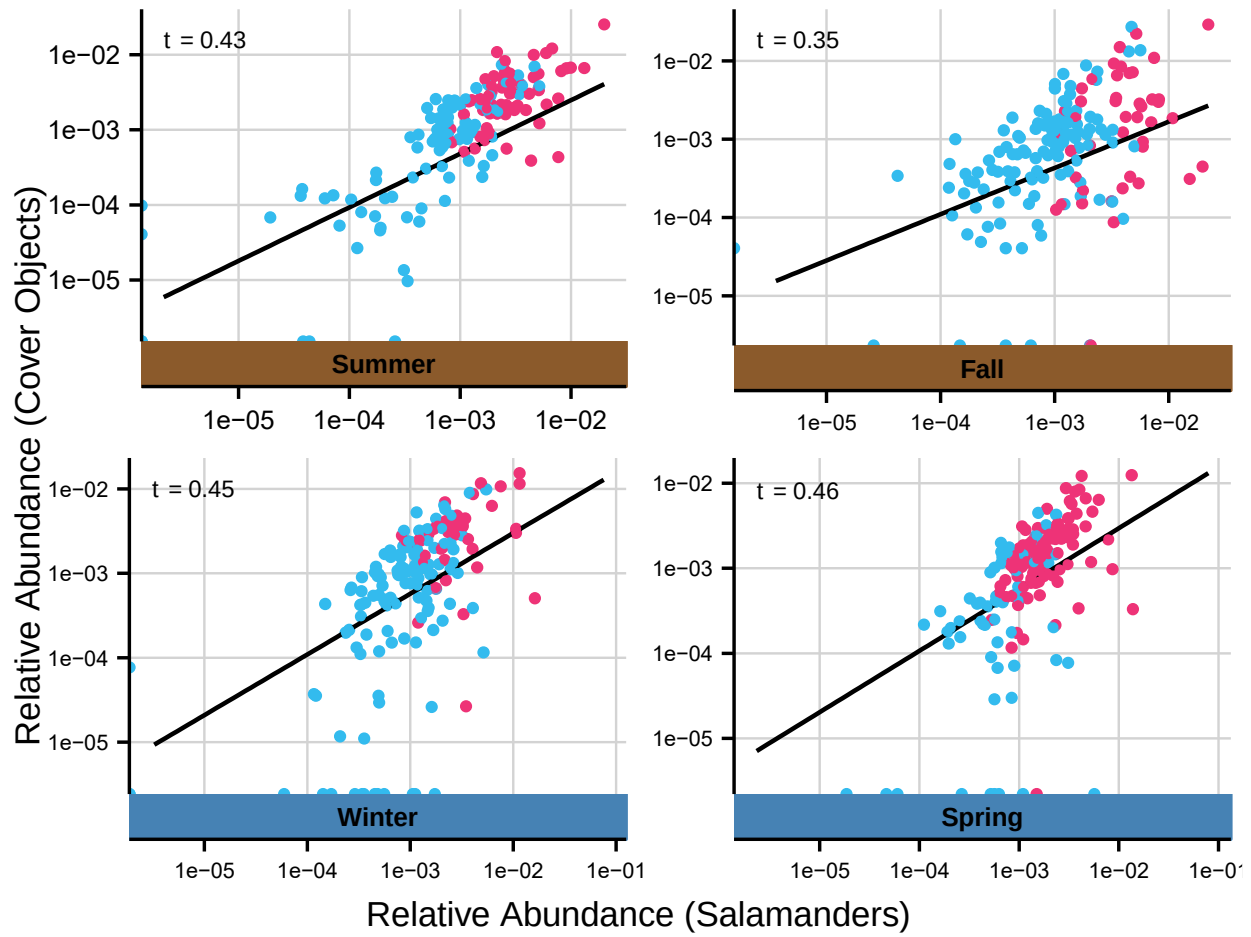


Figure 14: Relative abundance of the common 'core' microbiome on salamanders and cover objects. Red points denote core membership for that season, while blue indicate core OTUs for other seasons.

(5) constituting both the highest relative abundance on salamanders and core membership (**Figs. A9-A10**).

The common core associated with fall sampling contained 44 OTUs with an average relative abundance of 0.22 on salamanders and 0.17 on cover objects. These taxa consisted of 28 families, with Sphingobacteriaceae and Sphingomonadaceae containing three OTUs a piece, and only seven families containing more than one OTU. Community-level relative abundance again relied heavily on core members, with Chitinophagaceae being the most abundant taxonomic family and containing two core OTUs, followed by Comamonadaceae and Pseudomonadaceae represented by just a single core member each, and Sphingomonadaceae and Xanthobacteraceae containing 3 and 2 core OTUs, respectively. Thirty-seven of our 44 common core OTUs for fall sampling were among the top 100 most abundant OTUs. Winter sampling found 43 common taxa detected across 80% of salamanders and were equally abundant on salamanders and cover objects, averaging 0.16 relative abundance. These taxa spanned 27 families, and contained 34 of our top 100 most abundant OTUs in winter. The most abundant family during our winter sampling was again Chitinophagaceae which only contained a single core member, followed by Sphingomonadaceae (3), Pedosphaeraceae (2), and Comamonadaceae, with Comamonadaceae being the most represented family in our winter common core with six OTUs (**Figs. A11-A12**).

Twelve out of 164 OTUs (7%) were shared common core taxa across all four seasons. Seventeen OTUs were members of the common core across three seasons, while an additional 26 were members in two seasons. Taxa detected on 80% of individuals across all four sampling seasons had an average relative abundance of 0.084 (min: 0.03, max: 0.16). Of the twelve OTUs, only the family Sphingomonadaceae had three members, while the remaining nine families had one. One OTU was unidentified and result from BLASTn (Camacho et al., 2009) indicated the closest identified sequence (99% similarity) belonged to the genus *Devosia* in the family Devosiaceae. One hundred and nine OTUs were found only in a single season, meaning the majority (66%) of OTUs identified as a common core fluctuated seasonally and were not necessarily persistent on salamander hosts over time and habitats. Mirrored representation on cover objects indicates these taxa are predominately abundant and highly detectible on salamanders due to horizontal transmission from cover objects.

Temporal core captures wide-ranging microbes

We defined the temporal core as OTUs detected on 75% (3/4) or more salamanders within a site over the course of one year. For each of our nine sites, the temporal core ranged from 50 to 313 OTUs, comprised of 120 taxonomically identified families, with 546 unique OTUs (3% of total OTUs). Cumulative relative abundance of these OTUs on salamanders within a given site ranged from 0.09 to 0.60, with a mean of 0.33 (**Fig. 15**). The same temporal core showed a slightly lower cumulative relative abundance on cover objects (mean 0.27), but a similar distribution (min: 0.06, max 0.55). At each site, the top 100 most abundant OTUs on hosts over the course of the year were comprised of anywhere between 30 and 89 of our temporal core members. Ten families were responsible for an average of 0.25 relative abundance on salamanders across all sites (**Fig. A13**).

Only twelve out of the 546 OTUs were shared on salamanders across all sites. These twelve taxa comprised an average relative abundance of 0.07 on salamanders and 0.05 in cover objects. Nine of these taxa were also members of the 12 OTUs detected across all four seasons in the common core. These nine taxa came from seven different taxonomic families, with Sphingomonadaceae containing 3 OTUs and a single representative from Rizobiaceae, Chitinophagaceae, Microbacteriaceae, Xanthobacteraceae, and Xanthomonadaceae, and Devosiaceae. An additional 13 OTUs were determined to be in the temporal core for 8 sites, while seven and six sites each had 20 OTUs. Two hundred and fifty-three OTUs (46%) were only found in a single site, while 105 OTUs were shared only between two sites, highlighting the role of geographic location on host-associated communities in detection-based assessment. The virtually matched relative abundance on cover objects likely indicates most temporal core members possess widespread distribution within a site with, in most cases, less evidence for subsistence on amphibians.

Anti-Bd taxa more represented in terrestrial hosts and habitats

The defense functional core was identified by pairing reference sequences of our dataset to the database of 16S sequences from isolates capable of producing anti-Bd metabolites in challenge assays (Woodhams et al., 2015). We allowed up to a 3 base-pair mismatch of our sequences, which had an average length of 417 nucleotides, meaning our threshold selected sequences higher than a 99% sequence similarity to the database. This resulted in 38 OUT reference

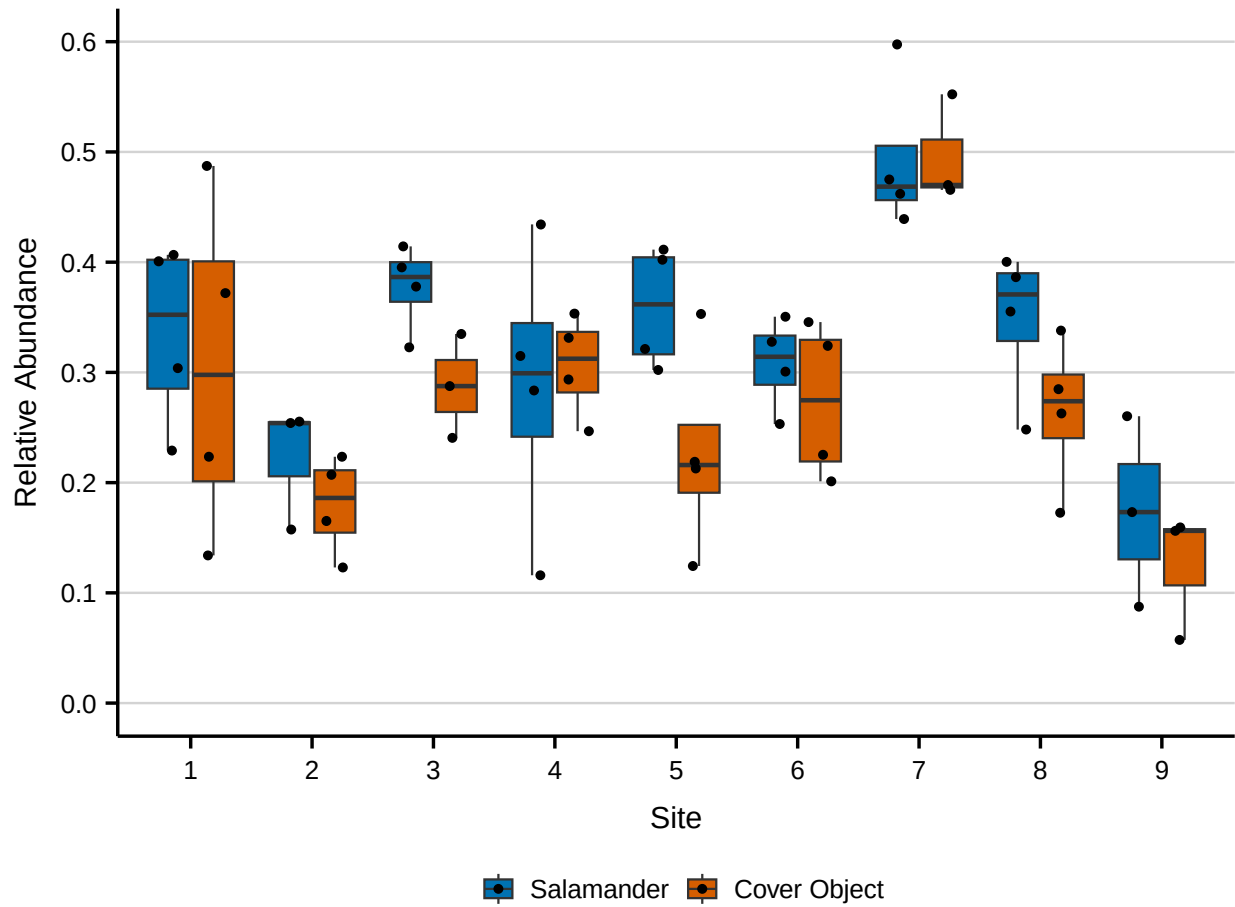


Figure 15: Relative abundance of the temporal core on salamanders and cover objects for each site.

sequence matches to 153 16S sequences in the database. Across 100 rarefactions, these OTUs averaged a collective 0.05 relative abundance on salamanders (min: 0.004, max: 0.23), and only 0.01 relative abundance in cover objects (min: 0, max: 0.20), signifying a potential high fidelity for functionally important taxa on salamander hosts. OTUs identified with anti-Bd capabilities were more prevalent and abundant in terrestrial habitats for both salamanders and cover objects. In other words, seasons where salamanders utilized a terrestrial habitat (Summer and Fall for 7/9 sites), were associated with a higher proportion of anti-fungal capable OTUs (**Fig. 16**). While these OTUs were less abundant in aquatic habitats, some persisted on salamanders over the course of the sampling seasons.

Our functional core consisted of five OTUs belonging to Pseudomonadaceae, four OTUs belonging to Xanthomonadaceae, Flavobacteriaceae, Enterobacteriaceae, and Moraxellaceae, five families with two anti-fungal OTUs, and seven families represented by a single OTU (**Table A4**). Of the 38 OTUs, three were detected on every salamander and belonged to the genera *Pseudomonas*, *Rhizobium*, and *Stenotrophomonas*. *Janthinobacterium* was additionally detected on 85% of salamanders. In total, 11 of 38 (29%) of OTUs were detected on half or more of salamanders, with only four OTUs being detected in only one sample. However, the majority of anti-Bd OTUs were also consistently detected in cover object samples. Only eight OTUs were not detected in cover object communities and those detected on every salamander were also detected on 22, 26, and 9 cover objects, suggesting that while salamanders could facilitate the maintenance of these symbionts between aquatic and terrestrial habitat transitions, they are not specific to amphibian hosts.

Non-random associations uncovered by a host-associated core

The host-associated core was defined as taxa showing a positive correlation with hosts. We identified OTUs using the ‘strassoc’ function in *indicspecies*, and selected OTUs with an average point biserial correlation coefficient higher than 0.3 across 1000 rarefactions. In total, 58 OTUs possessed a r_{pb} greater than 0.3. Average cumulative relative abundance of these taxa was 0.10 on salamanders (min: 0.02, max: 0.26) and 0.02 in cover objects (min 0.002, max: 0.10) (**Fig. 17**). Beijerinckiaceae and Sphingobacteriaceae were the two most prevalent families represented, each containing five OTUs. Of the 58 OTUs, only five were detected on every

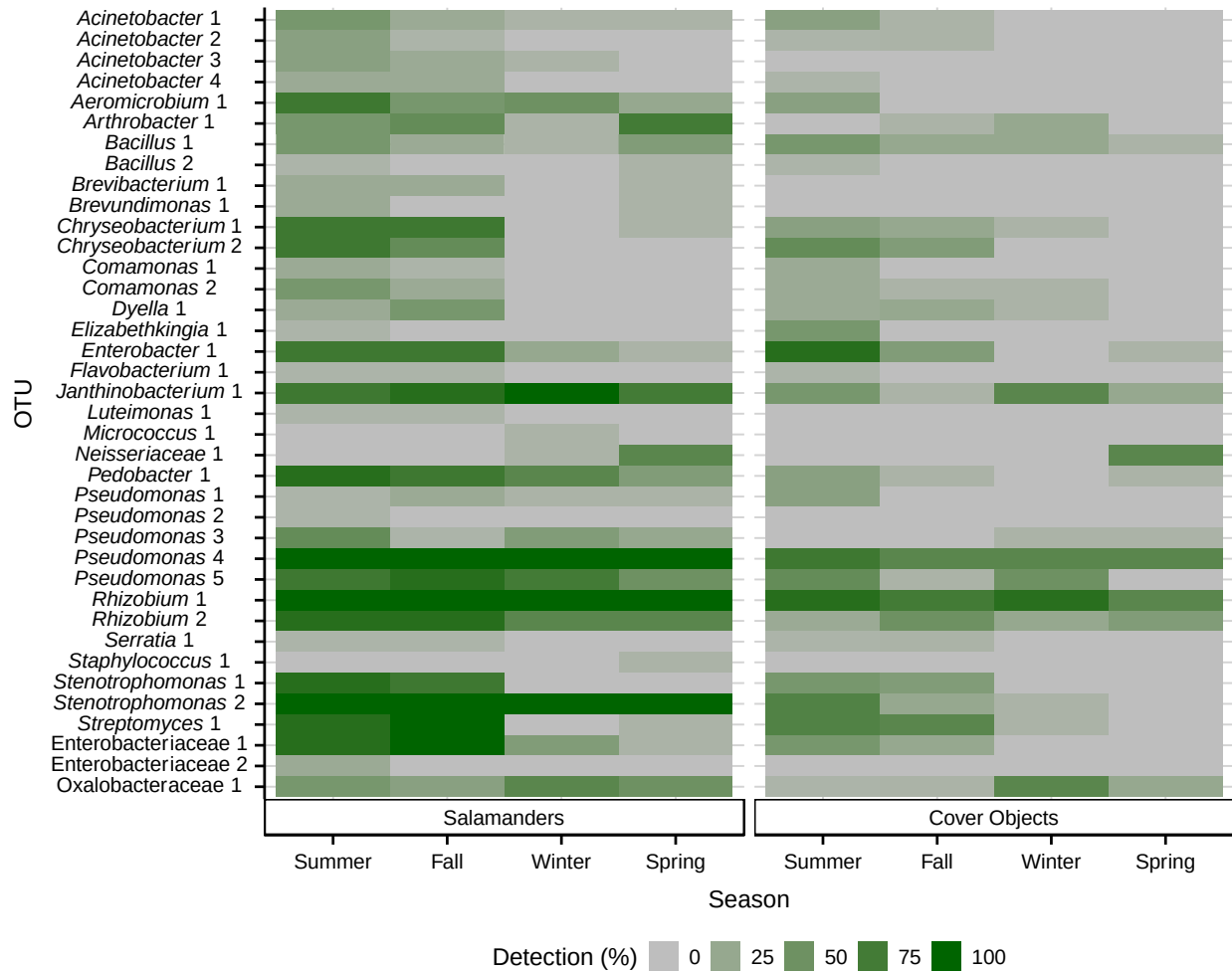


Figure 16: Detection of defensive OTUs on salamander and cover objects.

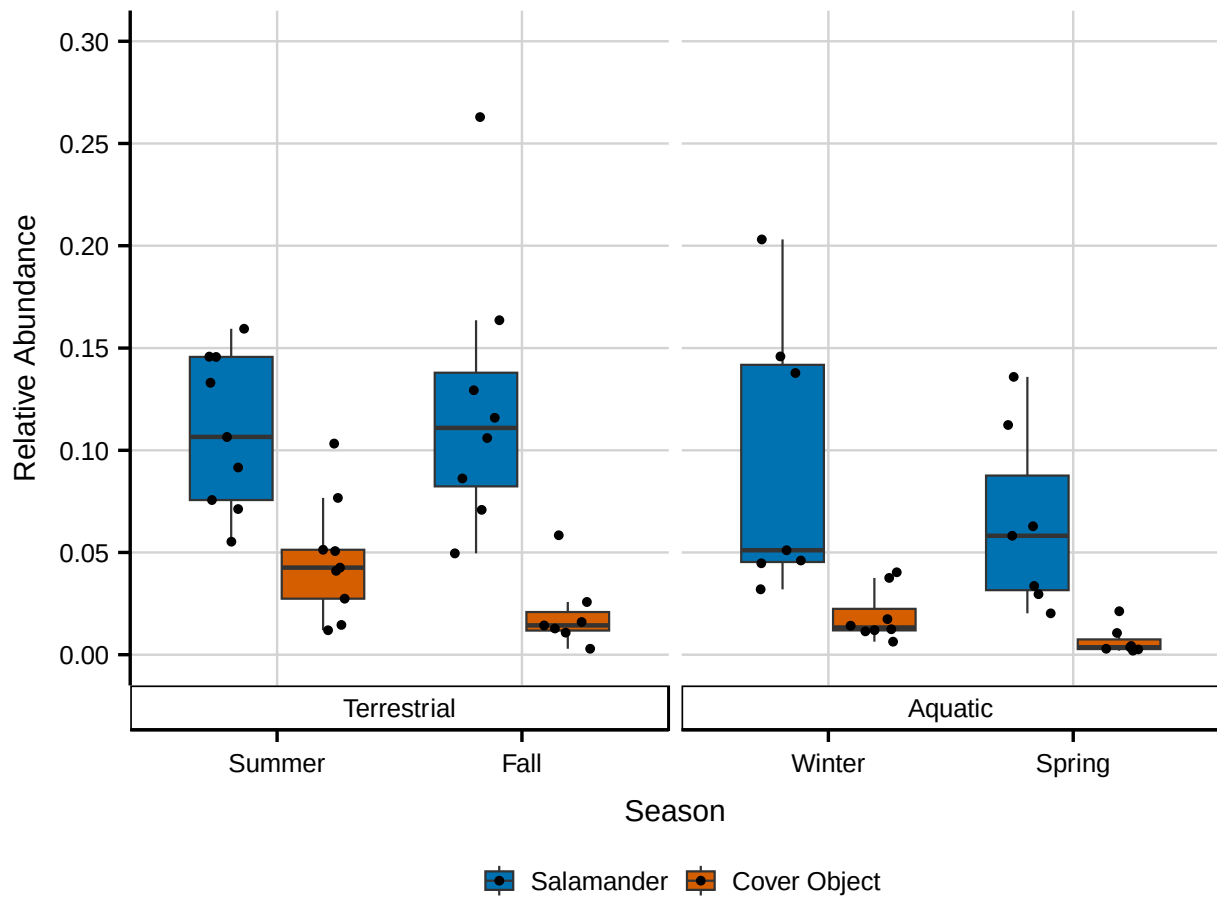


Figure 17: Relative abundance of OTUs showing statistical affinity for salamanders.

salamander and belonged to the genera *Pseudomonas*, *Salinibacterium*, *Sphingomonas*, *Rhizobium*, and *Stenotrophomonas*. Notably, these OTUs were additionally detected in between nine and thirty cover objects. Three of these five OTUs (*Pseudomonas*, *Rhizobium*, and *Stenotrophomonas*) were also in the other three core definitions. In total, 8 OTUs (14%) of the host-associated core were also capable of chytrid inhibition.

Kernel density estimates of observed and simulated null distributions produced contrasting patterns. Randomized communities exhibited a trimodal distribution of indicator species correlations, with the highest density centered on zero, while our observed samples showed a bimodal distribution with relatively low density around zero and peaks of higher density around 0.1 and -0.1, meaning a larger fraction of OTUs were moderately associated with either salamanders or cover objects than expected by chance (**Fig. 18**). This observed bimodality was concentrated in either a slightly negative or slightly positive association range and stems from many OTUs having detection on only a few salamanders and no cover objects, or *vice versa*. OTUs negatively associated with salamanders could additionally reflect microbes with low relative abundance in cover objects and dormant microbes with low dispersal probability. In many cases, OTUs positively associated with salamander skin were not exclusive to salamander-associated communities but instead showed strong associations through differential abundance. For example, nineteen of our indicator OTUs were detected in ten or more cover objects, while 22 were found on two or fewer. In this regard, taxa in the host-associated core fall on a spectrum between OTUs disproportionately abundant on salamanders with similar detection rates on cover objects, and OTUs disproportionately detected on salamanders.

Discussion

External microbiomes are highly susceptible to immigration from environmental reservoirs, making it unclear how specific communities are to a host and which symbionts could be candidates for more intimate associations. We have previously shown that salamander-associated communities are differentiated by microhabitat utilization, with some evidence for microbes showing tolerance for the ‘salamander condition’. Even between co-occurring hosts, communities are highly heterogeneous, with differentiation consistent with neutral processes. In this study, we assessed the temporal constancy of skin bacterial assemblages of *Eurycea* cf.

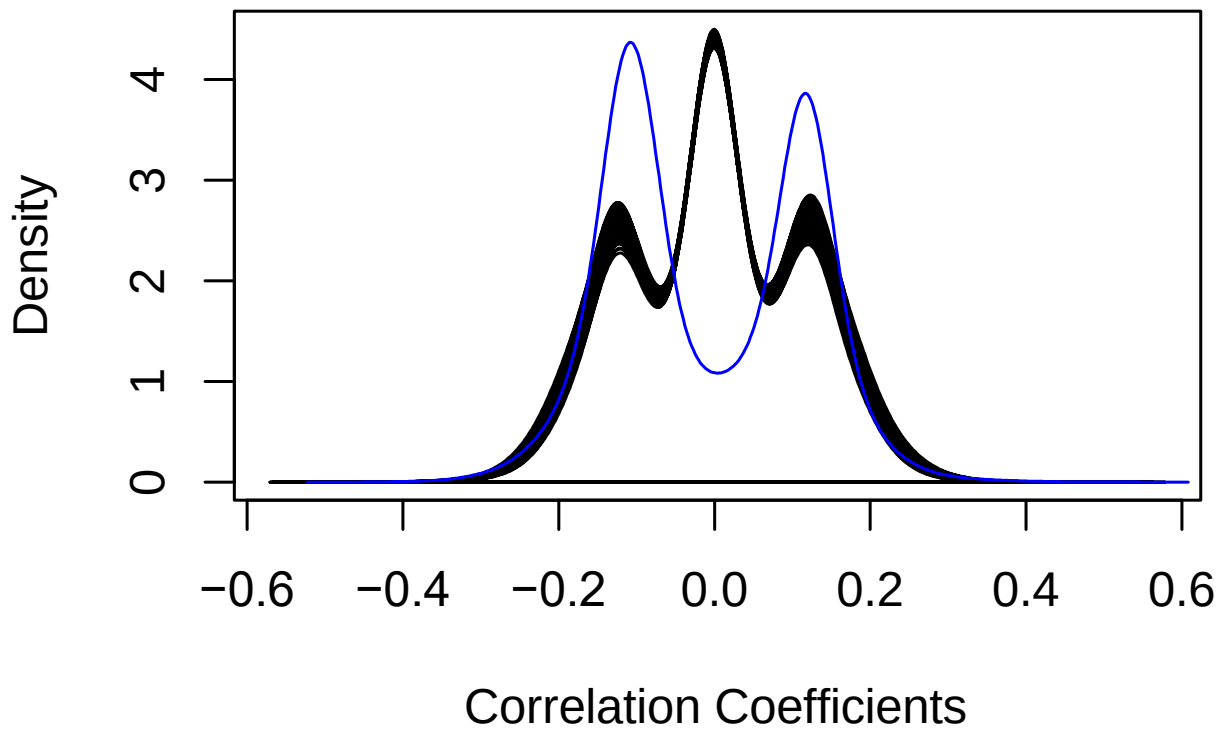


Figure 18: Distribution of observed (blue) and simulated (black) correlation coefficients.

wilderae over the course of four seasons as hosts move between aquatic and terrestrial habitats. We used taxonomic and phylogenetic turnover to assess community patterns and tested whether a non-random component of the skin microbiome remains consistent over habitat transitions using commonly applied ‘core’ microbiome definitions. Our results found high seasonal turnover of skin assemblages, particularly when salamanders shifted between aquatic and terrestrial habitats. In general, the most common bacteria on salamander skin were also common on cover objects, suggesting that a simplistic concept of a core microbiome as those taxa that are common on hosts is of limited value. However, there are bacteria with weak to modest statistical associations with salamander skin relative to cover objects, including some taxa with antifungal metabolites that could provide defensive benefits to the hosts. Thus, it is possible that there is a defensive functional core providing important or even essential services on salamander skin.

Seasonality and habitat utilization had little effect on the alpha diversity of *E. cf. wilderae*, as we found subtle differences regarding the effective number of species between seasons or habitats, especially when taking relative abundance into account. The consistency of diversity seasonally and across habitats contrasts with seasonal studies on anurans, which typically find a decrease in alpha diversity of skin samples during cooler months (Hernández-Gómez & Hua, 2023; Le Sage et al., 2021; Tong et al., 2020). That said, the overwintering of *Rana dybowskii* occurs under ice where hosts undergo documented reduced metabolic rates. Similarly, the reduced alpha diversity observed in *Lithobates palustris* occurred in March, when amphibians had the lowest ambient body temperatures. *Eurycea bislineata*, a close relative to *E. cf. wilderae*, is known to be mobile and partake in nest guarding and foraging during the winter months in aquatic habitats (Ashton & Ashton, 1978). While activity is likely less, movement within microbe rich streams would still enable potential colonization from cover objects to hosts, which is additionally supplemented by flowing water. The observed increase in community evenness and turnover in the sampling season associated with changing habitats suggests salamander hosts are acquiring microbes year-round, even in the colder months. Thus, hosts are not metabolically reduced in such a way that prohibits the sustenance of resident populations, and microbes are not metabolically compromised to prevent dispersal and establishment on hosts in Southern Appalachian streams.

Bacterial communities on salamanders displayed high taxonomic and phylogenetic turnover, mirroring the results of other temporal studies of amphibians at varying sampling frequencies (Bletz, Perl, Bobowski, et al., 2017; Ellison et al., 2021; Sabino Pinto et al., 2017; Walke et al., 2021). While some of this could be attributed to the lack of repeated sampling of the same individuals, Bletz et al. (2017) observed equally high turnover rates temporally in the *T. cristatus* microbiome despite sampling the same individuals. Pairwise turnover appears to be consistently high between salamander hosts, and taxonomic and phylogenetic turnover in this study were significantly higher when hosts underwent habitat shifts. External microbiomes are sensitive to immigration, and host effects appear to be less influential than stochastic immigration from environmental reservoirs. While unconstrained ordinations of salamander samples show clustering associated with habitat, salamander-associated communities most resemble communities found beneath their immediate cover object, emphasizing the importance of immigration in shaping salamander-associated communities. Host-associated communities showed a subtle increase in evenness when hosts shifted habitats, consistent with habitat changes acting as an intermediate disturbance affecting both taxa represented and equity of abundances. This is consistent with stochastic immigration from a new regional pool (in the new habitat) coupled with declines and local extinction of resident species. Hosts remaining in the same habitat for consecutive sampling seasons had more uneven bacterial communities with shifts in relative abundance consistent with competition and continued immigration from the same or similar regional pool (in the same habitat). As a result, temporal constancy is restricted to broad-ranging taxa and a few OTUs displaying affinity for salamander conditions in otherwise transient communities. As such, any disturbance impacting the reservoir communities (land use change), or host habitat utilization will also be reflected in host-associated assemblages.

A ‘core microbiome’ describes an attempt to identify the ecologically and functionally important microbes associated with a host or environment and is often referenced for identifying important symbionts. Our results indicate phenomenological definitions of a common and temporal core microbiome based on the most abundant or prevalent taxa found on amphibian skin offer limited promise for identifying which microbes are candidates for potential positive associations with hosts, and the spatial/temporal trends of those associates. Instead, these definitions more broadly represent the most abundant microbes in reservoirs. We propose

identifying symbionts using statistical procedures coupled with thorough sampling of both hosts and environmental reservoirs to parse signatures of positive associations from environmental colonization.

Temporal trends in anti-fungal bacteria associated with amphibian hosts are relevant to conservation biology given the global decline of amphibians owing to Bd/Bsal chytridiomycosis. The Blue Ridge ecoregion is of particular concern due to high amphibian richness and density, introduction risk, Bsal climatic suitability, and species susceptibility (Grisnik et al., 2023). Chytrid pathogens are more prevalent in aquatic environments and show evidence of temperature dependence making hosts particularly susceptible during cooler months in aquatic environments (Carter et al., 2021). Members of *Eurycea* have shown species and population variation in susceptibility and lethality to Bsal, ranging from susceptible yet experiencing no mortality (*Eurycea lucifuga*) to populational variability in susceptibility and mortality (*Eurycea wilderae*), with some variability confounded by captive-rearing (Carter et al., 2020). In the present study, temporal trends of microbes matching isolates associated with chytrid-inhibiting properties indicate varying patterns of environmental transmission and persistence. We found both a decrease in prevalence (detection) and relative abundance of anti-Bd on aquatically captured salamanders, with the relative abundance of defensive OTUs peaking on hosts in terrestrial sampling seasons (particularly fall) and then declining in the aquatic season. This suggests antifungal capabilities are more prevalent in terrestrial habitats in the Blue Ridge and that defensive OTUs are capable of colonizing the hosts from reservoirs.

Defensive OTUs were more scarce in aquatic habitats, and those that were detected were far less abundant. Despite the large turnover of the *Eurycea* skin microbiome seasonally and a decrease in anti-Bd richness on hosts in aquatic habitats, we found evidence for the persistence of defensive OTUs on salamander skin even when they were extremely rare in environmental samples. Salamander skin appears to actively or passively favor bacteria with antifungal metabolites. Twenty-one out of thirty-eight defensive OTUs showed lower temporal variability on salamanders than cover objects, and eight OTUs were statistically identified to have positive associations with hosts. Of these eight, four were in the top six most abundant anti-Bd taxa and belonged to *Pseudomonas*, *Stenotrophomonas*, *Rhizobium*, and *Janthinobacterium*. While some defensive OTUs may colonize hosts from aquatic habitats, others show persistence on hosts

through affinity for salamander skin in the aquatic environment after colonizing from terrestrial reservoirs.

Five OTUs in our dataset (0.027% of all OTUs) were consistent across all four core definitions, indicating they are commonly found within a sampling period and across time and space while also having statistical associations with salamander skin and potential functional relevance to hosts. These OTUs belonged to the genera *Janthinobacterium*, *Stenotrophomonas*, *Pseudomonas*, and *Rhizobium*. *Janthinobacterium* isolated from a plethodontid salamander was the first documented host-associated isolate capable of inhibiting Bd in vitro and is commonly found in amphibian microbiome studies (Harris et al., 2009; Muletz et al., 2014). Observed bioactivity against Bd in *Janthinobacterium* isolates is hypothesized to be linked to violacein production, with three variable genes (*vioB*, *vioC*, and *vioD*) potentially leading to strain level differences in inhibition (Wax et al., 2023). Members of *Stenotrophomonas* have also been linked to antifungal capabilities (Barnes et al., 2020; Becker et al., 2015) but have also been associated with Bd susceptibility (Bates et al., 2022). This genus is consistently a dominant taxon on Plethodontid salamander hosts in Southern Appalachia (Ch. 1) and has been also linked to venom production in cone snails and providing nutrients and plant growth hormones in the rhizosphere (Ryan et al., 2009; Torres et al., 2017). Given its bioactivity in lab experiments, high fidelity on salamander hosts, and low intra-genus diversity, this genus warrants further investigation regarding what mechanisms could facilitate symbiosis with hosts and potential benefits from association for both parties.

Pseudomonas has been associated with anti-fungal capabilities for amphibian hosts, with Becker (2015) finding 73% of strains capable of Bd inhibitions. Similarly, nearly 40% of *Pseudomonads* isolated from salamander skin had defensive properties (Barnes et al., 2020). Host benefits attributed to *Pseudomonas* are not restricted to antifungal capabilities, with a recent study (Brunetti et al., 2022) finding *Pseudomonas* sp. resulting in sex-specific scents in frogs and isolates possessing genomic modifications adapted for anti-microbial peptide resistance. Even more, this isolate was also capable of metabolizing macrocyclic acylcarnitine, a major metabolite secreted by hosts, as its sole carbon and nitrogen source (Brunetti et al., 2023). *Pseudomonas* is a diverse clade, and tight associations are likely limited to select strains that could engage in reciprocal selection despite environmental transmission. Symbiont fitness could increase from

enhanced dispersal by associating with a host and niche expansion in the ability to metabolize host secretory products, while host fitness could increase through sex-specific scents, antifungal, or predator deterrent compounds (Vaelli et al., 2020). The genus *Rhizobium* is associated with nitrogen fixation capabilities in plants as an endosymbiont but can also exist as a free-living saprophyte with antifungal compounds in soils and low dispersal capabilities (Nemergut et al., 2013; Pawar et al., 2014). It has been found on other amphibians, ranging from Europe to North America (Costa et al., 2016; A. J. Douglas et al., 2020), and can produce antifungal compounds in association with bats to combat white-nose syndrome (Grisnik et al., 2020).

Our results suggest symbionts associated with salamander hosts are largely transient and are not ‘distinct’ from environmental reservoirs with the exception of a few non-random associations uncovered by statistical associations and defensive capabilities. These genera represent the best candidates for investigating the possibility of intimate associations in otherwise transient communities. Potential benefits of skin symbionts for hosts include defense against predators (Vaelli et al., 2020), defense against skin pathogens like Bd and Bsal (Flechas et al., 2012; Muletz et al., 2012b), defense against opportunistic saprophytes that can infect eggs when left untended (Walke et al., 2011), and chemical signals affecting courtship (Brunetti et al., 2019). Potential benefits to skin symbionts from hosts include nutrition and enemy-free space (Brunetti et al., 2023), but reciprocal benefits are not necessary, and perhaps not to be expected in very open systems such as skin. Whether there is a ‘functional core’ set of microbes that are critical to host resistance to disease is an important question for conservation, and the genetic and ecological underpinnings of skin-microbe interactions are keys for understanding coevolution in complex communities. Our results, in combination with previous studies, point towards a few specific bacterial taxa that should be investigated in greater depth regarding what promotes persistence of environmentally derived symbionts on amphibian skin.

CONCLUSION

This dissertation took a high resolution spatial and temporal investigation of the amphibian skin microbiome in the context of environmental reservoirs. In **Chapter one**, I sought to clarify the role of host ecology and phylogeny by evaluating the similarities and differences between salamanders and cover objects pertaining to habitat, microhabitat, and host species. I found that salamander-associated assemblages are differentiated by host species; however, differentiation is not consistent with host-mediated selection, but instead most reflects differences in microhabitat utilization. I additionally show that while cover objects are strongly differentiated by habitat, salamander-associated communities are largely heterogenous and harbor little similarities. Microbes showing statistical affinity for salamander skin appear to be generally associated with ‘salamander conditions’ rather than species-specific conditions. More broadly, communities on salamanders tend to be most similar to their immediate cover objects suggesting high reliance on environmental reservoirs and ecological processes rather than host-specific traits. With the expansion of scientific explorations of microbial communities associated with hosts, much attention has been given to the role of host traits in shaping communities, but my results emphasize the influence of environmental colonization. These results highlight the critical need for thorough, high-resolution sampling of source communities when assessing the specificity of external microbiomes associated with a particular host species.

In **Chapter two**, I assessed the signatures of deterministic and stochastic processes between hosts, other hosts, and cover objects in the context of priority effects, salamander-mediated selection, and random environmental transmission. Ecological null models revealed that assemblages on cover objects were comprised of taxonomically and phylogenetically similar microbes characteristic of the microhabitat occupied by host species. Salamanders-associated communities, contrastingly, were as different as expected by chance, reflecting idiosyncratic, rather than salamander-specific, conditions. Communities on salamanders were differentiated from communities on cover objects, but differences between host and free-living communities were along a predominately shared phylogeny and most prevalent at the lowest taxonomic levels. This indicates the salamander skin microbiome is a product of weak and random ecological

forces, apart from a small subset of microbes that is statistically associated with salamanders across habitats and sampling sites.

Finally, in **Chapter three**, I assessed the temporal constancy of the salamander skin microbiome by sampling a migratory salamander between aquatic and terrestrial habitats for the course of a year. I found high community turnover between samplings seasons which was exacerbated when hosts changed habitats, consistent with colonization from a different regional pool of microbes. Microbes consistently detected on salamanders during each sampling season were equally represented in source communities, as were microbes detectable on hosts over the course of a year. This corroborates results from previous chapters in that continuous environmental colonization is a consistent structuring component of external microbiomes. As such, changes to environmental reservoirs (captive rearing or land-use change) will manifest changes in communities associated with amphibians. The seasonal dynamics of defensive microbes is particularly relevant since chytrid is most prevalent in aquatic habitats and during cooler months. I found potential defensive OTUs capable of inhibiting the growth of chytrid were more prevalent in terrestrial habitats but showed persistence on salamander hosts over time and space. Five of these of these were statistically associated with salamanders and readily detectable, providing evidence for some non-random associations in an otherwise transient community.

To conclude, host-microbe associations can have profound consequences for evolutionary trajectories of hosts by mediating physiology, behavior, and reproduction. In intimate relationships, hosts and symbionts can engage in reciprocal interactions promoting co-dependence. Environmentally acquired symbionts are more puzzling from an evolutionary perspective but can still engage in positive benefits for hosts. We show that while most microbes found on salamander skin are consistent with environmental colonization from cover objects, a few taxa showing consistent affinity for salamander over time and space show promise for future investigation. Reciprocal benefits leading to co-evolutionary patterns are likely not to be expected for these symbionts, but mechanisms detailing persistence and partner benefits will enhance understanding of how engaging in symbiotic relations could promote mutual benefits for both parties.

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APPENDIX

Table A1: Multi-level pattern analysis identifying indicator species for groups.

Indicator Species (multipatt)	OTUs	Species and Cover Objects	All Salamanders
<i>D. gvnigeusgwotli</i>	68	11	5
<i>D. conanti</i>	29	7	-
<i>D. monticola</i>	49	8	-
<i>E. cf. wilderae</i>	23	5	-
<i>D. conanti</i> (Cover)	94	-	-
<i>D. monticola</i> (Cover)	223	-	-
<i>D. gvnigeusgwotli</i> (Cover)	239	-	-
<i>E. cf. wilderae</i> (Cover)	121	-	-

Table A2: Variance Partitioning of salamanders and cover objects for each dissimilarity. Only values greater than zero are shown.

Dissimilarity	Comparison	Samples Used	Adjusted r^2
Weighted Unifrac	All Samples	Site	0.029
Weighted Unifrac	All Samples	Type	0.026
Weighted Unifrac	All Samples	Habitat	0.041
Weighted Unifrac	All Samples	Site x Habitat	0.001
Weighted Unifrac	Cover Objects	Site	0.039
Weighted Unifrac	Cover Objects	Habitat	0.048
Weighted Unifrac	Cover Objects	Species	0.006
Weighted Unifrac	Cover Objects	Site x Habitat	0.058
Weighted Unifrac	Cover Objects	Site x Species	0.021
Weighted Unifrac	Cover Objects	Habitat x Species	0.094
Weighted Unifrac	Salamanders	Site	0.021
Weighted Unifrac	Salamanders	Species	0.012
Weighted Unifrac	Salamanders	Site x Habitat	0.007
Weighted Unifrac	Salamanders	Site x Species	0.005
Weighted Unifrac	Salamanders	Habitat x Species	0.015
Sorensen	All Samples	Site	0.028
Sorensen	All Samples	Type	0.028
Sorensen	All Samples	Habitat	0.045
Sorensen	All Samples	Site x Habitat	0.002
Sorensen	Cover Objects	Site	0.026
Sorensen	Cover Objects	Habitat	0.046
Sorensen	Cover Objects	Species	0.009
Sorensen	Cover Objects	Site x Habitat	0.038
Sorensen	Cover Objects	Site x Species	0.011
Sorensen	Cover Objects	Habitat x Species	0.079
Sorensen	Salamanders	Site	0.024
Sorensen	Salamander	Habitat	0.002
Sorensen	Salamanders	Species	0.008
Sorensen	Salamanders	Site x Habitat	0.004
Sorensen	Salamanders	Site x Species	0.006
Sorensen	Salamanders	Habitat x Species	0.008

Table A2 continued

Morisita-Horn	All Samples	Site	0.05
Morisita-Horn	All Samples	Type	0.037
Morisita-Horn	All Samples	Habitat	0.065
Morisita-Horn	Cover Objects	Site	0.045
Morisita-Horn	Cover Objects	Habitat	0.077
Morisita-Horn	Cover Objects	Species	0.011
Morisita-Horn	Cover Objects	Site x Habitat	0.064
Morisita-Horn	Cover Objects	Site x Species	0.016
Morisita-Horn	Cover Objects	Habitat x Species	0.145
Morisita-Horn	Salamanders	Site	0.036
Morisita-Horn	Salamanders	Habitat	0.004
Morisita-Horn	Salamanders	Species	0.011
Morisita-Horn	Salamanders	Site x Habitat	0.006
Morisita-Horn	Salamanders	Site x Species	0.02
Morisita-Horn	Salamanders	Habitat x Species	0.01

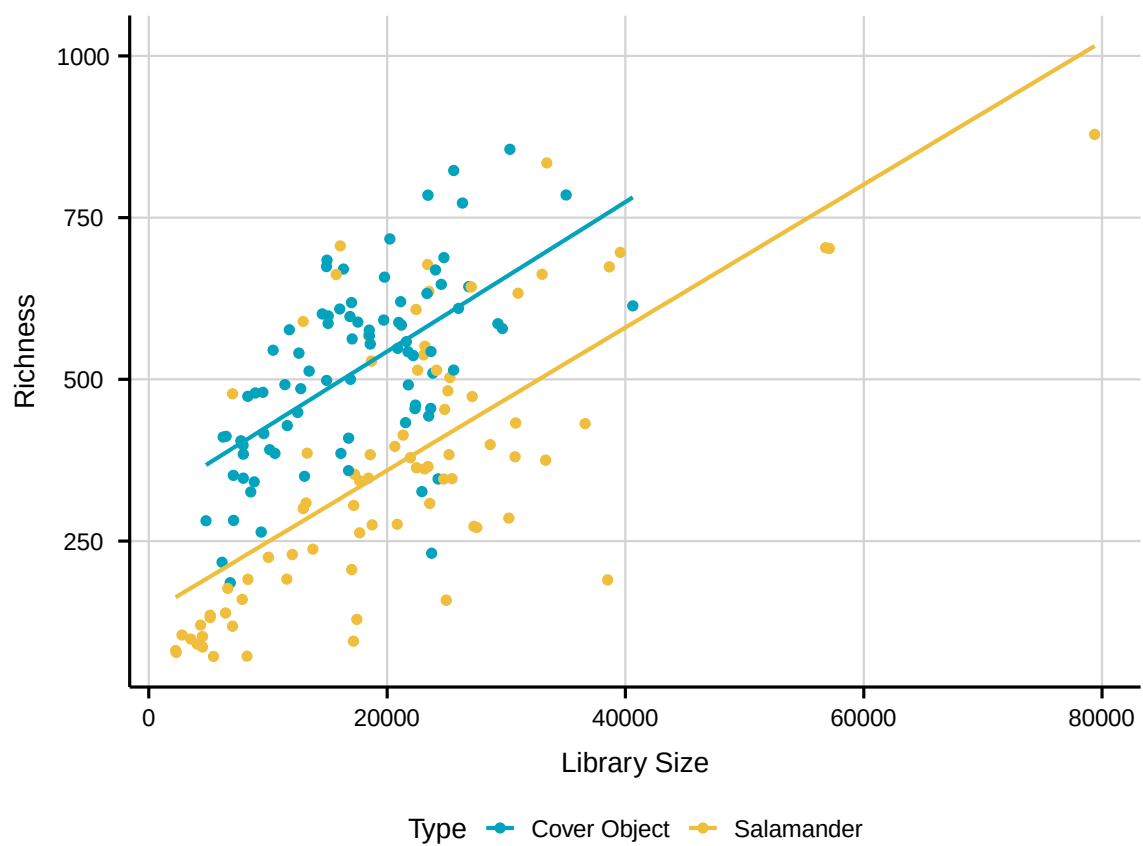


Figure A1: Scatterplot of richness and sequence variants for salamander and cover objects.

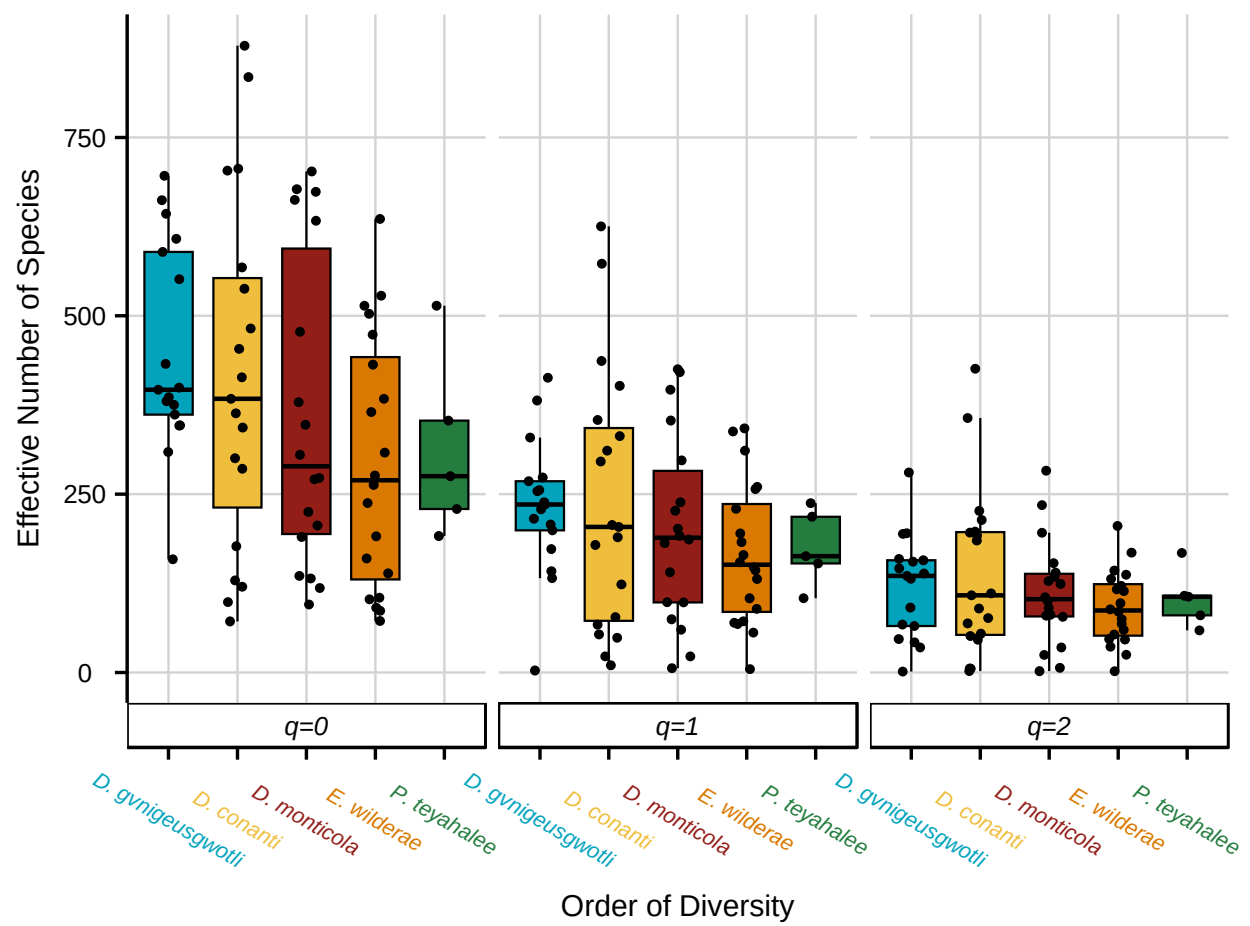


Figure A2: Alpha diversity estimations for 500 rarefactions. Statistical comparisons found no significant differences between species at each order of q for either habitat or species.

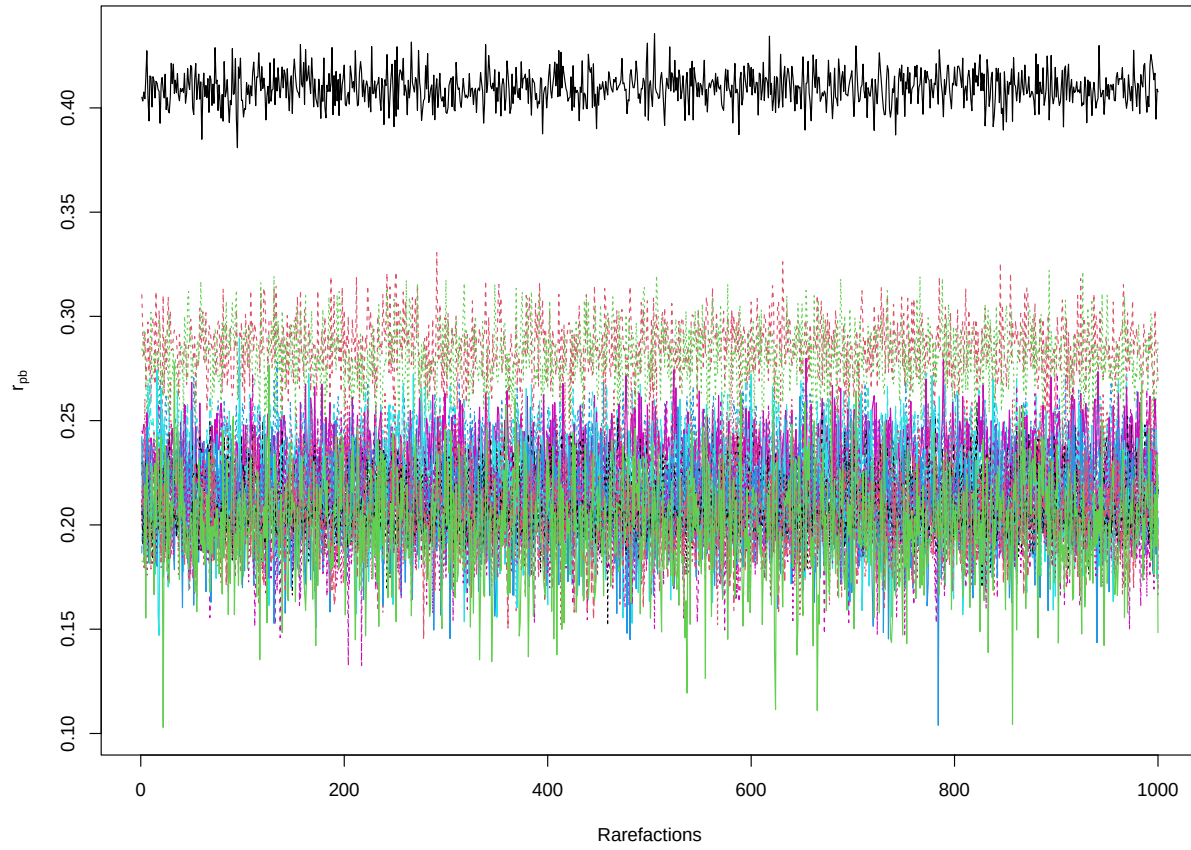


Figure A3: Fluctuation of point biserial correlation coefficients (r_{pb}) for 1000 rarefactions of the 21 sequence variants with an average $r_{pb} > .20$. The top black line corresponds to the sequence variant belonging to the genus *Stenotrophomonas*.

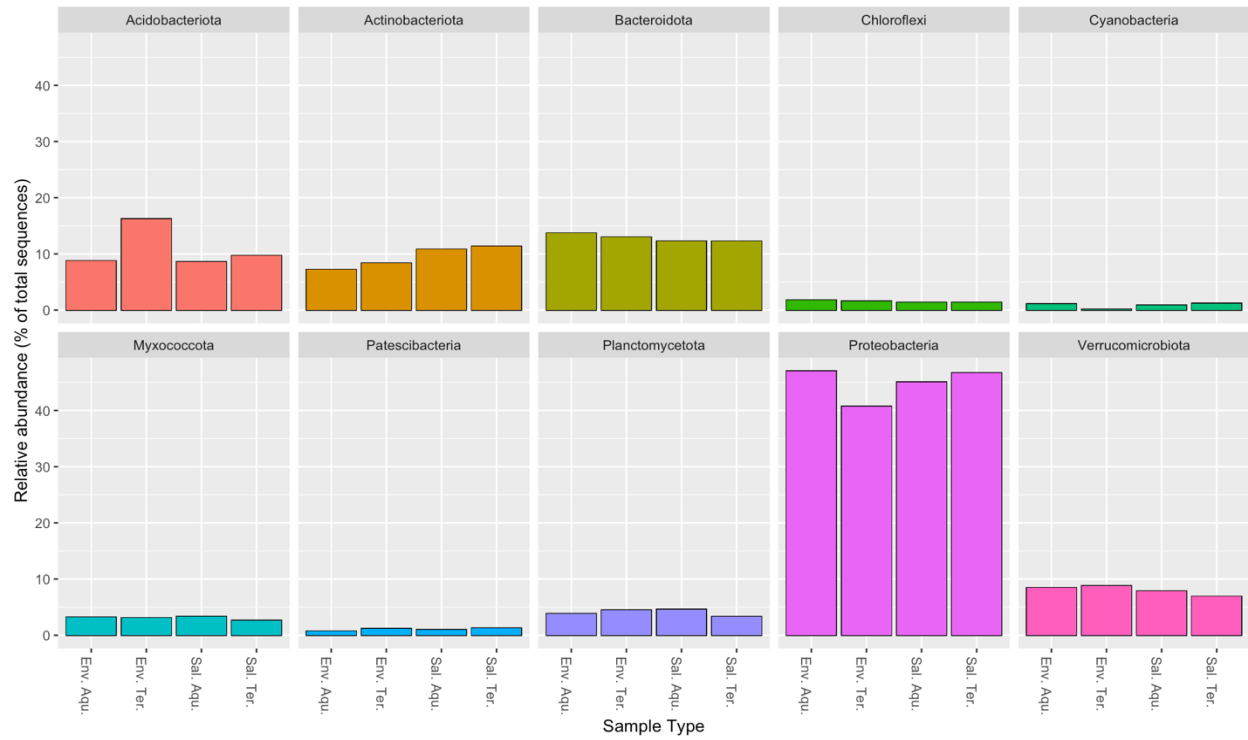


Figure A4: Facet plot of the 15 most abundant phyla in all sample types. Colors correspond to phylum.

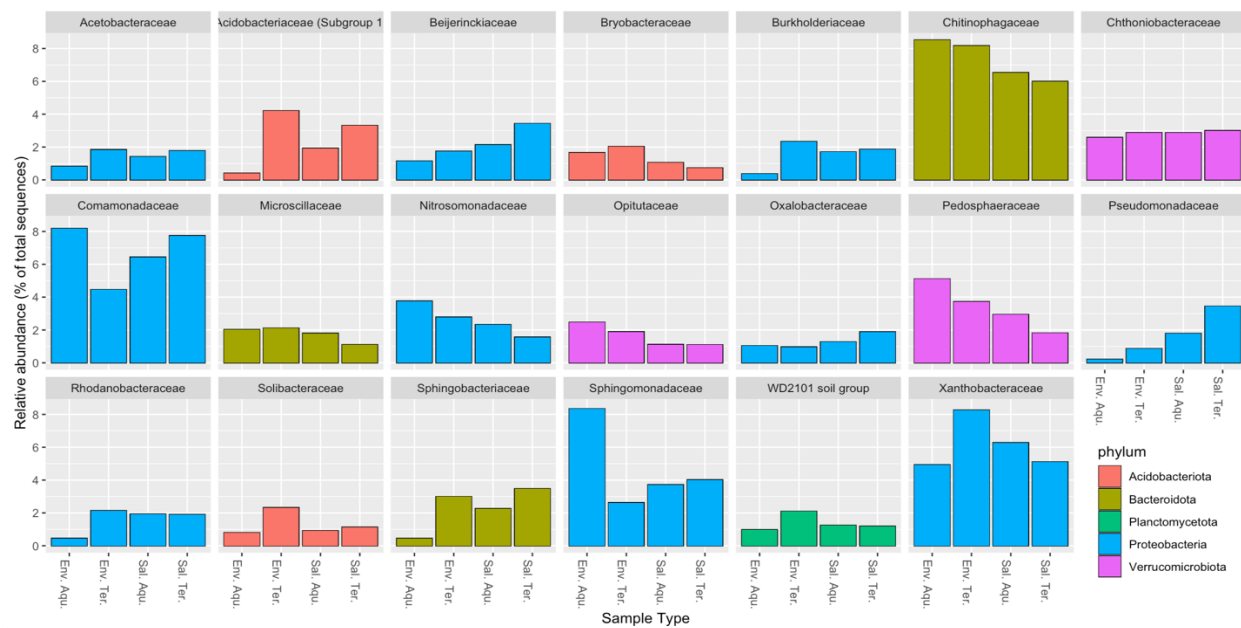


Figure A5: Twenty most abundant families found in sample types. Colors correspond to phylum.

Table A3: Point biserial correlation coefficient used for identifying OTUs associated with salamanders.

Family	Genus	Value	Group
Xanthobacteraceae	<i>Tardiphaga</i>	0.2459	All Salamanders
Beijerinckiaceae	<i>Roseiarcus</i>	0.2163	All Salamanders
Nocardiodaceae	<i>Nocardioides</i>	0.2055	All Salamanders
Nocardiodaceae	<i>Nocardioides</i>	0.2227	All Salamanders
Microbacteriaceae	NA	0.2439	All Salamanders
Microbacteriaceae	NA	0.2267	All Salamanders
Microbacteriaceae	NA	0.2784	All Salamanders
Acidobacteriaceae (Subgroup 1)	<i>Terriglobus</i>	0.2316	All Salamanders
Acidobacteriaceae (Subgroup 1)	<i>Granulicella</i>	0.2016	All Salamanders
Weeksellaceae	<i>Chryseobacterium</i>	0.2094	All Salamanders
Chitinophagaceae	<i>Puia</i>	0.2109	All Salamanders
Comamonadaceae	<i>Comamonas</i>	0.2122	All Salamanders
Pseudomonadaceae	<i>Pseudomonas</i>	0.2037	All Salamanders
Comamonadaceae	<i>Rhizobacter</i>	0.2702	All Salamanders/Aquatic Salamanders
Acetobacteraceae	<i>Acidiphilium</i>	0.3149	All Salamanders/Aquatic Salamanders
Beijerinckiaceae	<i>Methylobacterium-Methylobacterium</i>	0.3329	All Salamanders/Aquatic Salamanders
Beijerinckiaceae	<i>Methylobacterium-Methylobacterium</i>	0.3876	All Salamanders/Aquatic Salamanders
Beijerinckiaceae	<i>Methylobacterium-Methylobacterium</i>	0.4030	All Salamanders/Aquatic Salamanders
Beijerinckiaceae	NA	0.3314	All Salamanders/Aquatic Salamanders
Hyphomicrobiaceae	<i>Hyphomicrobium</i>	0.3049	All Salamanders/Aquatic Salamanders
Sphingomonadaceae	<i>Sphingomonas</i>	0.3235	All Salamanders/Aquatic Salamanders
Sphingomonadaceae	NA	0.3350	All Salamanders/Aquatic Salamanders

Table A3 Continued

Microbacteriaceae	NA	0.2572	All Salamanders/Aquatic Salamanders
Mycobacteriaceae	<i>Mycobacterium</i>	0.3942	All Salamanders/Aquatic Salamanders
Corynebacteriaceae	<i>Corynebacterium</i>	0.3707	All Salamanders/Aquatic Salamanders
Xanthomonadaceae	<i>Stenotrophomonas</i>	0.3767	All Salamanders/Aquatic Salamanders
Moraxellaceae	<i>Acinetobacter</i>	0.3731	All Salamanders/Aquatic Salamanders
Pseudomonadaceae	<i>Pseudomonas</i>	0.3687	All Salamanders/Aquatic Salamanders
NA	NA	0.4457	All Salamanders/Aquatic Salamanders
Paracaedibacteraceae	<i>Candidatus Finniella</i>	0.2538	All Salamanders/Terrestrial Salamanders
Xanthobacteraceae	NA	0.3460	All Salamanders/Terrestrial Salamanders
NA	NA	0.3663	All Salamanders/Terrestrial Salamanders
Rhodocyclaceae	<i>Ferribacterium</i>	0.2590	Aquatic Salamanders
Comamonadaceae	NA	0.2588	Aquatic Salamanders
Comamonadaceae	<i>Delftia</i>	0.2562	Aquatic Salamanders
Comamonadaceae	<i>Comamonas</i>	0.2537	Aquatic Salamanders
Comamonadaceae	NA	0.2664	Aquatic Salamanders
Sphingomonadaceae	NA	0.2834	Aquatic Salamanders
NA	NA	0.2584	Aquatic Salamanders
Acetobacteraceae	NA	0.2675	Aquatic Salamanders
Acetobacteraceae	NA	0.2714	Aquatic Salamanders
Beijerinckiaceae	<i>Methylobacterium-Methylobacterium</i>	0.2722	Aquatic Salamanders
Beijerinckiaceae	<i>Methylobacterium-Methylobacterium</i>	0.2513	Aquatic Salamanders
Beijerinckiaceae	<i>Methylobacterium-Methylobacterium</i>	0.2945	Aquatic Salamanders
NA	NA	0.2648	Aquatic Salamanders
Beijerinckiaceae	1174-901-12	0.3206	Aquatic Salamanders

Table A3 Continued

Beijerinckiaceae	1174-901-12	0.2654	Aquatic Salamanders
Beijerinckiaceae	1174-901-12	0.2618	Aquatic Salamanders
Beijerinckiaceae	1174-901-12	0.2666	Aquatic Salamanders
Beijerinckiaceae	NA	0.2710	Aquatic Salamanders
Sphingomonadaceae	NA	0.2588	Aquatic Salamanders
Sphingomonadaceae	NA	0.2755	Aquatic Salamanders
Rhizobiaceae	NA	0.2986	Aquatic Salamanders
NA	NA	0.2505	Aquatic Salamanders
Polyangiaceae	NA	0.2600	Aquatic Salamanders
Propionibacteriaceae	<i>Cutibacterium</i>	0.2770	Aquatic Salamanders
Nocardiodaceae	NA	0.2565	Aquatic Salamanders
Brevibacteriaceae	<i>Brevibacterium</i>	0.2569	Aquatic Salamanders
Microbacteriaceae	NA	0.2618	Aquatic Salamanders
Microbacteriaceae	NA	0.2848	Aquatic Salamanders
Microbacteriaceae	NA	0.2622	Aquatic Salamanders
Acidothermaceae	<i>Acidothermus</i>	0.2731	Aquatic Salamanders
Mycobacteriaceae	<i>Mycobacterium</i>	0.2607	Aquatic Salamanders
Mycobacteriaceae	<i>Mycobacterium</i>	0.2816	Aquatic Salamanders
Corynebacteriaceae	<i>Corynebacterium</i>	0.2718	Aquatic Salamanders
Corynebacteriaceae	<i>Corynebacterium</i>	0.2845	Aquatic Salamanders
Sphingobacteriaceae	NA	0.2834	Aquatic Salamanders
Sphingobacteriaceae	<i>Mucilaginibacter</i>	0.2574	Aquatic Salamanders
Bacteroidaceae	<i>Bacteroides</i>	0.2550	Aquatic Salamanders
Ruminococcaceae	<i>Angelakisella</i>	0.2577	Aquatic Salamanders
Staphylococcaceae	<i>Macrococcus</i>	0.3051	Aquatic Salamanders
NA	NA	0.3070	Aquatic Salamanders
NA	NA	0.3079	Aquatic Salamanders
NA	NA	0.2814	Aquatic Salamanders
NA	NA	0.2725	Aquatic Salamanders
Acidobacteriaceae (Subgroup 1)	NA	0.2624	Aquatic Salamanders
Acidobacteriaceae (Subgroup 1)	<i>Granulicella</i>	0.2809	Aquatic Salamanders
Flavobacteriaceae	NA	0.2503	Aquatic Salamanders
Flavobacteriaceae	<i>Flavobacterium</i>	0.2885	Aquatic Salamanders
Flavobacteriaceae	<i>Flavobacterium</i>	0.3464	Aquatic Salamanders

Table A3 Continued

Crocinitomicaceae	<i>Fluviicola</i>	0.2508	Aquatic Salamanders
Chitinophagaceae	<i>Aurantisolimonas</i>	0.2574	Aquatic Salamanders
Chitinophagaceae	<i>Parafilimonas</i>	0.2503	Aquatic Salamanders
Spirosomaceae	<i>Flectobacillus</i>	0.2929	Aquatic Salamanders
NA	NA	0.2563	Aquatic Salamanders
Comamonadaceae	<i>Schlegelella</i>	0.2524	Aquatic Salamanders
A21b	NA	0.3099	Aquatic Salamanders
Xanthomonadaceae	<i>Stenotrophomonas</i>	0.2553	Aquatic Salamanders
Pseudomonadaceae	<i>Pseudomonas</i>	0.2587	Aquatic Salamanders
Erwiniaceae	NA	0.3117	Aquatic Salamanders
NA	NA	0.2786	Aquatic Salamanders
Oxalobacteraceae	NA	0.2823	Terrestrial Salamanders
Oxalobacteraceae	NA	0.2826	Terrestrial Salamanders
Oxalobacteraceae	NA	0.2817	Terrestrial Salamanders
Nitrosomonadaceae	<i>Ellin6067</i>	0.2810	Terrestrial Salamanders
Xanthobacteraceae	<i>Rhodoplanes</i>	0.2865	Terrestrial Salamanders
Xanthobacteraceae	<i>Pseudolabrys</i>	0.2636	Terrestrial Salamanders
Xanthobacteraceae	<i>Rhodopseudomonas</i>	0.2617	Terrestrial Salamanders
Xanthobacteraceae	<i>Tardiphaga</i>	0.2660	Terrestrial Salamanders
Beijerinckiaceae	NA	0.2632	Terrestrial Salamanders
NA	NA	0.2515	Terrestrial Salamanders
Devosiaceae	<i>Devosia</i>	0.2923	Terrestrial Salamanders
Solirubrobacteraceae	<i>Conexibacter</i>	0.2543	Terrestrial Salamanders
Solirubrobacteraceae	NA	0.2667	Terrestrial Salamanders
BIrii41	NA	0.2536	Terrestrial Salamanders
BIrii41	NA	0.2618	Terrestrial Salamanders
NA	NA	0.3137	Terrestrial Salamanders
Nocardioideae	<i>Nocardioides</i>	0.2822	Terrestrial Salamanders
Cellulomonadaceae	<i>Cellulomonas</i>	0.2725	Terrestrial Salamanders
Acidothermaceae	<i>Acidothermus</i>	0.2508	Terrestrial Salamanders
Acidothermaceae	<i>Acidothermus</i>	0.2576	Terrestrial Salamanders
Frankiaceae	<i>Jatrophihabitans</i>	0.2882	Terrestrial Salamanders
Pseudonocardiaceae	NA	0.2574	Terrestrial Salamanders
Pseudonocardiaceae	NA	0.3111	Terrestrial Salamanders
Pseudonocardiaceae	<i>Pseudonocardia</i>	0.2542	Terrestrial Salamanders

Table A3 Continued

Streptosporangiaceae	<i>Herbidospora</i>	0.2784	Terrestrial Salamanders
Chthonomonadaceae	<i>Chthonomonas</i>	0.2747	Terrestrial Salamanders
NA	NA	0.2815	Terrestrial Salamanders
Chthoniobacteraceae	<i>Chthoniobacter</i>	0.2833	Terrestrial Salamanders
Xiphinematobacteraceae	Candidatus <i>Xiphinematobacter</i>	0.2666	Terrestrial Salamanders
Xiphinematobacteraceae	Candidatus <i>Xiphinematobacter</i>	0.2600	Terrestrial Salamanders
Simkaniaceae	Candidatus <i>Rhabdochlamydia</i>	0.2532	Terrestrial Salamanders
NA	NA	0.2503	Terrestrial Salamanders
Pirellulaceae	Pir4 lineage	0.2568	Terrestrial Salamanders
NA	NA	0.2640	Terrestrial Salamanders
Microscillaceae	NA	0.2714	Terrestrial Salamanders
Chitinophagaceae	NA	0.2571	Terrestrial Salamanders
NA	NA	0.2784	Terrestrial Salamanders
Unknown Family_4	<i>Acidibacter</i>	0.2937	Terrestrial Salamanders
Rhodanobacteraceae	<i>Luteibacter</i>	0.2690	Terrestrial Salamanders
Rhodanobacteraceae	<i>Rhodanobacter</i>	0.2886	Terrestrial Salamanders
Moraxellaceae	<i>Acinetobacter</i>	0.2667	Terrestrial Salamanders
Moraxellaceae	NA	0.2640	Terrestrial Salamanders

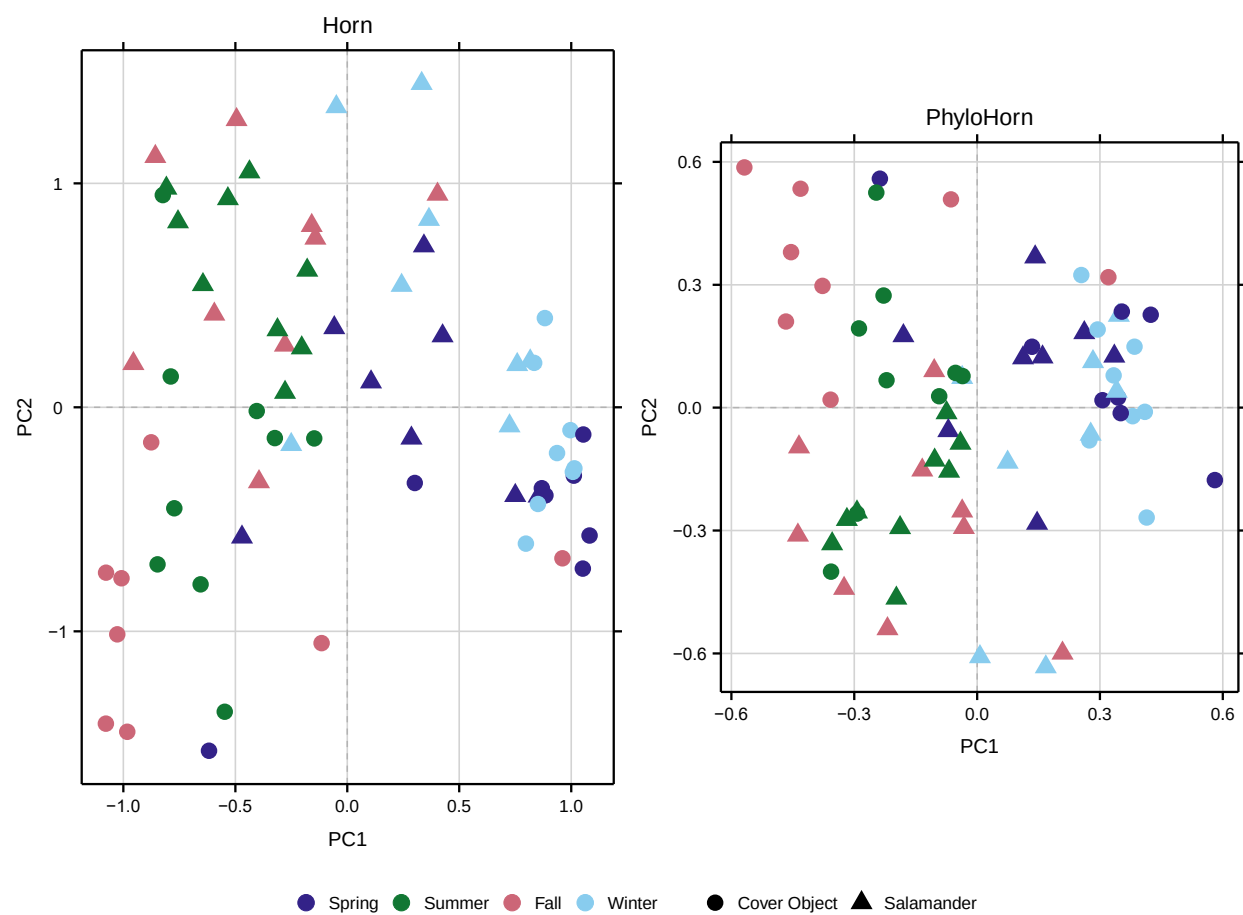


Figure A6: Unconstrained ordination of Horn and PhyloHorn dissimilarities between salamanders and cover objects, seasonally.

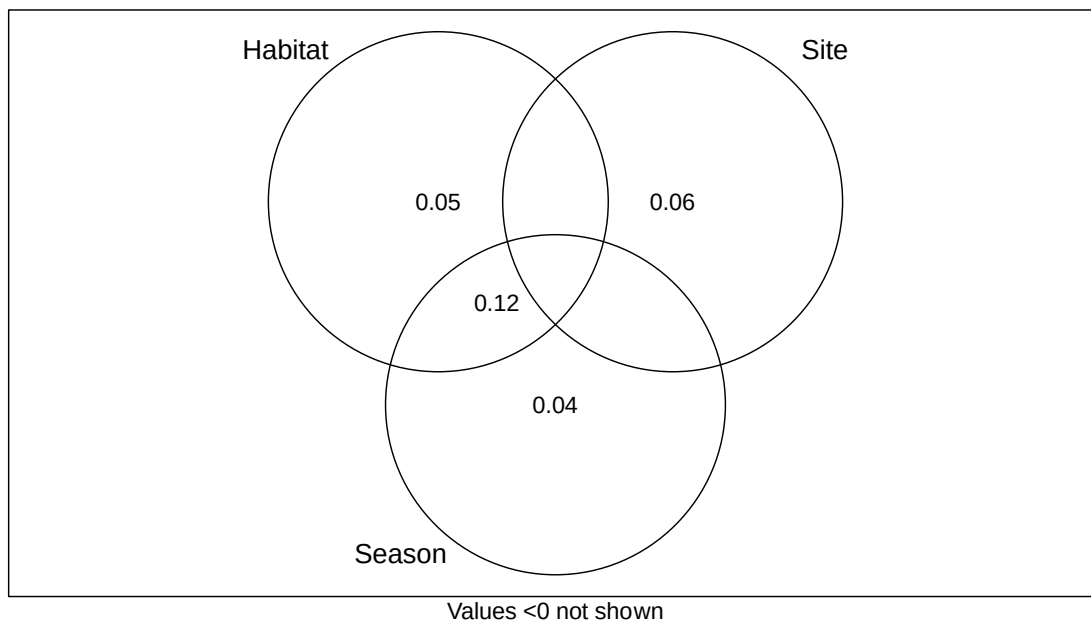


Figure A7: Variance partitioning of the Horn dissimilarity for salamander-associated assemblages.

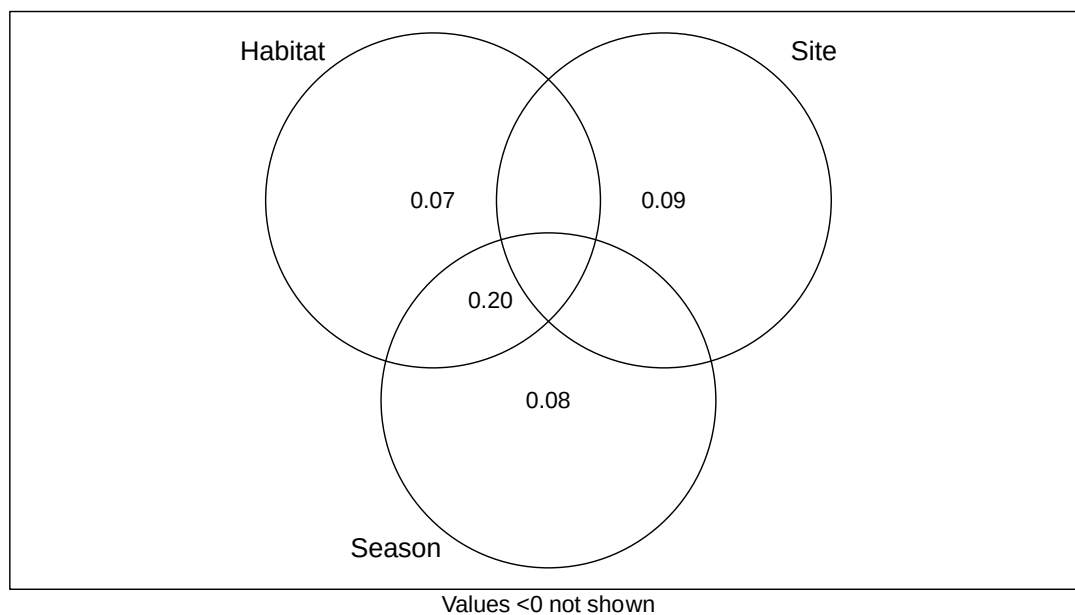


Figure A8: Variance partitioning of the PhyloHorn dissimilarity for salamander-associated assemblages.



Figure A9: Heatmap showing relative abundance of ‘common core’ OTUs during spring sampling. Highlighted families are families with common OTUs shared across all sampling seasons.



Figure A10: Heatmap showing relative abundance of ‘common core’ OTUs during our summer sampling. Highlighted families are families with common OTUs shared across all sampling seasons.

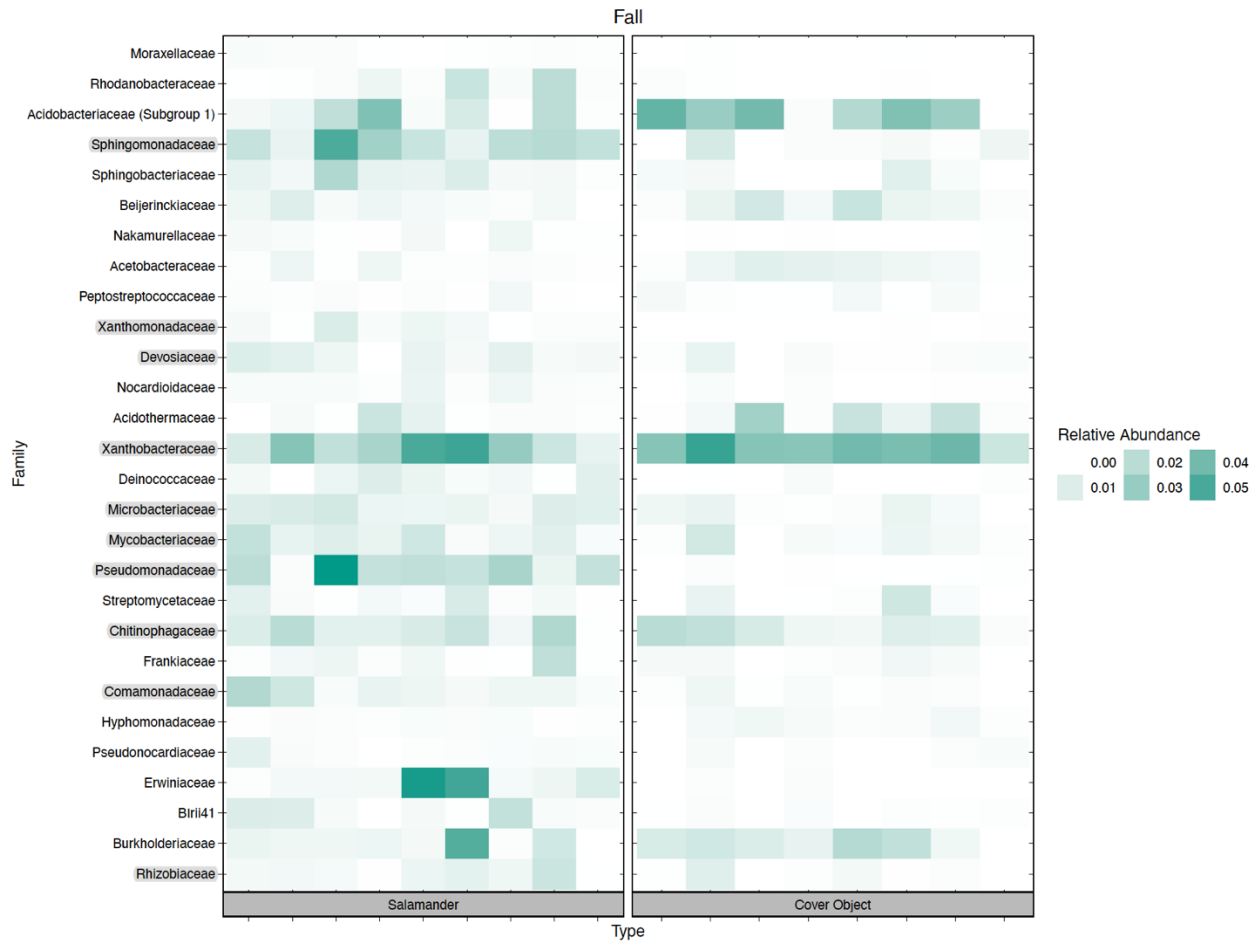


Figure A11: Heatmap showing relative abundance of 'common core' OTUs during the fall sampling. Highlighted families are families with common OTUs shared across all sampling seasons.

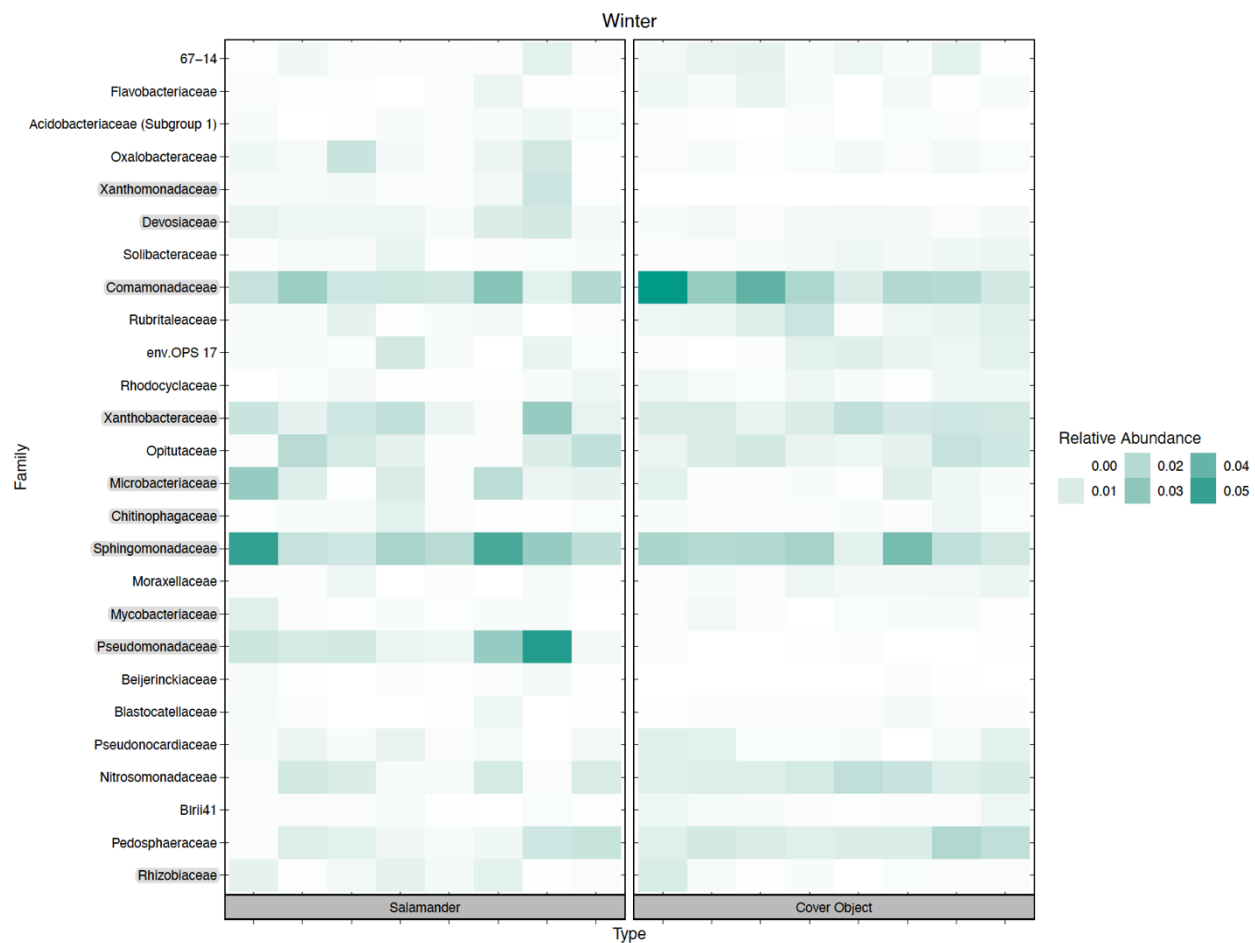


Figure A12: Heatmap showing relative abundance of ‘common core’ OTUs during our winter sampling. Highlighted families are families with common OTUs shared across all sampling seasons.

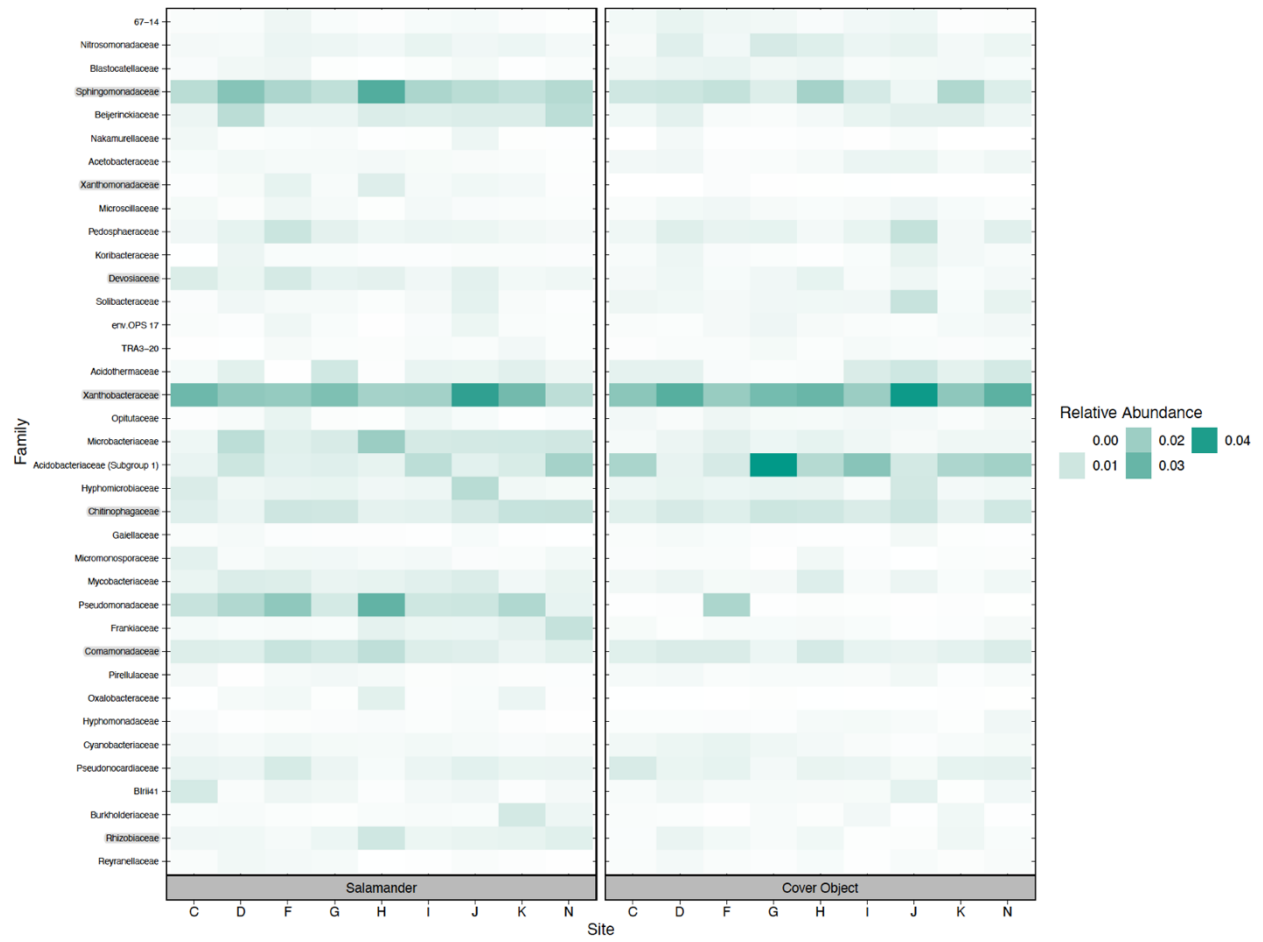


Figure A13: Heatmap of relative abundance of temporal core OTUs at each site.

Table A4: OTUs associated with anti-fungal capabilities and the detection on salamanders.

Order	Family	Genus	Proportion Detected on Hosts
Pseudomonadales	Pseudomonadaceae	<i>Pseudomonas</i>	1
Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	1
Xanthomonadales	Xanthomonadaceae	<i>Stenotrophomonas</i>	1
Burkholderiales	Oxalobacteraceae	<i>Janthinobacterium</i>	0.85
Rhizobiales	Rhizobiaceae	<i>Rhizobium</i>	0.76
Pseudomonadales	Pseudomonadaceae	<i>Pseudomonas</i>	0.74
Sphingobacteriales	Sphingobacteriaceae	<i>Pedobacter</i>	0.68
Enterobacteriales	Enterobacteriaceae	<i>Bacteria</i>	0.62
Actinomycetales	Streptomycetaceae	<i>Streptomyces</i>	0.53
Enterobacteriales	Enterobacteriaceae	<i>Enterobacter</i>	0.50
Actinomycetales	Nocardioidaceae	<i>Aeromicrobium</i>	0.50
Actinomycetales	Micrococcaceae	<i>Arthrobacter</i>	0.47
Burkholderiales	Oxalobacteraceae	NA	0.47
Xanthomonadales	Xanthomonadaceae	<i>Stenotrophomonas</i>	0.44
Flavobacteriales	Flavobacteriaceae	<i>Chryseobacterium</i>	0.44
Flavobacteriales	Flavobacteriaceae	<i>Chryseobacterium</i>	0.35
Pseudomonadales	Pseudomonadaceae	<i>Pseudomonas</i>	0.32
Bacillales	Bacillaceae	<i>Bacillus</i>	0.29
Pseudomonadales	Moraxellaceae	<i>Acinetobacter</i>	0.24
Burkholderiales	Comamonadaceae	<i>Comamonas</i>	0.18
Neisseriales	Neisseriaceae	NA	0.18
Pseudomonadales	Moraxellaceae	<i>Acinetobacter</i>	0.18
Xanthomonadales	Xanthomonadaceae	<i>Dyella</i>	0.18
Actinomycetales	Brevibacteriaceae	<i>Brevibacterium</i>	0.15
Pseudomonadales	Pseudomonadaceae	<i>Pseudomonas</i>	0.15
Pseudomonadales	Moraxellaceae	<i>Acinetobacter</i>	0.12
Pseudomonadales	Moraxellaceae	<i>Acinetobacter</i>	0.12
Burkholderiales	Comamonadaceae	<i>Comamonas</i>	0.09
Caulobacteriales	Caulobacteraceae	<i>Brevundimonas</i>	0.09
Bacillales	Bacillaceae	<i>Bacillus</i>	0.06
Enterobacteriales	Enterobacteriaceae	<i>Serratia</i>	0.06
Xanthomonadales	Xanthomonadaceae	<i>Luteimonas</i>	0.06
Flavobacteriales	Flavobacteriaceae	<i>Flavobacterium</i>	0.06
Enterobacteriales	Enterobacteriaceae	NA	0.06

Table A4 Continued

Pseudomonadales	Pseudomonadaceae	<i>Pseudomonas</i>	0.03
Bacillales	Staphylococcaceae	<i>Staphylococcus</i>	0.03
Flavobacteriales	Flavobacteriaceae	<i>Elizabethkingia</i>	0.03
Actinomycetales	Micrococcaceae	<i>Micrococcus</i>	0.03

VITA

Benjamin Holt is originally from Knoxville, Tennessee, and received a B.S. in Biology from East Tennessee State University as well as a minor in Geographical Information Systems. While not doing fieldwork, his hobbies include hiking with his beloved Catahoula, Stella, and fly fishing. He uses a plethora of field and laboratory techniques in tandem with molecular and computational technologies to answer an array of questions spanning ecology and evolutionary biology, particularly organisms in freshwater environments.