The role of priority effects in the assembly of the amphibian microbiome

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Community assembly in the amphibian microbiome

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Academic Abstract:

Communities are a critical link that impact how species-level population dynamics translate into ecosystem functions, and thus, understanding community assembly is an important goal of ecology. Variation in the relative importance of the four processes of drift, selection, speciation, and dispersal likely govern much of the variation that is observed in community structure across landscapes. Microbial communities provide critical functions across an array of environments, but only recently have technological advances in DNA sequencing allowed us to study these communities with higher resolution. My dissertation research has investigated community assembly in host-associated microbial communities, with a focus on understanding how stochasticity in dispersal that leads to priority affects can impact bacterial community assembly in amphibian embryos. In chapter 1, I experimentally show that priority effects resulting from stochastic dispersal can be observed in the microbiome of newly-hatched hourglass treefrog (Dendropsophus ebraccatus) tadpoles. Changes in microbiome composition due to priority effects could be observed in a simple two bacteria system and when the inoculation by the initial bacteria is followed by a more diverse community inoculum. Outcomes of my two taxa system in co-culture do not strictly mirror those observed in treefrog embryos, highlighting that priority effect outcomes are context dependent. Additionally, these results provide support that priority effects do not benefit all bacterial species equally and the magnitude of these effects will be dependent on the traits of individual colonists. In chapter 2 I demonstrate that priority effects are not unique to the hourglass treefrog system but can be observed in spring peeper (*Pseudacris crucifer*) tadpoles as well. This study demonstrates the applicability of priority effects in increasing the abundance of target probiotic taxa; a benefit to amphibian populations facing threats by a lethal fungal pathogen. By treating embryos with a priority inoculation of *Janthinobacterium lividum*, a bacterial species known to inhibit fungal pathogen growth, I increased the relative abundance of *J. lividum* on newly hatched tadpoles. I also provide evidence that closely-related species of bacteria can effectively co-exist regardless of priority inoculation. An understanding of variation in the amphibian microbiome across life stages in the wild is required to better understand the long-term impacts of priority effects in embryos. My final chapter, therefore, examined compositional changes in the microbiomes of locally occurring amphibians in Virginia across the egg, tadpole, and juvenile developmental stages. In this study, I show characterize the initial egg microbiome across amphibian species and demonstrate that egg microbiomes, are distinct between species but are more similar across species than tadpole or juvenile microbiomes. Additionally, I show that minor differences in host environment can lead to differences in the microbiome structure of conspecific tadpoles. Overall, my dissertation empirically demonstrates the role of dispersal, and more specifically priority effects, in the assembly of the vertebrate microbiome.

General Abstract:

An ecological community is a set of species that occur at a given site. Communities have been a fundamental focus of ecological research, as communities serve to link the population dynamics of individual species to ecosystem level processes provided by species. Microbial communities, in particular, are of interest because of the wide range of important functions they provide across a variety of systems, yet relatively little is known about how these communities initially come together and are maintained. This is particularly true for the microbial communities that live in and on plants and animals, which are called "host-associated" communities. Host-associated microbial communities contribute many important functions to their hosts, including guiding host development, assisting with nutrient assimilation, and providing disease resistance. Four processes are thought to govern how ecological communities assemble across landscapes at local sites or habitat patches: selection, dispersal, speciation, and drift. Variation in the relative importance of these processes is thought to drive the variation in community composition across sites, or in the case of host-associated microbial communities, across hosts. Selection occurs at a local level when environmental variables or the presence of other species impact where a species occurs. Dispersal of individuals among habitat patches can also impact what species occur at a local site, and speciation gives rise to new species in communities over time. Drift is the stochastic, or random, element of species abundance that is driven by variation in the birth and death rates of a population at a site. I have investigated the assembly of host-associated microbial communities using amphibians as a study system. In chapter 1, I experimentally demonstrate that stochasticity in dispersal that impacts which species arrive first to a site (priority effects) can be observed in the host-associated bacterial communities of newly-hatched treefrog (*Dendropsophus ebraccatus*) tadpoles. This can be observed in a simplified system where only two bacterial species are used, and also when a single bacterial species arrives and is followed by a more diverse community of bacteria. However, not every bacterial species is able to take advantage of priority, and these results seem to be context dependent, as the outcomes in treefrog embryos do not exactly mirror the outcomes when the bacteria are grown in a nutrient broth together. In chapter 2, I show that priority effects are not unique to the hourglass treefrog system; priority effects can also be observed in spring peeper (*Pseudacris crucifer*) tadpoles. In this study, I also demonstrated that we may be able to apply our knowledge of priority effects to benefit amphibian populations threatened by a potentially lethal fungal pathogen by manipulating the abundances of bacteria on the skin during development. Priority treatment of embryos with *Janthinobacterium lividum*, a bacterial species known for its ability to inhibit growth of this fungal pathogen, resulted in increased relative abundance of *J. lividum* in the tadpoles following hatching. Additionally, I found that even closely-related bacterial species can have differing abilities to take advantage of priority effects and can co-exist on tadpoles. To determine long-term impacts of priority effects in embryos requires an understanding of the variation associated with amphibians in the wild across different life stages. My final chapter, therefore, focused on examining changes in the bacterial communities associated with locally occurring amphibians in Virginia across the egg, tadpole, and juvenile stages of development. Specifically, I characterize the initial communities associated with eggs across different species, including predicted associations with algal symbionts, and examine patterns of host-associated communities among species and across development. Overall, my dissertation showcases the role that dispersal, but more specifically priority effects, can play in the development of the vertebrate microbiome.

Dedication:

This work is dedicated to my late father, Robert Earl Jones, a constant source of support and love throughout my life. I am deeply saddened that he will not see the conclusion of this journey, but he never showed anything but full confidence that I would get to where I am now.

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Chapter 2: Colonization order of bacterial isolates on treefrog embryos impacts microbiome structure in tadpoles

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Chapter 3: Priority Effects Alter Microbiome Composition and Increase Abundance of Probiotic Taxa in Treefrog Tadpoles

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Introduction:

A community is a collection of different species that occupy the same location at the same time [1]. Community ecologists seek to understand the principles that guide the assembly and persistence of these communities. Despite such a simple definition, communities are complex. The wide range of potential organisms, environmental variables, and study scales challenges our ability to form general laws regarding communities [2]. Community ecology may be lacking in generality, but understanding the local dynamics of individual communities facilitates our understanding of complex issues involving networks of communities [3].

Networks of communities, referred to as metacommunities, can be defined as a collection of communities connected by the dispersal of multiple species [4]. Focusing only on local community dynamics may ignore the effects of factors, such as regional species diversity or source-sink dynamics, that link communities across the landscape [5]. Structure within metacommunities has been studied in the context of four metacommunity paradigms: patch-dynamics, species sorting, mass-effects, and neutral models. While these paradigms can be used independently, they are not mutually exclusive and may vary in importance along a gradient of temporal scale [6]. The patch-dynamic model is defined as identical patches that contain communities whose composition is determined by tradeoffs that species make between competitive ability and dispersal. The species-sorting model describes heterogenous patches and emphasizes the effects of environmental suitability and dispersal. Mass-effect models also feature heterogeneous patches, but with dispersal being viewed as the driving factor in local community composition. Neutral theory, often used as a null hypothesis, assumes all species to be equivalent and places an emphasis on drift as the determining factor of community composition [5].

In pursuit of providing a more accessible and repeatable framework for the study of community ecology, Vellend (2010) [7] proposed that community composition is driven by four main processes: selection, speciation, dispersal, and drift. The relative importance of these processes across a landscape can drive the variation observed in community structure. While the four metacommunity frameworks also rely on variation in these processes, this more explicit focus on the processes themselves allows for a more direct comparison across studies and scales. Stochasticity in dispersal, for example can influence differences in community composition between otherwise equivalent habitat patches [8,9]. Because early arriving taxa may monopolize resources (niche preemption) or alter the environment (niche modification), subsequently colonizing taxa may be negatively or beneficially affected. These relatively understudied consequences of colonization order, commonly known as priority effects, are likely important for structuring communities in a range of systems [10–14]. For example, priority effects influence nectar microbial metacommunities across generations [15], human infant neurodevelopment [16], and invasion outcomes in plant communities [17].

Historically, research into communities, and thus the principles derived from the research, has focused on communities of animals and plants rather than microbial communities. New technological advancements, such as 16S rRNA gene amplicon sequencing, have facilitated an increase in our understanding of bacterial community composition, which was previously limited by a researcher's ability to culture target organisms [18]. Improvements in data processing have allowed researchers to move away from clustering bacterial sequences into operational taxonomic units based on a similarity threshold (usually 97%), to amplicon sequence variants, allowing for the cataloging of unique sequences. Traits of bacterial communities that previously limited their use in

community ecology, such as their small size [19] and spatial scale [20], can now be viewed as advantageous in testing ecological theory.

Within the context of community assembly, simplified bacterial communities can be used to understand and observe interactions among taxa. Although synthetic communities may not possess the magnitude of diversity found in natural systems, they allow for clearer tests of assumptions from ecological models [21] and can be more precisely manipulated [22]. Studies involving community subsets continue to grow in the field of synthetic biology, but still retain a great deal of untapped potential [23,24]. Using synthetic communities, the four drivers of community assembly proposed by Vellend (2010) [7] can be controlled in ways that would be impractical in a natural system. For example, synthetic communities also allow us to manipulate and observe dispersal under controlled conditions, which is generally unlikely in natural environments.

Our ability to better observe microbial communities has led to an increased understanding of how microbes influence their environment and vice versa. Interest in developing probiotic applications targeted at disease treatment or prevention have contributed to the continued focus on host-associated microbial communities. Within the human microbiome, many links exist between bacterial community composition and host health [25]. For example, changes in the composition of microbial communities within the human gut are correlated with the ability of the bacterial pathogen, *Clostridium difficile*, to invade the gut, resulting in colitis [26]. There are many additional examples of the importance of host-associated microbial communities beyond the human gut. For example, symbiotic bacteria within the plant rhizosphere can promote plant growth and suppress disease [27,28]. Even among studies seeking to understand disease susceptibility without a specific focus on the effects of host-associated microbes, experimental outcomes can be partially tied to microbial community composition [29].

The fungal pathogen, *Batrachochytrium dendrobatidis* (Bd), first described in 1999 [30], has been implicated in worldwide amphibian population declines and even extinctions [31–33]. The lethality of Bd is likely due to its ability to disrupt critical osmoregulation within amphibian skin, creating an electrolyte imbalance that eventually results in death [34]. Despite its potential for lethality, Bd does not always cause mortality or extinctions. The likelihood of Bd infection occurring and resulting in mortality events varies with host species [35], temperature [36], the presence of organisms capable of predating Bd zoospores [37], and the composition of host-associated bacterial communities [38–42]. Many of these factors cannot be reasonably altered in nature; however, reducing susceptibility to Bd through bacterial probiotic augmentation is possible in a laboratory setting [40,43], and holds promise as a potential conservation measure.

Certain members of the amphibian skin microbiome, such as *Janthinobacterium lividum* and *Serratia marcescens* produce anti-fungal compounds that can inhibit the growth of Bd[44,45]. The potential to combat Bd has, therefore, led to research dedicated to understanding the amphibian skin microbiome. Bacterial skin communities are more similar within, than between, amphibian species (e.g. [46]). Differences in community composition have also been observed between life stages within the same species [47–49]. Recent research has placed an emphasis on the environmental source pool as a determining factor of amphibian skin bacterial community composition [47,50–53].

We have developed a broad understanding of the functional roles of amphibian microbial communities, such as its effects on thermal tolerance, and disease resistance [54–56]); however our understanding of how these communities assemble lags. Function within microbial communities is

dependent on composition, therefore, developing a thorough understanding of community assembly is important when attempting to leverage the microbiome to improve host health. With my dissertation research, I aimed to conduct empirical studies within the framework of community assembly theory to better understand the mechanisms behind bacterial community assembly in the amphibian microbiome. First, in chapter two, I experimentally determined if priority effects could be detected in the amphibian microbiome, and whether bacterial priority effects *in vitro* reflected those *in vivo*. In chapter two, I expanded this understanding of priority effects to investigate potential bacterial probiotic applications, and to test whether I could observe priority effects between two closely-related bacterial isolates. Additionally, I examined potential vertical transfer of bacteria from parents to offspring and how rearing location can affect community composition. Lastly, understanding how priority effects contribute to differences in amphibian microbiome composition requires an understanding of natural community trajectories. In chapter three, therefore, I surveyed local amphibians to better understand differences in the bacterial communities associated with different amphibian species over the course of development, and to establish an understanding of variation across species in the bacterial communities associated with amphibian embryos.

(1) Fauth, J. E.; Bernardo, J.; Camara, M.; Resetarits, W. J.; Van Buskirk, J.; McCollum, S. A. Simplifying the Jargon of Community Ecology: A Conceptual Approach. *The American Naturalist* **1996**, *147* (2), 282–286. https://doi.org/10.1086/285850.

(2) Lawton, J. H. Are There General Laws in Ecology? *Oikos* **1999**, *84* (2), 177. https://doi.org/10.2307/3546712.

(3) Simberloff, D. Community Ecology: Is It Time to Move On?: (An American Society of Naturalists Presidential Address). *The American Naturalist* **2004**, *163* (6), 787–799. https://doi.org/10.1086/420777.

(4) Wilson, D. S. Complex Interactions in Metacommunities, with Implications for Biodiversity and Higher Levels of Selection. *Ecology* **1992**, *73* (6), 1984–2000. https://doi.org/10.2307/1941449.

(5) Leibold, M. A.; Holyoak, M.; Mouquet, N.; Amarasekare, P.; Chase, J. M.; Hoopes, M. F.; Holt, R. D.; Shurin, J. B.; Law, R.; Tilman, D.; Loreau, M.; Gonzalez, A. The Metacommunity Concept: A Framework for Multi-Scale Community Ecology: The Metacommunity Concept. *Ecology Letters* **2004**, *7* (7), 601–613. https://doi.org/10.1111/j.1461-0248.2004.00608.x.

(6) Stroud, J. T.; Bush, M. R.; Ladd, M. C.; Nowicki, R. J.; Shantz, A. A.; Sweatman, J. Is a Community Still a Community? Reviewing Definitions of Key Terms in Community Ecology. *Ecology and Evolution* **2015**, *5* (21), 4757–4765. https://doi.org/10.1002/ece3.1651.

(7) Vellend, M. Conceptual Synthesis in Community Ecology. *The Quarterly Review of Biology* **2010**, *85* (2), 183–206. https://doi.org/10.1086/652373.

(8) Chase, J. M. Stochastic Community Assembly Causes Higher Biodiversity in More Productive Environments. *Science* **2010**, *328* (5984), 1388–1391. https://doi.org/10.1126/science.1187820.

(9) Evans, S.; Martiny, J. B. H.; Allison, S. D. Effects of Dispersal and Selection on Stochastic Assembly in Microbial Communities. *The ISME Journal* **2017**, *11* (1), 176–185. https://doi.org/10.1038/ismej.2016.96.

(10) Cheong, J. Z. A.; Johnson, C. J.; Wan, H.; Liu, A.; Kernien, J. F.; Gibson, A. L. F.; Nett, J. E.; Kalan, L. R. Priority Effects Dictate Community Structure and Alter Virulence of Fungal-Bacterial Biofilms. *ISME J* **2021**, *15* (7), 2012–2027. https://doi.org/10.1038/s41396-021-00901-5.

(11) Debray, R.; Herbert, R. A.; Jaffe, A. L.; Crits-Christoph, A.; Power, M. E.; Koskella, B. Priority Effects in Microbiome Assembly. *Nat Rev Microbiol* **2021**. https://doi.org/10.1038/s41579-021-00604-w.

(12) Devevey, G.; Dang, T.; Graves, C. J.; Murray, S.; Brisson, D. First Arrived Takes All: Inhibitory Priority Effects Dominate Competition between Co-Infecting Borrelia Burgdorferi Strains. *BMC Microbiology* **2015**, *15* (1), 61. https://doi.org/10.1186/s12866-015-0381-0.

(13) Fukami, T. Historical Contingency in Community Assembly: Integrating Niches, Species Pools, and Priority Effects. *Annual Review of Ecology, Evolution, and Systematics* **2015**, *46* (1), 1–23. https://doi.org/10.1146/annurev-ecolsys-110411-160340.

(14) Ridout, M. E.; Schroeder, K. L.; Hunter, S. S.; Styer, J.; Newcombe, G. Priority Effects of Wheat Seed Endophytes on a Rhizosphere Symbiosis. *Symbiosis* **2019**, *78* (1), 19–31. https://doi.org/10.1007/s13199-019-00606-6.

(15) Toju, H.; Vannette, R. L.; Gauthier, M.-P. L.; Dhami, M. K.; Fukami, T. Priority Effects Can Persist across Floral Generations in Nectar Microbial Metacommunities. *Oikos* **2018**, *127* (3), 345–352. https://doi.org/10.1111/oik.04243.

(16) Zhou, L.; Qiu, W.; Wang, J.; Zhao, A.; Zhou, C.; Sun, T.; Xiong, Z.; Cao, P.; Shen, W.; Chen, J.; Lai, X.; Zhao, L.; Wu, Y.; Li, M.; Qiu, F.; Yu, Y.; Xu, Z. Z.; Zhou, H.; Jia, W.; Liao, Y.; Retnakaran, R.; Krewski, D.; Wen, S. W.; Clemente, J. C.; Chen, T.; Xie, R.; He, Y. Effects of Vaginal Microbiota Transfer on the Neurodevelopment and Microbiome of Cesarean-Born Infants: A Blinded Randomized Controlled Trial. *Cell Host & Microbe* **2023**. https://doi.org/10.1016/j.chom.2023.05.022.

(17) Goodale, K. M.; Wilsey, B. J. Priority Effects Are Affected by Precipitation Variability and Are Stronger in Exotic than Native Grassland Species. *Plant Ecol* **2018**, *219* (4), 429–439. https://doi.org/10.1007/s11258-018-0806-6.

(18) Amann, R. I.; Ludwig, W.; Schleifer, K.-H. Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation. *MICROBIOL. REV.* **1995**, *59*, 33.

(19) Poretsky, R.; Rodriguez-R, L. M.; Luo, C.; Tsementzi, D.; Konstantinidis, K. T. Strengths and Limitations of 16S RRNA Gene Amplicon Sequencing in Revealing Temporal Microbial Community Dynamics. *PLOS ONE* **2014**, *9* (4), e93827. https://doi.org/10.1371/journal.pone.0093827.

(20) Nemergut, D. R.; Schmidt, S. K.; Fukami, T.; O'Neill, S. P.; Bilinski, T. M.; Stanish, L. F.; Knelman, J. E.; Darcy, J. L.; Lynch, R. C.; Wickey, P.; Ferrenberg, S. Patterns and Processes of Microbial Community Assembly. *Microbiology and Molecular Biology Reviews* **2013**, *77* (3), 342–356. https://doi.org/10.1128/MMBR.00051-12.

(21) Friedman, J.; Higgins, L. M.; Gore, J. Community Structure Follows Simple Assembly Rules in Microbial Microcosms. *Nature Ecology & Evolution* **2017**, *1* (5). https://doi.org/10.1038/s41559-017-0109.

(22) Jessup, C. M.; Kassen, R.; Forde, S. E.; Kerr, B.; Buckling, A.; Rainey, P. B.; Bohannan, B. J. M. Big Questions, Small Worlds: Microbial Model Systems in Ecology. *Trends in Ecology & Evolution* **2004**, *19* (4), 189–197. https://doi.org/10.1016/j.tree.2004.01.008.

(23) Brenner, K.; You, L.; Arnold, F. H. Engineering Microbial Consortia: A New Frontier in Synthetic Biology. *Trends in Biotechnology* **2008**, *26* (9), 483–489. https://doi.org/10.1016/j.tibtech.2008.05.004.

(24) Fredrickson, J. K. Ecological Communities by Design. *Science* **2015**, *348* (6242), 1425–1427. https://doi.org/10.1126/science.aab0946.

(25) Clemente, J. C.; Ursell, L. K.; Parfrey, L. W.; Knight, R. The Impact of the Gut Microbiota on Human Health: An Integrative View. *Cell* **2012**, *148* (6), 1258–1270. https://doi.org/10.1016/j.cell.2012.01.035.

(26) Britton, R. A.; Young, V. B. Role of the Intestinal Microbiota in Resistance to Colonization by Clostridium Difficile. *Gastroenterology* **2014**, *146* (6), 1547–1553. https://doi.org/10.1053/j.gastro.2014.01.059.

(27) Chaparro, J. M.; Sheflin, A. M.; Manter, D. K.; Vivanco, J. M. Manipulating the Soil Microbiome to Increase Soil Health and Plant Fertility. *Biol Fertil Soils* **2012**, *48* (5), 489–499. https://doi.org/10.1007/s00374-012-0691-4.

(28) Mendes, R.; Kruijt, M.; Bruijn, I. de; Dekkers, E.; Voort, M. van der; Schneider, J. H. M.; Piceno, Y. M.; DeSantis, T. Z.; Andersen, G. L.; Bakker, P. A. H. M.; Raaijmakers, J. M. Deciphering the Rhizosphere Microbiome for Disease-Suppressive Bacteria. *Science* **2011**, *332* (6033), 1097–1100. https://doi.org/10.1126/science.1203980.

(29) Velazquez, E. M.; Nguyen, H.; Heasley, K. T.; Saechao, C. H.; Gil, L. M.; Rogers, A. W. L.; Miller, B. M.; Rolston, M. R.; Lopez, C. A.; Litvak, Y.; Liou, M. J.; Faber, F.; Bronner, D. N.; Tiffany, C. R.; Byndloss, M. X.; Byndloss, A. J.; Bäumler, A. J. Endogenous Enterobacteriaceae Underlie Variation in Susceptibility to Salmonella Infection. *Nature Microbiology* **2019**, 1. https://doi.org/10.1038/s41564-019-0407-8.

(30) Longcore, J. E.; Pessier, A. P.; Nichols, D. K. Batrachochytrium Dendrobatidis Gen. et Sp. Nov., a Chytrid Pathogenic to Amphibians. *Mycologia* **1999**, *91* (2), 219. https://doi.org/10.2307/3761366.

(31) Berger, L.; Speare, R.; Daszak, P.; Green, D. E.; Cunningham, A. A.; Goggin, C. L.; Slocombe, R.; Ragan, M. A.; Hyatt, A. D.; McDonald, K. R.; Hines, H. B.; Lips, K. R.; Marantelli, G.; Parkes, H. Chytridiomycosis Causes Amphibian Mortality Associated with Population Declines in the Rain Forests of Australia and Central America. *Proc Natl Acad Sci U S A* **1998**, *95* (15), 9031–9036.

(32) Lips, K. R.; Brem, F.; Brenes, R.; Reeve, J. D.; Alford, R. A.; Voyles, J.; Carey, C.; Livo, L.; Pessier, A. P.; Collins, J. P. Emerging Infectious Disease and the Loss of Biodiversity in a Neotropical Amphibian Community. *Proc Natl Acad Sci U S A* **2006**, *103* (9), 3165–3170. https://doi.org/10.1073/pnas.0506889103.

(33) Scheele, B. C.; Pasmans, F.; Skerratt, L. F.; Berger, L.; Martel, A.; Beukema, W.; Acevedo, A. A.; Burrowes, P. A.; Carvalho, T.; Catenazzi, A.; Riva, I. D. la; Fisher, M. C.; Flechas, S. V.; Foster, C. N.; Frías-Álvarez, P.; Garner, T. W. J.; Gratwicke, B.; Guayasamin, J. M.; Hirschfeld, M.; Kolby, J. E.; Kosch, T. A.; Marca, E. L.; Lindenmayer, D. B.; Lips, K. R.; Longo, A. V.; Maneyro, R.; McDonald, C. A.; Mendelson, J.;

Palacios-Rodriguez, P.; Parra-Olea, G.; Richards-Zawacki, C. L.; Rödel, M.-O.; Rovito, S. M.; Soto-Azat, C.; Toledo, L. F.; Voyles, J.; Weldon, C.; Whitfield, S. M.; Wilkinson, M.; Zamudio, K. R.; Canessa, S. Amphibian Fungal Panzootic Causes Catastrophic and Ongoing Loss of Biodiversity. *Science* **2019**, *363* (6434), 1459–1463. https://doi.org/10.1126/science.aav0379.

(34) Voyles, J.; Young, S.; Berger, L.; Campbell, C.; Voyles, W. F.; Dinudom, A.; Cook, D.; Webb, R.; Alford, R. A.; Skerratt, L. F.; Speare, R. Pathogenesis of Chytridiomycosis, a Cause of Catastrophic Amphibian Declines. *Science* **2009**, *326* (5952), 582–585. https://doi.org/10.1126/science.1176765.

(35) Wagner, N.; Neubeck, C.; Guicking, D.; Finke, L.; Wittich, M.; Weising, K.; Geske, C.; Veith, M. No Evidence for Effects of Infection with the Amphibian Chytrid Fungus on Populations of Yellow-Bellied Toads. *Diseases of Aquatic Organisms* **2017**, *123* (1), 55–65. https://doi.org/10.3354/dao03090.

(36) Voyles, J.; Johnson, L. R.; Briggs, C. J.; Cashins, S. D.; Alford, R. A.; Berger, L.; Skerratt, L. F.; Speare, R.; Rosenblum, E. B. Temperature Alters Reproductive Life History Patterns in Batrachochytrium Dendrobatidis, a Lethal Pathogen Associated with the Global Loss of Amphibians. *Ecol Evol* **2012**, *2* (9), 2241–2249. https://doi.org/10.1002/ece3.334.

(37) Schmeller, D. S.; Blooi, M.; Martel, A.; Garner, T. W. J.; Fisher, M. C.; Azemar, F.; Clare, F. C.; Leclerc, C.; Jäger, L.; Guevara-Nieto, M.; Loyau, A.; Pasmans, F. Microscopic Aquatic Predators Strongly Affect Infection Dynamics of a Globally Emerged Pathogen. *Current Biology* **2014**, *24* (2), 176–180. https://doi.org/10.1016/j.cub.2013.11.032.

(38) Becker Matthew H.; Walke Jenifer B.; Cikanek Shawna; Savage Anna E.; Mattheus Nichole; Santiago Celina N.; Minbiole Kevin P. C.; Harris Reid N.; Belden Lisa K.; Gratwicke Brian. Composition of Symbiotic Bacteria Predicts Survival in Panamanian Golden Frogs Infected with a Lethal Fungus. *Proceedings of the Royal Society B: Biological Sciences* **2015**, *282* (1805), 20142881. https://doi.org/10.1098/rspb.2014.2881.

(39) Jani, A. J.; Knapp, R. A.; Briggs, C. J. Epidemic and Endemic Pathogen Dynamics Correspond to Distinct Host Population Microbiomes at a Landscape Scale. *Proceedings of the Royal Society B: Biological Sciences* **2017**, *284* (1857), 20170944. https://doi.org/10.1098/rspb.2017.0944.

(40) Kueneman, J. G.; Woodhams, D. C.; Harris, R.; Archer, H. M.; Knight, R.; McKenzie, V. J. Probiotic Treatment Restores Protection against Lethal Fungal Infection Lost during Amphibian Captivity. *Proceedings of the Royal Society B: Biological Sciences* **2016**, *283* (1839), 20161553. https://doi.org/10.1098/rspb.2016.1553.

(41) Kueneman, J. G.; Woodhams, D. C.; Van Treuren, W.; Archer, H. M.; Knight, R.; McKenzie, V. J. Inhibitory Bacteria Reduce Fungi on Early Life Stages of Endangered Colorado Boreal Toads (*Anaxyrus Boreas*). *The ISME Journal* **2016**, *10* (4), 934–944. https://doi.org/10.1038/ismej.2015.168.

(42) Walke, J. B.; Becker, M. H.; Loftus, S. C.; House, L. L.; Teotonio, T. L.; Minbiole, K. P. C.; Belden, L. K. Community Structure and Function of Amphibian Skin Microbes: An Experiment with Bullfrogs Exposed to a Chytrid Fungus. *PLoS ONE* **2015**, *10* (10), e0139848. https://doi.org/10.1371/journal.pone.0139848.

(43) Harris, R. N.; Brucker, R. M.; Walke, J. B.; Becker, M. H.; Schwantes, C. R.; Flaherty, D. C.; Lam, B. A.; Woodhams, D. C.; Briggs, C. J.; Vredenburg, V. T.; Minbiole, K. P. C. Skin Microbes on Frogs Prevent

Morbidity and Mortality Caused by a Lethal Skin Fungus. *The ISME Journal* **2009**, *3* (7), 818–824. https://doi.org/10.1038/ismej.2009.27.

(44) Madison, J. D.; Berg, E. A.; Abarca, J. G.; Whitfield, S. M.; Gorbatenko, O.; Pinto, A.; Kerby, J. L. Characterization of Batrachochytrium Dendrobatidis Inhibiting Bacteria from Amphibian Populations in Costa Rica. *Front. Microbiol.* **2017**, *8*. https://doi.org/10.3389/fmicb.2017.00290.

(45) Woodhams, D. C.; LaBumbard, B. C.; Barnhart, K. L.; Becker, M. H.; Bletz, M. C.; Escobar, L. A.; Flechas, S. V.; Forman, M. E.; Iannetta, A. A.; Joyce, M. D.; Rabemananjara, F.; Gratwicke, B.; Vences, M.; Minbiole, K. P. C. Prodigiosin, Violacein, and Volatile Organic Compounds Produced by Widespread Cutaneous Bacteria of Amphibians Can Inhibit Two Batrachochytrium Fungal Pathogens. *Microb Ecol* **2018**, *75* (4), 1049–1062. https://doi.org/10.1007/s00248-017-1095-7.

(46) Belden, L. K.; Hughey, M. C.; Rebollar, E. A.; Umile, T. P.; Loftus, S. C.; Burzynski, E. A.; Minbiole, K. P. C.; House, L. L.; Jensen, R. V.; Becker, M. H.; Walke, J. B.; Medina, D.; Ibáñez, R.; Harris, R. N. Panamanian Frog Species Host Unique Skin Bacterial Communities. *Front Microbiol* **2015**, *6*. https://doi.org/10.3389/fmicb.2015.01171.

(47) Kueneman, J. G.; Laura Wegener Parfrey; Woodhams, D. C.; Archer, H. M.; Knight, R.; McKenzie, V. J. The Amphibian Skin-Associated Microbiome across Species, Space and Life History Stages. *Molecular Ecology* **2014**, *23* (6), 1238–1250. https://doi.org/10.1111/mec.12510.

(48) Prest, T. L.; Kimball, A. K.; Kueneman, J. G.; McKenzie, V. J. Host-Associated Bacterial Community Succession during Amphibian Development. *Molecular Ecology* **2018**, *27* (8), 1992–2006. https://doi.org/10.1111/mec.14507.

(49) Sabino-Pinto, J.; Galán, P.; Rodríguez, S.; Bletz, M. C.; Bhuju, S.; Geffers, R.; Jarek, M.; Vences, M. Temporal Changes in Cutaneous Bacterial Communities of Terrestrial- and Aquatic-Phase Newts (Amphibia): Temporal Changes in Skin Microbial Communities. *Environmental Microbiology* **2017**, *19* (8), 3025–3038. https://doi.org/10.1111/1462-2920.13762.

(50) Bletz, M. C.; Archer, H.; Harris, R. N.; McKenzie, V. J.; Rabemananjara, F. C. E.; Rakotoarison, A.; Vences, M. Host Ecology Rather Than Host Phylogeny Drives Amphibian Skin Microbial Community Structure in the Biodiversity Hotspot of Madagascar. *Front. Microbiol.* **2017**, *8*. https://doi.org/10.3389/fmicb.2017.01530.

(51) Hughey, M. C.; Pena, J. A.; Reyes, R.; Medina, D.; Belden, L. K.; Burrowes, P. A. Skin Bacterial Microbiome of a Generalist Puerto Rican Frog Varies along Elevation and Land Use Gradients. *PeerJ* **2017**, *5*, e3688. https://doi.org/10.7717/peerj.3688.

(52) Kueneman, J. G.; Bletz, M. C.; McKenzie, V. J.; Becker, C. G.; Joseph, M. B.; Abarca, J. G.; Archer, H.; Arellano, A. L.; Bataille, A.; Becker, M.; Belden, L. K.; Crottini, A.; Geffers, R.; Haddad, C. F. B.; Harris, R. N.; Holden, W. M.; Hughey, M.; Jarek, M.; Kearns, P. J.; Kerby, J. L.; Kielgast, J.; Kurabayashi, A.; Longo, A. V.; Loudon, A.; Medina, D.; Nuñez, J. J.; Perl, R. G. B.; Pinto-Tomás, A.; Rabemananjara, F. C. E.; Rebollar, E. A.; Rodríguez, A.; Rollins-Smith, L.; Stevenson, R.; Tebbe, C. C.; Asensio, G. V.; Waldman, B.; Walke, J. B.; Whitfield, S. M.; Zamudio, K. R.; Chaves, I. Z.; Woodhams, D. C.; Vences, M. Community Richness of Amphibian Skin Bacteria Correlates with Bioclimate at the Global Scale. *Nature Ecology & Evolution* **2019**, *3* (3), 381. https://doi.org/10.1038/s41559-019-0798-1.

(53) Loudon, A. H.; Venkataraman, A.; Van Treuren, W.; Woodhams, D. C.; Parfrey, L. W.; McKenzie, V. J.; Knight, R.; Schmidt, T. M.; Harris, R. N. Vertebrate Hosts as Islands: Dynamics of Selection, Immigration, Loss, Persistence, and Potential Function of Bacteria on Salamander Skin. *Front. Microbiol.* **2016**, *7*. https://doi.org/10.3389/fmicb.2016.00333.

(54) Becker, M. H.; Brucker, R. M.; Schwantes, C. R.; Harris, R. N.; Minbiole, K. P. C. The Bacterially Produced Metabolite Violacein Is Associated with Survival of Amphibians Infected with a Lethal Fungus. *Applied and Environmental Microbiology* **2009**, *75* (21), 6635–6638. https://doi.org/10.1128/AEM.01294-09.

(55) Fontaine, S. S.; Mineo, P. M.; Kohl, K. D. Experimental Manipulation of Microbiota Reduces Host Thermal Tolerance and Fitness under Heat Stress in a Vertebrate Ectotherm. *Nat Ecol Evol* **2022**, *6* (4), 405–417. https://doi.org/10.1038/s41559-022-01686-2.

(56) Knutie, S. A.; Wilkinson, C. L.; Kohl, K. D.; Rohr, J. R. Early-Life Disruption of Amphibian Microbiota Decreases Later-Life Resistance to Parasites. *Nature Communications* **2017**, *8* (1), 86. https://doi.org/10.1038/s41467-017-00119-0.

Title: Colonization order of bacterial isolates on treefrog embryos impacts microbiome structure in tadpoles

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Abstract:

Priority effects, or impacts of colonization order, may have lasting influence on ecological community composition. The embryonic microbiome is subject to stochasticity in colonization order of bacteria. Stochasticity may be especially impactful for embryos developing in bacteria-rich environments, such as the embryos of many amphibians. To determine if priority effects experienced as embryos impacted bacterial community composition in newly hatched tadpoles, we selectively inoculated the embryos of lab-raised hourglass treefrogs, *Dendropsophus ebraccatus*, with bacteria initially isolated from the skin of wild *D. ebraccatus* adults over two days. First, embryos were inoculated with two bacteria in alternating sequences. Next, we evaluated the outcomes of priority effects in an *in vitro* co-culture assay absent of host factors. We then performed a second embryo experiment, inoculating embryos with one of three bacteria on the first day and a community of five target bacteria on the second. Through 16S rRNA gene amplicon sequencing, we observed relative abundance shifts in tadpole bacteria communities due to priority effects. Our results suggest that the initial bacterial source pools of embryos shape

bacterial communities at later life stages, however the magnitude of those changes is dependent on the host environment and the identity of bacterial colonists.

Introduction:

Understanding the mechanisms that influence the assembly of ecological communities is a central goal of ecology [1]. Community assembly is generally governed by the processes of dispersal, drift, selection, and speciation [2]; however, the great diversity of species, environmental variables, scales of study, and stochastic events lead to idiosyncrasies within communities. Stochasticity in dispersal, for example, can result in differences in colonization order, causing otherwise equivalent habitats to develop differing community structures. These consequences of colonization order, commonly referred to as priority effects [3,4], are being explored in a variety of systems [5–9]. Priority effects are likely to be regulated by fitness differences between taxa (as defined in [10]) and the presence or absence of destabilizing, stabilizing, or equalizing forces [5,11]. Mechanistically, priority effects are broadly divided into two categories: niche preemption and niche modification [9,12]. Priority effects through niche preemption occur when early arriving species deplete resources, thus removing access to those resources for later arriving species. Priority effects through niche modification occur when early arriving species modify environmental conditions, therefore enhancing or hindering colonization by later arriving species [12].

Experimentally investigating priority effects is necessary for developing a more thorough understanding of how ecological communities assemble. In practice, however, experimentally manipulating the assembly of natural communities can be a challenge. Historically, these challenges have been alleviated by studying plant and microbial communities [13–15]. For example, niche preemption is a demonstrated mechanism of priority effects in both the exclusion of native

species by invasive plants [16,17] and between closely related fungal taxa [18]. In addition, niche modification can alter the success rates of later arriving colonists in both plant [19] and microbial communities [20,21].

Host-associated bacterial communities are nearly ubiquitous among plant and animal hosts. These symbiotic bacteria have multiple effects on their hosts, including playing key roles in development and host defense (e.g. [22–24]). Experimental investigations of priority effects in the microbiome are rare in vertebrate systems, and tend to focus on inoculations of germ-free mice [25–27]. Amphibian embryos, which often develop externally, provide a good system for understanding the initial assembly of host-associated bacterial communities. The skin microbiome of adult frogs is well-studied due to its role in mitigating host susceptibility to disease [28–31] and many of the dominant taxa are readily culturable [32,33]. These skin communities also differ among species [34,35] and display intraspecific variation based on geographic location [33] and life stage [32,36]. This intraspecific variation, and prior studies [33,37–40], led us to hypothesize that amphibian microbiomes are strongly influenced by environmental source pools, rather than having a deterministic final composition, and thus, priority effects are likely important during establishment of these communities.

In the current study, we experimentally investigated the role of priority effects during frog embryonic development on the structure of bacterial communities on newly-hatched tadpoles. First, we inoculated the embryos of lab-raised frogs with two bacterial isolates in alternating order and examined whether differences in colonization order would impact the composition of bacterial communities after hatching, at the tadpole stage (Figure 1). We then used the same isolates in an *in vitro* co-culture assay to gain insight into potential outcomes from our inoculations in an environment free of host influence. Finally, to expand our results to a

broader community of relevant bacterial taxa, we completed a second inoculation experiment in which embryos were exposed to one of three isolates on the first day and a small synthetic community inoculum on the second day.

Methods:

Study Overview:

Dendropsophus ebraccatus is a small treefrog native to the Neotropics, including Panama. We conducted two inoculation experiments in this system, in June 2019 and September 2021. For both experiments, treefrog embryos were placed in individual wells of 12-well cell culture plates (Corning; volume per well=6.9 ml) and inoculated once a day for two days. Each treatment received a different two-day inoculation regimen. For our experiments, we focused on bacterial isolates in our culture collection (obtained from wild adults) that dominate the relative abundance of the adult *D. ebraccatus* skin community [41]. A reanalysis of our previously published field survey data [34] highlighted a potential inverse relationship between the relative abundances of *Stenotrophomonas* and *Acinetobacter* within the skin communities of adult *D. ebraccatus* (Appendix A: Figure S1), which could be driven by priority effects. This inverse pattern motivated the isolate choices for this experiment. For the first experiment, we selected our most abundant isolates within these two genera: *Acinetobacter* (C23M, hereafter isolate [A]) and *Stenotrophomonas* (C25H, [S]) [41]. For the second inoculation experiment, we expanded this list to include *Erwinia* (C32N, [E]), *Ochrobactrum* (C22A, [O]), and *Achromobacter* (C26N, [C]).

Frog Collection:

Eggs for both experiments were obtained from adult *D. ebraccatus* maintained in captivity since 2016 on the Vassar College campus (Fig 1A). We obtained eggs from two breeding pairs for the first experiment, and from one pair for the second experiment. Detailed methods for frog breeding and egg collection can be found in Appendix A. All animal work was approved by the Vassar College Institutional Animal Care and Use Committee (Protocols #19-03B and #18-12B).

Monoculture inoculum preparation:

We used frozen glycerol stocks from our isolate collection to inoculate R2A agar plates with individual isolates (Fig 1B). We then started 1% tryptone broth cultures for each isolate which were placed on a shaker to incubate overnight at room temperature. The next day, 500 µl of broth from each isolate was transferred to individual, sterile 1.5 ml microcentrifuge tubes and spun at 5,000 rpm for ten minutes to pellet down the bacteria. The broth solution was then removed and replaced with 500 µl of sterilized spring water. Tubes were then briefly vortexed to resuspend the pellets. This process was repeated once more to remove any remaining broth, and then, using a haemocytometer to count the cells, we created standardized inoculation solutions for each isolate at 10^6 cells/ml. We chose this density based on estimates of naturally-occurring freshwater bacteria [42,43]. Samples (500 µl) of each monoculture were collected for sequencing. All work was completed in a biosafety hood.

Community inoculum preparation:

To standardize the second inoculum across treatments, the community inoculum included all five chosen isolates. Broth cultures were started as outlined above for all five target isolates. We then added 100 µl of each of the five overnight isolate solutions to five 1.5 ml microcentrifuge tubes

(500 µl total/tube). We replaced the broth with sterilized spring water following the protocol for the individual isolate cultures. The five individual tubes were combined, vortexed, and diluted to 10^6 cells/ml to provide a community stock solution. Samples (500 µl) of each monoculture and the community inoculum were collected for sequencing.

Experiment 1:

In our first experiment (Fig 1C), we placed eggs on autoclaved window screen suspended over a container and rinsed them with sterilized spring water to reduce any bacteria present. Once rinsed, eggs were placed within individual wells in lidded, 12-well culture plates and inoculated with 3 ml of a treatment solution (N=15/treatment). Inoculation treatments were as follows: the same isolate on both days (SS or AA), a different isolate on each day (alternating priority: SA or AS), an isolate and then sterile spring water (SW), or a control treatment of sterile spring water on both days (WW). This procedure was continued until we had completed 15 replicates for each of the 6 treatments, totaling 8 plates. On the eighth plate, we dedicated two wells to our *Acinetobacter* and *Stenotrophomonas* inoculum without embryos to assess the ability of our isolates to persist in sterile spring water over the course of the experiment. Once all plates were prepared, they were placed in an environmental chamber at 26.6 °C with a 12 hour light schedule. After 24 hours, the plates were returned to the biosafety hood. All liquid was removed from each well and replaced with a fresh aliquot of the second designated inoculate. After completing this for all samples, the well-plates were returned to the incubator. Samples were checked daily to assess the condition of embryos; 48 hours after the second inoculation, tadpoles had begun to hatch. Upon hatching, tadpoles were individually removed from wells, rinsed in sterile spring water, and flash frozen in 1.5 ml microcentrifuge tubes. Samples were stored at -80 °C until processing. This process was repeated after an additional 24 hours, until all

viable samples had been removed (36 tadpoles out of 90 total embryos). Final sample sizes were 6 per treatment. Additionally, 20 µl of solution from each of the 6 embryo-free wells was plated onto individual R2A agar plates. Observed growth confirmed that the isolates remained viable in the sterile water solution for the duration of the experiment.

In vitro co-culture trials

To complement our initial tadpole experiment, we conducted co-culture trials with isolates A and S within a 96-well plate (Fig 1D). Because community interactions and the strength of priority effects can be affected by nutrient availability [44,45], we included two nutrient concentrations (Full LB broth or 1:20 diluted LB broth) to gain a broader understanding of our co-culture outcomes, especially as amphibian skin may represent a more nutrient poor environment than that of standard LB media. Treatments for co-culturing (N=5 per nutrient concentration) were as follows: A single isolate (A or S), equal initial inoculations of both isolates (A=S), uneven inoculations of both isolates (A_High or S_High), and an initial inoculation followed by a second inoculation of the other isolate after a six-hour delay, such as would occur with priority effects (AS, SA).

To set up the plate for co-culture trials, isolates S and A were initially grown on R2A plates and then single colonies were transferred to LB broth and left overnight. The next day, we vortexed the broth cultures and transferred 1 ml of each broth to individual, sterile 1.5 ml microcentrifuge tubes for inoculations. Each well of the 96-well plate was filled with 190 µl of LB broth or diluted LB broth. Single isolate wells (A, S) received 5 µl of the overnight broth culture of the target isolate. Wells that required both isolates (A=S) received 5 µl of each broth culture. Our uneven treatments (A_High, S_High) consisted of 7.5 µl of one isolate and 2.5 µl of

the other. For our treatment in which the secondary inoculation was delayed (AS, SA), the wells received 5 µl of the first inoculum and then 5 µl of the second six hours later.

After all wells were inoculated, the plate was covered and placed within a plate reader at 26 °C. Every 30 minutes, the plates were shaken within the plate reader for 30 seconds. After 6 hours, the plate was removed from the plate reader and then returned after the specified wells (AS, SA) received their second inoculum. After 24 hours had passed since the initial inoculation, the content of each well was pipetted into a 1.5 µl microcentrifuge tube and frozen prior to DNA extraction.

Experiment 2:

The set up for the second experiment was similar to experiment 1, with minor changes (Fig 1E). On day one, embryos (N=20/treatment) were inoculated with one of three isolates (A, S, or E). On the second day, the initial inoculum from all experimental wells was replaced with the community solution of all 5 isolates (A, S, E, O, C). Control wells received an aliquot of sterile water on both days. Additionally, two wells without embryos received an inoculum of the community inoculum to monitor compositional changes over the course of the experiment. On day four of the experiment, free-swimming tadpoles were collected as described for experiment 1. The remaining viable tadpoles (N=3) were collected on day five (Final N: S=20, A=15, E=17, Control=11).

Tadpole experiments DNA extraction, amplification, and sequencing:

For both experiments, DNA was extracted from whole body tadpole samples using the DNeasy blood and tissue extraction kit (Qiagen, Inc, Valencia, CA, USA). We followed the manufacturer's protocols, but included a 1 hr pretreatment with 180 µl of enzymatic lysis buffer

followed by a 30-minute incubation at 70 °C. At the beginning of the pretreatment, samples were mechanically ground for 5 s with a sterile pestle (Fisher Scientific, Waltham, MA). We also included DNA from pure cultures of each isolate for experiment 1, and from each isolate and community inoculum samples for experiment 2 in their respective sequencing runs. DNA from isolates and inoculum was extracted using the same methods as the tadpole samples, aside from the grinding process. For all samples, we targeted the V4 region of the 16S rRNA gene and amplified it using the 515F and barcoded 806R primer pair and PCR conditions developed by Caporaso et. al. [46]. Reactions for each sample were run in triplicate, combined after PCR, and quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA). 200 ng of DNA from each sample was used to create a pooled sample for each experiment. Pooled samples were cleaned using the QIAquick PCR purification kit according to manufacturer instructions (Qiagen, Inc., Valencia, CA, USA). The final elution was sent to the Molecular Biology Core Facilities of the Dana Farber Cancer Institute at Harvard University (Cambridge, MA, USA) to be sequenced on an Illumina MiSeq instrument using a 250 base-pair single-end strategy, with one run for each experiment. Raw sequence data are available at: https://doi.org/10.7294/19170056.

Co-culture DNA extraction, amplification, and sequencing:

Isolate co-culture samples were extracted using the DNeasy blood and tissue extraction kit (Qiagen, Inc, Valencia, CA, USA), following manufacturer's instructions. Amplicon libraries were prepared for sequencing on an Illumina iSeq 100 at Vassar College following Illumina's two-step, tailed PCR approach. In brief, an initial PCR targeted the V4 region of the 16S rRNA gene using unbarcoded versions of the 515F and 806R primer pair with Illumina overhang adapter sequences appended, and a second PCR added Illumina index sequences and sequencing

adapters from the Nextera Index kit (Detailed in Appendix A). 75 ng of DNA from each sample was used to create a pooled sample. The pooled sample was cleaned using AMPure XP beads according to manufacturer's instructions, diluted to a sequencing concentration of 75 pM, spiked with 10% PhiX, and sequenced using with a 150 bp single-end strategy. Raw sequence data are available at https://doi.org/10.7294/19170056

We used the Qiime2 pipeline [47] to process our sequence data, including demultiplexing, denoising, and dereplication. Each experimental dataset was processed separately. We used Dada2 [48] to denoise our sequences, with our quality score parameter set to 11. We filtered out any reads with a frequency of less than 0.01% across all samples. We used scikit-learn [49,50] to apply taxonomy to our representative sequences based on the SILVA taxonomic classifier [51]. Tree construction was performed using RAxML [52] and the tree was rooted at the midpoint. Our final table for the first tadpole experiment contained a total of 364 unique amplicon sequence variants (ASVs) across our 41 samples. The final table for the second experiment contained 183 unique ASVs across 85 samples. Tables were imported into the R v4.1.0 environment [53] for further processing. In R, we filtered out any ASVs that were not bacteria, any ASVs identified as chloroplasts or mitochondria, and any ASVs unidentifiable at the phylum level of classification. Many of the ASVs that were filtered out, when run through NCBI BLAST, were frog mitochondrial sequences.

We identified the corresponding ASVs for our isolates through analysis of our isolate-only amplicon samples. For the first experiment, ASV 91 (Genus *Stenotrophomonas*) was identified as isolate S and ASV 155 (Genus *Acinetobacter*) was identified as isolate A. For the second experiment, ASVs 23 and 24 (Genus *Stenotrophomonas)* corresponded to isolate S, ASV

25 (*Acinetobacter)* to isolate A, ASV 31 (*Erwinia)* to isolate E, ASV116 (*Ochrobactrum)* to isolate O, and ASV67 (*Achromobacter)* to isolate C.

Of note within this study is the expected presence of background taxa. Embryo-associated bacteria may originate in the cloaca of the parents, as in other systems [54]. Because our eggs were not exposed to antibiotics and were only rinsed with sterile water, we expected to see some level of non-target bacteria within our results. While we cannot eliminate the possibility of contamination, the absence of any clear growth of other bacteria from isolates cultured prior to the experiment, or from wells cultured after the experiment, supports that contamination was unlikely. To address this, we focus our primary analyses on differences among treatments that would demonstrate the presence of priority effects, regardless of this background.

Our co-culture samples were processed using the same Qiime2 pipeline[47] outlined above. Our final table contained 23 unique ASVs. Of these ASVs, six were unidentified at the Kingdom level and filtered out. The remaining 17 ASVs all corresponded to either *Stenotrophomonas* or *Acinetobacter* and were clustered at the genus level for analysis.

Statistical Analyses:

All statistical analyses were conducted in the R v4.1.0 environment [53]. For our tadpole beta diversity analyses, we converted raw count data from our ASV tables to relative abundances as a method of normalization. ASV tables rarified to the minimum per-experiment sequencing depth were used for all tadpole alpha diversity analyses. To create a complete picture of differences in alpha diversity, we used three metrics: richness (the number of ASVs per sample), Faith's phylogenetic diversity, and the effective number of species (Hill numbers, derived from the Shannon diversity index). Both species richness and Shannon diversity were calculated using

phyloseq (v.1.28.0; [ref 38]). To calculate the effective number of species, we used the equation [exp(Shannon diversity)] [56]. Faith's phylogenetic diversity values were calculated using the picante package (v1.8) [57]. Our two inoculation experiment datasets were analyzed separately.

To determine whether we could detect the presence of priority effects within our tadpole bacterial communities (Experiment 1), we first investigated differences in the relative abundances of our isolates between our SA and AS treatments. We used a linear model fitted with a normal distribution to determine if our two isolates differed in relative abundance between treatments. To ascertain the presence of community level differences between our SA and AS treatments, we first performed a PERMANOVA analysis [function adonis2; vegan package v2.5-5 [58]] based on 10000 permutations using a Bray-Curtis matrix based on ASV relative abundances. To determine if our two treatment groups differed in dispersion, we conducted a beta dispersion analysis using the betadisper function [vegan package v2.5-5 [58]]. Next, we performed a distance-based redundancy analysis (DB-RDA; function capscale; vegan package) to determine which ASVs were the strongest contributors to the observed differences in beta diversity among our SA and AS treatments. Model selection for our DB-RDA was conducted through forward and reverse stepwise methods (function ordistep; vegan package).

To test for overall effects of our bacterial inoculation treatments on the microbiome of our tadpoles, we assessed differences in alpha diversity and beta diversity among our treatments and between treatments and the control group. For ASV richness, phylogenetic diversity and the effective number of species, linear models were fit using negative binomial, normal, and lognormal distributions, respectively. As above, we used a linear model fitted with a normal distribution to determine if our two isolates differed in relative abundance among all treatment groups. Differences in community structure (beta diversity) were analyzed using PERMANOVA

as above. We used Bray-Curtis dissimilarity matrices to assess community level differences in the relative abundances of ASVs.

To detect priority effects in the co-culture trials (a non-host environment), we assessed differences in the relative abundances between our AS and SA treatments. Next, we sought to determine if differing initial abundances (A_High, S_High, A=S) influenced final relative abundance. In addition, to understand the potential role of nutrient concentrations, we assessed each of these scenarios under a high and low nutrient concentration. Differences in relative abundance were analyzed using linear models fitted with lognormal distributions.

To determine if giving a bacterial colonist priority influenced the ultimate bacterial community of tadpoles when followed by a community inoculum (Experiment 2), we investigated differences in the community composition among our experimental treatments (A/S/E). To this end, we performed a PERMANOVA as above. Next, we used our rarified table to perform pairwise Wilcoxon tests to assess differences in relative abundances of our isolates between treatments.

We determined overall effects of our inoculation treatments on the tadpole microbiome by assessing differences in alpha and beta diversity among our treatments and the control group. We performed a PERMANOVA as above, this time including the control group. Alpha diversity values for our samples were calculated as above. We then ran linear models using a negative binomial distribution for ASV richness and lognormal distributions for Faith's phylogenetic diversity and the effective number of species. In addition, we calculated the mean percentage of our community isolates present among our treatments to understand how community composition was influenced by our inoculum.

Results:

Experiment 1

A total of 157 unique ASVs were observed within our newly-hatched tadpoles. Individual tadpoles had a range of 18-55 unique ASVs. Samples were dominated by ASVs from the phylum Proteobacteria (88 total ASVs, 88% average relative abundance). Two other Phyla averaged over 1% of the total relative abundance: Phyla Bacteroidetes (25 total ASVs, 6% average relative abundance) and Firmicutes (20 total ASVs, 5% average relative abundance). The remaining three phyla averaged below 1%: Actinobacteria (18 total ASVs), Patescibacteria (4 total ASVs), and Chlamydiae (2 total ASVs). Of the 157 unique ASVs, we observed five taxa as the core community that were present in 90% of our tadpole samples. These core taxa were from four genera: *Clostridium sensu stricto 9* (ASV33 and ASV34), *Vogesella (*ASV116*), Pseudomonas* (ASV144), and *Acinetobacter* (Isolate A [ASV155]).

Focusing on our AS and SA treatments, colonization order impacted the structure of the experimental communities. Treatment AS, in which *Acinetobacter* was inoculated first, became enriched in *Acinetobacter* (b=0. 580, SE=0.143, t=2.25, p=0.0479) relative to treatment SA (Fig 2A). This increase in relative abundance due to priority in colonization was not observed for *Stenotrophomonas* (b=-0.0298, SE=1.16, t=-0.094, p=0.927) (Fig 2A). Treatments SA and AS also differed from one another in terms of community structure (PERMANOVA: Bray-Curtis Pseudo-F=3.09, R^2=0.236, p=0.00820) (Fig 2B). Our AS and SA treatments did not, however, differ in group dispersion (betadisper: p adj=0.172)

Several other taxa in the community also responded differently in our AS and SA treatments. Our DB-RDA model selection identified a set of five ASVs that most strongly

contributed to the differences in community structure revealed by our PERMANOVA analysis of the Bray-Curtis distance matrix. We were able to explain 88% of the total variation in relative abundances using these five identified ASVs (ASV155 [Isolate A], Genus *Acinetobacter*; ASV116, Genus *Vogsella*; ASV144, Genus *Pseudomonas*; ASV153, Genus *Acinetobacter*; ASV129, Genus *Aeromonas*) as our constrained variables. The first two axes of this ordination explained 66% of the total variation (CAP1=0.436, CAP2=0.221) (Fig 2C). Linear models showed that two of the identified non-inoculant taxa had significantly lower relative abundances in treatment AS compared to SA (ASV116, Genus *Vogsella*|b=-0.0530, SE=0.802, t=-2.60, p=0.0267; ASV144, Genus *Pseudomonas*|b=-0.0414, SE=0.786, t=-2.41, p=0.0370) and one taxon showed an increase in AS compared to SA (ASV129, Genus *Aeromonas* |b=1.13, SE=0.592, t=3.53, p=0.00543)(Fig 2D).

When looking across all treatment groups, ASV richness did not differ by treatment (Chisq=6.23, df=5, P =0.285) (Appendix A: Fig S2A). Phylogenetic diversity (Pr(>F)=0.820) and effective number of species (Pr(>F)=0.257) were also similar across treatments (Appendix A: Fig S2B & C). Compared to the control treatment WW, both treatments with A as the initial isolate (AA, AS) showed an increase in the relative abundance of isolate A (AA |b=0.908, SE=11.7, t=5.18, p=0.000112; AS|b=0.642, SE=11.7, t=3.66, p=0.00232) (Fig 2A). Isolate S did not show a similar increase when it was the first inoculum (SS|b=-0.00118, SE=0.0911, t=-0.276, p=0.786; SA|b=0.0133, SE=0.240, t=1.18, p=0.252; SW|b=0.00234, SE=0.122, t=0.410, p=0.686) (Figure 2A). Neither *Acinetobacter* (SA vs SS | b=-0.00156, SE=0.0486, t=-1.13, p=0.284), nor *Stenotrophomonas* (AS vs AA | b= 0.0199, SE=0.253, t=-0.655, p=0.527) showed an increase in relative abundance when used as the second inoculum compared to the mono-isolate treatments (Figure 2A) or to the control (AS vs WW | b=-0.000439, SE=0.896, t=-0.408,

p=0.692; SA vs WW | b=0.142, SE=111, t=0.452, p=0.661). Community structure varied among treatments (PERMANOVA: Bray-Curtis Pseudo-F=2.44, R2=0.289, p=0.0002) (Appendix A: Fig S3).

Co-culture trial

Among our co-culture samples, *Acinetobacter* (AS|b=3.55 SE= 0.119, t=11.1, p=3.95e-6) and *Stenotrophomonas* (SA|b=1.48, SE=0.0303, t=18.1, p=8.82e-8) increased in relative abundance when given colonization priority in our high nutrient LB broth environment, but not within the low nutrient environment (Fig 4). In the high nutrient environment, when compared to the equal treatment (A=S), increased initial abundance led to an increase in relative abundance for *Stenotrophomonas* (S_High|b=1.26, SE=0.0405, t=5.351, p=1.73e-4), but not *Acinetobacter* (A_High) (Fig 3). Also in the high nutrient environment, *Stenotrophomonas* had a higher relative abundance in the SA treatment than in the S_High treatment (b=1.06, SE=0.0310, t=4.54, p=0.0019), however no significant difference was detected between the *Acinetobacter* favored treatments (AS and A_High) (Fig 3). Neither isolate showed a relative abundance increase between treatments in the low nutrient environment.

Experiment 2

Within our newly hatched tadpoles, we observed a total of 117 unique ASVs. Individual tadpoles possessed a range of 30-78 unique ASVs. Within these samples, only three phyla reached abundances higher than one percent. ASVs from the community inoculation made up 8.65% of the control, 63.0% of the *Stenotrophomonas*, 71.3% of the *Acinetobacter*, and 59.8% of the *Erwinia* treatments.

Changing the initial inoculum resulted in differing community structure across treatments (Fig 4A) within our tadpoles. When *Acinetobacter* was the first inoculum, tadpoles became enriched in *Acinetobacter* compared to treatments in which *Stenotrophomonas* or *Erwinia* served as the first inoculum (pairwise.wilcox.test |A/E: p=0.015; A/S: p=0.0093) (Fig 4B). *Erwinia* similarly increased in relative abundance compared to the other two treatments when given priority (pairwise.wilcox.test |E/A: p=0.0067; E/S p=0.0078) (Fig 4C). *Stenotrophomonas* only showed an increase in abundance when given priority compared to the treatment in which *Erwinia* was given priority (pairwise.wilcox.test |S/E: p=0.037, S/A: p=0.28) (Fig 4D). Neither of the two isolates present only in the community inoculation (*Ochrobactrum* and *Achromobacter*) saw an increase across experimental treatments, however all isolates saw an increase in relative abundance compared to the control (pairwise.wilcox.test| $p<0.005$) (Fig 4). We also observed a significant effect of treatment on overall community composition among experimentally treated tadpoles both with the control group included (PERMANOVA Bray-Curtis| Pseudo-F=8.54, $R^2=0.303$, $p<0.0005$) and without it (PERMANOVA Bray-Curtis| Pseudo-F=2.48, $R^2=0.092$, p=0.0016) (Fig 5).

Compared to our control, alpha diversity generally differed in our experimental treatments. ASV richness was significantly lower in our *Acinetobacter* and *Erwinia* treatments than in our control (Con/A: $p<0.0005$; Con/E: p=0.000355) (Fig 6A). Interestingly, our *Stenotrophomonas* treatment did not differ significantly from our control treatment (Con/S: p=0.053) (Fig 6A) but was significantly higher than the treatment in which *Acinetobacter* was inoculated first (emmeans| S/A: p=0.039) (Fig 6A). Faith's phylogenetic diversity was also significantly lower in our *Acinetobacter* and *Erwinia* treatments (Con/A: p=0.000499; Con/E: p=0.00173) (Fig 6B) compared to the control treatment, but not in our *Stenotrophomonas*

treatment (Con/S: p=0.110) (Fig 6B). The effective number of species was significantly lower in all treatments compared to the control (Con/A: p<0.0005; Con/E: p<0.0005; Con/S: p<0.0005) (Fig 6C). Neither phylogenetic diversity, nor the effective number of species, differed among the three experimental treatments.

Discussion:

Our results demonstrate that priority effects can be observed within the vertebrate host-associated microbiome. Altering the order in which frog embryos were inoculated with bacteria led to differences in the subsequent bacterial communities of tadpoles under two experimental conditions. Importantly, in our experiments, embryos remained exposed to their second inoculum until they developed into free-swimming tadpoles. Embryos were, therefore, exposed to the second inoculum for a longer period than the initial inoculum. Despite this, priority effects were still observed regardless of the second inoculum being a single isolate (experiment 1) or a broader community (experiment 2), suggesting that colonization order had a greater impact on community composition than exposure time during the early stages of development.

In experiment 1, we saw an increase in the relative abundance of our *Acinetobacter* isolate when it was introduced first, but not our *Stenotrophomonas* isolate. These results were consistent in the second experiment when only looking at the A and S priority treatments. When comparing the S and E priority treatments in experiment 2, however, the relative abundance of *Stenotrophomonas* increased when it was given priority. Essentially, the host environment created when *Acinetobacter* is given priority does not alter *Stenotrophomonas* relative abundance compared to when *Stenotrophomonas* is given priority. On the other hand, *Erwinia* created an

environment in which the proportion of our *Stenotrophomonas* isolate was comparatively limited. Our results, therefore, demonstrate that priority effects may benefit the initial isolate but interspecific competition among subsequent colonists may still allow dominant species to reach appreciable levels of abundance within the remaining niche space.

We also saw evidence of priority effects in our co-culture trials. When given colonization priority, both *Acinetobacter* and *Stenotrophomonas* increased in relative abundance within our higher nutrient broth environment. While increased relative growth for *Stenotrophomonas* was observed within our co-cultures, we did not observe this in the first embryo inoculation experiment. Our culture conditions differed from that of the embryo experiment due to resource differences, potential host regulatory mechanisms, and the presence of non-target bacteria that may have originated from inside the embryo. When given a proportional advantage without priority access, *Stenotrophomonas*, but not *Acinetobacter*, increased in relative abundance. Additionally, *Acinetobacter* showed no shift in relative abundance due to priority in the low nutrient environment. The observed priority effects of *Acinetobacter* may be dependent on having priority access to resources during some initial growth window (niche preemption), while *Stenotrophomonas* can outcompete *Acinetobacter* due to a higher growth rate or carrying capacity. This is further supported by the relative abundance of *Stenotrophomonas* remaining statistically similar in our A and S treatments within our second experiment but differing in the E and S treatments. While we lean towards niche preemption as the best classification for the observed relative abundance outcomes [9,12], we cannot rule out niche modification, nor the scenario that resource availability influences the ability of *Acinetobacter* to modify its environment.

Between our alternating embryo treatments (SA/AS) within the first experiment, we identified five taxa that explained the observed differences in the relative abundances of taxa across treatments. Our potential community drivers were ASVs belonging to the genera *Pseudomonas*, *Acinetobacter*, *Aeromonas*, and *Vogesella*. Among treatments in which our *Acinetobacter* isolate was the first inoculum and exhibited increased relative abundance, we saw lower relative abundances of the *Pseudomonas* ASV. We emphasize that the relative abundances of any taxa within a community are only a comparison to the relative abundances of other taxa. Therefore, we can only speculate about direct relationships between these taxa based on our current data. Previous research has, however, pointed to an interesting relationship between strains of *Acinetobacter* and *Pseudomonas*, in which *Acinetobacter* outcompetes, but then supports the growth of, *Pseudomonas* [59]. If such a relationship existed within our system, this could explain the increase of *Pseudomonas* in treatments that were comparatively lacking in *Acinetobacter*, as well as the persistence of *Pseudomonas* within communities dominated by *Acinetobacter*. Further experimentation would need to be conducted with isolated strains of these bacteria to understand the specific nature of these interactions in our system, especially given the wide range of niches that can be occupied by bacteria within the genus *Pseudomonas* [60,61].

Environmental source pools can influence the composition of host-associated bacterial communities [33,37,62], and may be especially critical during the initial assembly of microbes on vertebrate embryos. For instance, we know that adult *D. ebraccatus* skin bacterial community composition varies based on geographic location [34]. In addition, *D. ebraccatus* is a species capable of laying eggs terrestrially on leaves or within aquatic habitats [63]. Eggs that develop on leaves are presumably exposed to a different consortium of bacteria than those developing in an aquatic environment. These differing egg environments have previously been implicated in

survival outcomes [64], which may tie into variation in the composition of the associated microbiome. Though not directly comparable, the differences in the communities among treatments within the same experiment, as well as the differences in which genera were dominant between experiments, further supports the idea that environmental source pools have a large impact on the composition of host-associated bacterial communities. We cannot yet determine if these effects will persist through metamorphosis, however, our usage of isolates found on the skin of adult frogs suggests that these isolates may persist after metamorphosis. Overall, it is likely that a combination of priority effects and environmental source pools determine the resulting structure of the microbiome [25].

Results from both our co-culture trials and our embryo inoculation experiments suggest that our isolates are able to take advantage of colonization priority. Egg jelly is believed to lack the chemical defenses present on the skin of adult frogs [65]. Egg jelly should, therefore, exist as a relatively open niche for potential colonists [66], like the broth in our co-culture environment. As the embryo develops and hatches, however, our isolates are subject to increasing regulation by the host immune system [67–69]. Despite the activation of potential host regulatory mechanisms, we observed differences in community structure due to inoculations made early on in the embryo stage. By exposing the embryos to our isolates, we may have facilitated the modulation of host mucosal peptides, influencing the abundance of specific taxa [67,68,70] through niche modification. Future studies incorporating transcriptomics could elucidate more clearly the role of host regulation of the microbiome during development.

The present study joins a growing body of literature that seeks to understand the assembly of host-associated bacterial communities (e.g. [54,66,71–73]). Host-associated bacterial communities can be critical to host development [70,74,75], especially within certain developmental

windows [71,76,77]. We found that colonization order on embryos can noticeably impact later community composition, but that the magnitude of impact is dependent on characteristics of the colonizing taxa [78]. Rather than being ubiquitous, priority effects are likely the result of a combination of fitness differences among taxa (as defined by Chesson [10]) and the presence of stabilizing, destabilizing or equalizing forces [5,11]. The use of ecological theory [12,79] to determine the circumstances in which priority effects are likely to occur is critical to fully understanding the role of priority effects in community assembly. This framework, combined with experimental investigations at the community level, focused on either species (e.g. [71,80]) or strains [81], will inform research in a variety of host-associated systems.

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Competing Interests:

The authors declare no competing interests.

References:

(1) Zhou, J.; Ning, D. Stochastic Community Assembly: Does It Matter in Microbial Ecology? *Microbiology and Molecular Biology Reviews* **2017**, *81* (4). https://doi.org/10.1128/MMBR.00002-17.

(2) Vellend, M. Conceptual Synthesis in Community Ecology. *The Quarterly Review of Biology* **2010**, *85* (2), 183–206. https://doi.org/10.1086/652373.

(3) Drake, J. A. Communities as Assembled Structures: Do Rules Govern Pattern? *Trends in Ecology & Evolution* **1990**, *5* (5), 159–164. https://doi.org/10.1016/0169-5347(90)90223-Z.

(4) Drake, J. A. Community-Assembly Mechanics and the Structure of an Experimental Species Ensemble. *The American Naturalist* **1991**, *137* (1), 1–26. https://doi.org/10.1086/285143.

(5) Fukami, T.; Mordecai, E. A.; Ostling, A. A Framework for Priority Effects. *Journal of Vegetation Science* **2016**, *27* (4), 655–657. https://doi.org/10.1111/jvs.12434.

(6) Carlström, C. I.; Field, C. M.; Bortfeld-Miller, M.; Müller, B.; Sunagawa, S.; Vorholt, J. A. Synthetic Microbiota Reveal Priority Effects and Keystone Strains in the Arabidopsis Phyllosphere. *Nat Ecol Evol* **2019**, *3* (10), 1445–1454. https://doi.org/10.1038/s41559-019-0994-z.

(7) Hess, M. C. M.; Mesléard, F.; Buisson, E. Priority Effects: Emerging Principles for Invasive Plant Species Management. *Ecological Engineering* **2019**, *127*, 48–57. https://doi.org/10.1016/j.ecoleng.2018.11.011.

(8) Busse, A.; Schoreisz, J. J.; Petermann, J. S. Predators and Priority Effects Suggested as Potential Drivers of Microfauna Communities in a Community Transplantation Experiment along an Elevational Gradient. *Oikos* **2019**, *128* (7), 929–942. https://doi.org/10.1111/oik.05894.

(9) Debray, R.; Herbert, R. A.; Jaffe, A. L.; Crits-Christoph, A.; Power, M. E.; Koskella, B. Priority Effects in Microbiome Assembly. *Nat Rev Microbiol* **2021**. https://doi.org/10.1038/s41579-021-00604-w.

(10) Chesson, P. Mechanisms of Maintenance of Species Diversity. *Annual Review of Ecology and Systematics* **2000**, *31* (1), 343–366. https://doi.org/10.1146/annurev.ecolsys.31.1.343.

(11) Mordecai, E. A. Pathogen Impacts on Plant Communities: Unifying Theory, Concepts, and Empirical Work. *Ecological Monographs* **2011**, *81* (3), 429–441. https://doi.org/10.1890/10-2241.1.

(12) Fukami, T. Historical Contingency in Community Assembly: Integrating Niches, Species Pools, and Priority Effects. *Annual Review of Ecology, Evolution, and Systematics* **2015**, *46* (1), 1–23. https://doi.org/10.1146/annurev-ecolsys-110411-160340.

(13) Jessup, C. M.; Kassen, R.; Forde, S. E.; Kerr, B.; Buckling, A.; Rainey, P. B.; Bohannan, B. J. M. Big Questions, Small Worlds: Microbial Model Systems in Ecology. *Trends in Ecology & Evolution* **2004**, *19* (4), 189–197. https://doi.org/10.1016/j.tree.2004.01.008.

(14) Price, J. E.; Morin, P. J. Colonization History Determines Alternate Community States in a Food Web of Intraguild Predators. *Ecology* **2004**, *85* (4), 1017–1028. https://doi.org/10.1890/03-0157.

(15) Zhang, Q.-G.; Zhang, D.-Y. Colonization Sequence Influences Selection and Complementarity Effects on Biomass Production in Experimental Algal Microcosms. *Oikos* **2007**, *116* (10), 1748–1758. https://doi.org/10.1111/j.0030-1299.2007.15988.x.

(16) Dickson, T. L.; Hopwood, J. L.; Wilsey, B. J. Do Priority Effects Benefit Invasive Plants More than Native Plants? An Experiment with Six Grassland Species. *Biol Invasions* **2012**, *14* (12), 2617–2624. https://doi.org/10.1007/s10530-012-0257-2.

(17) Dawson, W.; Fischer, M.; van Kleunen, M. The Maximum Relative Growth Rate of Common UK Plant Species Is Positively Associated with Their Global Invasiveness: Growth Rates of Invasive Plants. *Global Ecology and Biogeography* **2011**, *20* (2), 299–306. https://doi.org/10.1111/j.1466-8238.2010.00599.x.

(18) Peay, K. G.; Belisle, M.; Fukami, T. Phylogenetic Relatedness Predicts Priority Effects in Nectar Yeast Communities. *Proc. R. Soc. B* **2012**, *279* (1729), 749–758. https://doi.org/10.1098/rspb.2011.1230.

(19) Corbin, J. D.; D'Antonio, C. M. Gone but Not Forgotten? Invasive Plants' Legacies on Community and Ecosystem Properties. *Invasive plant sci. manag.* **2012**, *5* (1), 117–124. https://doi.org/10.1614/IPSM-D-11-00005.1.

(20) Potnis, N.; Soto-Arias, J. P.; Cowles, K. N.; van Bruggen, A. H. C.; Jones, J. B.; Barak, J. D. Xanthomonas Perforans Colonization Influences Salmonella Enterica in the Tomato Phyllosphere. *Appl Environ Microbiol* **2014**, *80* (10), 3173–3180. https://doi.org/10.1128/AEM.00345-14.

(21) Chang, P. V. Chemical Mechanisms of Colonization Resistance by the Gut Microbial Metabolome. *ACS Chem Biol* **2020**, *15* (5), 1119–1126. https://doi.org/10.1021/acschembio.9b00813.

(22) Kohl, K. D.; Connelly, J. W.; Dearing, M. D.; Forbey, J. S. Microbial Detoxification in the Gut of a Specialist Avian Herbivore, the Greater Sage-Grouse. *FEMS Microbiol Lett* **2016**, *363* (14). https://doi.org/10.1093/femsle/fnw144.

(23) Sharma, V.; Smolin, J.; Nayak, J.; Ayala, J. E.; Scott, D. A.; Peterson, S. N.; Freeze, H. H. Mannose Alters Gut Microbiome, Prevents Diet-Induced Obesity, and Improves Host Metabolism. *Cell Reports* **2018**, *24* (12), 3087–3098. https://doi.org/10.1016/j.celrep.2018.08.064.

(24) Harris, R. N.; Brucker, R. M.; Walke, J. B.; Becker, M. H.; Schwantes, C. R.; Flaherty, D. C.; Lam, B. A.; Woodhams, D. C.; Briggs, C. J.; Vredenburg, V. T.; Minbiole, K. P. C. Skin Microbes on Frogs Prevent Morbidity and Mortality Caused by a Lethal Skin Fungus. *The ISME Journal* **2009**, *3* (7), 818–824. https://doi.org/10.1038/ismej.2009.27.

(25) Sprockett, D.; Fukami, T.; Relman, D. A. Role of Priority Effects in the Early-Life Assembly of the Gut Microbiota. *Nature Reviews Gastroenterology & Hepatology* **2018**, *15* (4), 197–205. https://doi.org/10.1038/nrgastro.2017.173.

(26) Martínez, I.; Maldonado-Gomez, M. X.; Gomes-Neto, J. C.; Kittana, H.; Ding, H.; Schmaltz, R.; Joglekar, P.; Cardona, R. J.; Marsteller, N. L.; Kembel, S. W.; Benson, A. K.; Peterson, D. A.; Ramer-Tait, A. E.; Walter, J. Experimental Evaluation of the Importance of Colonization History in Early-Life Gut Microbiota Assembly. *Elife* **2018**, *7*. https://doi.org/10.7554/eLife.36521.

(27) Lee, S. M.; Donaldson, G. P.; Mikulski, Z.; Boyajian, S.; Ley, K.; Mazmanian, S. K. Bacterial Colonization Factors Control Specificity and Stability of the Gut Microbiota. *Nature* **2013**, *501* (7467), 426–429. https://doi.org/10.1038/nature12447.

(28) Muletz, C. R.; Myers, J. M.; Domangue, R. J.; Herrick, J. B.; Harris, R. N. Soil Bioaugmentation with Amphibian Cutaneous Bacteria Protects Amphibian Hosts from Infection by Batrachochytrium Dendrobatidis. *Biological Conservation* **2012**, *152*, 119–126. https://doi.org/10.1016/j.biocon.2012.03.022.

(29) Woodhams, D. C.; LaBumbard, B. C.; Barnhart, K. L.; Becker, M. H.; Bletz, M. C.; Escobar, L. A.; Flechas, S. V.; Forman, M. E.; Iannetta, A. A.; Joyce, M. D.; Rabemananjara, F.; Gratwicke, B.; Vences, M.; Minbiole, K. P. C. Prodigiosin, Violacein, and Volatile Organic Compounds Produced by Widespread Cutaneous Bacteria of Amphibians Can Inhibit Two Batrachochytrium Fungal Pathogens. *Microb Ecol* **2018**, *75* (4), 1049–1062. https://doi.org/10.1007/s00248-017-1095-7.

(30) Knutie, S. A.; Wilkinson, C. L.; Kohl, K. D.; Rohr, J. R. Early-Life Disruption of Amphibian Microbiota Decreases Later-Life Resistance to Parasites. *Nature Communications* **2017**, *8* (1), 86. https://doi.org/10.1038/s41467-017-00119-0.

(31) Harrison, X. A.; Price, S. J.; Hopkins, K.; Leung, W. T. M.; Sergeant, C.; Garner, T. W. J. Diversity-Stability Dynamics of the Amphibian Skin Microbiome and Susceptibility to a Lethal Viral Pathogen. *Front. Microbiol.* **2019**, *10*. https://doi.org/10.3389/fmicb.2019.02883.

(32) Kueneman, J. G.; Laura Wegener Parfrey; Woodhams, D. C.; Archer, H. M.; Knight, R.; McKenzie, V. J. The Amphibian Skin-Associated Microbiome across Species, Space and Life History Stages. *Molecular Ecology* **2014**, *23* (6), 1238–1250. https://doi.org/10.1111/mec.12510.

(33) Kueneman, J. G.; Bletz, M. C.; McKenzie, V. J.; Becker, C. G.; Joseph, M. B.; Abarca, J. G.; Archer, H.; Arellano, A. L.; Bataille, A.; Becker, M.; Belden, L. K.; Crottini, A.; Geffers, R.; Haddad, Célio. F. B.; Harris, R. N.; Holden, W. M.; Hughey, M.; Jarek, M.; Kearns, P. J.; Kerby, J. L.; Kielgast, J.; Kurabayashi, A.; Longo, A. V.; Loudon, A.; Medina, D.; Nuñez, J. J.; Perl, R. G. B.; Pinto-Tomás, A.; Rabemananjara, F. C. E.; Rebollar, E. A.; Rodríguez, A.; Rollins-Smith, L.; Stevenson, R.; Tebbe, C. C.; Vargas Asensio, G.; Waldman, B.; Walke, J. B.; Whitfield, S. M.; Zamudio, K. R.; Zúñiga Chaves, I.; Woodhams, D. C.; Vences, M. Community Richness of Amphibian Skin Bacteria Correlates with Bioclimate at the Global Scale. *Nature Ecology & Evolution* **2019**, *3* (3), 381–389. https://doi.org/10.1038/s41559-019-0798-1.

(34) Belden, L. K.; Hughey, M. C.; Rebollar, E. A.; Umile, T. P.; Loftus, S. C.; Burzynski, E. A.; Minbiole, K. P. C.; House, L. L.; Jensen, R. V.; Becker, M. H.; Walke, J. B.; Medina, D.; Ibáñez, R.; Harris, R. N. Panamanian Frog Species Host Unique Skin Bacterial Communities. *Front Microbiol* **2015**, *6*. https://doi.org/10.3389/fmicb.2015.01171.

(35) McKenzie, V. J.; Bowers, R. M.; Fierer, N.; Knight, R.; Lauber, C. L. Co-Habiting Amphibian Species Harbor Unique Skin Bacterial Communities in Wild Populations. *The ISME Journal* **2012**, *6* (3), 588–596. https://doi.org/10.1038/ismej.2011.129.

(36) Sanchez, E.; Bletz, M. C.; Duntsch, L.; Bhuju, S.; Geffers, R.; Jarek, M.; Dohrmann, A. B.; Tebbe, C. C.; Steinfartz, S.; Vences, M. Cutaneous Bacterial Communities of a Poisonous Salamander: A Perspective from Life Stages, Body Parts and Environmental Conditions. *Microbial Ecology* **2017**, *73* (2), 455–465. https://doi.org/10.1007/s00248-016-0863-0.

(37) Estrada, A.; Hughey, M. C.; Medina, D.; Rebollar, E. A.; Walke, J. B.; Harris, R. N.; Belden, L. K. Skin Bacterial Communities of Neotropical Treefrogs Vary with Local Environmental Conditions at the Time of Sampling. *PeerJ* **2019**, *7*, e7044. https://doi.org/10.7717/peerj.7044.

(38) Jani, A. J.; Briggs, C. J. Host and Aquatic Environment Shape the Amphibian Skin Microbiome but Effects on Downstream Resistance to the Pathogen Batrachochytrium Dendrobatidis Are Variable. *Front. Microbiol.* **2018**, *9*. https://doi.org/10.3389/fmicb.2018.00487.

(39) Walke, J. B.; Becker, M. H.; Krinos, A.; Chang, E. A. B.; Santiago, C.; Umile, T. P.; Minbiole, K. P. C.; Belden, L. K. Seasonal Changes and the Unexpected Impact of Environmental Disturbance on Skin Bacteria of Individual Amphibians in a Natural Habitat. *FEMS Microbiology Ecology* **2021**, *97* (fiaa248). https://doi.org/10.1093/femsec/fiaa248.

(40) Hughey, M. C.; Pena, J. A.; Reyes, R.; Medina, D.; Belden, L. K.; Burrowes, P. A. Skin Bacterial Microbiome of a Generalist Puerto Rican Frog Varies along Elevation and Land Use Gradients. *PeerJ* **2017**, *5*, e3688. https://doi.org/10.7717/peerj.3688.

(41) Rebollar, E. A.; Bridges, T.; Hughey, M. C.; Medina, D.; Belden, L. K.; Harris, R. N. Integrating the Role of Antifungal Bacteria into Skin Symbiotic Communities of Three Neotropical Frog Species. *The ISME Journal* **2019**, *13* (7), 1763. https://doi.org/10.1038/s41396-019-0388-x.

(42) Simek, K.; Jürgens, K.; Nedoma, J.; Comerma, M.; Armengol, J. Ecological Role and Bacterial Grazing of Halteria Spp.: Small Freshwater Oligotrichs as Dominant Pelagic Ciliate Bacterivores. *Aquat. Microb. Ecol.* **2000**, *22*, 43–56. https://doi.org/10.3354/ame022043.

(43) Walker, E. D.; Merritt, R. W. Bacterial Enrichment in the Surface Microlayer of an Anopheles Quadrimaculatus (Diptera: Culicidae) Larval Habitat. *Journal of Medical Entomology* **1993**, *30* (6), 1050–1052. https://doi.org/10.1093/jmedent/30.6.1050.

(44) Pereira, F. C.; Berry, D. Microbial Nutrient Niches in the Gut: Microbial Nutrient Niches in the Gut. *Environmental Microbiology* **2017**, *19* (4), 1366–1378. https://doi.org/10.1111/1462-2920.13659.

(45) Kardol, P.; Souza, L.; Classen, A. T. Resource Availability Mediates the Importance of Priority Effects in Plant Community Assembly and Ecosystem Function. *Oikos* **2013**, *122* (1), 84–94. https://doi.org/10.1111/j.1600-0706.2012.20546.x.

(46) Caporaso, J. G.; Lauber, C. L.; Walters, W. A.; Berg-Lyons, D.; Huntley, J.; Fierer, N.; Owens, S. M.; Betley, J.; Fraser, L.; Bauer, M.; Gormley, N.; Gilbert, J. A.; Smith, G.; Knight, R. Ultra-High-Throughput Microbial Community Analysis on the Illumina HiSeq and MiSeq Platforms. *The ISME Journal* **2012**, *6* (8), 1621–1624. https://doi.org/10.1038/ismej.2012.8.

(47) Bolyen, E.; Rideout, J. R.; Dillon, M. R.; Bokulich, N. A.; Abnet, C. C.; Al-Ghalith, G. A.; Alexander, H.; Alm, E. J.; Arumugam, M.; Asnicar, F.; Bai, Y.; Bisanz, J. E.; Bittinger, K.; Brejnrod, A.; Brislawn, C. J.; Brown, C. T.; Callahan, B. J.; Caraballo-Rodríguez, A. M.; Chase, J.; Cope, E. K.; Da Silva, R.; Diener, C.; Dorrestein, P. C.; Douglas, G. M.; Durall, D. M.; Duvallet, C.; Edwardson, C. F.; Ernst, M.; Estaki, M.; Fouquier, J.; Gauglitz, J. M.; Gibbons, S. M.; Gibson, D. L.; Gonzalez, A.; Gorlick, K.; Guo, J.; Hillmann, B.; Holmes, S.; Holste, H.; Huttenhower, C.; Huttley, G. A.; Janssen, S.; Jarmusch, A. K.; Jiang, L.; Kaehler, B. D.; Kang, K. B.; Keefe, C. R.; Keim, P.; Kelley, S. T.; Knights, D.; Koester, I.; Kosciolek, T.; Kreps, J.; Langille, M. G. I.; Lee, J.; Ley, R.; Liu, Y.-X.; Loftfield, E.; Lozupone, C.; Maher, M.; Marotz, C.; Martin, B. D.; McDonald, D.; McIver, L. J.; Melnik, A. V.; Metcalf, J. L.; Morgan, S. C.; Morton, J. T.; Naimey, A. T.; Navas-Molina, J. A.; Nothias, L. F.; Orchanian, S. B.; Pearson, T.; Peoples, S. L.; Petras, D.; Preuss, M. L.; Pruesse, E.; Rasmussen, L. B.; Rivers, A.; Robeson, M. S.; Rosenthal, P.; Segata, N.; Shaffer, M.; Shiffer, A.; Sinha, R.; Song, S. J.; Spear, J. R.; Swafford, A. D.; Thompson, L. R.; Torres, P. J.; Trinh, P.; Tripathi, A.; Turnbaugh, P. J.; Ul-Hasan, S.; van der Hooft, J. J. J.; Vargas, F.; Vázquez-Baeza, Y.; Vogtmann, E.; von Hippel, M.; Walters, W.; Wan, Y.; Wang, M.; Warren, J.; Weber, K. C.; Williamson, C. H. D.; Willis, A. D.; Xu, Z. Z.; Zaneveld, J. R.; Zhang, Y.; Zhu, Q.; Knight, R.; Caporaso, J. G. Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2. *Nat Biotechnol* **2019**, *37* (8), 852–857. https://doi.org/10.1038/s41587-019-0209-9.

(48) Callahan, B. J.; McMurdie, P. J.; Rosen, M. J.; Han, A. W.; Johnson, A. J. A.; Holmes, S. P. DADA2: High-Resolution Sample Inference from Illumina Amplicon Data. *Nature Methods* **2016**, *13* (7), 581–583. https://doi.org/10.1038/nmeth.3869.

(49) Pedregosa, F.; Varoquaux, G.; Gramfort, A.; Michel, V.; Thirion, B.; Grisel, O.; Blondel, M.; Prettenhofer, P.; Weiss, R.; Dubourg, V.; Vanderplas, J.; Passos, A.; Cournapeau, D. Scikit-Learn: Machine Learning in Python. *MACHINE LEARNING IN PYTHON* **2011**, 6.

(50) Bokulich, N. A.; Kaehler, B. D.; Rideout, J. R.; Dillon, M.; Bolyen, E.; Knight, R.; Huttley, G. A.; Caporaso, J. G. Optimizing Taxonomic Classification of Marker-Gene Amplicon Sequences with QIIME 2's Q2-Feature-Classifier Plugin. *Microbiome* **2018**, *6* (1), 1–17. https://doi.org/10.1186/s40168-018-0470-z.

(51) Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glöckner, F. O. The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools. *Nucleic Acids Res* **2013**, *41* (D1), D590–D596. https://doi.org/10.1093/nar/gks1219.

(52) Stamatakis, A. RAxML Version 8: A Tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. *Bioinformatics* **2014**, *30* (9), 1312–1313. https://doi.org/10.1093/bioinformatics/btu033.

(53) R Core Team. R: A Language and Environment for Statistical Computing., 2021. https://www.R-project.org/.

(54) Lee, S.; La, T.-M.; Lee, H.-J.; Choi, I.-S.; Song, C.-S.; Park, S.-Y.; Lee, J.-B.; Lee, S.-W. Characterization of Microbial Communities in the Chicken Oviduct and the Origin of Chicken Embryo Gut Microbiota. *Sci Rep* **2019**, *9* (1), 6838. https://doi.org/10.1038/s41598-019-43280-w.

(55) McMurdie, P. J.; Holmes, S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE* **2013**, *8* (4), e61217. https://doi.org/10.1371/journal.pone.0061217.

(56) Jost, L. *Entropy and Diversity*. *Oikos* **2006**, *113* (2), 363–375. https://doi.org/10.1111/j.2006.0030-1299.14714.x.

(57) Kembel, S. W.; Cowan, P. D.; Helmus, M. R.; Cornwell, W. K.; Morlon, H.; Ackerly, D. D.; Blomberg, S. P.; Webb, C. O. Picante: R Tools for Integrating Phylogenies and Ecology. *Bioinformatics* **2010**, *26* (11), 1463–1464. https://doi.org/10.1093/bioinformatics/btq166.

(58) Dixon, P. VEGAN, a Package of R Functions for Community Ecology. *Journal of Vegetation Science* **2003**, *14* (6), 927–930. https://doi.org/10.1111/j.1654-1103.2003.tb02228.x.

(59) Christensen, B. B.; Haagensen, J. A. J.; Heydorn, A.; Molin, S. Metabolic Commensalism and Competition in a Two-Species Microbial Consortium. *AEM* **2002**, *68* (5), 2495–2502. https://doi.org/10.1128/AEM.68.5.2495-2502.2002.

(60) Özen, A. I.; Ussery, D. W. Defining the Pseudomonas Genus: Where Do We Draw the Line with Azotobacter? *Microb Ecol* **2012**, *63* (2), 239–248. https://doi.org/10.1007/s00248-011-9914-8.

(61) Gomila, M.; Peña, A.; Mulet, M.; Lalucat, J.; García-Valdés, E. Phylogenomics and Systematics in Pseudomonas. *Front. Microbiol.* **2015**, *6*. https://doi.org/10.3389/fmicb.2015.00214.

(62) Jones, K. R.; Walke, J. B.; Becker, M. H.; Belden, L. K.; Hughey, M. C. Time in the Laboratory, but Not Exposure to a Chytrid Fungus, Results in Rapid Change in Spring Peeper (Pseudacris Crucifer) Skin Bacterial Communities. *Ichthyology & Herpetology* **2021**, *109* (1). https://doi.org/10.1643/h2020077.

(63) Touchon, J. C.; Warkentin, K. M. Reproductive Mode Plasticity: Aquatic and Terrestrial Oviposition in a Treefrog. *Proceedings of the National Academy of Sciences* **2008**, *105* (21), 7495–7499. https://doi.org/10.1073/pnas.0711579105.

(64) Touchon, J. C.; Warkentin, K. M. Short- and Long-Term Effects of the Abiotic Egg Environment on Viability, Development and Vulnerability to Predators of a Neotropical Anuran: *Effects of Egg Environment through Metamorphosis*. *Functional Ecology* **2010**, *24* (3), 566–575. https://doi.org/10.1111/j.1365-2435.2009.01650.x.

(65) Walke, J. B.; Harris, R. N.; Reinert, L. K.; Rollins-Smith, L. A.; Woodhams, D. C. Social Immunity in Amphibians: Evidence for Vertical Transmission of Innate Defenses. *Biotropica* **2011**, *43* (4), 396–400. https://doi.org/10.1111/j.1744-7429.2011.00787.x.

(66) Hughey, M. C.; Delia, J.; Belden, L. K. Diversity and Stability of Egg-Bacterial Assemblages: The Role of Paternal Care in the Glassfrog Hyalinobatrachium Colymbiphyllum. *Biotropica* **2017**, *49* (6), 792–802. https://doi.org/10.1111/btp.12461.

(67) Woodhams, D. C.; Rollins-Smith, L. A.; Reinert, L. K.; Lam, B. A.; Harris, R. N.; Briggs, C. J.; Vredenburg, V. T.; Patel, B. T.; Caprioli, R. M.; Chaurand, P.; Hunziker, P.; Bigler, L. Probiotics Modulate a Novel Amphibian Skin Defense Peptide That Is Antifungal and Facilitates Growth of Antifungal Bacteria. *Microb Ecol* **2019**. https://doi.org/10.1007/s00248-019-01385-9.

(68) Küng, D.; Bigler, L.; Davis, L. R.; Gratwicke, B.; Griffith, E.; Woodhams, D. C. Stability of Microbiota Facilitated by Host Immune Regulation: Informing Probiotic Strategies to Manage Amphibian Disease. *PLoS ONE* **2014**, *9* (1), e87101. https://doi.org/10.1371/journal.pone.0087101.

(69) Rollins-Smith, L. A. Global Amphibian Declines, Disease, and the Ongoing Battle between Batrachochytrium Fungi and the Immune System. *Herpetologica* **2020**, *76* (2), 178–188. https://doi.org/10.1655/0018-0831-76.2.178.

(70) Fallet, M.; Montagnani, C.; Petton, B.; Dantan, L.; de Lorgeril, J.; Comarmond, S.; Chaparro, C.; Toulza, E.; Boitard, S.; Escoubas, J.-M.; Vergnes, A.; Le Grand, J.; Bulla, I.; Gueguen, Y.; Vidal-Dupiol, J.; Grunau, C.; Mitta, G.; Cosseau, C. Early Life Microbial Exposures Shape the Crassostrea Gigas Immune System for Lifelong and Intergenerational Disease Protection. *Microbiome* **2022**, *10* (1), 85. https://doi.org/10.1186/s40168-022-01280-5.

(71) Warne, R. W.; Kirschman, L.; Zeglin, L. Manipulation of Gut Microbiota during Critical Developmental Windows Affects Host Physiological Performance and Disease Susceptibility across Ontogeny. *Journal of Animal Ecology* **2019**, *88* (6), 845–856. https://doi.org/10.1111/1365-2656.12973.

(72) Prest, T. L.; Kimball, A. K.; Kueneman, J. G.; McKenzie, V. J. Host-Associated Bacterial Community Succession during Amphibian Development. *Molecular Ecology* **2018**, *27* (8), 1992–2006. https://doi.org/10.1111/mec.14507.

(73) Wilson, K. M.; Rodrigues, D. R.; Briggs, W. N.; Duff, A. F.; Chasser, K. M.; Bielke, L. R. Evaluation of the Impact of in Ovo Administered Bacteria on Microbiome of Chicks through 10 Days of Age. *Poultry Science* **2019**, *98* (11), 5949–5960. https://doi.org/10.3382/ps/pez388.

(74) Heijtz, R. D.; Wang, S.; Anuar, F.; Qian, Y.; Björkholm, B.; Samuelsson, A.; Hibberd, M. L.; Forssberg, H.; Pettersson, S. Normal Gut Microbiota Modulates Brain Development and Behavior. *PNAS* **2011**, *108* (7), 3047–3052. https://doi.org/10.1073/pnas.1010529108.

(75) Sommer, F.; Bäckhed, F. The Gut Microbiota — Masters of Host Development and Physiology. *Nature Reviews Microbiology* **2013**, *11* (4), 227–238. https://doi.org/10.1038/nrmicro2974.

(76) Arrieta, M.-C.; Stiemsma, L. T.; Dimitriu, P. A.; Thorson, L.; Russell, S.; Yurist-Doutsch, S.; Kuzeljevic, B.; Gold, M. J.; Britton, H. M.; Lefebvre, D. L.; Subbarao, P.; Mandhane, P.; Becker, A.; McNagny, K. M.; Sears, M. R.; Kollmann, T.; Investigators, the C. S.; Mohn, W. W.; Turvey, S. E.; Finlay, B. B. Early Infancy Microbial and Metabolic Alterations Affect Risk of Childhood Asthma. *Science Translational Medicine* **2015**, *7* (307), 307ra152-307ra152. https://doi.org/10.1126/scitranslmed.aab2271.

(77) Hild, B.; Dreier, M. S.; Oh, J. H.; McCulloch, J. A.; Badger, J. H.; Guo, J.; Thefaine, C. E.; Umarova, R.; Hall, K. D.; Gavrilova, O.; Rosshart, S. P.; Trinchieri, G.; Rehermann, B. Neonatal Exposure to a Wild-Derived Microbiome Protects Mice against Diet-Induced Obesity. *Nat Metab* **2021**, *3* (8), 1042–1057. https://doi.org/10.1038/s42255-021-00439-y.

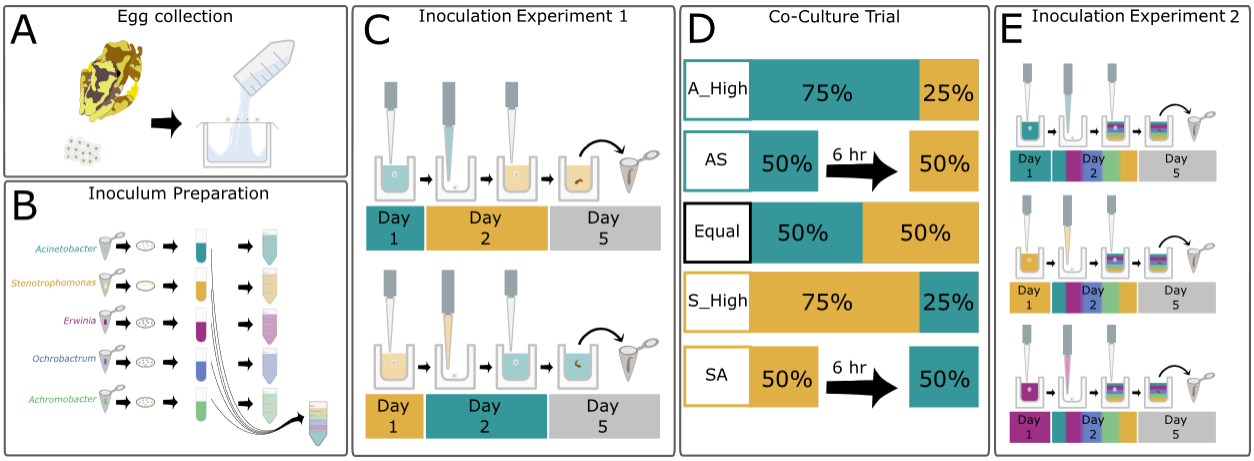
(78) Goodale, K. M.; Wilsey, B. J. Priority Effects Are Affected by Precipitation Variability and Are Stronger in Exotic than Native Grassland Species. *Plant Ecol* **2018**, *219* (4), 429–439. https://doi.org/10.1007/s11258-018-0806-6.

(79) Costello, E. K.; Stagaman, K.; Dethlefsen, L.; Bohannan, B. J. M.; Relman, D. A. The Application of Ecological Theory Toward an Understanding of the Human Microbiome. *Science* **2012**, *336* (6086), 1255–1262. https://doi.org/10.1126/science.1224203.

(80) Leopold, D. R.; Busby, P. E. Host Genotype and Colonist Arrival Order Jointly Govern Plant Microbiome Composition and Function. *Current Biology* **2020**, *30* (16), 3260-3266.e5. https://doi.org/10.1016/j.cub.2020.06.011.

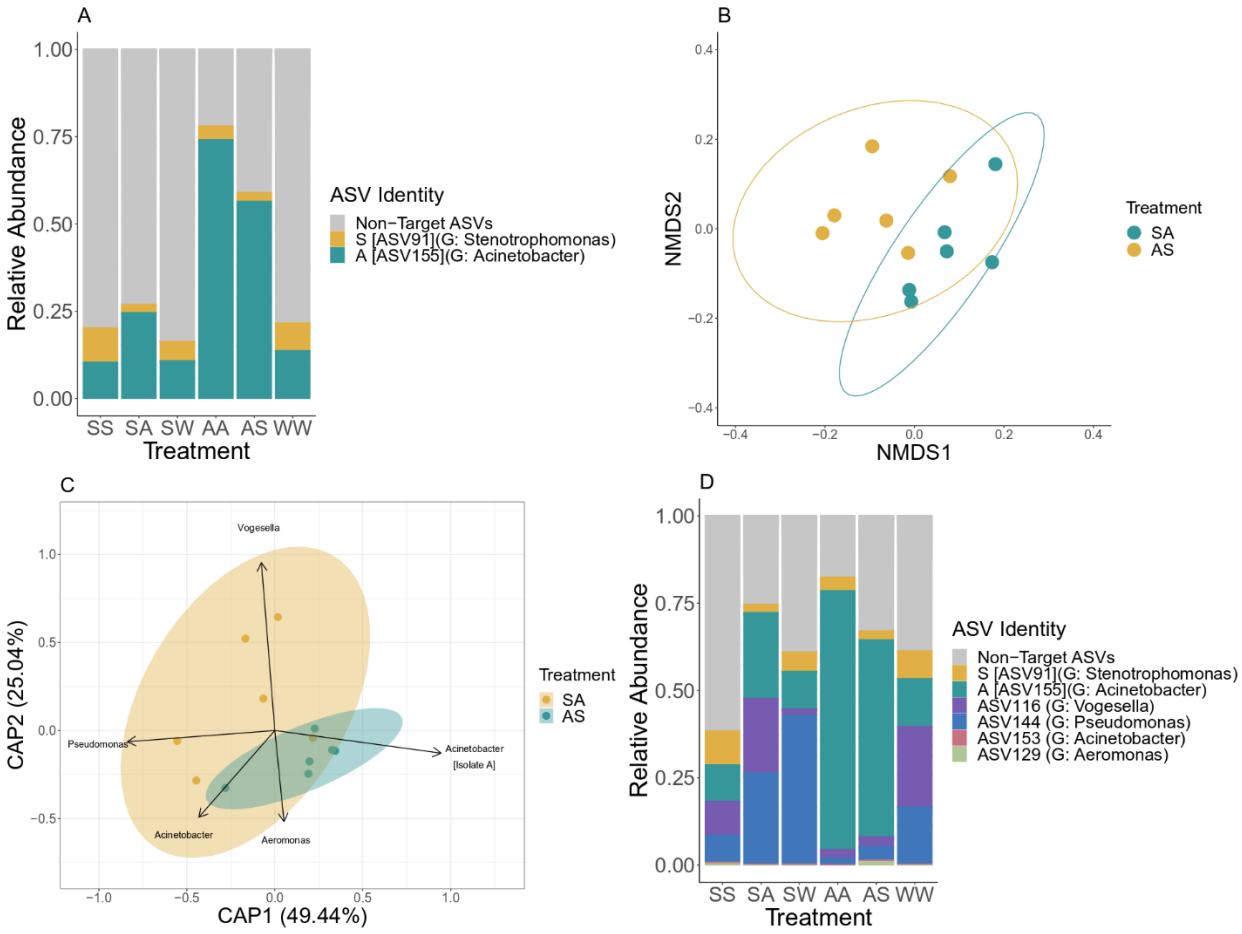
(81) Devevey, G.; Dang, T.; Graves, C. J.; Murray, S.; Brisson, D. First Arrived Takes All: Inhibitory Priority Effects Dominate Competition between Co-Infecting Borrelia Burgdorferi Strains. *BMC Microbiology* **2015**, *15* (1), 61. https://doi.org/10.1186/s12866-015-0381-0.

Figure 1



Conceptual diagram of experimental framework. **A)** Eggs were collected from breeding pairs left overnight and then rinsed with sterile water. **B)** Stock cultures of bacteria were plated onto R2A media. Overnight broth cultures were made from agar cultures. Final inoculation solutions were made the morning of egg collection. **C)** Inoculation Experiment 1: Eggs were placed into wells and inoculated sequentially over a two-day period. Eggs received their initial inoculation on day one. On day 2, liquid from the previous inoculation was removed and replaced with the second inoculation. Eggs remained in the second inoculum until they are free-swimming tadpoles, at which point they were collected, and the experiment was terminated. **D)** Co-culture Trial: All treatment groups included within our co-culture trial. Each of these experimental treatments was conducted under full and diluted broth conditions. **E)** Inoculation Experiment 2: Following the single isolate inoculation on day one, all liquid was removed and replaced with a synthetic community inoculum made of five culture solutions in equal volume.

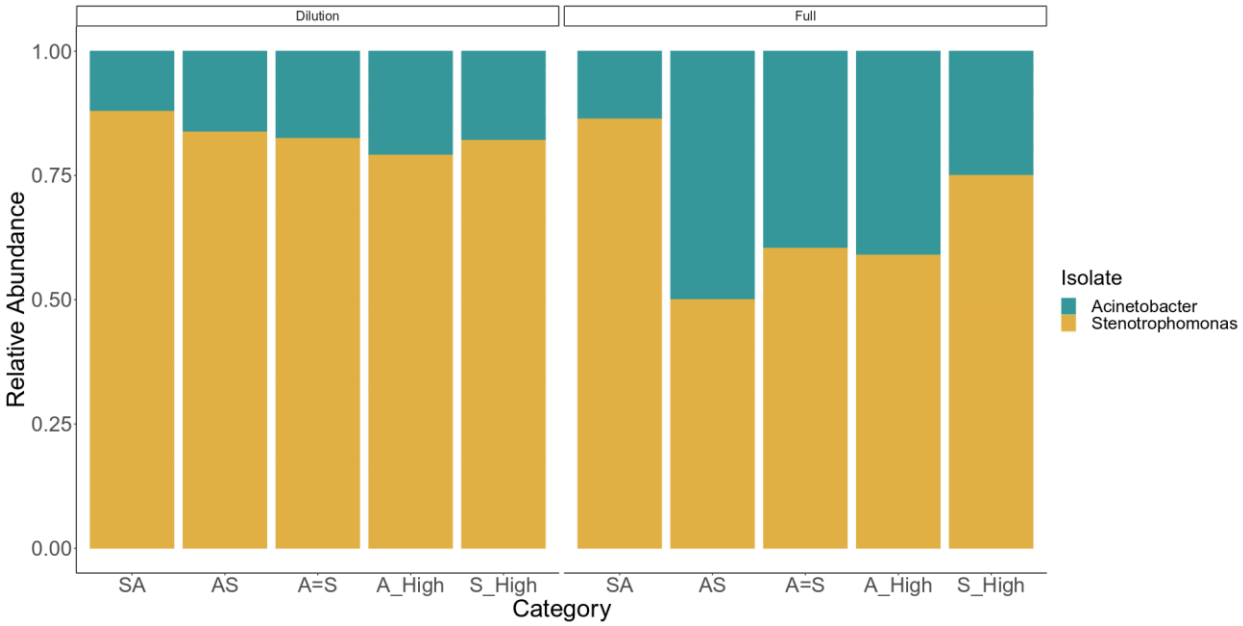
Figure 2



A) Relative abundance of target isolates from tadpole samples in test of priority effects (experiment 1). Treatments with isolate A (*Acinetobacter*) as the first inoculum showed a higher relative abundance of Isolate A than treatments that used isolate S (*Stenotrophomonas*) as the first inoculum and the control. This same increase in relative abundance due to being the first inoculum was not observed in the case of isolate S. **B)** NMDS Ordinations of whole-tadpole bacterial communities (experiment 1). Communities differed between treatments SA (*Stenotrophomonas* > *Acinetobacter*) and AS (*Acinetobacter* > *Stenotrophomonas*) in terms of relative abundance as measured through a Bray-Curtis dissimilarity matrix. **C)** Drivers of differences in community composition

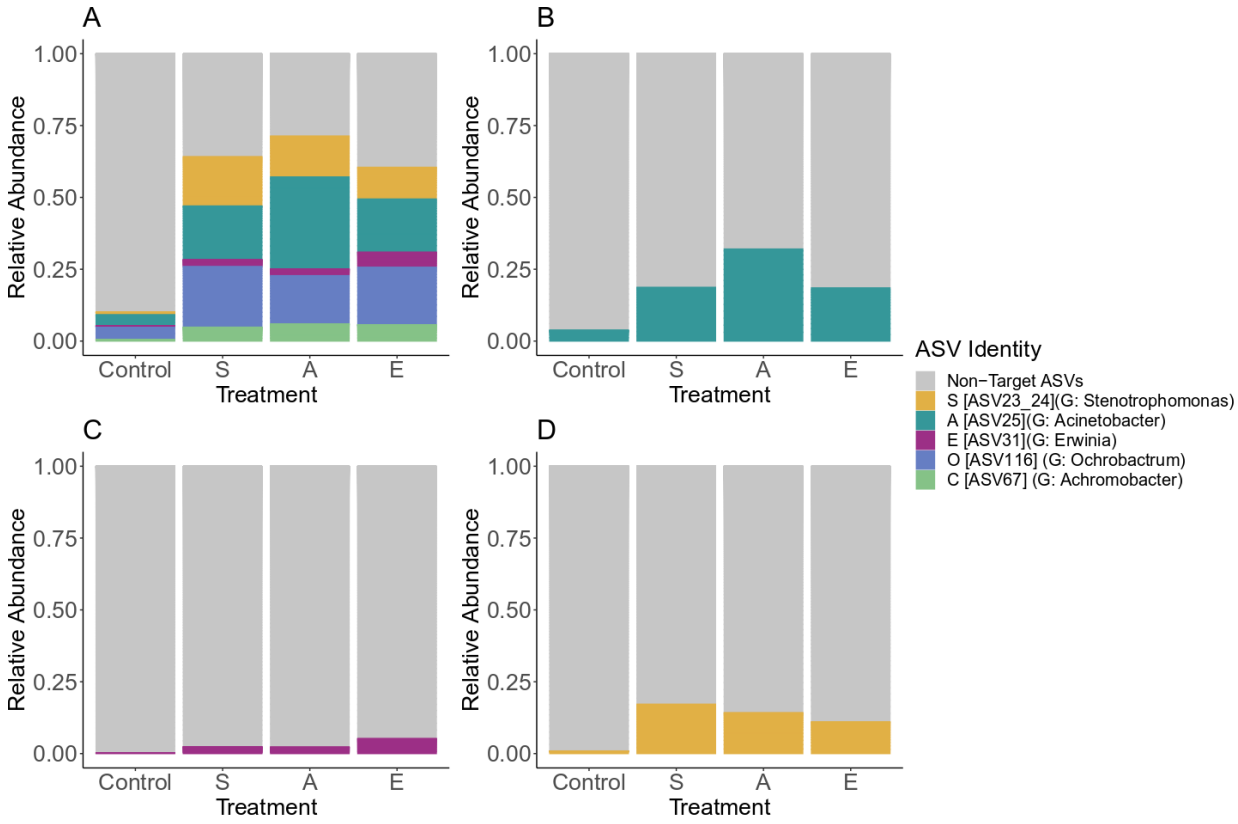
identified through a distance-based redundancy analysis using a Bray-Curtis dissimilarity matrix (experiment 1). The five taxa identified (Isolate A [ASV155], Genus *Acinetobacter*; ASV116, Genus *Vogesella*; ASV144, Genus *Pseudomonas*; ASV153, Genus *Acinetobacter*; ASV129, Genus *Aeromonas*) significantly explained differences in community structure among the AS (*Acinetobacter* > *Stenotrophomonas*) and SA (*Stenotrophomonas* > *Acinetobacter*) treatments. The percentage of the total variance explained by each axis is included in parentheses. Combined, these taxa explained 88% of the observed community variation in terms of the relative abundances of taxa. **D)** Relative abundances of the identified drivers of community composition combined with our two isolates (experiment 1). Both isolates are included in this figure, however, Isolate S was not identified as a driver of community composition during our analysis. ASV116 (Genus *Vogesella*), ASV144 (Genus *Pseudomonas*), and ASV129 (Genus *Aeromonas*) showed differential abundance between the AS (*Acinetobacter* > *Stenotrophomonas*) and SA (*Stenotrophomonas* > *Acinetobacter*) treatments.

Figure 3



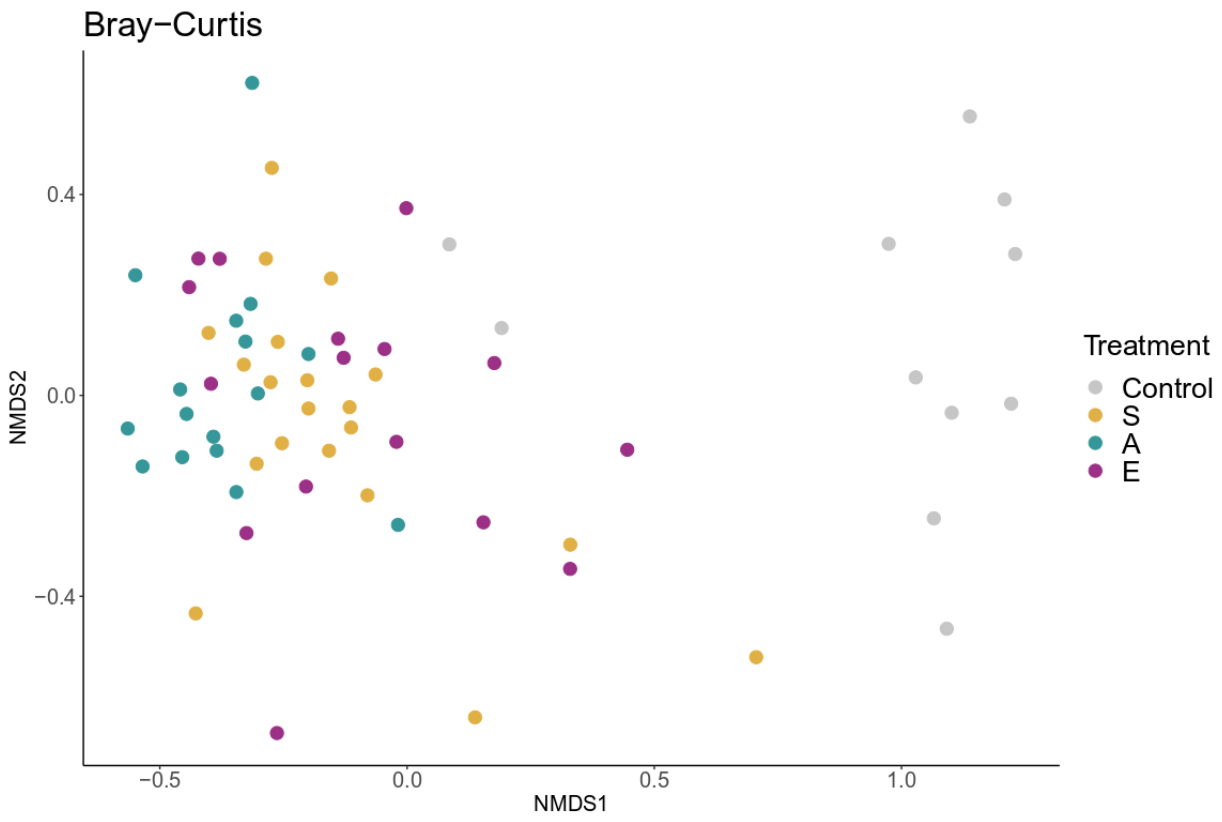
Relative abundances of our isolates within our co-culture well plates as measured by 16S rRNA sequences.

Figure 4



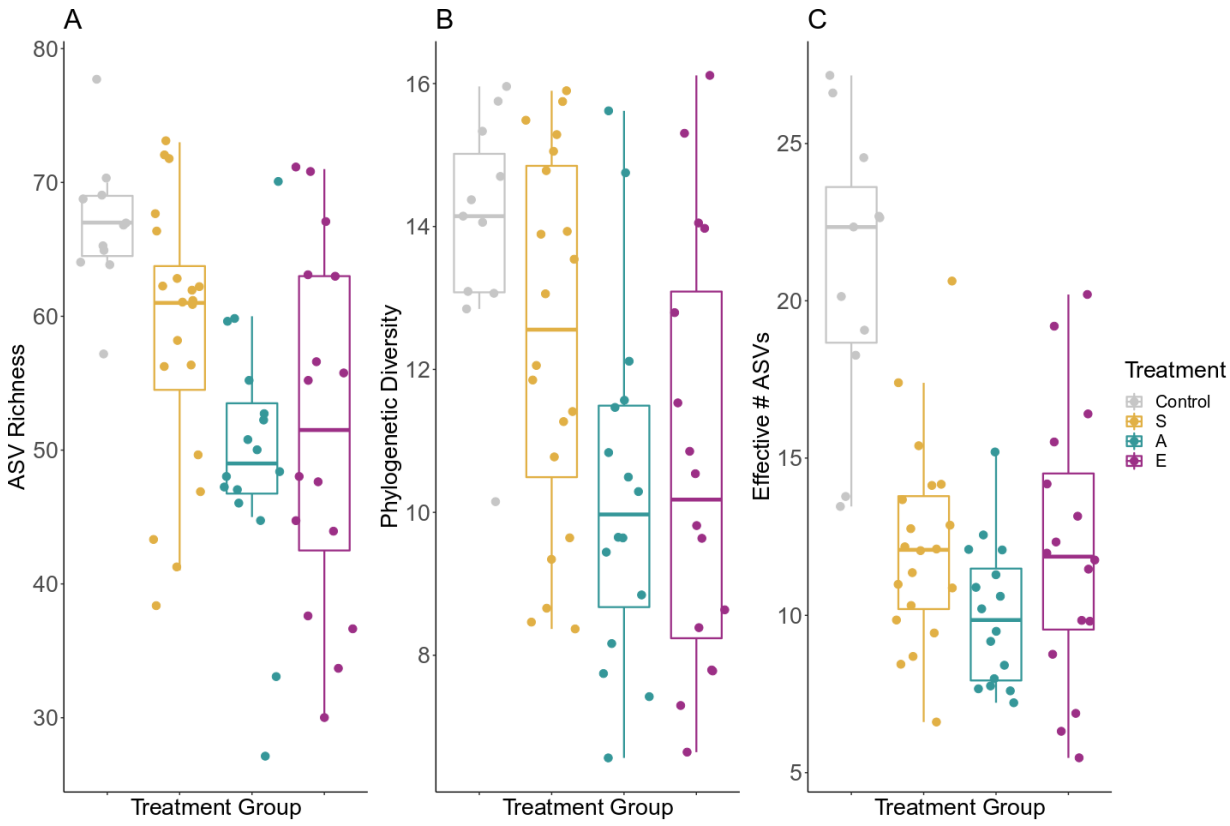
Relative abundances of our isolates across treatments (experiment 2). Treatments A and E showed a universal increase in their initial isolates relative abundance compared to other treatments. Treatment S showed an increase in *Stenotrophomonas* compared to treatment E and the control.

Figure 5



NMDS ordination of whole-tadpole bacterial communities (experiment 2). Communities differed between treatments independent of the inclusion of control samples based on a Bray-Curtis dissimilarity matrix.

Figure 6



Alpha diversity of whole-tadpole bacterial communities (experiment 2) differed between treatments in measures of **A)** ASV richness (A|E vs Con, S vs A), **B)** Phylogenetic diversity (A|E vs Con), and **C)** Effective number of species (A|E|S vs Con).

Title: Priority Effects Alter Microbiome Composition and Increase Abundance of Probiotic Taxa in Treefrog Tadpoles

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Abstract:

Host-associated microbial communities, like other ecological communities, may be impacted by the colonization order of taxa through priority effects. Developing embryos and their associated microbiomes are subject to stochasticity during colonization by bacteria. For amphibian embryos, many of which develop externally in bacteria rich environments, this stochasticity may be particularly impactful. The amphibian microbiome can mitigate lethal outcomes from disease for their hosts, however this is dependent on microbiome composition. In a series of studies, we examined the assembly of the bacterial community in spring peeper (*Pseudacris crucifer*) embryos and tadpoles. First, we reared spring peeper embryos from identified mating pairs in either lab or field environments to examine the relative impact of environment and parentage on the composition of the embryo and tadpole bacterial communities. Second, we completed an experimental inoculation of spring peeper embryos to determine if priority effects: (1) could be used to increase the relative abundance of *Janthinobacterium lividum*, an amphibian-associated bacteria with antifungal properties, and (2): would lead to observed differences in the relative abundances of two closely-related bacteria from the genus *Pseudomonas*. Using 16S rRNA gene amplicon sequencing to assess bacterial communities, we found differences in community composition based on both rearing location and parentage in embryos and tadpoles. In the inoculation experiment, we found that priority inoculation could increase the relative abundance of *J. lividum*, but did not find that the *Pseudomonas* isolates were able to prevent colonization by a closely-related isolate when given priority. These results highlight the importance of environmental source pools and parentage in determining microbiome composition, while also providing novel methods for the administration of a known amphibian probiotic.

Introduction:

Host-associated microbial communities are common in the natural world. These symbiotic communities of microorganisms are capable of helping or hindering their host organisms in myriad ways [1–4]. Bacterial communities, in particular, have been a popular subject of study due to their role in host health and development [5–7]. For example, bacterial community structure has been associated with detoxification of harmful compounds within the host gut [8,9], improving plant health and fertility [4,10], and limiting susceptibility to disease [11–14]. As community composition may impact the ultimate function of these communities, understanding how the communities initially establish, or "assemble", is an important goal in microbial ecology.

The assembly of ecological communities is broadly thought to be governed by the four processes of selection, dispersal, speciation and drift [15]. Variation in the relative importance of these factors across communities and sites can be driven by environmental conditions, the scale of study and the species that are present [16,17]. Stochasticity can also lead to differences in community composition between otherwise equivalent habitat patches [18,19]. In the case of dispersal, stochasticity in arrival order in a habitat can result in priority effects, leading ultimately to differences in community structure. Priority effects are thought to occur when fitness differences between taxa are low in an environment lacking stabilizing forces [20,21]. Early arriving colonists may monopolize resources (niche preemption) or modify the environment (niche modification), therefore enhancing or

hindering the colonization potential of later arriving species [20]. We may, therefore, expect priority effects under scenarios where multiple closely-related taxa colonize a given environment due to similarities in fitness and niche breadth. Priority effects are likely important for structuring communities across a range of systems [22–25], and may offer a potential mechanism for understanding differences in host-associated microbial communities.

Experimental investigations of priority effects in host-associated microbial communities are still relatively rare in vertebrates, and have mainly focused on the gut microbiome of germ-free mice [26]. Amphibians, many of which have externally developing embryos, offer an interesting alternative system in which to explore priority effects within the vertebrate microbiome [27,28]. Studies on the amphibian microbiome have demonstrated the influence of the gut microbiome on host physiological traits, such as fat acquisition, temperature tolerance, and disease susceptibility [29,30]. Additionally, investigations into amphibian skin microbiome composition have shown that intraspecific variation in community structure exists across sites [31–34], highlighting the importance of environmental source pools in dictating community composition [34–36]. Prior experiments suggest that the relative abundances of individual bacterial taxa on tadpoles can be altered via priority effects during embryonic inoculation [27].

Many studies have focused on the role of the amphibian microbiome in reducing host susceptibility to the fungal skin pathogen, *Batrachochytrium dendrobatidis* (Bd) [3,30,37,38]. Amphibian-associated bacterial communities may contribute to lowered rates of infection [39], and may reduce morbidity or mortality when infections occur [40]. Specific taxa within the community, such as *Janthinobacterium lividum* or *Serratia marcescens*, produce antifungal compounds that aid in limiting Bd infections [38,41]. For example, the antifungal compound violacein is produced by *J. lividum* found on amphibian skin, making isolates of this genus of interest for probiotic applications [38]. Leveraging the ability of probiotic bacteria to reduce Bd associated mortality is, therefore, a central goal of amphibian conservation [39,41,42]. Understanding which life stages and under what conditions bacteria can best be incorporated into symbiotic communities is important for implementation of any probiotic strategy for conservation.

In the current study, we investigated the impact of priority effects during early embryonic development on the bacterial communities associated with newly-hatched spring peeper (*Peudacris crucifer*) tadpoles. We first performed a differential rearing study to understand how the egg laying environment and parentage might affect the microbiomes of embryos, and if any differences due to location and parentage would persist post-hatching. We then conducted experimental inoculations to answer two major questions. First, we wanted to understand if priority effects might serve as a potential mechanism to increase the relative abundance of *J. lividum* due to its production of the antifungal compound violacein. Second, we aimed to develop a better understanding of whether the relative abundances of two closely-related bacterial isolates would shift due to priority effect-derived exclusion [21] using isolates from the genus *Pseudomonas*.

Methods:

Overview of studies:

Spring peepers, *Pseudacris crucifer*, are small treefrogs native to the eastern US. In our differential rearing study, we collected breeding pairs of free-living adult spring peepers. A subset of these pairs were bred in the lab and a subset were left to breed in pond enclosures. Adult pairs bred in the lab were swabbed to characterize their skin microbiomes. Egg samples from each group

were collected and a set of tadpole samples were collected from each location post hatching. Bacterial communities from samples were assessed using 16S rRNA gene amplicon sequencing.

In our inoculation experiment, spring peeper embryos were placed in individual wells of 12-well cell culture plates and inoculated over two days. We exposed embryos to either one of three individual bacterial isolates or sterile water, followed 24 hours later by sterile water or a community inoculum containing 5 bacterial isolates (details below). After hatching, we assessed the whole-body bacterial communities of tadpoles using 16S rRNA gene amplicon sequencing (Figure 1). To consider the potential impact of vertical transmission from parents to eggs in our experimental design, we also compared our tadpole microbiomes to those of eggs and swabs from the parent frogs.

We have previously isolated bacteria from the skin of wild caught adult spring peepers in Virginia [43]. For the present study, we selected three isolates from those frozen stocks to be used as initial inoculum, and two additional bacteria to be used as part of the synthetic community inoculum, similar to the design used in Jones et al. [27]. For our initial inoculum, we selected a *Janthinobacterium lividum* isolate (HP6A, Isolate J) and two *Pseudomonas* isolates that could be distinguished from each other based on their 16S rRNA gene sequences (HP12K, Isolate P1 and HP3I, Isolate P2). Our *Pseudomonas* isolates were chosen to gain insight into the outcomes of priority effects between closely-related taxa. Additionally, from our set of spring peeper isolates, we selected a *Flavobacterium* (HP3M, Isolate F) and a *Microbacterium* (HP3T, Isolate M) for use in our synthetic community, as we were able to clearly distinguish them with 16S gene sequencing.

Differential Rearing Study:

Pairs of adult spring peepers were collected from ponds on the campus of Vassar College (Poughkeepsie, NY, USA) on 10 March 2016. Three pairs were placed in field breeding enclosures (one pair per enclosure). Field enclosures consisted of 61L x 40.6W x 35.2H cm plastic Sterilite containers with the bottoms cut out. Enclosures were pushed down into the mud at the bottom of the pond, the pair was placed inside, and then the lid was fastened on top to prevent the pair from escaping. Pairs were left overnight to breed. Enclosures were checked the following morning, and three eggs from each pair (family) were collected and preserved to characterize bacterial community diversity. Eggs were placed individually in 1.5 ml centrifuge tubes on ice until transfer to -80 C. Additional eggs were placed individually in cylindrical window screen mesh bags (40 cm tall X 12 cm diameter) within the pond to continue development. The inner space of these bags was maintained by a PVC ring at bottom and a plastic float at top. Two leaves from the pond were added to each bag to provide food and cover. Bags were closed by folding over the mesh at the top and securing with a clothespin. After one month, surviving tadpoles were collected by gloved hand and preserved by flash freezing.

Six pairs of spring peepers were returned to the lab and placed in 35.6L x 20.3W x 12.4H cm plastic Sterilite containers with lids to breed. Containers were autoclaved ahead of time and contained 300 ml of sterile spring water (Poland Spring® brand water). Containers were elevated slightly so that the water pooled at one end of the container. Pairs remained in their containers for 48 hours (females were still laying eggs after 24 hours). After 48 hours, all adults were swabbed in the laboratory and then released at the site of capture. Swabbing was completed using sterile swabs (MW113; Medical Wire Equipment & Co. Ltd.); each frog was swabbed using our standard swabbing procedure of 10 bidirectional passes (upward and downward stroke) on their ventral side, and five

passes in a single direction on each hind thigh and on each hide foot. Three eggs from each family were individually preserved in 1.5 ml centrifuge tubes. Three additional eggs were placed individually in the wells of cell culture plates containing 4 ml sterile spring water. Eggs were checked daily until hatching; hatchlings were preserved two days post hatching when yolk absorption was complete. All samples were immediately placed on ice and preserved at – 80 C until further processing.

Inoculation Experiment:

The night prior to the start of the experiment, amplexed pairs (N=5) of adult spring peepers were collected from Virginia Tech's Kentland Farm research area (Montgomery County, VA, USA). Pairs were placed individually in lidded, 5-gallon buckets with water from the collection site and left overnight. The following morning, we returned to the collection site. Amplexed pairs were separated, rinsed with sterile water, and each individual was swabbed as above with a sterile swab (MW113; Medical Wire Equipment & Co. Ltd.) to characterize their skin bacterial communities. Swabs were stored on ice in 1.5 ml microcentrifuge tubes until they could be stored at -80°C. After swabbing, adults were released at the site of collection. From each of the five resulting egg masses, we initially collected 10 eggs/mass and placed them together in a sterile 50 ml tube. This was repeated to give us two tubes containing 50 eggs each. Eggs in each tube were then rinsed three times with sterile water before being placed on ice and transported to the lab for experimental inoculation. Water from the final rinse was retained to prevent eggs from drying out. We also collected a set of 10 eggs from each individual amplexed pair for sequencing. These eggs were rinsed with sterile water three times in 15 ml Falcon tubes with enough water retained to cover the eggs before being stored on ice for transport to the lab and stored at -80 °C.

We inoculated R2A agar plates with individual isolates from frozen glycerol stocks. Broth cultures were started from a single colony on the agar plates in 1% tryptone and incubated overnight at room temperature on a shaker. The morning of the experiment, 1000 µl of broth from each isolate was transferred to individual, sterile 1.5 ml microcentrifuge tubes and centrifuged at 5,000 rpm for 10 min. The broth was pipetted out, leaving the pelleted bacteria. We then pipetted 500 µl of sterile water into each tube and vortexed tubes to resuspend the bacteria. This process was repeated, and the resulting solution was then quantified using a haemocytometer. We then created standardized inoculation solutions for each isolate at 10^6 cells/ml. This concentration of bacteria was chosen based on similarity to what eggs might be exposed to in a pond [44,45]. We collected 500 µl of each inoculum solution for sequencing.

To prepare the community inoculum for day two (the day after the initial inoculations were completed), 1% tryptone broth cultures were created for each of our five isolates. The next day, 200 µl of each broth culture was added to five 1.5 ml microcentrifuge tubes. We then replaced the broth with water and created standardized solutions as done for the individual isolates. We collected 500 µl of each monoculture and the community inoculum for sequencing.

We randomly assigned eggs to one of the four bacterial treatment groups or a control treatment (N=20/treatment) and set up the experiment in our biosafety hood. Eggs were removed from field collection tubes, placed in sterile petri dishes, and rinsed with sterile water within 30 minutes of transport from the field. Eggs were then transferred to a second petri dish and rinsed with sterile water again. We then transferred eggs to individual wells within 12-well, lidded cell

culture plates (N=9 total plates). Embryos for treatments P1, P2, and J each received 3 ml of an inoculum of their respective isolate. Embryos assigned to the community-only and control treatments were given an inoculum of sterile water. Once all wells were treated, lids were returned to the plates, and the plates were placed within a sterilized plastic container. The container was then transported to our animal care room (26 °C with a 12 hour on/off light schedule). After 24 hours, the well-plates were returned to the biosafety hood. Liquid from the prior day's inoculation was removed via pipette and replaced with a fresh inoculum. All experimental treatments (P1, P2, J, community-only) were inoculated with our community inoculum. Our control treatment received a new inoculum of sterile water. Additionally, one empty well received an inoculum of our community inoculation to understand outcomes within our inoculum community in the absence of a host. After day two inoculations were complete, well plates were returned to the animal care room. We monitored eggs daily for signs of hatching. Tadpoles began to hatch 48 hours after the second inoculation, and all surviving embryos had hatched by 72 hours after the second inoculation. Upon hatching, tadpoles were removed from wells using sterile 1000 µl pipettes (N/treatment: J=13, P1=16, P2=12, Community-only=12, Control=13). We collected 500 µl of our non-host community sample on day 5 (72 hours post second inoculation) via pipette. Hatchlings were rinsed in sterile water, flash frozen in individual 1.5 ml microcentrifuge tubes, and stored at -80 °C until processing.

DNA extraction, amplification, and sequencing:

DNA was extracted from each experiment and sequenced separately. DNA for the differential rearing study was extracted from whole body tadpoles (n=23), egg samples (n=25), and adult skin swabs (n=12). DNA for the inoculation experiment was extracted from whole body tadpoles (n=66), individual isolate and community inoculum samples (N=10), pooled field-collected egg samples (N=5) and parental swabs (N=10) using the DNeasy blood and tissue extraction kit (Qiagen, Inc, Valencia, CA, USA). We followed the manufacturer's protocols and included a 1 hr pretreatment with 180 µl of enzymatic lysis buffer followed by a 30-minute incubation at 70 °C. Eggs and tadpoles were mechanically ground for 30 s with sterile pestles. DNA from inoculum samples and swabs was extracted using the same methods, except there was no grinding step. We targeted the V4 region of the 16S rRNA gene in all samples and amplified it using the 515F and barcoded 806R primer pair developed by Caporaso et al. [46]. PCR reactions were 25 µl, and consisted of 0.5 µl of each primer; 9.5 µl ultra clean PCR grade water; 12.5 µl GoTaq G2 Master Mix (Promega, Madison, WI, USA); and 2 µl genomic DNA. Our thermocycler protocol was: 3 minutes at 94 °C, then 34 cycles at 94 °C for 45 s, 50 °C for 60 s, 72 °C for 90 s, and then a final extension for 10 minutes at 72 °C. Reactions were run in triplicate for each sample, with an additional negative control that did not include DNA template. Triplicates were combined following PCR amplification. We used a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA) to quantify the combined PCR products. We combined 200ng of DNA from each sample to create the pooled samples for this experiment. Three sequencing runs were completed in total. Differential rearing samples were included on a single run. Inoculation samples were sequenced separately, with tadpoles and isolate samples in one sequencing run, and inoculation parent swabs and egg samples as part of another run. We cleaned our pooled samples using the QIAquick PCR purification kit, following the manufacturer's instructions (Qiagen, Inc., Valencia, CA, USA). Amplicon sequencing was completed at the Dana Farber Cancer Center at Harvard University on an Illumina Mi-Seq instrument with a 250 bp single-end strategy.

Amphibian bacterial community data processing:

We used a Qiime2 [47] pipeline to process our sample sequence data, including demultiplexing, denoising, and dereplication. Our two datasets ((1) experimental inoculation and (2) differential rearing) were processed individually using the same methods. We denoised our sequences using Dada2 [48] with a quality score set at 11. ASVs with a frequency less than 0.01% across all samples were filtered out. To apply taxonomy to our ASVs, we used scikit-learn [49] and the SILVA 138.1 [50] taxonomic classifier. We created a tree with a root at the midpoint using RAxML [51]. The final inoculation experiment table contained a total of 973 ASVs across 118 samples. The final differential rearing dataset contained 663 ASVs across 60 samples. Tables were then imported into the R v4.1.0 [52] environment for further processing and analysis. Once imported into R, we filtered out any ASVs that were not identified as bacteria, were identified as chloroplasts or mitochondria, and those that were unidentifiable at the phylum level. The filtered inoculation experiment table contained 545 ASVs and the differential rearing table contained 562 ASVs across our samples.

For the experimental dataset, we identified the ASVs corresponding to our isolates through analysis of our isolate-only amplicon samples. Our *Janthinobacterium* isolate was identified as ASV225 and ASV226, *Pseudomonas* 1 as ASV86, *Pseudomonas* 2 as ASV74, *Flavobacterium* as ASV369, and *Microbacterium* as ASV23. Within our experimental tadpole samples, we observed the expected presence of background taxa [27]. Our eggs were thoroughly rinsed; however, we did not apply antibiotics due to the potential host interactions. We, therefore, expected to observe some level of background taxa, potentially originating from the parent frogs. To minimize the impact of background taxa, our analyses focus on the proportional abundance of our target isolates to determine the presence of priority effects.

Statistical Analyses:

All statistical analyses were conducted in the R v4.1.0 environment [52].

Differential Rearing Study:

Differences in microbiome structure between life stages and rearing environment were determined through PERMANOVA analyses with 1000 permutations using Bray-Curtis and Jaccard matrices.

We used a negative binomial mixed model and a generalized mixed model with a log link function to identify differences between differential rearing samples in species richness or the effective number of species, respectively, including family as a random effect. We used a generalized linear model with a log link function for our phylogenetic diversity analysis because including a random effect in our model generated singularity errors. Estimated marginal means [function emmeans; package emmeans v1.8.3] were used to determine pairwise differences between groups.

We determined ASV overlap between life stages by identifying ASVs found on each stage using a rarified table (1467 reads/sample). We looked for adult-egg, egg-tadpole and tadpole-adult overlap, determined the percentage of total observed ASVs shared between stages and the relative proportion of the community occupied by shared ASVs.

Inoculation Experiment:

To assess whether our embryo treatments impacted bacterial community structure of tadpoles based on relative abundance, we first converted the raw count data from our ASV tables to relative abundances as a method of normalization. We then performed a PERMANOVA analysis with 1000 permutations using a Bray-Curtis matrix and visualized this with an NMDS ordination. We then used pairwise PERMANOVA analyses with Benjamini-Hochberg adjusted p-values to determine pairwise differences between treatment groups. We repeated these methods to understand differences in community composition between our lab and pond raised samples in our lab/pond dataset.

To determine if treatments impacted individual isolate relative abundance, we used a negative binomial fitted linear model followed by a comparison of estimated marginal means between treatments [function emmeans; package emmeans v1.8.3]. To ascertain the presence of an inverse relationship between the relative abundances of our *Pseudomonas* isolates, we used two negative binomial linear models: one between P1 and P2 and one across all treatments.

We used three metrics to create a complete picture of differences in alpha diversity: Species richness (the number of ASVs per sample), Faith's phylogenetic diversity, and the effective number of species (Hill numbers calculated using the Shannon diversity index and the equation [exp(Shannon diversity)][53]. To determine if treatment had an overall effect on species richness, we used negative binomial linear models. Differences in phylogenetic diversity and the effective number of species due to treatment were determined using generalized linear models with a log link function.

Estimates of relative abundance differences for individual isolates and all alpha diversity analyses were conducted using ASV tables rarefied to the minimum per sample sequencing depth (1549 reads).

We determined ASV overlap as above for adult-egg and adult-tadpole samples (rarefied table 1511 reads/sample.

Results:

Differential Rearing Study:

Among our differential rearing samples, we saw differences in the bacterial communities due to rearing location, life stage, family, and the interaction between location and life stage in terms of both relative abundance and in the presence/absence of taxa ([Bray-Curtis] Location: R^2=0.22862, F=24.3043, Pr(>F)=9.999e-05; Life stage: R^2=0.10806, F=11.4882, Pr(>F)=9.999e-05; Family: R^2=0.25010, F=3.7983, Pr(>F)=9.999e-05, Location*Life stage: R^2=0.06517, F=6.9286, Pr(>F)=9.999e-05 | [Jaccard] Location: R^2=0.14677 F=11.7230, Pr(>F)=9.999e-05; Life stage: R^2=0.08103, F=6.4720, Pr(>F)=9.999e-05; Family: R^2=0.25186, F=2.8738, Pr(>F)=9.999e-05, Location*Life stage: R^2=0.05711, F=4.5619, Pr(>F)=9.999e-05)(Figure 2).

For all alpha diversity metrics, pond samples had significantly higher values than lab samples (pond vs lab eggs: species richness p<0.0001, effective number of species p<0.0001, phylogentic diversity p<0.0001 | pond vs lab tadpoles: species richness p<0.0001, effective number of species p<0.0015, phylogentic diversity p<0.0001). Lab eggs showed significantly higher values of species richness and phylogenetic diversity than lab tadpoles, however there was no difference in the effective number of species. (lab tadpoles vs eggs: species richness p<0.0001, phylogentic diversity p=0.0048, effective number of species p=0.5454). Pond eggs showed significantly higher

phylogenetic diversity than pond tadpoles, but did not differ in species richness or the effective number of species (pond tadpoles vs eggs: species richness p=0.9305, effective number of species p=0.1718, phylogentic diversity p=0.0450) (Figure 3).

We found that 84% of the ASVs found on eggs were also found on adult samples and these shared ASVs made up 98% of the relative abundance in egg-associated communities (Figure 4A). Similarly, 89% of the ASVs found on tadpoles were also found on eggs and represented 99% of the overall relative abundance in tadpole-associated communities (Figure 4A).

Experimental Inoculation:

Individual tadpoles possessed between 21-169 unique ASVs. Tadpoles were dominated by ASVs belonging to the phylum Proteobacteria (71%), followed by Bacteroidota (20%), Firmicutes (4%) and Actinobacteriota (2%) (Figure S1). ASVs from our community inoculum made up 7% of the relative abundance of control samples, 31% of the community-only treatment, 27% of treatment P1, 32% of treatment P2, and 33% of treatment J (Figure 5A).

Altering which inoculum embryos were initially exposed to resulted in differing community structure, based on relative abundance, among our tadpole treatments (Adonis2; Bray-Curtis| R^2=0.15545, F=2.807, Pr(>F)=9.999e-05) (Figure 5A, Figure 6). Looking at pairwise differences, treatment J was distinct from all other treatments (J/P1 adjusted p= 0.0163, J/P2 adjusted p=0.0184, J/Community adjusted p=0.00460). Treatments P1 and P2 were not significantly different from each other (P1/P2 adjusted p=0.953) and between those treatments, only P1 was different from the community inoculum (P1/Community adjusted p= 0.0441). All treatments were significantly different from the control (All treatments adjusted p=<0.005) When given priority, the relative abundance of isolate J became enriched in our tadpole samples compared to treatments in which it was only part of the community inoculum (emmeans| J/Community: p=0.0461, J/P2: p=0.0240, J/P1: p=0.0013) (Figure 5B). Isolate P2 only showed a significant increase in its relative abundance when comparing the P2 and J treatments (emmeans| P2/J: p=0.0206) (Figure 5C). We did not observe a significant difference in the relative abundance of isolate P1 between the P1 and community-only treatments; however, we saw a difference between treatments P1 and J (emmeans| P1/J: p=0.0121)(Figure 5D). We did not observe an inverse relationship between the relative abundances of the closely-related *Pseudomonas* isolates P1 and P2 when considering only the P1 and P2 treatments (Pr(>|z|)=0.787) , or across all treatments (Pr(>|z|)=0.112). Isolate F, which was only present in the community inoculum, showed differential abundance when the community-only treatment was compared to treatment J (emmeans| Community/J: p=0.0134), but not to P1 or P2. Isolate M did not show a significant difference in its relative abundances across any treatments. Neither species richness (Pr(>F)=0.1218), the effective number of species (Pr(>F)=0.1358) nor phylogenetic diversity (Pr(>F)=0.4986) differed among tadpole treatments (Figure 7).

We found a total of 427 unique ASVs in our egg samples. We found that 90% of these ASVs were also found in the adult skin swabs. Shared ASVs made up 89% of the relative abundance of adult skin swab samples and 97% of our egg sample communities (Figure 4B). Between our adult swabs and tadpole samples, we found that 80% of tadpole ASVs were also found on adult swabs. These shared ASVs made up 89% of the tadpole and 77% of the adult swab microbiome in terms of relative abundance. Among these ASVs, those that represented over 1% of the total relative abundance included isolates J, P1, and P2, and ASVs from the genera *Rhodoferax* (1), *Undibacterium*

(2), *Arcicella* (1), *Fibrella (*1), *Flavobacterium* (1), *Parasediminibacterium* (1), *Rheinheimera* (1), and the family Comamonadaceae (1, lowest level of classification) (Figure 4D).

Discussion:

Our results highlight the potential of priority effects as a mechanism for increasing the relative abundance of target bacteria within the vertebrate microbiome. We inoculated eggs during a critical developmental window [28] and saw significant increases in relative abundance, based on priority, for our *Janthinobacterium lividum* isolate. This result suggests that priority effects may be a viable method of probiotic augmentation. To be effective, probiotic-derived metabolites need to reach a minimum inhibitory concentration (MIC). The MIC can be achieved in monoculture or through synergistic interactions between taxa [54,55]. Incorporating our community inoculum within our methods has highlighted our potential ability to successfully establish multiple bacteria within early tadpole communities. Our inoculation methods may, therefore, be capable of establishing synergistic communities, as well as singular probiotic taxa. Further experimentation would need to be completed to understand the longevity of the observed increase in relative abundance, and whether this increase in the relative abundance of a Bd-inhibitory taxa results in an increase in host protection from Bd [39,42,56]. Successful probiotic establishment may be further enhanced if combined with strategies learned from previous probiotic studies, such as the availability of target taxa within the environment [39,57].

Priority effects are most likely to be observed when "fitness" differences among taxa in a community are minor, and taxa share an overlap in resource use [21]. Devevey et al. (2015), for example, showed that with multiple strains of *Borrelia burgdorferi*, the first to colonize experimentally inoculated ticks could inhibit secondary strain colonization. Using two isolates from the same genus (*Pseudomonas*), we expected that competition would drive an inverse relationship in relative abundance between the priority treatments. *Pseudomonas* is one of the most diverse bacterial genera [58], so perhaps the lack of an inverse correlation in our study suggests that these isolates may not directly compete for niche space within this system. Host regulation or intraspecific competition may act as stabilizing forces that are preventing priority effects from being observed with these two *Pseudomonas* isolates [20,21].

Overall, by alternating the initial bacterial environment that frog embryos were exposed to, we saw differences in microbiome community structure. This was true for our experimental inoculations and between pond and lab raised samples. Differences in environmental conditions, and, therefore, the available bacteria in environmental source pools, contribute to intraspecific variation in bacterial community diversity across different populations of amphibians [35,36,59,60]. We saw consistency in the effective number of species between tadpole and egg samples raised in the same environments, as in prior work [61]. Because host regulation of bacteria is likely a less active component in early embryo microbiome assembly [62], this suggests the effective number of species could be driven by microbe-microbe interactions, potentially even at the tadpole stage. Additionally, relocating experimental tadpoles to a natural or semi-natural environment would provide a greater pool of bacteria within the environment, which may lead to alterations in composition [60].

In addition to acquiring bacteria from environmental source pools, some species receive symbionts through vertical transfer from parents. Whether this transpires broadly in frogs remains unknown, however, there is support for this idea from prior studies [62–64]. Hughey et al. (2017), for

example, found that eggs and adult skin of the neotropical glass frog *H. colymbiphyllum* harbored similar bacterial communities, even without continued paternal care. The overlap in ASV identity between our egg-adult and tadpole-adult samples in our data provides support for this idea. Additionally, within our differential rearing samples, we saw distinctions in microbiomes based on parentage at the egg and tadpole stage in both the lab and pond environments, suggesting that priority given to vertical transmitted bacteria or genetics may play a consistent role in shaping bacterial community composition. When coupled with prior research suggesting that amphibian-associated bacteria are relatively rare in the environment [65], it is possible that many of the egg-associated bacteria may originate from parents in our study system. Rinsing the eggs used in our inoculation experiment may have diminished any priority advantages provided to parentally transferred bacteria, facilitating the observation of priority effects in our probiotic inoculum.

Our study builds on earlier work that identified hatching as a critical developmental period for the microbiome [28,66], and improves our understanding of colonization by bacterial taxa during early life [27,67]. Priority effects may be a viable method of increasing the relative abundance of target taxa in future applied studies, but should also be taken into consideration when raising captive bred animals [67], and in studies seeking to understand intraspecific microbiome variation [68]. Continuing to empirically test priority effects across systems will be crucial to developing a thorough understanding of how and when priority effects impact microbiome composition and ultimately increase the ability of applied projects seeking to leverage the functional potential of the microbiome.

(1) Allender, M. C.; Baker, S.; Britton, M.; Kent, A. D. Snake Fungal Disease Alters Skin Bacterial and Fungal Diversity in an Endangered Rattlesnake. *Scientific Reports* **2018**, *8* (1), 12147. https://doi.org/10.1038/s41598-018-30709-x.

(2) Aoun, A.; Darwish, F.; Hamod, N. The Influence of the Gut Microbiome on Obesity in Adults and the Role of Probiotics, Prebiotics, and Synbiotics for Weight Loss. *Prev Nutr Food Sci* **2020**, *25* (2), 113–123. https://doi.org/10.3746/pnf.2020.25.2.113.

(3) Walke, J. B.; Belden, L. K. Harnessing the Microbiome to Prevent Fungal Infections: Lessons from Amphibians. *PLOS Pathogens* **2016**, *12*.

(4) Wei, Z.; Gu, Y.; Friman, V.-P.; Kowalchuk, G. A.; Xu, Y.; Shen, Q.; Jousset, A. Initial Soil Microbiome Composition and Functioning Predetermine Future Plant Health. *Sci. Adv.* **2019**, *5* (9), eaaw0759. https://doi.org/10.1126/sciadv.aaw0759.

(5) Moossavi, S.; Miliku, K.; Sepehri, S.; Khafipour, E.; Azad, M. B. The Prebiotic and Probiotic Properties of Human Milk: Implications for Infant Immune Development and Pediatric Asthma. *Front. Pediatr.* **2018**, *6*. https://doi.org/10.3389/fped.2018.00197.

(6) Rebollar, E. A.; Bridges, T.; Hughey, M. C.; Medina, D.; Belden, L. K.; Harris, R. N. Integrating the Role of Antifungal Bacteria into Skin Symbiotic Communities of Three Neotropical Frog Species. *The ISME Journal* **2019**, *13* (7), 1763. https://doi.org/10.1038/s41396-019-0388-x.

(7) Sommer, F.; Bäckhed, F. The Gut Microbiota — Masters of Host Development and Physiology. *Nature Reviews Microbiology* **2013**, *11* (4), 227–238. https://doi.org/10.1038/nrmicro2974.

(8) Ceja-Navarro, J. A.; Vega, F. E.; Karaoz, U.; Hao, Z.; Jenkins, S.; Lim, H. C.; Kosina, P.; Infante, F.; Northen, T. R.; Brodie, E. L. Gut Microbiota Mediate Caffeine Detoxification in the Primary Insect Pest of Coffee. *Nature Communications* **2015**, *6* (1), 7618. https://doi.org/10.1038/ncomms8618.

(9) Kohl, K. D.; Connelly, J. W.; Dearing, M. D.; Forbey, J. S. Microbial Detoxification in the Gut of a Specialist Avian Herbivore, the Greater Sage-Grouse. *FEMS Microbiol Lett* **2016**, *363* (14). https://doi.org/10.1093/femsle/fnw144.

(10) Chaparro, J. M.; Sheflin, A. M.; Manter, D. K.; Vivanco, J. M. Manipulating the Soil Microbiome to Increase Soil Health and Plant Fertility. *Biol Fertil Soils* **2012**, *48* (5), 489–499. https://doi.org/10.1007/s00374-012-0691-4.

(11) Becker, M. H.; Harris, R. N. Cutaneous Bacteria of the Redback Salamander Prevent Morbidity Associated with a Lethal Disease. *PLOS ONE* **2010**, *5* (6), e10957. https://doi.org/10.1371/journal.pone.0010957.

(12) Hild, B.; Dreier, M. S.; Oh, J. H.; McCulloch, J. A.; Badger, J. H.; Guo, J.; Thefaine, C. E.; Umarova, R.; Hall, K. D.; Gavrilova, O.; Rosshart, S. P.; Trinchieri, G.; Rehermann, B. Neonatal Exposure to a Wild-Derived Microbiome Protects Mice against Diet-Induced Obesity. *Nat Metab* **2021**, *3* (8), 1042–1057. https://doi.org/10.1038/s42255-021-00439-y.

(13) Hill, A. J.; Leys, J. E.; Bryan, D.; Erdman, F. M.; Malone, K. S.; Russell, G. N.; Applegate, R. D.; Fenton, H.; Niedringhaus, K.; Miller, A. N.; Allender, M. C.; Walker, D. M. Common Cutaneous Bacteria Isolated from Snakes Inhibit Growth of Ophidiomyces Ophiodiicola. *EcoHealth* **2018**, *15* (1), 109–120. https://doi.org/10.1007/s10393-017-1289-y.

(14) McKenzie, V. J.; Kueneman, J. G.; Harris, R. N. Probiotics as a Tool for Disease Mitigation in Wildlife: Insights from Food Production and Medicine. *Annals of the New York Academy of Sciences* **2018**, *1429* (1), 18–30. https://doi.org/10.1111/nyas.13617.

(15) Vellend, M. Conceptual Synthesis in Community Ecology. *The Quarterly Review of Biology* **2010**, *85* (2), 183–206. https://doi.org/10.1086/652373.

(16) Lawton, J. H. Are There General Laws in Ecology? *Oikos* **1999**, *84* (2), 177. https://doi.org/10.2307/3546712.

(17) Simberloff, D. Community Ecology: Is It Time to Move On?: (An American Society of Naturalists Presidential Address). *The American Naturalist* **2004**, *163* (6), 787–799. https://doi.org/10.1086/420777.

(18) Chase, J. M. Stochastic Community Assembly Causes Higher Biodiversity in More Productive Environments. *Science* **2010**, *328* (5984), 1388–1391. https://doi.org/10.1126/science.1187820.

(19) Evans, S.; Martiny, J. B. H.; Allison, S. D. Effects of Dispersal and Selection on Stochastic Assembly in Microbial Communities. *The ISME Journal* **2017**, *11* (1), 176–185. https://doi.org/10.1038/ismej.2016.96.

(20) Fukami, T. Historical Contingency in Community Assembly: Integrating Niches, Species Pools, and Priority Effects. *Annual Review of Ecology, Evolution, and Systematics* **2015**, *46* (1), 1–23. https://doi.org/10.1146/annurev-ecolsys-110411-160340.

(21) Fukami, T.; Mordecai, E. A.; Ostling, A. A Framework for Priority Effects. *Journal of Vegetation Science* **2016**, *27* (4), 655–657. https://doi.org/10.1111/jvs.12434.

(22) Carlström, C. I.; Field, C. M.; Bortfeld-Miller, M.; Müller, B.; Sunagawa, S.; Vorholt, J. A. Synthetic Microbiota Reveal Priority Effects and Keystone Strains in the Arabidopsis Phyllosphere. *Nat Ecol Evol* **2019**, *3* (10), 1445–1454. https://doi.org/10.1038/s41559-019-0994-z.

(23) Devevey, G.; Dang, T.; Graves, C. J.; Murray, S.; Brisson, D. First Arrived Takes All: Inhibitory Priority Effects Dominate Competition between Co-Infecting Borrelia Burgdorferi Strains. *BMC Microbiology* **2015**, *15* (1), 61. https://doi.org/10.1186/s12866-015-0381-0.

(24) Hess, M. C. M.; Mesléard, F.; Buisson, E. Priority Effects: Emerging Principles for Invasive Plant Species Management. *Ecological Engineering* **2019**, *127*, 48–57. https://doi.org/10.1016/j.ecoleng.2018.11.011.

(25) Jacobsen, R. M.; Birkemoe, T.; Sverdrup-Thygeson, A. Priority Effects of Early Successional Insects Influence Late Successional Fungi in Dead Wood. *Ecology and Evolution* **2015**, *5* (21), 4896–4905. https://doi.org/10.1002/ece3.1751.

(26) Debray, R.; Herbert, R. A.; Jaffe, A. L.; Crits-Christoph, A.; Power, M. E.; Koskella, B. Priority Effects in Microbiome Assembly. *Nat Rev Microbiol* **2021**. https://doi.org/10.1038/s41579-021-00604-w.

(27) Jones, K. R.; Hughey, M. C.; Belden, L. K. Colonization Order of Bacterial Isolates on Treefrog Embryos Impacts Microbiome Structure in Tadpoles. *Proceedings of the Royal Society B* **2023**. https://doi.org/10.1098/rspb.2023.0308.

(28) Warne, R. W.; Kirschman, L.; Zeglin, L. Manipulation of Gut Microbiota during Critical Developmental Windows Affects Host Physiological Performance and Disease Susceptibility across Ontogeny. *Journal of Animal Ecology* **2019**, *88* (6), 845–856. https://doi.org/10.1111/1365-2656.12973.

(29) Fontaine, S. S.; Mineo, P. M.; Kohl, K. D. Experimental Manipulation of Microbiota Reduces Host Thermal Tolerance and Fitness under Heat Stress in a Vertebrate Ectotherm. *Nat Ecol Evol* **2022**, *6* (4), 405–417. https://doi.org/10.1038/s41559-022-01686-2.

(30) Knutie, S. A.; Wilkinson, C. L.; Kohl, K. D.; Rohr, J. R. Early-Life Disruption of Amphibian Microbiota Decreases Later-Life Resistance to Parasites. *Nature Communications* **2017**, *8* (1), 86. https://doi.org/10.1038/s41467-017-00119-0.

(31) Antwis, R. E.; Haworth, R. L.; Engelmoer, D. J. P.; Ogilvy, V.; Fidgett, A. L.; Preziosi, R. F. Ex Situ Diet Influences the Bacterial Community Associated with the Skin of Red-Eyed Tree Frogs (Agalychnis Callidryas). *PLOS ONE* **2014**, *9* (1), e85563. https://doi.org/10.1371/journal.pone.0085563.

(32) Estrada, A.; Hughey, M. C.; Medina, D.; Rebollar, E. A.; Walke, J. B.; Harris, R. N.; Belden, L. K. Skin Bacterial Communities of Neotropical Treefrogs Vary with Local Environmental Conditions at the Time of Sampling. *PeerJ* **2019**, *7*, e7044. https://doi.org/10.7717/peerj.7044.

(33) Kruger, A. Frog Skin Microbiota Vary With Host Species and Environment but Not Chytrid Infection. *Frontiers in Microbiology* **2020**, *11*.

(34) Wagener, C.; du Plessis, M.; Measey, J. Invasive Amphibian Gut Microbiota and Functions Shift Differentially in an Expanding Population but Remain Conserved Across Established Populations. *Microb Ecol* **2021**. https://doi.org/10.1007/s00248-021-01896-4.

(35) Jones, K. R.; Walke, J. B.; Becker, M. H.; Belden, L. K.; Hughey, M. C. Time in the Laboratory, but Not Exposure to a Chytrid Fungus, Results in Rapid Change in Spring Peeper (Pseudacris Crucifer) Skin Bacterial Communities. *Ichthyology & Herpetology* **2021**, *109* (1). https://doi.org/10.1643/h2020077.

(36) Kueneman, J. G.; Bletz, M. C.; McKenzie, V. J.; Becker, C. G.; Joseph, M. B.; Abarca, J. G.; Archer, H.; Arellano, A. L.; Bataille, A.; Becker, M.; Belden, L. K.; Crottini, A.; Geffers, R.; Haddad, Célio. F. B.; Harris, R. N.; Holden, W. M.; Hughey, M.; Jarek, M.; Kearns, P. J.; Kerby, J. L.; Kielgast, J.; Kurabayashi, A.; Longo, A. V.; Loudon, A.; Medina, D.; Nuñez, J. J.; Perl, R. G. B.; Pinto-Tomás, A.; Rabemananjara, F. C. E.; Rebollar, E. A.; Rodríguez, A.; Rollins-Smith, L.; Stevenson, R.; Tebbe, C. C.; Vargas Asensio, G.; Waldman, B.; Walke, J. B.; Whitfield, S. M.; Zamudio, K. R.; Zúñiga Chaves, I.; Woodhams, D. C.; Vences, M. Community Richness of Amphibian Skin Bacteria Correlates with Bioclimate at the Global Scale. *Nature Ecology & Evolution* **2019**, *3* (3), 381–389. https://doi.org/10.1038/s41559-019-0798-1.

(37) Antwis, R. E.; Preziosi, R. F.; Harrison, X. A.; Garner, T. W. J. Amphibian Symbiotic Bacteria Do Not Show a Universal Ability To Inhibit Growth of the Global Panzootic Lineage of Batrachochytrium Dendrobatidis. *Appl. Environ. Microbiol.* **2015**, *81* (11), 3706–3711. https://doi.org/10.1128/AEM.00010-15.

(38) Becker, M. H.; Brucker, R. M.; Schwantes, C. R.; Harris, R. N.; Minbiole, K. P. C. The Bacterially Produced Metabolite Violacein Is Associated with Survival of Amphibians Infected with a Lethal Fungus. *Applied and Environmental Microbiology* **2009**, *75* (21), 6635–6638. https://doi.org/10.1128/AEM.01294-09.

(39) Muletz, C. R.; Myers, J. M.; Domangue, R. J.; Herrick, J. B.; Harris, R. N. Soil Bioaugmentation with Amphibian Cutaneous Bacteria Protects Amphibian Hosts from Infection by Batrachochytrium Dendrobatidis. *Biological Conservation* **2012**, *152*, 119–126. https://doi.org/10.1016/j.biocon.2012.03.022.

(40) Harris, R. N.; Brucker, R. M.; Walke, J. B.; Becker, M. H.; Schwantes, C. R.; Flaherty, D. C.; Lam, B. A.; Woodhams, D. C.; Briggs, C. J.; Vredenburg, V. T.; Minbiole, K. P. C. Skin Microbes on Frogs Prevent Morbidity and Mortality Caused by a Lethal Skin Fungus. *The ISME Journal* **2009**, *3* (7), 818–824. https://doi.org/10.1038/ismej.2009.27.

(41) Madison, J. D.; Ouellette, S. P.; Schmidt, E. L.; Kerby, J. L. Serratia Marcescens Shapes Cutaneous Bacterial Communities and Influences Survival of an Amphibian Host. *Proceedings of the Royal Society B: Biological Sciences* **2019**, *286* (1914), 20191833. https://doi.org/10.1098/rspb.2019.1833.

(42) Becker, M. H.; Brophy, J. A. N.; Barrett, K.; Bronikowski, E.; Evans, M.; Glassey, E.; Kaganer, A. W.; Klocke, B.; Lassiter, E.; Meyer, A. J.; Muletz-Wolz, C. R.; Fleischer, R. C.; Voigt, C. A.; Gratwicke, B. Genetically Modifying Skin Microbe to Produce Violacein and Augmenting Microbiome Did Not Defend Panamanian Golden Frogs from Disease. *ISME COMMUN.* **2021**, *1* (1), 1–10. https://doi.org/10.1038/s43705-021-00044-w.

(43) Walke, J. B.; Becker, M. H.; Hughey, M. C.; Swartwout, M. C.; Jensen, R. V.; Belden, L. K. Most of the Dominant Members of Amphibian Skin Bacterial Communities Can Be Readily Cultured. *Applied and Environmental Microbiology* **2015**, *81* (19), 6589–6600. https://doi.org/10.1128/AEM.01486-15.

(44) Simek, K.; Jürgens, K.; Nedoma, J.; Comerma, M.; Armengol, J. Ecological Role and Bacterial Grazing of Halteria Spp.: Small Freshwater Oligotrichs as Dominant Pelagic Ciliate Bacterivores. *Aquat. Microb. Ecol.* **2000**, *22*, 43–56. https://doi.org/10.3354/ame022043.

(45) Walker, E. D.; Merritt, R. W. Bacterial Enrichment in the Surface Microlayer of an Anopheles Quadrimaculatus (Diptera: Culicidae) Larval Habitat. *Journal of Medical Entomology* **1993**, *30* (6), 1050–1052. https://doi.org/10.1093/jmedent/30.6.1050.

(46) Caporaso, J. G.; Lauber, C. L.; Walters, W. A.; Berg-Lyons, D.; Huntley, J.; Fierer, N.; Owens, S. M.; Betley, J.; Fraser, L.; Bauer, M.; Gormley, N.; Gilbert, J. A.; Smith, G.; Knight, R. Ultra-High-Throughput Microbial Community Analysis on the Illumina HiSeq and MiSeq Platforms. *The ISME Journal* **2012**, *6* (8), 1621–1624. https://doi.org/10.1038/ismej.2012.8.

(47) Bolyen, E.; Rideout, J. R.; Dillon, M. R.; Bokulich, N. A.; Abnet, C.; Al-Ghalith, G. A.; Alexander, H.; Alm, E. J.; Arumugam, M.; Asnicar, F.; Bai, Y.; Bisanz, J. E.; Bittinger, K.; Brejnrod, A.; Brislawn, C. J.; Brown, C. T.; Callahan, B. J.; Caraballo-Rodríguez, A. M.; Chase, J.; Cope, E.; Silva, R. D.; Dorrestein, P. C.; Douglas, G. M.; Durall, D. M.; Duvallet, C.; Edwardson, C. F.; Ernst, M.; Estaki, M.; Fouquier, J.; Gauglitz, J. M.; Gibson, D. L.; Gonzalez, A.; Gorlick, K.; Guo, J.; Hillmann, B.; Holmes, S.; Holste, H.; Huttenhower, C.; Huttley, G.; Janssen, S.; Jarmusch, A. K.; Jiang, L.; Kaehler, B.; Kang, K. B.; Keefe, C. R.; Keim, P.; Kelley, S. T.; Knights, D.; Koester, I.; Kosciolek, T.; Kreps, J.; Langille, M. G.; Lee, J.; Ley, R.; Liu, Y.-X.; Loftfield, E.; Lozupone, C.; Maher, M.; Marotz, C.; Martin, B. D.; McDonald, D.; McIver, L. J.; Melnik, A. V.; Metcalf, J. L.; Morgan, S. C.; Morton, J.; Naimey, A. T.; Navas-Molina, J. A.; Nothias, L. F.; Orchanian, S. B.; Pearson, T.; Peoples, S. L.; Petras, D.; Preuss, M. L.; Pruesse, E.; Rasmussen, L. B.; Rivers, A.; Michael S Robeson, I. I.; Rosenthal, P.; Segata, N.; Shaffer, M.; Shiffer, A.; Sinha, R.; Song, S. J.; Spear, J. R.; Swafford, A. D.; Thompson, L. R.; Torres, P. J.; Trinh, P.; Tripathi, A.; Turnbaugh, P. J.; Ul-Hasan, S.; Hooft, J. J. van der; Vargas, F.; Vázquez-Baeza, Y.; Vogtmann, E.; Hippel, M. von; Walters, W.; Wan, Y.; Wang, M.; Warren, J.; Weber, K. C.; Williamson, C. H.; Willis, A. D.; Xu, Z. Z.; Zaneveld, J. R.; Zhang, Y.; Zhu, Q.; Knight, R.; Caporaso, J. G. *QIIME 2: Reproducible, Interactive, Scalable, and Extensible Microbiome Data Science*; e27295v2; PeerJ Inc., 2018. https://doi.org/10.7287/peerj.preprints.27295v2.

(48) Callahan, B. J.; McMurdie, P. J.; Rosen, M. J.; Han, A. W.; Johnson, A. J. A.; Holmes, S. P. DADA2: High-Resolution Sample Inference from Illumina Amplicon Data. *Nature Methods* **2016**, *13* (7), 581–583. https://doi.org/10.1038/nmeth.3869.

(49) Pedregosa, F.; Varoquaux, G.; Gramfort, A.; Michel, V.; Thirion, B.; Grisel, O.; Blondel, M.; Prettenhofer, P.; Weiss, R.; Dubourg, V.; Vanderplas, J.; Passos, A.; Cournapeau, D. Scikit-Learn: Machine Learning in Python. *MACHINE LEARNING IN PYTHON* **2011**, 6.

(50) Glöckner, F. O. The SILVA Database Project: An ELIXIR Core Data Resource for High-Quality Ribosomal RNA Sequences. *Biodiversity Information Science and Standards* **2019**, *3*, e36125. https://doi.org/10.3897/biss.3.36125.

(51) Stamatakis, A. RAxML Version 8: A Tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. *Bioinformatics* **2014**, *30* (9), 1312–1313. https://doi.org/10.1093/bioinformatics/btu033.

(52) R Core Team. R: A Language and Environment for Statistical Computing., 2021. https://www.R-project.org/.

(53) Jost, L. *Entropy and Diversity*. *Oikos* **2006**, *113* (2), 363–375. https://doi.org/10.1111/j.2006.0030-1299.14714.x.

(54) Brucker, R. M.; Harris, R. N.; Schwantes, C. R.; Gallaher, T. N.; Flaherty, D. C.; Lam, B. A.; Minbiole, K. P. C. Amphibian Chemical Defense: Antifungal Metabolites of the Microsymbiont Janthinobacterium Lividum on the Salamander Plethodon Cinereus. *J Chem Ecol* **2008**, *34* (11), 1422–1429. https://doi.org/10.1007/s10886-008-9555-7.

(55) Loudon, A. H.; Holland, J. A.; Umile, T. P.; Burzynski, E. A.; Minbiole, K. P. C.; Harris, R. N. Interactions between Amphibians' Symbiotic Bacteria Cause the Production of Emergent Anti-Fungal Metabolites. *Front. Microbiol.* **2014**, *5*. https://doi.org/10.3389/fmicb.2014.00441.

(56) Woodhams, D. C.; Rollins-Smith, L. A.; Reinert, L. K.; Lam, B. A.; Harris, R. N.; Briggs, C. J.; Vredenburg, V. T.; Patel, B. T.; Caprioli, R. M.; Chaurand, P.; Hunziker, P.; Bigler, L. Probiotics Modulate a Novel Amphibian Skin Defense Peptide That Is Antifungal and Facilitates Growth of Antifungal Bacteria. *Microb Ecol* **2020**, *79* (1), 192–202. https://doi.org/10.1007/s00248-019-01385-9.

(57) Rebollar, E. A.; Simonetti, S. J.; Shoemaker, W. R.; Harris, R. N. Direct and Indirect Horizontal Transmission of the Antifungal Probiotic Bacterium Janthinobacterium Lividum on Green Frog (Lithobates Clamitans) Tadpoles. *Appl. Environ. Microbiol.* **2016**, *82* (8), 2457–2466. https://doi.org/10.1128/AEM.04147-15.

(58) Peix, A.; Ramírez-Bahena, M.-H.; Velázquez, E. Historical Evolution and Current Status of the Taxonomy of Genus Pseudomonas. *Infection, Genetics and Evolution* **2009**, *9* (6), 1132–1147. https://doi.org/10.1016/j.meegid.2009.08.001.

(59) Belden, L. K.; Hughey, M. C.; Rebollar, E. A.; Umile, T. P.; Loftus, S. C.; Burzynski, E. A.; Minbiole, K. P. C.; House, L. L.; Jensen, R. V.; Becker, M. H.; Walke, J. B.; Medina, D.; Ibáñez, R.; Harris, R. N. Panamanian Frog Species Host Unique Skin Bacterial Communities. *Front Microbiol* **2015**, *6*. https://doi.org/10.3389/fmicb.2015.01171.

(60) Jani, A. J.; Briggs, C. J. Host and Aquatic Environment Shape the Amphibian Skin Microbiome but Effects on Downstream Resistance to the Pathogen Batrachochytrium Dendrobatidis Are Variable. *Front. Microbiol.* **2018**, *9*. https://doi.org/10.3389/fmicb.2018.00487.

(61) Flechas, S. V.; Acosta-González, A.; Escobar, L. A.; Kueneman, J. G.; Sánchez-Quitian, Z. A.; Parra-Giraldo, C. M.; Rollins-Smith, L. A.; Reinert, L. K.; Vredenburg, V. T.; Amézquita, A.; Woodhams, D. C. Microbiota and Skin Defense Peptides May Facilitate Coexistence of Two Sympatric Andean Frog Species with a Lethal Pathogen. *The ISME Journal* **2019**, *13* (2), 361. https://doi.org/10.1038/s41396-018-0284-9.

(62) Hughey, M. C.; Delia, J.; Belden, L. K. Diversity and Stability of Egg-Bacterial Assemblages: The Role of Paternal Care in the Glassfrog Hyalinobatrachium Colymbiphyllum. *Biotropica* **2017**, *49* (6), 792–802. https://doi.org/10.1111/btp.12461.

(63) Banning, J. L.; Weddle, A. L.; Wahl III, G. W.; Simon, M. A.; Lauer, A.; Walters, R. L.; Harris, R. N. Antifungal Skin Bacteria, Embryonic Survival, and Communal Nesting in Four-Toed Salamanders,

Hemidactylium Scutatum. *Oecologia* **2008**, *156* (2), 423–429. https://doi.org/10.1007/s00442-008-1002-5.

(64) Prest, T. L.; Kimball, A. K.; Kueneman, J. G.; McKenzie, V. J. Host-Associated Bacterial Community Succession during Amphibian Development. *Molecular Ecology* **2018**, *27* (8), 1992–2006. https://doi.org/10.1111/mec.14507.

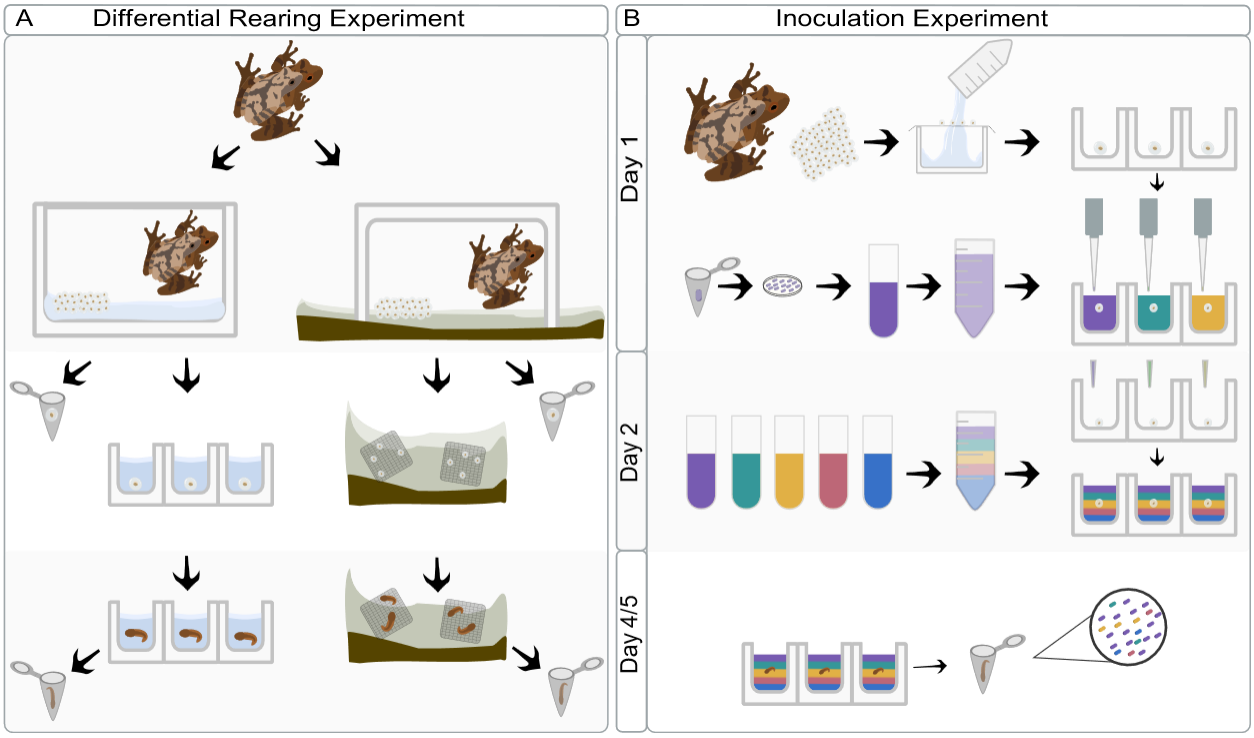
(65) Walke, J. B.; Becker, M. H.; Loftus, S. C.; House, L. L.; Cormier, G.; Jensen, R. V.; Belden, L. K. Amphibian Skin May Select for Rare Environmental Microbes. *The ISME Journal* **2014**, *8* (11), 2207–2217. https://doi.org/10.1038/ismej.2014.77.

(66) Warne, R. W.; Kirschman, L.; Zeglin, L. Manipulation of Gut Microbiota Reveals Shifting Community Structure Shaped by Host Developmental Windows in Amphibian Larvae. *Integrative and Comparative Biology* **2017**, *57* (4), 786–794. https://doi.org/10.1093/icb/icx100.

(67) Fallet, M.; Montagnani, C.; Petton, B.; Dantan, L.; de Lorgeril, J.; Comarmond, S.; Chaparro, C.; Toulza, E.; Boitard, S.; Escoubas, J.-M.; Vergnes, A.; Le Grand, J.; Bulla, I.; Gueguen, Y.; Vidal-Dupiol, J.; Grunau, C.; Mitta, G.; Cosseau, C. Early Life Microbial Exposures Shape the Crassostrea Gigas Immune System for Lifelong and Intergenerational Disease Protection. *Microbiome* **2022**, *10* (1), 85. https://doi.org/10.1186/s40168-022-01280-5.

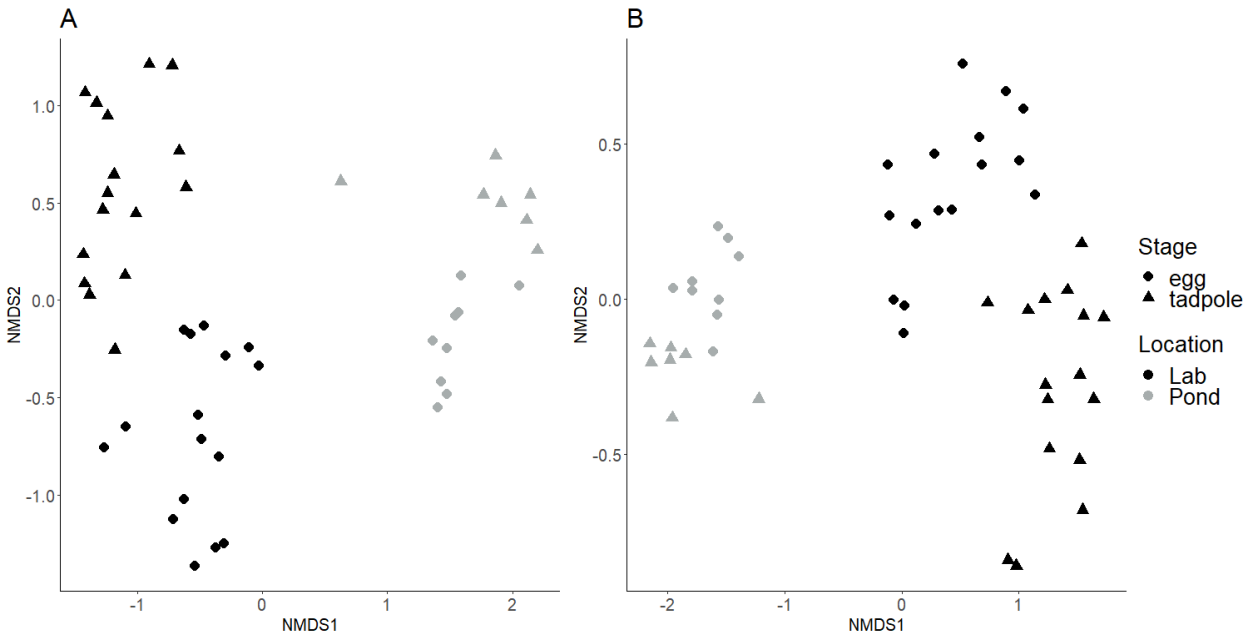
(68) Ellegaard, K. M.; Engel, P. Genomic Diversity Landscape of the Honey Bee Gut Microbiota. *Nat Commun* **2019**, *10* (1), 446. https://doi.org/10.1038/s41467-019-08303-0.

Figure 1



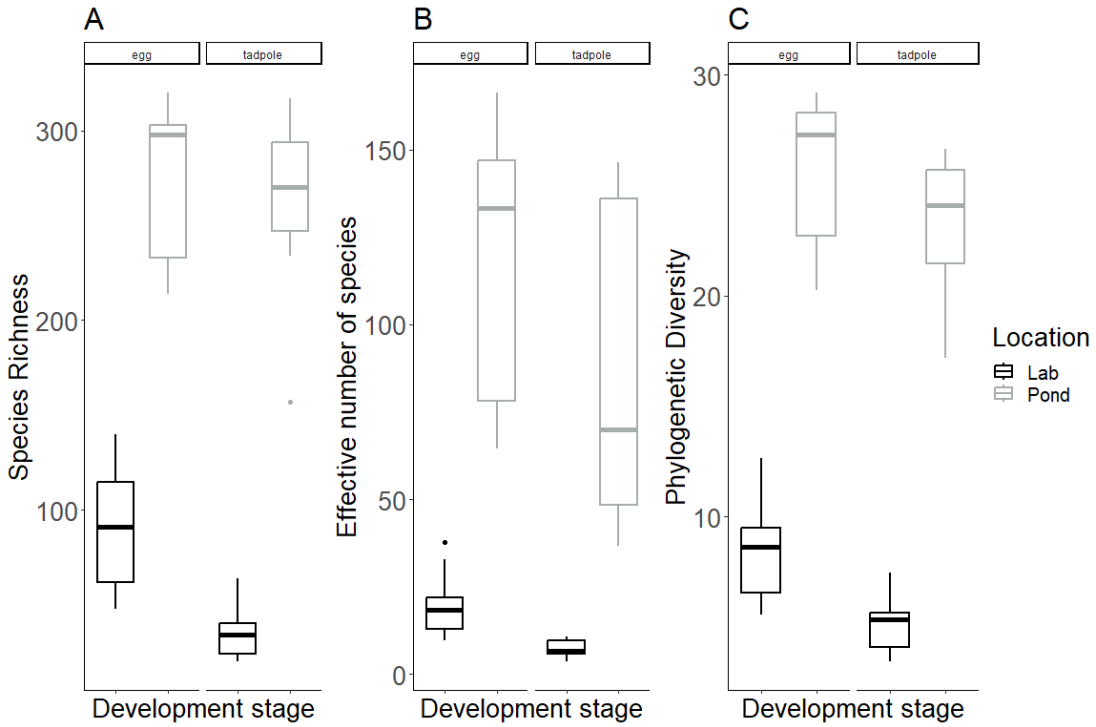
Conceptual diagram describing overview of the experimental design. A) Differential Rearing Experiment: Amplexed pairs of adult spring peepers were obtained in the field. Pairs were then left to breed in containers located in either the lab or the field. A subset of eggs were collected from each environment for processing and a subset were left to hatch into tadpoles. Lab eggs were reared in well plates and field eggs in mesh enclosures. Tadpoles were later collected and processed. B) Inoculation Experiment: Eggs were collected from breeding pairs and rinsed with sterilized water. Overnight broth cultures were created from plated stock cultures and used to inoculate eggs in well plates. On the second day, the initial inoculum was removed and replaced with a community inoculum made from broth cultures of five plated isolates. Eggs remained in the second inoculum until they developed into free-swimming tadpoles. Tadpoles were then collected and the experiment concluded.

Figure 2



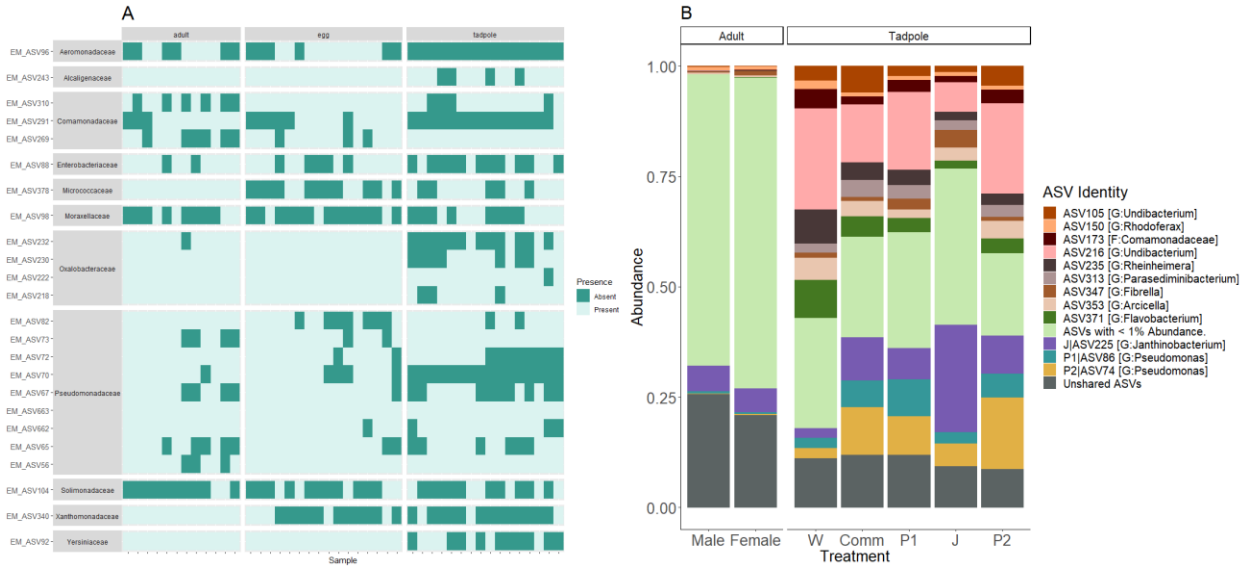
NMDS ordinations of whole egg and tadpole samples from the differential rearing experiment. Communities differed based on life stage, rearing location, and parentage in terms of **A)** Bray-curtis and **B)** Jaccard distance matrices.

Figure 3



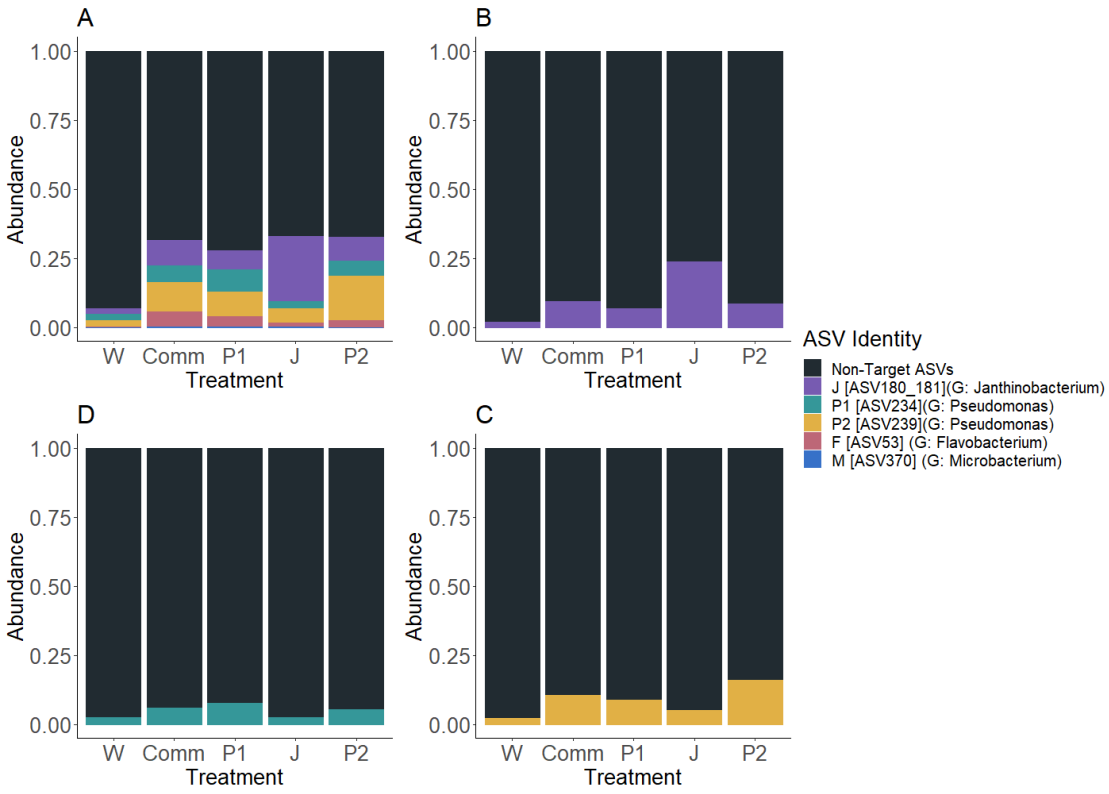
Alpha diversity of whole egg and tadpole bacterial communities from the differential rearing experiment. Life stage and rearing location impacted **A)** species richness, **B)** the effective number of species, and **C)** phylogenetic diversity.

Figure 4



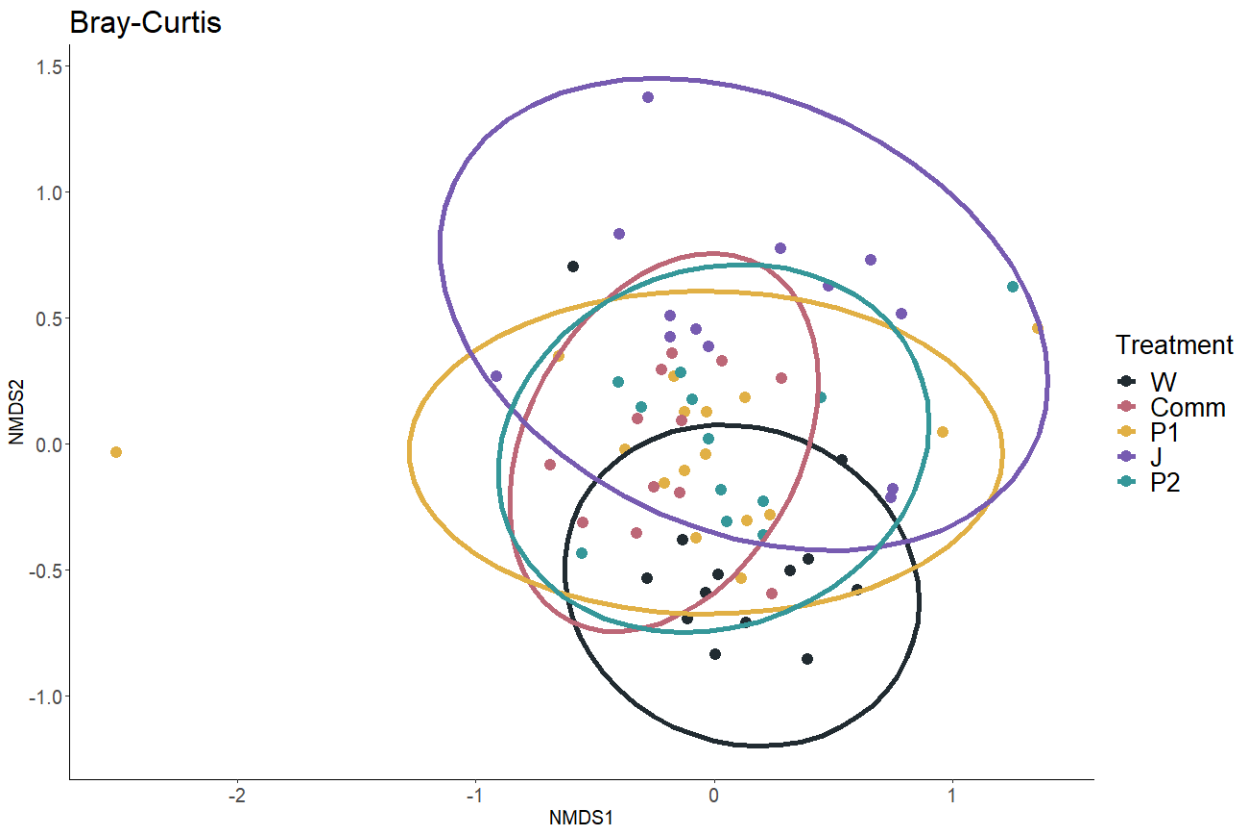
A) Heatmap of ASV presence or absence according to life stages in whole eggs, whole body tadpoles and adult skin swabs from the differential rearing experiment. **B)** Relative abundance plot of ASV overlap between whole body tadpoles and adult skin swabs from samples in the inoculation experiment. ASVs representing isolates are labeled with the isolate letter.

Figure 5



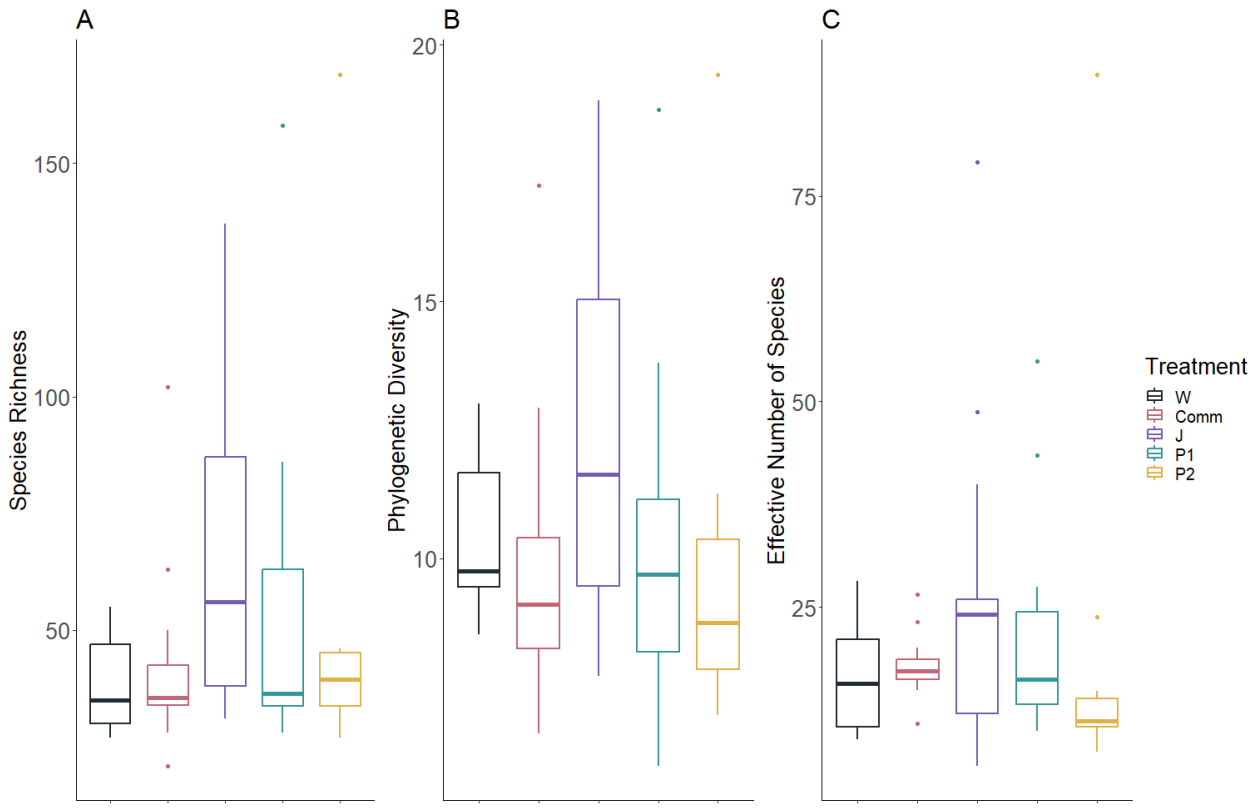
Relative abundances of isolates across treatments from the inoculation experiment. Treatment J showed an increase in the relative abundance of isolate J compared to all other treatments. Treatments P1 and P2 only showed increased initial isolate abundances when compared to treatment J or W.

Figure 6



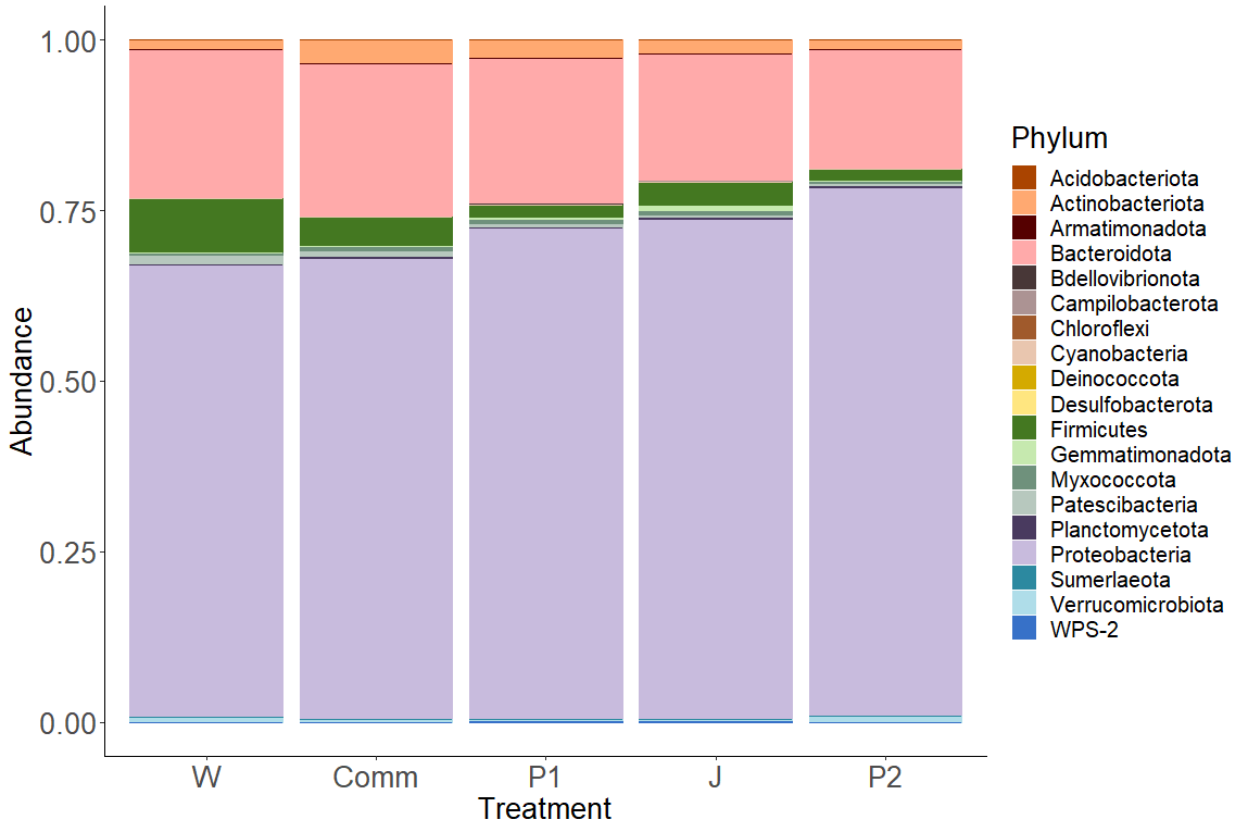
NMDS plot created using a Bray-Curtis distance matrix of samples from the inoculation experiment. The relative abundances of taxa in tadpole communities differed due to treatment.

Figure 7



Bar plot of alpha diversity metrics among inoculation experiment samples. Samples did not differ in A) species richness, B) phylogenetic diversity, nor C) the effective number of species due to treatment.

Figure S1



Relative abundance bar plot of phylum level representation among experimental inoculation samples.

Title: Virginia Amphibian Microbiome Structure Differs Among Species and Across Development

Authors: Jones, K. R., Duong, T., Sacci, O., Gregory, C., Belden L. K.

Abstract:

Host-associated microbial communities are nearly ubiquitous and essential to the growth and development of a variety of host organisms. The assembly of these communities on hosts is the result of a combination of factors, including host-specific factors and environmental availability of symbionts. In the case of some species, such as Ambystomatid amphibians, symbionts essential to development are quickly acquired during embryonic development in the environment, while other species may have symbionts horizontally transferred from parents. Unlike most other vertebrate taxa, many amphibians undergo metamorphosis, facilitating drastic physiological changes. While it is understood that metamorphosis impacts the composition of amphibian-associated microbial communities, we do not have a complete understanding of these impacts among amphibians occurring in close proximity. To more thoroughly characterize the bacterial communities associated with amphibians, and to understand how these communities vary across species and development, we collected eggs from five locally occurring amphibians and whole-body samples from tadpoles and juveniles from four of these species. Additionally, we reared one species, spring peepers (*Pseudacris crucifer*), in outdoor 1000L mesocosms to determine how stochasticity among habitat patches may affect the microbiome of conspecifics. Through 16S rRNA gene amplicon sequencing, we found distinct bacterial communities across amphibian species and development. Additionally, we observed differences in community composition among mesocosms for our mesocosm-reared spring peeper tadpoles. The results of this study serve to deepen our understanding of natural shifts in amphibian-associated bacterial communities over time, that begin very early in development with variation in the bacterial communities associated with embryos. This study also highlights the role of stochasticity and environmental source pools in the development of the bacterial communities among individuals within a species.

Introduction:

Microbes are nearly ubiquitous and have adapted to live in a great diversity of environments. Among these environments, host-associated microbial communities, or "microbiomes", are of great scientific interest due to their functional significance for their hosts. Microbiome composition influences host behavior, health, and physiology across a variety of host taxa. Microbiome composition, however, is influenced by host ecology, health, and developmental stage, as well as the environment. Despite the functional importance of the microbiome, we are still developing a thorough understanding of the factors that influence community composition across the life span of the host.

Dispersal and selection (as defined in [1]) are two of the major factors influencing macro ecological community assembly and stability, and these factors appear to apply to host-associated microbial communities as well. Studies investigating the role of environmental source pools in host microbiome composition have highlighted how bacterial dispersal from the environment may affect the composition of host-associated communities [2–4]. Additionally, differences in community composition between cohabitating species and between species and their environment showcase how selection by the host influences which taxa are present within the microbiome [5,6]. In amphibians, for example, hosts can be extremely selective in which bacteria they accumulate from the environment[6] and host specific traits lead to the development of species specific microbiomes[7]. Even within cohabitating conspecifics, traits

such as host genetics [8,9] and physiological changes [10–13] can act to influence the composition of the microbiome.

Environmental source pools are a determining factor in microbiome development. Large changes in environmental conditions, such as changing seasons, relocation to captivity or geographic gradients have been associated with differences in microbiome composition[14–16]. Small changes in environmental conditions, however, may also impact microbiome composition. In the field of ecology, mesocosms are often employed as a semi-controlled way to mimic natural environmental conditions, however stochasticity in colonizing taxa can cause equivalent habitat patches to develop different communities. In the case of macro-ecological communities this may be controlled through mesh netting or other barriers, however it is more difficult for researchers to control the effects that stochasticity may have on the microbial communities within mesocosm environments, leading to potential consequences in studies examining host-associated microbiomes.

Host-associated microbiomes often play important roles in host development. To acquire symbionts critical to development, some species vertically transfer critcal symbionts to offspring [17–19], while others may quickly accumulate symbionts from the environment during early development[20]. For organisms that produce large, aquatic egg masses, like those of the wood frogs in this study, it may be difficult for all embryos to access oxygen [21]. The embryos of the spotted salamander, *Ambystoma maculatum*, overcome this challenge by associating with the green algae, *Oophila amblystomatis* [22]. *O. amblystomatis* is well studied within the *A. maculatum* system; however, it has also been found associated with frog embryos, including wood frogs and northern red-legged frogs (*Rana aurora*), and other salamander species, including northwestern salamanders (*A. gracile*). It has not been idenftified previously in Jefferson salamander eggs [22,23]. As most work within the *O. amblystomatis* symbiosis system has been done with the ambystomatid salamanders for which it is named, our understanding of host specificity within this system remains insufficiently studied [22–24].

Amphibians are relatively unique among vertebrates as most undergo metamorphosis, resulting in drastic developmental changes. In many amphibians, this transition is marked by a keratinization of the skin [25,26], a shift in diet [27], and a transition from an aquatic to a more terrestrial lifestyle. These physiological and lifestyle shifts make frogs an interesting study system in which to explore changes in microbiome composition. Prior surveys have shown that bacteria inhabiting the amphibian gut shift along with changes in diet and development [28]. For those bacteria present on the skin, the transition from tadpole to juvenile presents a similar shift as the skin environment changes, influencing host-microbe interactions [13,26,29]. In the case of the fungal pathogen *Batrachochytrium dendrobatidis,* for example, the keratinization of the skin following metamorphosis allows the fungus to expand from the mouthparts of tadpoles to the skin of juvenile frogs [26]. Additionally, changes in host ecology, such as moving from an aquatic to a terrestrial lifestyle changes which bacteria are available in the host environment, and ultimately impact the composition of the host-associated microbial communities.

In this study, we characterized the bacterial communities associated with Virginia amphibians. First, we wanted to understand how frog associated communities shift over the course of development as hosts mature from embryos into juvenile frogs and if patterns in community structure could be observed between species across development. Second, we wanted to understand how bacterial communities associated with these species are initially structured among eggs, including potential associated with *O. amblystomatis.* Lastly, we sought to determine if stochasticity associated with minor environmental

differences may lead to observable differences in bacterial community composition among conspecifics by raising spring peeper tadpoles in an outdoor mesocosm experiment.

Methods:

Egg Collection:

From March through April 2022, we collected eggs of five amphibian species from two field sites located in Blacksburg, Virginia, USA: wood frogs (*Lithobates sylvaticus*, n=6), American toads (*Anaxyrus americanus* n=6), gray treefrogs (*Hyla versicolor*, n=8), and Jefferson salamanders (*Ambystoma jeffersonianum*, n=8)). A sample consisted of 10 eggs from a single, naturally-laid clutch. In addition, spring peeper (*Pseudacris crucifer*, n=12) eggs were collected by placing amplexed pairs overnight in buckets with pond water. Eggs were collected by hand using a new pair of gloves for each collection point, placed into sterile 50ml tubes, gently rinsed twice with 50ml sterile DI water, and then stored on ice until returned to the lab. In the lab, eggs were rinsed once more, drained of excess water, and stored at -70 C.

Tadpole and juvenile collection:

For frog species aside from spring peepers (which were raised in pond enclosures and mesocosms), tadpoles (*Lithobates sylvaticus*, n=9, *Bufo americanus* n=9, *Hyla versicolor*, n=10) and juvenile metamorphs (*Lithobates sylvaticus*, n=8, *Bufo americanus*, n=9, *Hyla versicolor*, n=13) were collected from our two field sites. Tadpoles were collected using sterile gloves and a dipnet and were placed into a sterile 1 L Nalgene container along with water from the site. In the lab, tadpoles were weighed, examined to determine developmental stage (Gosner 1960), rinsed and flash frozen in 1.5 ml microcentrifuge tubes using a slurry of dry ice and ethanol. Juveniles (Gosner stage >44) were collected in individual sterile petri dishes and stored on ice until brought to the lab. In the lab, juveniles were weighed, rinsed and flash frozen as above. Frozen samples were stored at -70 C until DNA extraction.

Pond enclosure and mesocosm study

In March 2022, 1000L cattle tank mesocosms at Virginia Tech (Montgomery County, VA) were rinsed and filled with ~500L of dechlorinated city water. Tanks were then seeded with 300g of dried mixed hardwood leaves and 1L of zooplankton and phytoplankton collected from a pond near the site. Tanks were then left covered with mesh lids to allow for the development of a semi-natural pond habitat. These types of mesocosms have commonly been used to mimic pond environments in ecological studies ([30,31]).

Additionally, two mesh field enclosures (46L x 24W x 22H) were placed in a nearby pond. These enclosures were seeded with 50g of dried mixed hardwood leaves. To acquire eggs for the field enclosures, at the end of March, three amplexed pairs of spring peepers were collected at Kentland Farm. Individual pairs were collected by hand using a new set of sterile gloves for each pair. Upon collection, pairs were placed into sterile, lidded, five-gallon buckets with ~3 cm of water from the collection site that were tilted to ensure both wet and dry areas were available. The following morning, buckets were checked for the presence of eggs. We transferred eggs from each bucket to falcon tubes to ensure that all clutches would be represented in both enclosures. Eggs (n=50/enclosure) were placed in each enclosure. To characterize the initial egg bacterial communities, a subset of eggs from the buckets

(10/clutch, n=3) were collected in sterile 1.5 ml microcentrifuge tubes and placed on ice until transfer to -70 C in the lab.

To acquire eggs to seed the mesocosms, in April, 2022, we collected four additional amplexed pairs of adult spring peepers at Kentland farm. We collected eggs from these pairs as above. Eggs were pooled together, randomized into sets of 50, and then gently placed within one of five cattle tanks (n=50/tank) and each tank was covered with a mesh lid. To characterize the initial egg bacterial communities, a subset of eggs from these pairs (n=4) were collected as above.

In May 2022, we collected six tadpoles from one of the field enclosures using sterilized dipnets. Tadpoles were placed in a 1 L Nalgene container along with pond water and stored on ice until returned to the lab. Once in the lab, tadpoles were weighed, examined to determine developmental stage [32], rinsed with ~20 ml sterile water and flash frozen as above.

Mesocosms were monitored daily to check for free-swimming tadpoles. Five weeks later (May), six tadpoles were collected from each tank using sterilized dipnets (total n=24). Tadpoles from each tank were placed in 1 L Nalgene containers along with tank water and placed on ice until they were taken to the lab to be processed as above. Additionally, styrofoam floats were placed in each tank in preparation for emerging juveniles.

In June, ~1 month after sampling tadpoles, juvenile metamorphs began to emerge in the mesocosms. Juveniles were collected as they emerged, with the final juvenile sampled in July. Using sterile gloves and dipnets, juveniles were collected (n=7) and transferred to individual petri dishes on ice. Upon arriving in the lab, juveniles were weighed, rinsed with ~20 ml of sterile water and then flash frozen within 1.5 ml microcentrifuge tubes as with the tadpole samples.

Sample DNA extraction, amplification, and sequencing:

DNA extraction from whole body amphibians and eggs was conducted using the DNeasy blood and tissue extraction kit (Qiagen, Inc, Valencia, CA, USA). We followed the manufacturer's protocols, including a 1 hr pretreatment with 180 µl of enzymatic lysis buffer followed by a 30-minute incubation with proteinase k at 70 C. Prior to pretreatment incubation, whole body amphibian and egg samples were mechanically ground with a sterile pestle (Fisher Scientific, Waltham, MA). Due to size, the gray treefrog and wood frog eggs were mechanically ground in sets of 10, and then 500 µl of the homogenized mixture was used in DNA extraction. We targeted the V4 region of the 16S rRNA gene and used the 515F and barcoded 806R primer pair developed by Caporaso et al.[33] for amplification. Each 25 µl PCR reaction contained 0.5 µl of each primer, 9.5 µl ultra clean PCR grade water, 12.5 µl Promega Master Mix, and 2 µl genomic DNA. The thermocycler settings were: 3 minutes at 94 C, then 34 cycles at 94 C for 45 s, 50 C for 60 s, 72 C for 90 s, and final extension for 10 minutes at 72 C. Reactions were run in triplicate for each sample, along with a negative control that did not contain DNA template. Triplicates were combined after PCR. The combined PCR products were quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA). The samples (total n=137 were split across two sequencing runs. Each pooled sample for sequencing contained 200ng of DNA from each sample in the pool. We cleaned the two pooled samples using the QIAquick PCR purification kit according to manufacturer instructions (Qiagen, Inc., Valencia, CA, USA). Sequencing was done on an Illumina MiSeq instrument using a 250 base-pair single-end strategy by the Molecular Biology Core Facilities of the Dana

Farber Cancer Institute at Harvard University (Cambridge, MA, USA). Raw sequence data are available upon request.

We processed our sequence data using the Qiime2 [34] pipeline, including demultiplexing, denoising, and dereplication. We denoised our sequences using DADA2 [35], with the quality score set at 11. After this step, the two amplicon sequence variants (ASV) tables from our runs were merged. Any ASVs with with less than 0.01% of reads across all samples were filtered out. The final table for our 137 samples contained a total of 828 unique ASVs. We assigned taxonomy to the ASVs with scikit-learn and the SILVA taxonomic classifier version 138 [36]. We constructed a tree with a midpoint root using RAxML [37]. We imported tables into R v4.1.0 for further processing, including filtering out any ASVs that were not bacteria, any ASVs identified as chloroplasts or mitochondria, and any ASVs that could not be identified at the phylum level. Prior to statistical analysis, one sample containing only three reads was removed. We created an additional dataset for the egg samples only that retained the chloroplast reads, as some amphibian embryos develop a symbiotic relationship with algae and we wanted to screen for that.

Statistical Analysis:

All statistical analyses were conducted in the R environment. To evaluate differences in bacterial community composition among the four species for which we had samples from all life stages (wood frogs, spring peepers, toads, gray treefrogs), we first converted raw ASV count data to relative abundances. Next, we investigated differences in community composition due to species and life stage using 1000 permutation PERMANOVA (function: Adonis2; package Vegan v2.6-4 [38]) analyses on Bray-Curtis (relative abundance) and Jaccard (presence/absence) distance matrices, and visualized these data using NMDS ordinations. To determine if egg samples showed greater within-life stage similarity than tadpole or juvenile samples, we analyzed differences in dispersion between life stages (function Betadisper; package Vegan). Bray-Curtis and Jaccard based PERMANOVA analyses were also applied to understand compositional differences within our egg samples based on species and also for individual species based on life stage (results in supplement).

For our spring peeper samples, we similarly used PERMANOVA analyses on Bray-Curtis and Jaccard matrices to understand differences in community structure due to mesocosm ID. Mesocosm IDs were set as follows: cattle tanks 1, 2, 3, and 4 or pond enclosure 1.

To gain a better understanding of differences in: (1) bacterial communities across species and life stages among frog samples, (2) species egg samples, and (3) between spring peeper tadpoles raised in different environments, we used three alpha diversity metrics: richness (the number of ASVs per sample), Faith's phylogenetic diversity, and the effective number of species (Hill numbers, derived from the Shannon diversity index). Species richness and Shannon diversity were calculated using phyloseq [39]. The effective number of species was calculated using the equation [exp(Shannon diversity)][40]. We used the picante package to calculate Faith's phylogenetic diversity values. To determine if we could observe differences in species richness among our sample groups, we used an ANOVA [function Anova; package car v3.1-1] on a negative binomial linear model. Similarly, effects on phylogenetic diversity and the effective number of species were examined using ANOVAs run on generalized linear models with a log link.

For our egg stage specific analyses, we used an egg only table containing all five species in which chloroplasts were not removed. Differences in community composition were determined using Bray-

Curtis and Jaccard distance matrices to perform PERMANOVA analyses. We used this same table to determine if the algal symbiont *Oophila amblystomatis* could be associated with our egg samples.

To understand whether local amphibian eggs possessed a shared group of core taxa (ASVs consisting of at least 0.01% relative abundance found on at least 75% of target samples), we used the microbiome package.

Results:

Our whole-body frog samples possessed between 32-284 ASVs. The observed number of ASVs phylogenetic diversity, and effective number of species associated with frogs differed by species, life stage and the interaction between the two(Anova|[Species richness] species: Pr(>Chisq) < 2.2e-16, life stage: Pr(>Chisq) < 2.2e-16, interaction: Pr(>Chisq) < 2.2e-16; [phylogenetic diversity]species: Pr(>Chisq) < 2.2e-16, life stage: Pr(>Chisq) = 1.466e-12, interaction: Pr(>Chisq) < 2.2e-16; [Effective number of species] species: Pr(>Chisq) < 2.2e-16, life stage: Pr(>Chisq) < 2.2e-16, interaction: Pr(>Chisq) < 2.2e-16) (Figure 1).

The bacteria associated with local frogs differed in relative abundance and the identity of taxa due to life stage, amphibian species, and the interaction between the two ([Life Stage] Bray-Curtis: R^2=0.16129, F= 22.901, Pr(>F) = 0.001 | Jaccard: R^2= 0.15133, F= 24.426, Pr(>F) = 0.001; [amphibian species] Bray-Curtis: R^2= 0.20881, F= 19.766, Pr(>F) = 0.001 | Jaccard: R^2= 0.26505, F= 28.522, Pr(>F) = 0.001; [interaction] Bray-Curtis: R^2= 0.22493, F= 10.646, Pr(>F) = 0.001 | Jaccard: R^2= 0.22739, F= 12.235, Pr(>F) = 0.001) (Figure 2, 3). Additionally, frog egg samples were significantly less dispersed in terms of both relative abundance and presence/absence of taxa compared to both tadpoles and juveniles (TukeyHSD| [eggs/tadpoles] Bray-Curtis: p= 5.83e-5, Jaccard: p= 2.2e-6; [eggs/juveniles] Bray-Curtis: p= 0.0372, Jaccard: p= 2.39e-4) (Figure S1).

Among frog and salamander samples, species richness found on eggs ranged from 48-213 ASVs. Egg communities differed based on amphibian species in bacterial relative abundances and presence/absence (Bray-Curtis: R^2=0.61482, F= 13967, Pr(>F) = 0.001 | Jaccard: R^2= 0.66273, F= 17.193, Pr(>F) = 0.001) (Figure 4A,4B). Proteobacteria was the phylum with the highest relative abundance on all egg samples , followed by Bacteroidota and Cyanobacteria before differentiating based on amphibian species (Proteobacteria: Jefferson salamander 83%, Gray Treefrogs 78%, Spring peepers 53%, Toads 65%, Wood Frogs 70%; Bacteroidota: Jefferson salamander 12%, Gray Treefrogs 14%, Spring peepers 23%, American Toads 10%, Wood Frogs 7%; Cyanobacteria: Jefferson salamander 3%, Gray Treefrogs 3%, Spring peepers 16%, Toads 17%, Wood Frogs 21%) (Figure 4C). Among the Cyanobacteria, we identified 3 ASVs that corresponded to *Oophila amblystomatis* within our wood frog and Jefferson salamander samples (Figure S2). Our egg samples harbored 15 bacteria that could be considered core to their bacterial communities. These ASVs belonged to phylum Proteobacteria (13/15 ASVs) and Bacteroidota (2/15 ASVs). These core ASVs represented 21% of the total community relative abundance across all eggs and ranged from 10-41% on individual amphibian species(Figure S3).

Limiting analysis to just our spring peeper samples, the relative abundance and identity of taxa present within the microbiome differed by life stage (Bray: R^2=0.37355, F= 13.715, Pr(>F) = 0.001 | Jaccard: R^2=0.35243, F= 12.518, Pr(>F) = 0.001). Spring peeper life stages also differed in species richness,

phylogenetic diversity and the effective number of species (species richness: Pr(>Chisq)=2.2e-16, phylogenetic diversity: Pr(>Chisq)=2.2e-16 effective number of species: Pr(>Chisq)=7.229e-12). Further subsetting our data to include only our reared tadpole samples, mesocosm ID influenced both the relative abundance and presence/absence of taxa with or without the inclusion of the pond enclosure samples ([no enclosure] Bray-Curtis R^2=0.72782, F=16.713, Pr(>F) = 0.001 | Jaccard: R^2=0.6321, F= 10.738, Pr(>F) = 0.001; [enclosure included] Bray: R^2=0.66274, F= 13.1, Pr(>F) = 0.001 | Jaccard: R^2=0.52072, F= 7.243, Pr(>F) = 0.001) (Figure 5). The number of observed ASVs, phylogenetic diversity, and the effective number of species also differed by mesocosm ID (species richness: Pr(>Chisq) = 2.397e-14) (phylogenetic diversity: Pr(>Chisq) = 7.731e-09) (effective number of species: Pr(>Chisq) < 2.2e-16) (Figure 6). These differences in alpha diversity remained significant with the removal of the enclosure samples.

Discussion:

The results of this study serve to emphasize the impacts of species and life stage on the bacterial communities associated with amphibians. Hatching and metamorphosis are major developmental events in terms of changing host physiology and also microbiome development [41,41,42]. Changes in host physiology, such as the transition from egg jelly to tadpole skin or the keratinization of skin as tadpoles metamorphose into juvenile frogs, may contribute to these compositional differences in the microbiome. Similarly, differences in host ecology, physiological characteristics, and genetics contribute to differences among host species, leading to differences in microbiome composition [7,13,43]. With this in mind, we expected to see similarities at the phylum level between species within developmental stages. Belden et al. (2015), for example, saw Proteobacteria as the dominant phyla in skin communities consistently across different frog species in Panama and Virginia. Among our tadpole samples, however, we did not observe any consistently dominant bacterial phyla, suggesting a potentially strong influence of individual host species characteristics. Because this study uses whole body samples, it is unclear whether these differences are driven by bacteria from a particular body site. Based on the dominance of Proteobacteria in prior work, we could speculate that compositional differences are due to gut communities, however, confirming this would require additional work. Similarly, metamorphosis did not lead to easily observable patterns at the phylum level, nor a significant change in dispersion compared to tadpole samples.

Within this study we also sought to develop a broader understanding of egg-associated communities across locally co-occurring species. Compared to later life stages, the bacterial communities within our egg samples were the least dispersed in terms of relative abundance and presence/absence of taxa. This results supports the idea that egg jelly and young embryos may be a relatively open niche for bacterial colonists [19]. Looking across species at the phylum level, eggs communities were consistently dominated by Proteobacteria and Bacteroidota. That we see differences among species, however, suggests that some degree of specificity influences egg colonization scenarios. Adult frogs may supply embryos with initial suitable bacteria to mitigate some of the potential challenge associated with acquiring environmentally rare taxa [6], however demonstrating vertical transmission of functionally important bacteria would require further research [44,45].

We saw evidence of *Oophila amblystomatis* present within our wood frog eggs, but, unexpectedly, only at extremely low relative abundances within our Jefferson salamander eggs[22]. It is well known that *O.*

amblystomatis associates with the *A. maculatum* salamanders for which it was named, and it is hypothesized to associate with other members of the genus *Ambystoma*[22,24]. It may be the case that our Jefferson salamander egg samples were collected prior to the effective establishment of *O. amblystomatis*, that our methods were not efficient to accurately assess the presence of *O.amblystomatis* [46], or that the Jefferson salamanders present at our sample site do not strongly associate with *O. amblystomatis*. That we did find evidence of *O. amblystomatis* within our wood frog eggs, supports prior research and the idea that large egg mass producing amphibians can mitigate potential oxygen issues through the use of algal symbionts[22–24].

Environmental bacteria are likely an important source pool for host-associated microbiome composition in many host taxa [2,15,47]. In the field of amphibian ecology, many researchers employ the use of mesocosms to retain a semblance of the natural environment. Field enclosures (Bletz, Perl, et al., 2017; Walke et al., 2021) and mesocosms[30,48] have been used in studies of the amphibian microbiome to study shifts in microbial community structure over time in the presence of natural environmental source pools . Our tadpoles that were raised in cattle tanks developed a simplified microbiome relative to those raised in pond enclosures. Mean ASV richness in the cattle tanks was 62, relative to 98 in the field enclosures. There were also community structure differences in the microbiomes of tadpoles between our cattle tank and mesh pond enclosure environments. In mouse model systems, studies disregarding differences gut composition due to mouse vendor sources may result in difficulty reproducing results[51]. Deciphering if these differences in community composition had functional consequences between cattle tanks and enclosure reared tadpoles was beyond the scope of this study, however, these concerns may be of interest in future projects.

In addition to broad environmental source pool differences, stochastic processes such as priority effects can also cause otherwise equivalent habitat patches to develop different communities [52–54]. We set up our cattle tanks concurrently with the same materials to create equivalent environments for our tadpoles, yet we saw differences in the microbial communities associated with tadpoles even from different tanks. Speculating on the differences between tadpoles raised in different tanks, priority effects may have served as the driver of microbiome composition in this system at the level of host or tank [55,56].

Overall, this study joins the growing body of literature highlighting the complexity of factors that contribute to microbiome composition. The data presented reinforce our understanding of species level differences in amphibian microbiomes (eg. Belden et al., 2015), as well as the impact of developmental transitions on microbiome structure (eg. Kohl et al., 2013; Kowallik & Mikheyev, 2021). Additionally, priority effects may be a driving factor in intraspecific differences in microbiome composition after accounting for any differences in environment. As we continue to study the interplay between these factors, we can better understand and predict how a given set of circumstances may shape host-associated bacterial communities.

(1) Vellend, M. Conceptual Synthesis in Community Ecology. *The Quarterly Review of Biology* **2010**, *85* (2), 183–206. https://doi.org/10.1086/652373.

(2) Bates, K. A.; Friesen, J.; Loyau, A.; Butler, H.; Vredenburg, V. T.; Laufer, J.; Chatzinotas, A.; Schmeller, D. S. Environmental and Anthropogenic Factors Shape the Skin Bacterial Communities of a Semi-Arid Amphibian Species. *Microb Ecol* **2022**. https://doi.org/10.1007/s00248-022-02130-5.

(3) Clayton, J. B.; Vangay, P.; Huang, H.; Ward, T.; Hillmann, B. M.; Al-Ghalith, G. A.; Travis, D. A.; Long, H. T.; Van Tuan, B.; Van Minh, V.; Cabana, F.; Nadler, T.; Toddes, B.; Murphy, T.; Glander, K. E.; Johnson, T. J.; Knights, D. Captivity Humanizes the Primate Microbiome. *Proceedings of the National Academy of Sciences of the United States of America* **2016**, *113* (37), 10376–10381. https://doi.org/10.1073/pnas.1521835113.

(4) Kueneman, J. G.; Bletz, M. C.; McKenzie, V. J.; Becker, C. G.; Joseph, M. B.; Abarca, J. G.; Archer, H.; Arellano, A. L.; Bataille, A.; Becker, M.; Belden, L. K.; Crottini, A.; Geffers, R.; Haddad, Célio. F. B.; Harris, R. N.; Holden, W. M.; Hughey, M.; Jarek, M.; Kearns, P. J.; Kerby, J. L.; Kielgast, J.; Kurabayashi, A.; Longo, A. V.; Loudon, A.; Medina, D.; Nuñez, J. J.; Perl, R. G. B.; Pinto-Tomás, A.; Rabemananjara, F. C. E.; Rebollar, E. A.; Rodríguez, A.; Rollins-Smith, L.; Stevenson, R.; Tebbe, C. C.; Vargas Asensio, G.; Waldman, B.; Walke, J. B.; Whitfield, S. M.; Zamudio, K. R.; Zúñiga Chaves, I.; Woodhams, D. C.; Vences, M. Community Richness of Amphibian Skin Bacteria Correlates with Bioclimate at the Global Scale. *Nature Ecology & Evolution* **2019**, *3* (3), 381–389. https://doi.org/10.1038/s41559-019-0798-1.

(5) Kwong, W. K.; Engel, P.; Koch, H.; Moran, N. A. Genomics and Host Specialization of Honey Bee and Bumble Bee Gut Symbionts. *Proceedings of the National Academy of Sciences* **2014**, *111* (31), 11509–11514. https://doi.org/10.1073/pnas.1405838111.

(6) Walke, J. B.; Becker, M. H.; Loftus, S. C.; House, L. L.; Cormier, G.; Jensen, R. V.; Belden, L. K. Amphibian Skin May Select for Rare Environmental Microbes. *The ISME Journal* **2014**, *8* (11), 2207–2217. https://doi.org/10.1038/ismej.2014.77.

(7) Belden, L. K.; Hughey, M. C.; Rebollar, E. A.; Umile, T. P.; Loftus, S. C.; Burzynski, E. A.; Minbiole, K. P. C.; House, L. L.; Jensen, R. V.; Becker, M. H.; Walke, J. B.; Medina, D.; Ibáñez, R.; Harris, R. N. Panamanian Frog Species Host Unique Skin Bacterial Communities. *Front Microbiol* **2015**, *6*. https://doi.org/10.3389/fmicb.2015.01171.

(8) Bolnick, D. I.; Snowberg, L. K.; Caporaso, J. G.; Lauber, C.; Knight, R.; Stutz, W. E. Major Histocompatibility Complex Class IIb Polymorphism Influences Gut Microbiota Composition and Diversity. *Molecular Ecology* **2014**, *23* (19), 4831–4845. https://doi.org/10.1111/mec.12846.

(9) Kokou, F.; Sasson, G.; Nitzan, T.; Doron-Faigenboim, A.; Harpaz, S.; Cnaani, A.; Mizrahi, I. Host Genetic Selection for Cold Tolerance Shapes Microbiome Composition and Modulates Its Response to Temperature. *eLife* **2018**, *7*, e36398. https://doi.org/10.7554/eLife.36398.

(10) Dzidic, M.; Collado, M. C.; Abrahamsson, T.; Artacho, A.; Stensson, M.; Jenmalm, M. C.; Mira, A. Oral Microbiome Development during Childhood: An Ecological Succession Influenced by Postnatal Factors and Associated with Tooth Decay. *The ISME Journal* **2018**, *12* (9), 2292–2306. https://doi.org/10.1038/s41396-018-0204-z.

(11) Kohl, K. D.; Cary, T. L.; Karasov, W. H.; Dearing, M. D. Restructuring of the Amphibian Gut Microbiota through Metamorphosis. *Environmental Microbiology Reports* **2013**, *5* (6), 899–903. https://doi.org/10.1111/1758-2229.12092.

(12) Kowallik, V.; Mikheyev, A. S. Honey Bee Larval and Adult Microbiome Life Stages Are Effectively Decoupled with Vertical Transmission Overcoming Early Life Perturbations. *mBio* **2021**, *12* (6), e02966-21. https://doi.org/10.1128/mBio.02966-21.

(13) Kueneman, J. G.; Laura Wegener Parfrey; Woodhams, D. C.; Archer, H. M.; Knight, R.; McKenzie, V. J. The Amphibian Skin-Associated Microbiome across Species, Space and Life History Stages. *Molecular Ecology* **2014**, *23* (6), 1238–1250. https://doi.org/10.1111/mec.12510.

(14) Hughey, M. C.; Sokol, E. R.; Walke, J. B.; Becker, M. H.; Belden, L. K. Ecological Correlates of Large-Scale Turnover in the Dominant Members of Pseudacris Crucifer Skin Bacterial Communities. *Microb Ecol* **2019**. https://doi.org/10.1007/s00248-019-01372-0.

(15) Jones, K. R.; Walke, J. B.; Becker, M. H.; Belden, L. K.; Hughey, M. C. Time in the Laboratory, but Not Exposure to a Chytrid Fungus, Results in Rapid Change in Spring Peeper (Pseudacris Crucifer) Skin Bacterial Communities. *Ichthyology & Herpetology* **2021**, *109* (1). https://doi.org/10.1643/h2020077.

(16) Estrada, A.; Hughey, M. C.; Medina, D.; Rebollar, E. A.; Walke, J. B.; Harris, R. N.; Belden, L. K. Skin Bacterial Communities of Neotropical Treefrogs Vary with Local Environmental Conditions at the Time of Sampling. *PeerJ* **2019**, *7*, e7044. https://doi.org/10.7717/peerj.7044.

(17) Douglas, A. E. Nutritional Interactions in Insect-Microbial Symbioses: Aphids and Their Symbiotic Bacteria *Buchnera*. *Annu. Rev. Entomol.* **1998**, *43* (1), 17–37. https://doi.org/10.1146/annurev.ento.43.1.17.

(18) Trevelline, B. K.; MacLeod, K. J.; Knutie, S. A.; Langkilde, T.; Kohl, K. D. In Ovo Microbial Communities: A Potential Mechanism for the Initial Acquisition of Gut Microbiota among Oviparous Birds and Lizards. *Biology Letters* **2018**, *14* (7), 20180225. https://doi.org/10.1098/rsbl.2018.0225.

(19) Walke, J. B.; Harris, R. N.; Reinert, L. K.; Rollins-Smith, L. A.; Woodhams, D. C. Social Immunity in Amphibians: Evidence for Vertical Transmission of Innate Defenses. *Biotropica* **2011**, *43* (4), 396–400. https://doi.org/10.1111/j.1744-7429.2011.00787.x.

(20) Gensollen, T.; Iyer, S. S.; Kasper, D. L.; Blumberg, R. S. How Colonization by Microbiota in Early Life Shapes the Immune System. *Science* **2016**, *352* (6285), 539–544. https://doi.org/10.1126/science.aad9378.

(21) Seymour, R. S.; Bradford, D. F. Respiration of Amphibian Eggs. *Physiological Zoology* **1995**, *68* (1), 1–25. https://doi.org/10.1086/physzool.68.1.30163915.

(22) Yang, H.; Genot, B.; Duhamel, S.; Kerney, R.; Burns, J. A. Organismal and Cellular Interactions in Vertebrate–Alga Symbioses. *Biochemical Society Transactions* **2022**, *50* (1), 609–620. https://doi.org/10.1042/BST20210153.

(23) Kim, E.; Lin, Y.; Kerney, R.; Blumenberg, L.; Bishop, C. Phylogenetic Analysis of Algal Symbionts Associated with Four North American Amphibian Egg Masses. *PLOS ONE* **2014**, *9* (11), e108915. https://doi.org/10.1371/journal.pone.0108915.

(24) Anslan, S.; Sachs, M.; Rancilhac, L.; Brinkmann, H.; Petersen, J.; Künzel, S.; Schwarz, A.; Arndt, H.; Kerney, R.; Vences, M. Diversity and Substrate-Specificity of Green Algae and Other Micro-Eukaryotes Colonizing Amphibian Clutches in Germany, Revealed by DNA Metabarcoding. *Sci Nat* **2021**, *108* (4), 29. https://doi.org/10.1007/s00114-021-01734-0.

(25) Berger, L.; Speare, R.; Daszak, P.; Green, D. E.; Cunningham, A. A.; Goggin, C. L.; Slocombe, R.; Ragan, M. A.; Hyatt, A. D.; McDonald, K. R.; Hines, H. B.; Lips, K. R.; Marantelli, G.; Parkes, H.

Chytridiomycosis Causes Amphibian Mortality Associated with Population Declines in the Rain Forests of Australia and Central America. *Proc Natl Acad Sci U S A* **1998**, *95* (15), 9031–9036.

(26) Marantelli, G.; Berger, L.; Speare, R.; Keegan, L. Distribution of the Amphibian Chytrid Batrachochytrium Dendrobatidis and Keratin during Tadpole Development. *Pac. Conserv. Biol.* **2004**, *10* (3), 173. https://doi.org/10.1071/PC040173.

(27) Jenssen, T. A. Food Habits of the Green Frog, Rana Clamitans, before and during Metamorphosis. *Copeia* **1967**, *1967* (1), 214. https://doi.org/10.2307/1442196.

(28) Zhang, M.; Chen, H.; Liu, L.; Xu, L.; Wang, X.; Chang, L.; Chang, Q.; Lu, G.; Jiang, J.; Zhu, L. The Changes in the Frog Gut Microbiome and Its Putative Oxygen-Related Phenotypes Accompanying the Development of Gastrointestinal Complexity and Dietary Shift. *Frontiers in Microbiology* **2020**, *11*.

(29) Van Rooij, P.; Martel, A.; Haesebrouck, F.; Pasmans, F. Amphibian Chytridiomycosis: A Review with Focus on Fungus-Host Interactions. *Vet Res* **2015**, *46*. https://doi.org/10.1186/s13567-015-0266-0.

(30) Rowe, C. L.; Dunson, W. A. The Value of Simulated Pond Communities in Mesocosms for Studies of Amphibian Ecology and Ecotoxicology. *Journal of Herpetology* **1994**, *28* (3), 346. https://doi.org/10.2307/1564534.

(31) Warner, S. C.; Dunson, W. A.; Travis, J. Interaction of PH, Density, and Priority Effects on the Survivorship and Growth of Two Species of Hylid Tadpoles. *Oecologia* **1991**, *88* (3), 331–339. https://doi.org/10.1007/BF00317575.

(32) Gosner, K. L. A Simplified Table for Staging Anuran Embryos and Larvae with Notes on Identification. *Herpetologica* **1960**, *16* (3), 183–190.

(33) Caporaso, J. G.; Lauber, C. L.; Walters, W. A.; Berg-Lyons, D.; Huntley, J.; Fierer, N.; Owens, S. M.; Betley, J.; Fraser, L.; Bauer, M.; Gormley, N.; Gilbert, J. A.; Smith, G.; Knight, R. Ultra-High-Throughput Microbial Community Analysis on the Illumina HiSeq and MiSeq Platforms. *The ISME Journal* **2012**, *6* (8), 1621–1624. https://doi.org/10.1038/ismej.2012.8.

(34) Bolyen, E.; Rideout, J. R.; Dillon, M. R.; Bokulich, N. A.; Abnet, C.; Al-Ghalith, G. A.; Alexander, H.; Alm, E. J.; Arumugam, M.; Asnicar, F.; Bai, Y.; Bisanz, J. E.; Bittinger, K.; Brejnrod, A.; Brislawn, C. J.; Brown, C. T.; Callahan, B. J.; Caraballo-Rodríguez, A. M.; Chase, J.; Cope, E.; Silva, R. D.; Dorrestein, P. C.; Douglas, G. M.; Durall, D. M.; Duvallet, C.; Edwardson, C. F.; Ernst, M.; Estaki, M.; Fouquier, J.; Gauglitz, J. M.; Gibson, D. L.; Gonzalez, A.; Gorlick, K.; Guo, J.; Hillmann, B.; Holmes, S.; Holste, H.; Huttenhower, C.; Huttley, G.; Janssen, S.; Jarmusch, A. K.; Jiang, L.; Kaehler, B.; Kang, K. B.; Keefe, C. R.; Keim, P.; Kelley, S. T.; Knights, D.; Koester, I.; Kosciolek, T.; Kreps, J.; Langille, M. G.; Lee, J.; Ley, R.; Liu, Y.-X.; Loftfield, E.; Lozupone, C.; Maher, M.; Marotz, C.; Martin, B. D.; McDonald, D.; McIver, L. J.; Melnik, A. V.; Metcalf, J. L.; Morgan, S. C.; Morton, J.; Naimey, A. T.; Navas-Molina, J. A.; Nothias, L. F.; Orchanian, S. B.; Pearson, T.; Peoples, S. L.; Petras, D.; Preuss, M. L.; Pruesse, E.; Rasmussen, L. B.; Rivers, A.; Michael S Robeson, I. I.; Rosenthal, P.; Segata, N.; Shaffer, M.; Shiffer, A.; Sinha, R.; Song, S. J.; Spear, J. R.; Swafford, A. D.; Thompson, L. R.; Torres, P. J.; Trinh, P.; Tripathi, A.; Turnbaugh, P. J.; Ul-Hasan, S.; Hooft, J. J. van der; Vargas, F.; Vázquez-Baeza, Y.; Vogtmann, E.; Hippel, M. von; Walters, W.; Wan, Y.; Wang, M.; Warren, J.; Weber, K. C.; Williamson, C. H.; Willis, A. D.; Xu, Z. Z.; Zaneveld, J. R.; Zhang, Y.; Zhu, Q.; Knight, R.;

Caporaso, J. G. *QIIME 2: Reproducible, Interactive, Scalable, and Extensible Microbiome Data Science*; e27295v2; PeerJ Inc., 2018. https://doi.org/10.7287/peerj.preprints.27295v2.

(35) Callahan, B. J.; McMurdie, P. J.; Rosen, M. J.; Han, A. W.; Johnson, A. J. A.; Holmes, S. P. DADA2: High-Resolution Sample Inference from Illumina Amplicon Data. *Nature Methods* **2016**, *13* (7), 581–583. https://doi.org/10.1038/nmeth.3869.

(36) Glöckner, F. O. The SILVA Database Project: An ELIXIR Core Data Resource for High-Quality Ribosomal RNA Sequences. *Biodiversity Information Science and Standards* **2019**, *3*, e36125. https://doi.org/10.3897/biss.3.36125.

(37) Stamatakis, A. RAxML Version 8: A Tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. *Bioinformatics* **2014**, *30* (9), 1312–1313. https://doi.org/10.1093/bioinformatics/btu033.

(38) Dixon, P. VEGAN, a Package of R Functions for Community Ecology. *Journal of Vegetation Science* **2003**, *14* (6), 927–930. https://doi.org/10.1111/j.1654-1103.2003.tb02228.x.

(39) McMurdie, P. J.; Holmes, S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE* **2013**, *8* (4), e61217. https://doi.org/10.1371/journal.pone.0061217.

(40) Jost, L. *Entropy and Diversity*. *Oikos* **2006**, *113* (2), 363–375. https://doi.org/10.1111/j.2006.0030-1299.14714.x.

(41) Bataille, A.; Lee-Cruz, L.; Tripathi, B.; Waldman, B. Skin Bacterial Community Reorganization Following Metamorphosis of the Fire-Bellied Toad (Bombina Orientalis). *Microb Ecol* **2018**, *75* (2), 505–514. https://doi.org/10.1007/s00248-017-1034-7.

(42) Warne, R. W.; Kirschman, L.; Zeglin, L. Manipulation of Gut Microbiota during Critical Developmental Windows Affects Host Physiological Performance and Disease Susceptibility across Ontogeny. *Journal of Animal Ecology* **2019**, *88* (6), 845–856. https://doi.org/10.1111/1365-2656.12973.

(43) Bletz, M. C.; Archer, H.; Harris, R. N.; McKenzie, V. J.; Rabemananjara, F. C. E.; Rakotoarison, A.; Vences, M. Host Ecology Rather Than Host Phylogeny Drives Amphibian Skin Microbial Community Structure in the Biodiversity Hotspot of Madagascar. *Front. Microbiol.* **2017**, *8*. https://doi.org/10.3389/fmicb.2017.01530.

(44) Davidson, S. K.; Powell, R. J.; Stahl, D. A. Transmission of a Bacterial Consortium in Eisenia Fetida Egg Capsules. *Environmental Microbiology* **2010**, *12* (8), 2277–2288. https://doi.org/10.1111/j.1462-2920.2010.02231.x.

(45) Nyholm, S. V. In the Beginning: Egg–Microbe Interactions and Consequences for Animal Hosts. *Philosophical Transactions of the Royal Society B* **2020**. https://doi.org/10.1098/rstb.2019.0593.

(46) Jurga, E.; Graham, L.; Bishop, C. D. Oophila Is Monophyletic within a Three-Taxon Eukaryotic Microbiome in Egg Masses of the Salamander Ambystoma Maculatum. *Symbiosis* **2020**, *81* (2), 187–199. https://doi.org/10.1007/s13199-020-00693-w.

(47) Hyde, E. R.; Navas-Molina, J. A.; Song, S. J.; Kueneman, J. G.; Ackermann, G.; Cardona, C.; Humphrey, G.; Boyer, D.; Weaver, T.; Mendelson, J. R.; McKenzie, V. J.; Gilbert, J. A.; Knight, R. The Oral

and Skin Microbiomes of Captive Komodo Dragons Are Significantly Shared with Their Habitat. *mSystems* **2016**, *1* (4), e00046-16. https://doi.org/10.1128/mSystems.00046-16.

(48) Hernández-Gómez, O.; Wuerthner, V.; Hua, J. Amphibian Host and Skin Microbiota Response to a Common Agricultural Antimicrobial and Internal Parasite. *Microb Ecol* **2020**, *79* (1), 175–191. https://doi.org/10.1007/s00248-019-01351-5.

(49) Bletz, M. C.; Perl, R. G. B.; Bobowski, B. T.; Japke, L. M.; Tebbe, C. C.; Dohrmann, A. B.; Bhuju, S.; Geffers, R.; Jarek, M.; Vences, M. Amphibian Skin Microbiota Exhibits Temporal Variation in Community Structure but Stability of Predicted Bd-Inhibitory Function. *ISME J* **2017**, *11* (7), 1521–1534. https://doi.org/10.1038/ismej.2017.41.

(50) Walke, J. B.; Becker, M. H.; Krinos, A.; Chang, E. A. B.; Santiago, C.; Umile, T. P.; Minbiole, K. P. C.; Belden, L. K. Seasonal Changes and the Unexpected Impact of Environmental Disturbance on Skin Bacteria of Individual Amphibians in a Natural Habitat. *FEMS Microbiology Ecology* **2021**, *97* (3), fiaa248. https://doi.org/10.1093/femsec/fiaa248.

(51) Ericsson, A. C.; Franklin, C. L. The Gut Microbiome of Laboratory Mice: Considerations and Best Practices for Translational Research. *Mamm Genome* **2021**, *32* (4), 239–250. https://doi.org/10.1007/s00335-021-09863-7.

(52) Devevey, G.; Dang, T.; Graves, C. J.; Murray, S.; Brisson, D. First Arrived Takes All: Inhibitory Priority Effects Dominate Competition between Co-Infecting Borrelia Burgdorferi Strains. *BMC Microbiology* **2015**, *15* (1), 61. https://doi.org/10.1186/s12866-015-0381-0.

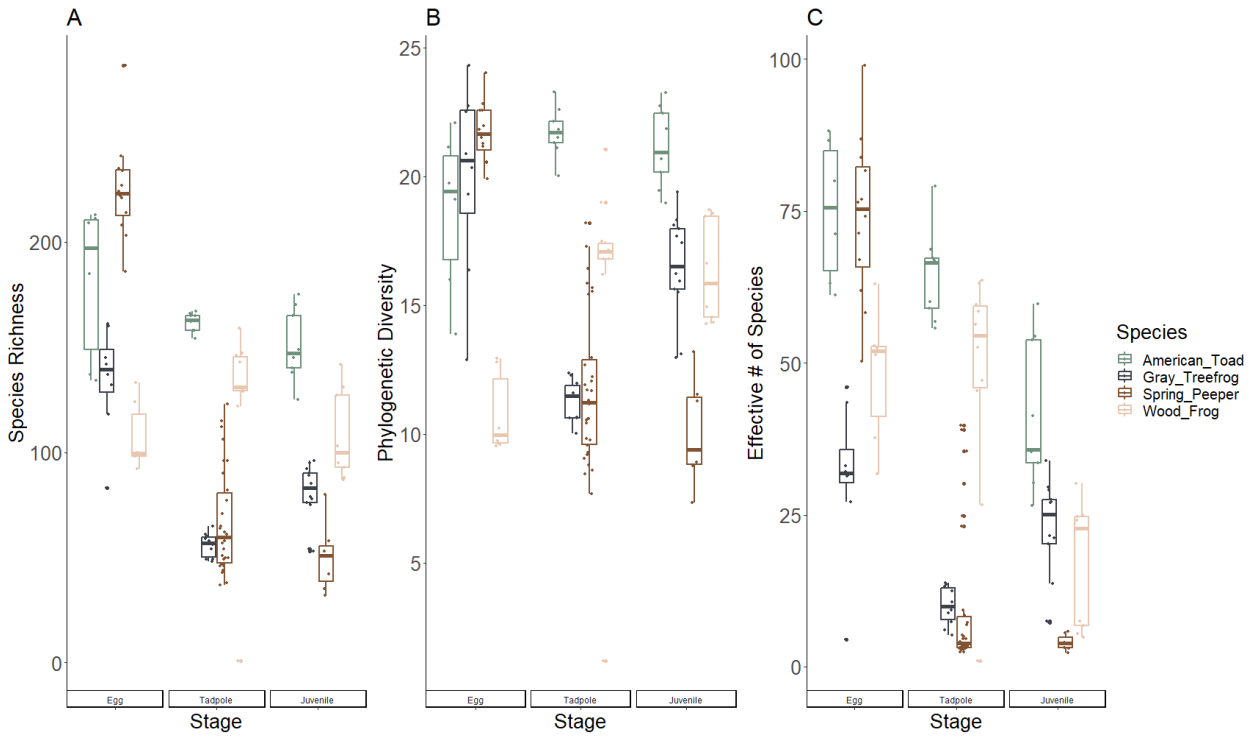
(53) Fukami, T. Historical Contingency in Community Assembly: Integrating Niches, Species Pools, and Priority Effects. *Annual Review of Ecology, Evolution, and Systematics* **2015**, *46* (1), 1–23. https://doi.org/10.1146/annurev-ecolsys-110411-160340.

(54) Jones, K. R.; Hughey, M. C.; Belden, L. K. Colonization Order of Bacterial Isolates on Treefrog Embryos Impacts Microbiome Structure in Tadpoles. *Proceedings of the Royal Society B* **2023**. https://doi.org/10.1098/rspb.2023.0308.

(55) Chase, J. M. Stochastic Community Assembly Causes Higher Biodiversity in More Productive Environments. *Science* **2010**, *328* (5984), 1388–1391. https://doi.org/10.1126/science.1187820.

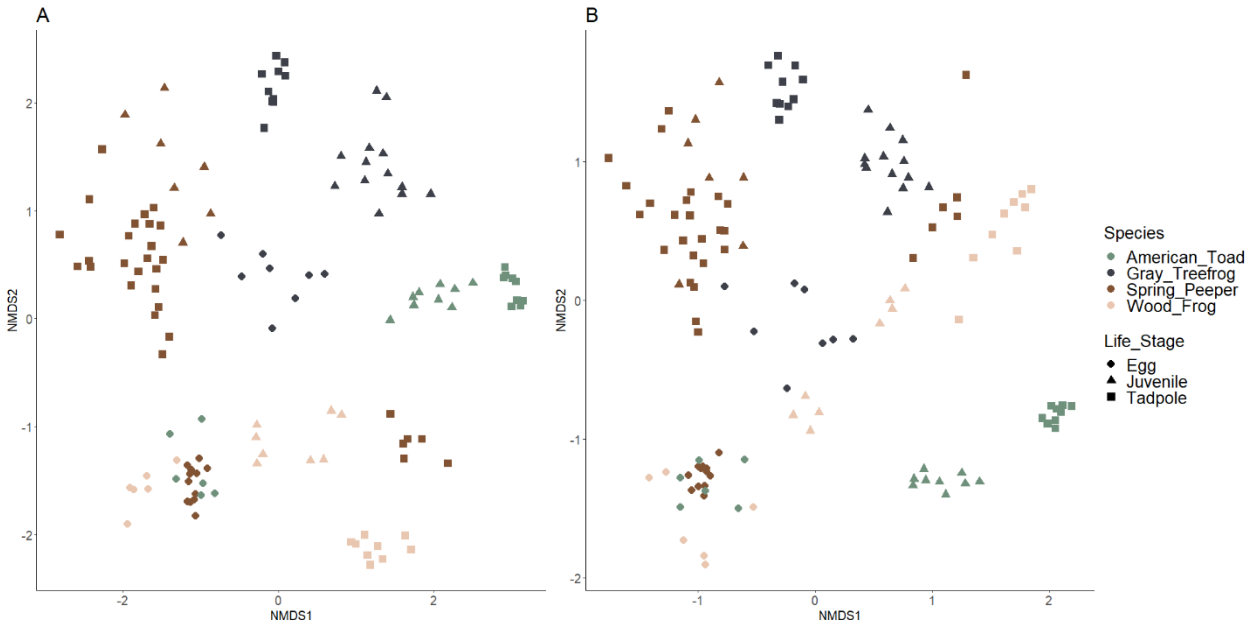
(56) Ellegaard, K. M.; Engel, P. Genomic Diversity Landscape of the Honey Bee Gut Microbiota. *Nat Commun* **2019**, *10* (1), 446. https://doi.org/10.1038/s41467-019-08303-0.

Figure 1



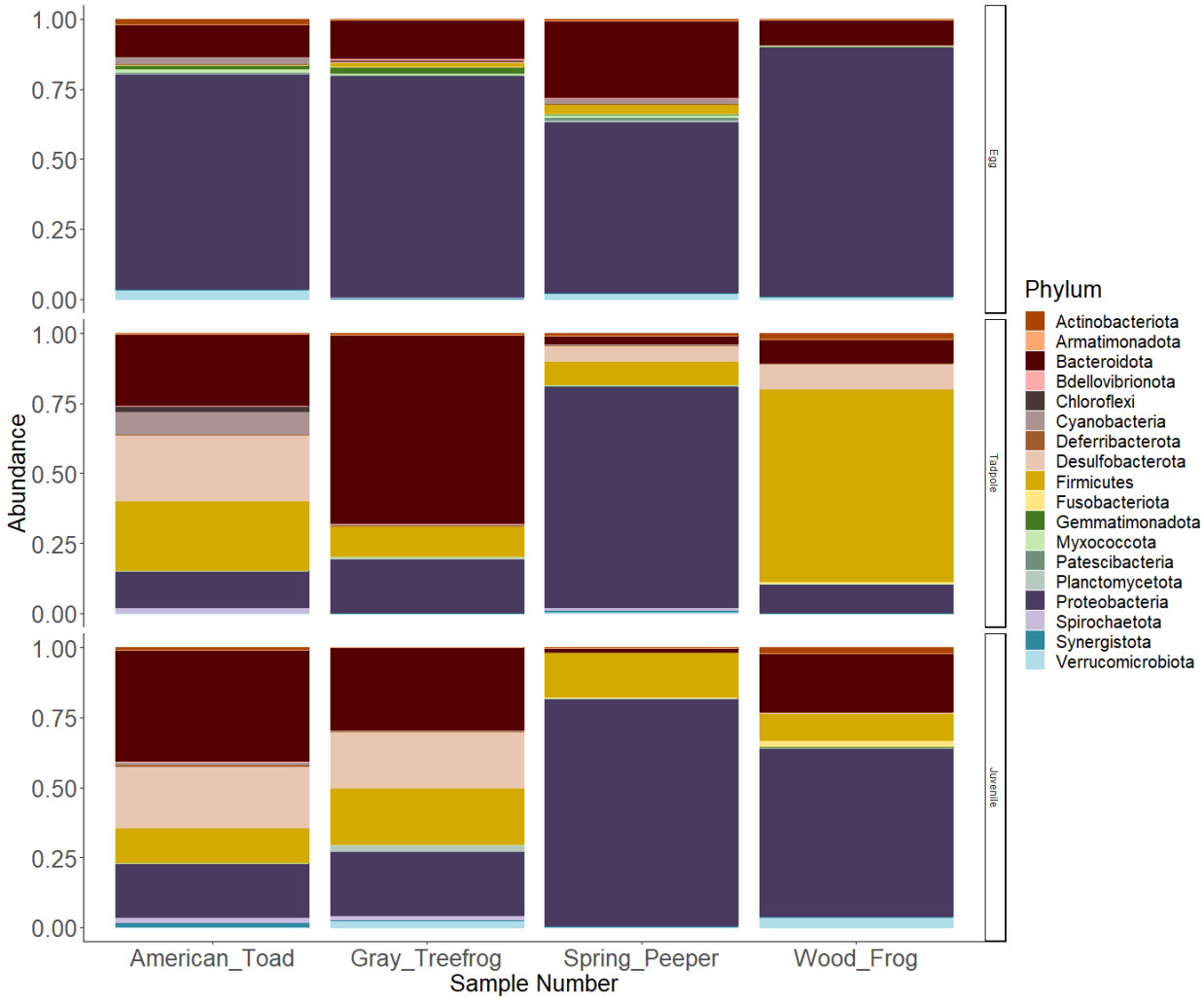
Alpha diversity metrics across species and life stages. Whole-body frog samples differed in A) species richness, B) phylogenetic diversity, and C) the effective number of species due to both species and life stage.

Figure 2



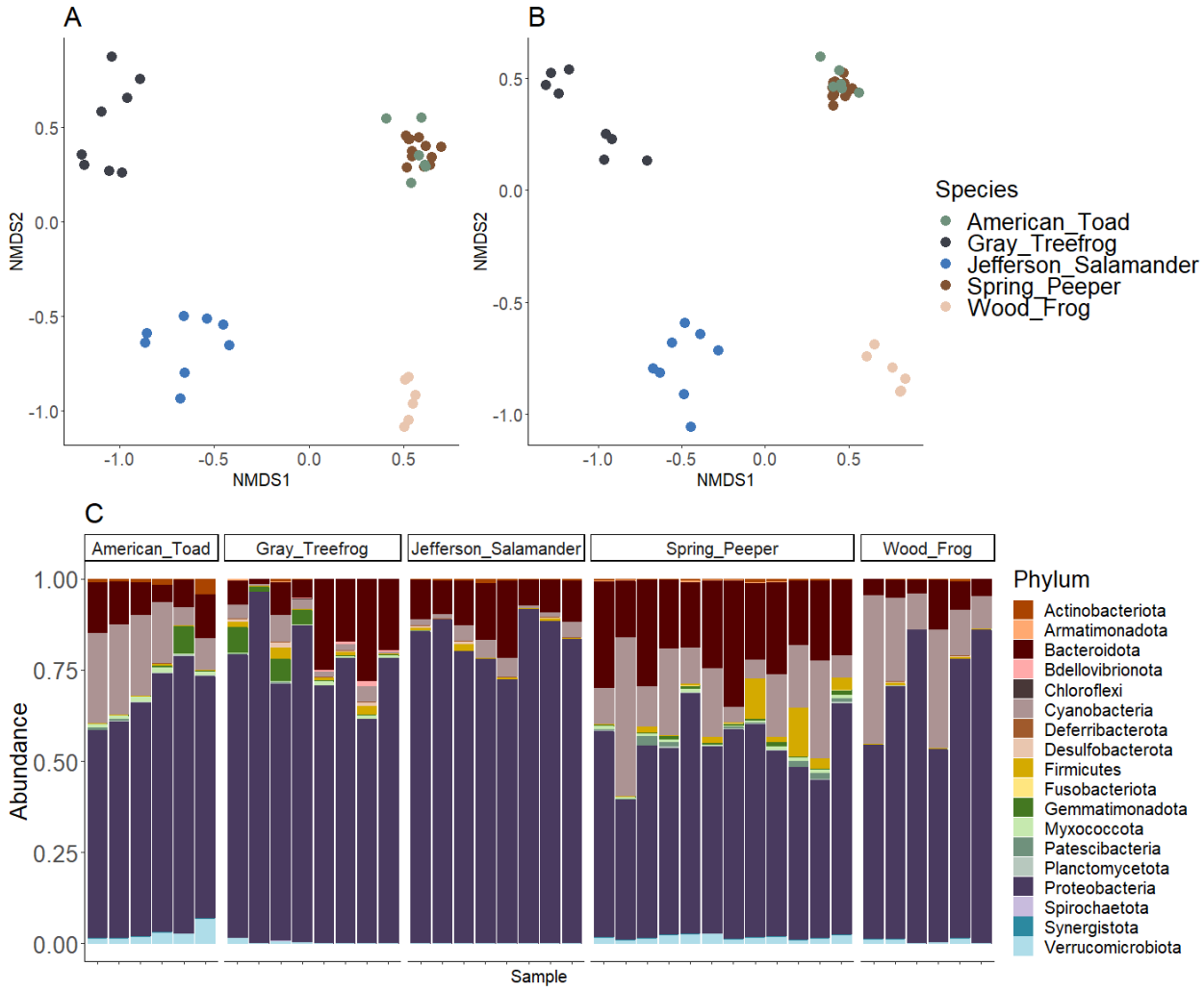
NDMS ordinations of whole-body frog bacterial communities. Communities differed in terms of the A) relative abundance and B) presence/absence of taxa across life stages and among species.

Figure 3



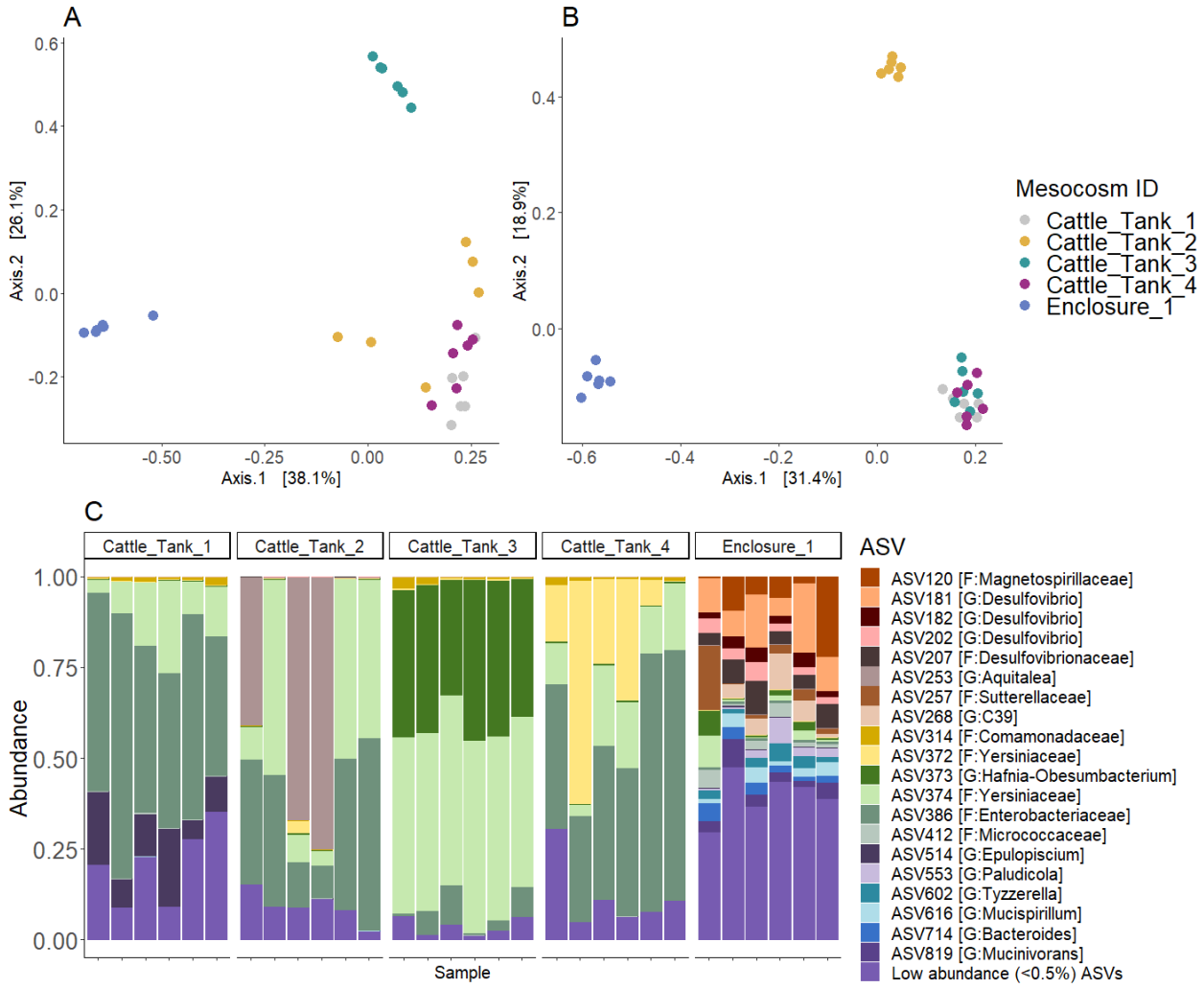
Phylum level relative abundance bar plot of bacterial communities associated with whole-body frog samples across species and developmental stages.

Figure 4



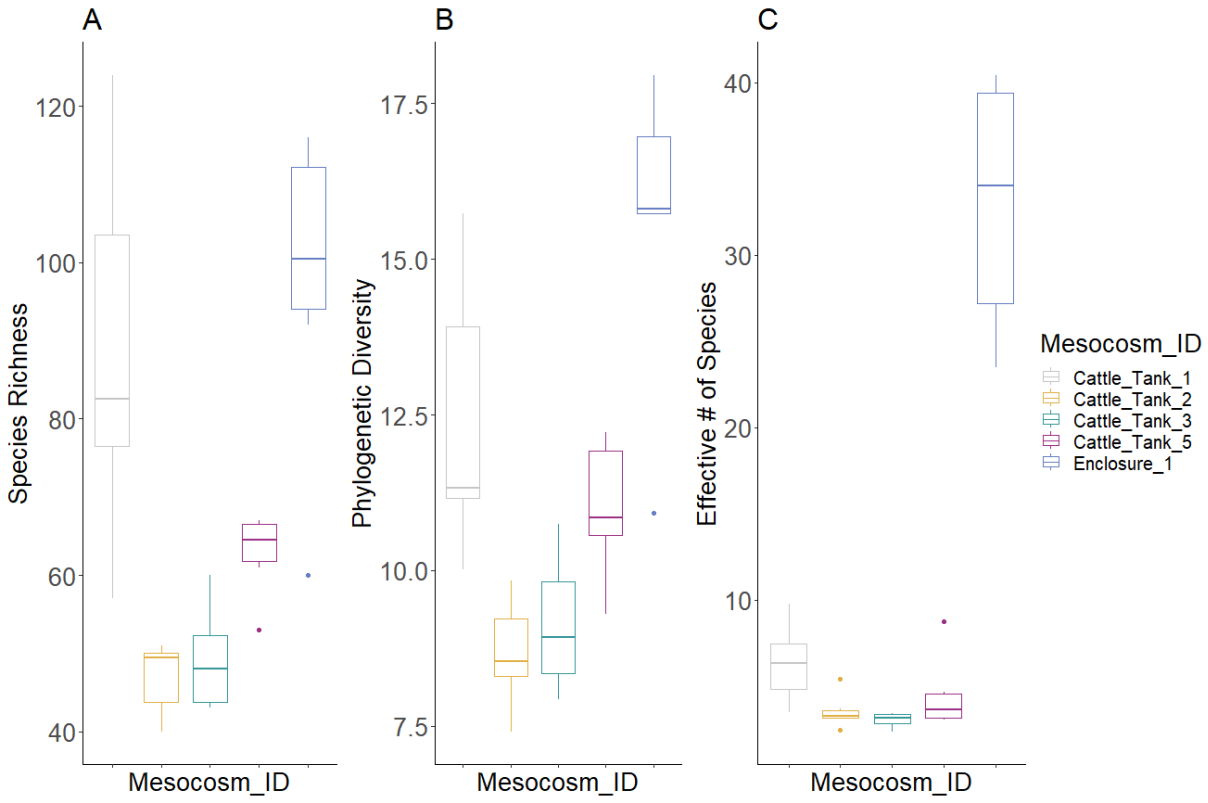
Investigating egg samples independent of other developmental stages show that egg communities differ due to life stage in terms of A) relative abundance and the B) presence/absence of taxa. C) Phylum level relative abundance plot of bacterial communities associated with egg samples.

Figure 5



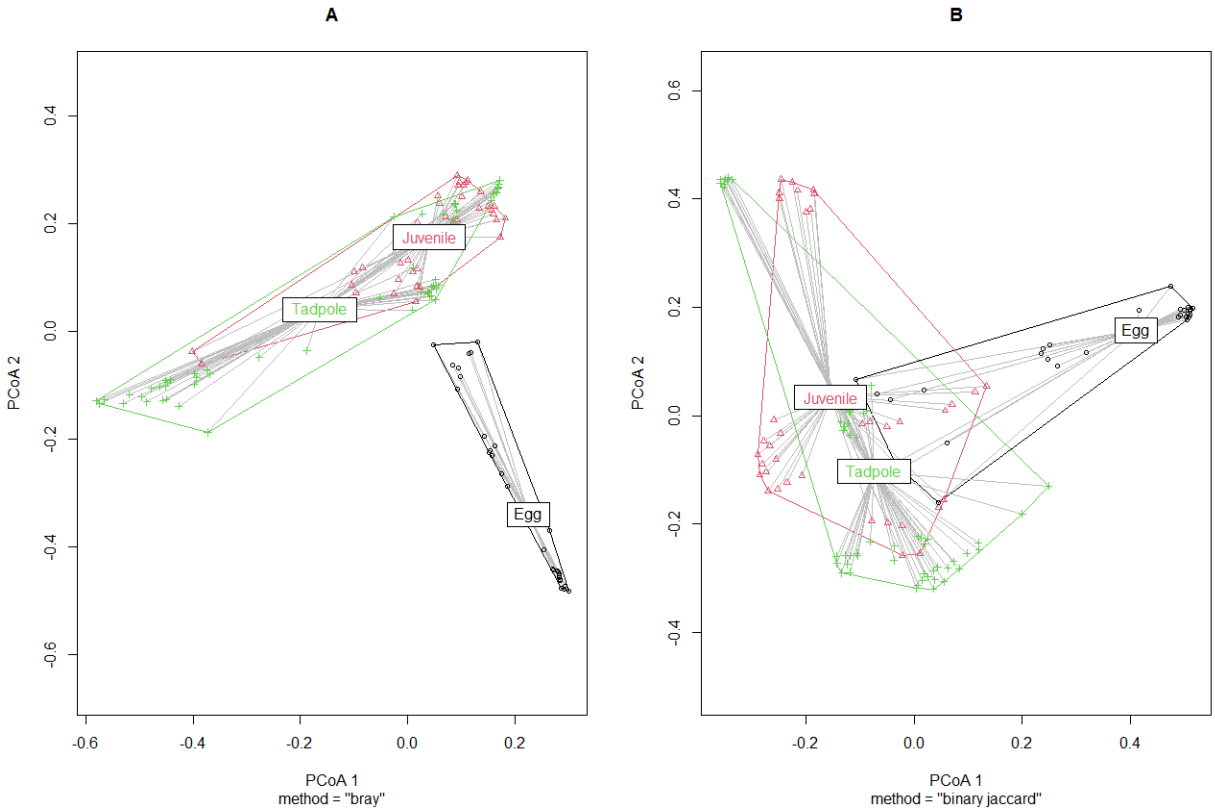
Looking solely at spring peeper tadpole samples, associated bacterial communities differed due to mesocosm ID both in the A) relative abundances and B) the presence/absence of taxa. C) ASV level relative abundance bar plot of bacteria associated with spring peeper tadpoles.

Figure 6



Alpha diversity metrics for spring peeper tadpole samples. Samples differed in A) species richness, B) phylogenetic diversity, and C) the effective number of species due based on mesocosm ID.

Figure S1

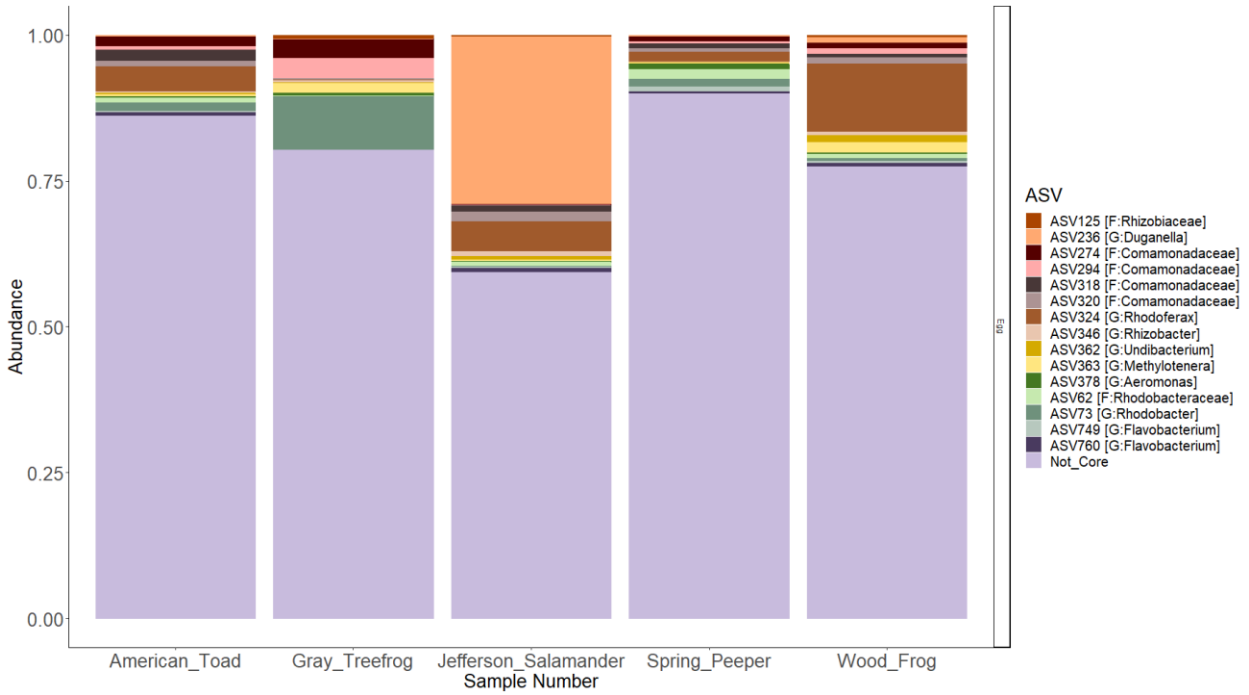


Ordination of betadisper output. Bacterial communities associated with frog eggs were significantly less dispersed than those of tadpoles or juveniles both in terms of A) relative abundance and B) presence/absence of taxa.

Figure S2

Barplot showing the relative abundance of *Oophila amblystomatis* identified ASVs among amphibian egg samples by species.

Figure S3



Barplot showing the relative abundance of ASVs considered to be core (ASVs minumum 0.01% relative abundance found on at least 75% of samples) to amphibian eggs.

Synthesis:

In my dissertation, I sought to develop a greater understanding of the basic ecological question: How do communities form and change over time? As our understanding of the functional roles of microbial communities increases [1–4], the ability to direct the formation and maintenance of these communities will similarly increase in importance [5,6]. Priority effects, though gaining popularity [7], represent a relatively untapped mechanism with which we can potentially shape both environmental and host microbiomes. Not only does my dissertation research serve as a proof of concept for directing microbiome composition through priority effects, but it also provides empirical evidence that these methods are effective across two host species, albeit with certain caveats. Additionally, my studies provide information on the interplay between parentage and environment in affecting the microbiome during vertebrate development [8–10].

In chapters one and two, I showed that it is possible to observe priority effects during embryonic colonization of two amphibian species. Priority effects are expected to occur when fitness differences between taxa are low and stabilizing forces are absent[11]. If one species is comparatively more fit than the other and resource requirements are similar, the less fit species will be excluded. Stabilizing forces are processes that increase negative interactions between conspecifics, which can include mechanisms such as intraspecific competition or frequency-dependent predation[12]. The initial colonist may inhibit [13] or promote the growth of subsequent taxa [14], however much of the research concerning priority effects focuses on inhibitory relationships [15]. In chapter 1, eggs (*in vivo*) and broth (*in vitro*) were inoculated with either *Acinetobacter* or *Stenotrophomonas* as priority taxa. While *Acinetobacter* saw an increase in relative abundance due to priority in both the *in vivo* and *in vitro* experiments, *Stenotrophomonas* only saw an advantage due to priority in the *in vitro* scenario. In both scenarios, however, we observed an impact of priority effects on ultimate community composition, as well as coexistence between isolates.

Interestingly, priority effects are often described as opposing the coexistence of taxa (e.g. [15,16] despite the facilitation of subsequent colonizing taxa being a potential outcome. In the case of non-homogeneous host-associated microbiomes, however, this dichotomy may miss the many circumstances in which priority effects lead to differential relative abundances of colonizing taxa or community level shifts. Early arriving taxa may gain advantage in abundance through niche preemption without consuming all available space or resources. Subsequent colonists may not be able to invade the primary colonist in areas it has already dominated, but there still opportunities for subsequent taxa to persist at significantly lower abundances. In this scenario, which may describe the outcomes in chapters 1 and 2, we would not only see priority effects, but also the coexistence of multiple taxa. Inhibition through priority effects will continue to be a relevant process in studies of microbial community assembly, however attention must also be directed towards proportional shifts in community abundances due to priority effects, even though these shifts may be more difficult to detect.

Through the inoculation experiments in chapters one and two, I show that priority effects hold promise as a potential method of increasing the relative abundances of target bacteria. Not only was this method successful in two different host species with different bacterial isolates, the outcomes from chapter one demonstrate consistent shifts in community composition based on the initial taxa. *Janthinobacterium lividum*, well known for its ability to inhibit the amphibian pathogen *Batrachochytrium dendrobatidis* [17], may be suited to probiotic applications taking advantage of

priority effect scenarios based on the relative abundance increase seen in the chapter two inoculations when given priority. For both *in vivo* inoculations in chapter one, *Acinetobacter* and *Stenotrophomonas* showed consistent outcomes from being given priority. Experimentation would still be needed to determine if individual isolates can take advantage of being given priority in each host species, however once identified, these results suggest that community outcomes may be relatively consistent.

While my inoculation experiments showed that the relative abundances of individual isolates can be increased through priority effects, it is unknown how permanent these changes are. In chapter one's first inoculation experiment, embryos exposed to isolates had no access to the initial isolate after the first 24 hours. For the *Acinetobacter* isolate, relative abundances were increased at the conclusion of the experiment, 4-5 days later. One explanation for this is that early colonizing bacteria may prime the host immune system in its favor [14,18]. There is, therefore, reason to believe that community structure might remain over the course of development.

At the time of sampling in my inoculation experiments (shortly after hatching--extremely early in development), the regulatory mechanisms of the host may be limited. As development progresses, host organisms may begin to regulate members of the microbiome through additional mechanisms, such as the production of skin peptides [19] or through physiological changes associated with shifts in microhabitat use [20]. Additionally, within these experiments, tadpoles were collected immediately after hatching, and did not have access to a natural diet, which would likely impact microbiome structure [21]. Even if maintained throughout the tadpole stage, microbiome composition would need to be maintained through metamorphosis, which can include skin keratinization [22,23], dietary shifts [24] and a shift to a terrestrial lifestyle rather than an aquatic one. Because I used bacteria that originated from the skin of adult frogs [25], these developmental changes may be less impactful than if environmental bacteria had been used.

The consequences of transferring experimentally inoculated tadpoles to environments that are greater sources of bacterial taxa are an interesting consideration. First, prior research [26] and unpublished studies within the Belden lab have indicated that the bacteria that are associated with amphibian skin are relatively rare within the environment. It is, therefore, possible that established bacteria from inoculation experiments would not be competing with a constant deluge of potentially successful bacterial colonists. Second, data from my third chapter has shown that, under a whole-body analysis, differences in microbiome composition at the phylum level that are host-species specific can exist for amphibians across development. While it cannot be concluded if these phylum level differences are present in the skin, gut or both in my whole-body samples, it implies a degree of specificity within host-species. Spring peeper microbiomes from this dissertation and other work [27] are consistently dominated by Proteobacteria, for example. Host organisms, either through active processes like diet [21], skin sloughing (cite) or antimicrobial peptide secretion [19] or more passive processes like skin composition (e.g. pH, moisture content, keratinization, etc.) or habitat use [27,28], are likely filtering out unsuitable taxa from the environment and maintaining suitable symbionts [26]. Choosing taxa that occupy the skin of adult frogs, therefore, should increase the likelihood that increased abundances of target taxa on tadpoles are retained if raised in a more natural, bacteria rich environment. In future studies, I hope to explore the longevity of these changes. Specifically, in natural or semi-natural settings, do the bacteria given priority remain over time, do community level impacts remain if the abundance of priority taxa wane over time, or do the impacts of priority effects become undetectable as tadpoles develop?

A recurring question in my research centers on the importance of environmental vs vertical transmission. Prior studies have also provided insight into this question, however our understanding of vertical transmission remains unclear [8,10]. While not the focus, I attempted to address this question in chapter two. First, I investigated the impact of parentage on the microbiome. Specifically, I attempted to determine if I could identify differences in microbial community structure between eggs from different parents. The results from this showed under identical environmental conditions, parentage led to microbiome compositional differences in eggs and tadpoles from both the lab and pond environments. While this provides support for parental transmission, it is unknown to what degree these differences are determined by family genetic structure rather than parental transmission. I also investigated the degree of overlap in ASVs between adults and eggs, finding a high presence of adult ASVs present among egg samples, providing some support for vertical transfer of microbes.

The research results presented in my dissertation improve our ability to understand and manipulate the amphibian microbiome. The external embryonic development of the amphibian system facilitated my ability to perform embryonic inoculations, however the conclusions drawn from this study can be applied to other host-associated communities. Experimental investigations of priority effects on host-associated communities using individual microbes are rare, although studies continue to appear in different systems, such as investigating bacterial strain exclusion in ticks and rhizosphere symbiont growth in wheat [13,29]. Strain level diversity within conspecific hosts may be facilitated by priority effects, as has been proposed in bees [30] and demonstrated in ticks [13]. Studies investigating early life microbial exposure have also shown lasting effects on host health and development [31–34], persisting through host generations in some cases [35]. Understanding more specifically how individual bacteria contribute to observed outcomes in these systems may be facilitated by selective inoculations of individual isolates during critical developmental periods. Systems in which the microbiome is well-studied and is of low relative complexity, such as those found associated with honey bees, would lend themselves well to understanding microbial priority effects, particularly between strains of closely related bacteria [30,36–38]. Though ecology rarely has firm laws [39], empirically investigating priority effects in a variety of systems will continue to be key in understanding when colonization history matters, and its relative importance in the establishment of host-associated microbial communities.

1. Brugman S, Ikeda-Ohtsubo W, Braber S, Folkerts G, Pieterse CMJ, Bakker PAHM. 2018 A Comparative Review on Microbiota Manipulation: Lessons From Fish, Plants, Livestock, and Human Research. *Front. Nutr.* **5**. (doi:10.3389/fnut.2018.00080)

2. Chaparro JM, Sheflin AM, Manter DK, Vivanco JM. 2012 Manipulating the soil microbiome to increase soil health and plant fertility. *Biol Fertil Soils* **48**, 489–499. (doi:10.1007/s00374-012-0691-4)

3. Fontaine SS, Mineo PM, Kohl KD. 2022 Experimental manipulation of microbiota reduces host thermal tolerance and fitness under heat stress in a vertebrate ectotherm. *Nat Ecol Evol* **6**, 405–417. (doi:10.1038/s41559-022-01686-2)

4. Knezevic J, Starchl C, Tmava Berisha A, Amrein K. 2020 Thyroid-Gut-Axis: How Does the Microbiota Influence Thyroid Function? *Nutrients* **12**, 1769. (doi:10.3390/nu12061769)

5. Trevelline BK, Fontaine SS, Hartup BK, Kohl KD. 2019 Conservation biology needs a microbial renaissance: a call for the consideration of host-associated microbiota in wildlife management practices. *Proceedings of the Royal Society B: Biological Sciences* **286**, 20182448. (doi:10.1098/rspb.2018.2448)

6. Walke JB, Belden LK. 2016 Harnessing the Microbiome to Prevent Fungal Infections: Lessons from Amphibians. *PLOS Pathogens* **12**.

7. Debray R, Herbert RA, Jaffe AL, Crits-Christoph A, Power ME, Koskella B. 2021 Priority effects in microbiome assembly. *Nat Rev Microbiol* (doi:10.1038/s41579-021-00604-w)

8. Hughey MC, Delia J, Belden LK. 2017 Diversity and stability of egg-bacterial assemblages: The role of paternal care in the glassfrog Hyalinobatrachium colymbiphyllum. *Biotropica* **49**, 792–802. (doi:10.1111/btp.12461)

9. Sylvain F-É, Derome N. 2017 Vertically and horizontally transmitted microbial symbionts shape the gut microbiota ontogenesis of a skin-mucus feeding discus fish progeny. *Scientific Reports* **7**, 5263. (doi:10.1038/s41598-017-05662-w)

10. Walke JB, Harris RN, Reinert LK, Rollins-Smith LA, Woodhams DC. 2011 Social Immunity in Amphibians: Evidence for Vertical Transmission of Innate Defenses. *Biotropica* **43**, 396–400. (doi:10.1111/j.1744-7429.2011.00787.x)

11. Fukami T, Mordecai EA, Ostling A. 2016 A framework for priority effects. *Journal of Vegetation Science* **27**, 655–657. (doi:10.1111/jvs.12434)

12. Chesson P. 2000 Mechanisms of Maintenance of Species Diversity. *Annual Review of Ecology and Systematics* **31**, 343–366. (doi:10.1146/annurev.ecolsys.31.1.343)

13. Devevey G, Dang T, Graves CJ, Murray S, Brisson D. 2015 First arrived takes all: inhibitory priority effects dominate competition between co-infecting Borrelia burgdorferi strains. *BMC Microbiology* **15**, 61. (doi:10.1186/s12866-015-0381-0)

14. Potnis N, Soto-Arias JP, Cowles KN, van Bruggen AHC, Jones JB, Barak JD. 2014 Xanthomonas perforans Colonization Influences Salmonella enterica in the Tomato Phyllosphere. *Appl Environ Microbiol* **80**, 3173–3180. (doi:10.1128/AEM.00345-14)

15. Fukami T. 2015 Historical Contingency in Community Assembly: Integrating Niches, Species Pools, and Priority Effects. *Annual Review of Ecology, Evolution, and Systematics* **46**, 1–23. (doi:10.1146/annurev-ecolsys-110411-160340)

16. Grainger TN, Letten AD, Gilbert B, Fukami T. 2019 Applying modern coexistence theory to priority effects. *PNAS* **116**, 6205–6210. (doi:10.1073/pnas.1803122116)

17. Becker MH, Brucker RM, Schwantes CR, Harris RN, Minbiole KPC. 2009 The Bacterially Produced Metabolite Violacein Is Associated with Survival of Amphibians Infected with a Lethal Fungus. *Applied and Environmental Microbiology* **75**, 6635–6638. (doi:10.1128/AEM.01294-09)

18. Halliday FW, Umbanhowar J, Mitchell CE. 2017 Interactions among symbionts operate across scales to influence parasite epidemics. *Ecology Letters* **20**, 1285–1294. (doi:10.1111/ele.12825)

19. Woodhams DC *et al.* 2019 Probiotics Modulate a Novel Amphibian Skin Defense Peptide That Is Antifungal and Facilitates Growth of Antifungal Bacteria. *Microb Ecol* (doi:10.1007/s00248-019-01385-9)

20. Bletz MC, Archer H, Harris RN, McKenzie VJ, Rabemananjara FCE, Rakotoarison A, Vences M. 2017 Host Ecology Rather Than Host Phylogeny Drives Amphibian Skin Microbial Community Structure in the Biodiversity Hotspot of Madagascar. *Front. Microbiol.* **8**. (doi:10.3389/fmicb.2017.01530)

21. Antwis RE, Haworth RL, Engelmoer DJP, Ogilvy V, Fidgett AL, Preziosi RF. 2014 Ex situ Diet Influences the Bacterial Community Associated with the Skin of Red-Eyed Tree Frogs (Agalychnis callidryas). *PLOS ONE* **9**, e85563. (doi:10.1371/journal.pone.0085563)

22. Berger L *et al.* 1998 Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc Natl Acad Sci U S A* **95**, 9031–9036.

23. Marantelli G, Berger L, Speare R, Keegan L. 2004 Distribution of the amphibian chytrid Batrachochytrium dendrobatidis and keratin during tadpole development. *Pac. Conserv. Biol.* **10**, 173. (doi:10.1071/PC040173)

24. Jenssen TA. 1967 Food Habits of the Green Frog, Rana clamitans, before and during Metamorphosis. *Copeia* **1967**, 214. (doi:10.2307/1442196)

25. Walke JB, Becker MH, Hughey MC, Swartwout MC, Jensen RV, Belden LK. 2015 Most of the Dominant Members of Amphibian Skin Bacterial Communities Can Be Readily Cultured. *Applied and Environmental Microbiology* **81**, 6589–6600. (doi:10.1128/AEM.01486-15)

26. Walke JB, Becker MH, Loftus SC, House LL, Cormier G, Jensen RV, Belden LK. 2014 Amphibian skin may select for rare environmental microbes. *The ISME Journal* **8**, 2207–2217. (doi:10.1038/ismej.2014.77)

27. Hughey MC, Sokol ER, Walke JB, Becker MH, Belden LK. 2019 Ecological Correlates of Large-Scale Turnover in the Dominant Members of Pseudacris crucifer Skin Bacterial Communities. *Microb Ecol* **78**, 832–842. (doi:10.1007/s00248-019-01372-0)

28. Kueneman JG *et al.* 2019 Community richness of amphibian skin bacteria correlates with bioclimate at the global scale. *Nature Ecology & Evolution* **3**, 381. (doi:10.1038/s41559-019-0798-1)

29. Ridout ME, Schroeder KL, Hunter SS, Styer J, Newcombe G. 2019 Priority effects of wheat seed endophytes on a rhizosphere symbiosis. *Symbiosis* **78**, 19–31. (doi:10.1007/s13199-019-00606-6)

30. Ellegaard KM, Engel P. 2019 Genomic diversity landscape of the honey bee gut microbiota. *Nat Commun* **10**, 446. (doi:10.1038/s41467-019-08303-0)

31. Arrieta M-C *et al.* 2015 Early infancy microbial and metabolic alterations affect risk of childhood asthma. *Science Translational Medicine* **7**, 307ra152-307ra152. (doi:10.1126/scitranslmed.aab2271)

32. Cho I *et al.* 2012 Antibiotics in early life alter the murine colonic microbiome and adiposity. *Nature* **488**, 621–626. (doi:10.1038/nature11400)

33. Hild B *et al.* 2021 Neonatal exposure to a wild-derived microbiome protects mice against diet-induced obesity. *Nat Metab* **3**, 1042–1057. (doi:10.1038/s42255-021-00439-y)

34. Warne RW, Kirschman L, Zeglin L. 2019 Manipulation of gut microbiota during critical developmental windows affects host physiological performance and disease susceptibility across ontogeny. *Journal of Animal Ecology* **88**, 845–856. (doi:10.1111/1365-2656.12973)

35. Fallet M *et al.* 2022 Early life microbial exposures shape the Crassostrea gigas immune system for lifelong and intergenerational disease protection. *Microbiome* **10**, 85. (doi:10.1186/s40168-022-01280-5)

36. Engel P, Stepanauskas R, Moran NA. 2014 Hidden Diversity in Honey Bee Gut Symbionts Detected by Single-Cell Genomics. *PLOS Genetics* **10**, e1004596. (doi:10.1371/journal.pgen.1004596)

37. Koch H, Abrol DP, Li J, Schmid-Hempel P. 2013 Diversity and evolutionary patterns of bacterial gut associates of corbiculate bees. *Mol Ecol* **22**, 2028–2044. (doi:10.1111/mec.12209)

38. Steele MI, Moran NA. 2021 Evolution of Interbacterial Antagonism in Bee Gut Microbiota Reflects Host and Symbiont Diversification. *mSystems* **6**, e00063-21. (doi:10.1128/mSystems.00063-21)

39. Lawton JH. 1999 Are There General Laws in Ecology? *Oikos* **84**, 177. (doi:10.2307/3546712)

Appendix A

Supplemental Material For:

Title: Colonization order of bacterial isolates on treefrog embryos impacts microbiome structure in tadpoles.

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Supplementary Methods:

Adult skin microbiome data:

D. ebraccatus samples (N=35) from a 2012 survey of the skin bacterial microbiomes of 3 Panamanian frog species (1) were re-analyzed to provide insight into which bacterial isolates to use for the present study. Sample collection, processing and sequencing information are provided in the original study (1). Forward 250bp reads (16S rRNA gene amplicons) were used. The Qiime2(2) pipeline was used to re-process the data, including demultiplexing, denoising, and dereplication. To denoise the sequences, we used Dada2 (3) with a quality score of 11. Any reads with a frequency less than 0.01% across all samples were removed. Scikit-learn (4) was used to apply taxonomy to sequences through the use of the SILVA taxonomic classifier (5). We constructed our tree using RAxML (6) and rooted the tree at its midpoint. Final tables from Qiime2 were imported into the R v4.1.0 (7) environment for further processing. Any ASVs that could not be identified at the phylum level, as well as any mitochondria or chloroplast sequences, were removed. Our final table contained 469 unique ASVs across 35 samples.

Statistical Analysis and results:

All statistical analyses were completed within the R environment (v4.1.0). Our analysis was limited to genera that were represented by isolates available within our culture library. Two of our isolates with the highest relative abundance on the skin of adult frogs belonged to the genera *Acinetobacter* and *Stenotrophomonas* (1). To examine the potential relationship between *Acinetobacter* and *Stenotrophomonas* ASVs within the skin communities of wild adult frogs, we first grouped ASVs at the genus level. We then used Spearman's rank correlation to determine if a relationship existed between these two

genera across our samples. Looking across our field samples, *Acinetobacter* and *Stenotrophomonas* relative abundance within the community are inversely correlated (Spearman's S= 59909, Rho= -0.509, p = 2.44e-05) (Fig S1).

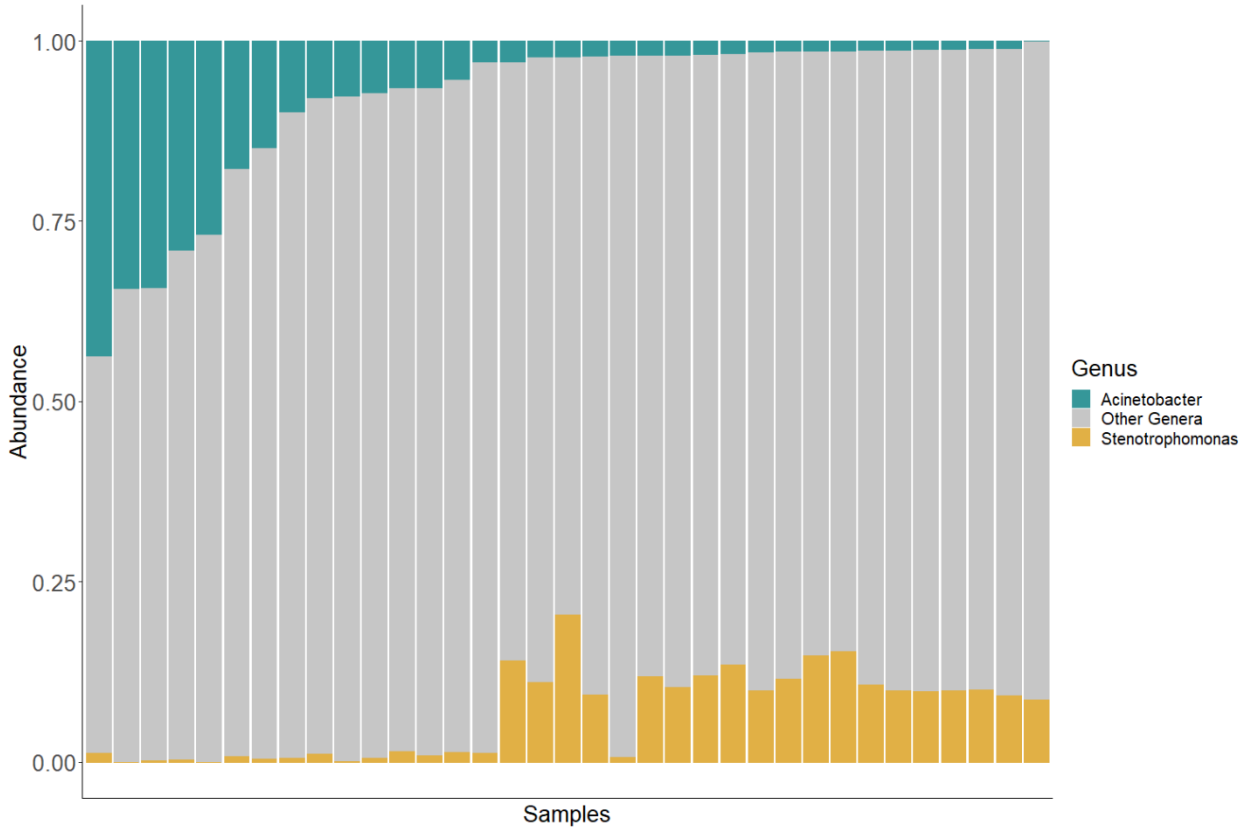
Frog breeding and egg collection:

The adults used for the first experiment were from the F1 generation from adults initially collected in Panama. Adults were housed in groups of four, separated by sex, unless chosen for breeding. We placed 10 female and 12 male frogs into a 50W x 50L x 90H cm breeding enclosure with a simulated rain system at midday. All frogs were handled with new sterile gloves. In the evening, amplexed pairs were collected and placed in Ziploc bags containing 50 ml of sterile spring water for the remainder of the night. The next morning, two pairs had produced sufficient eggs (>40) for the experiment. All adult frogs were returned to their enclosures while the eggs were relocated to the laboratory.

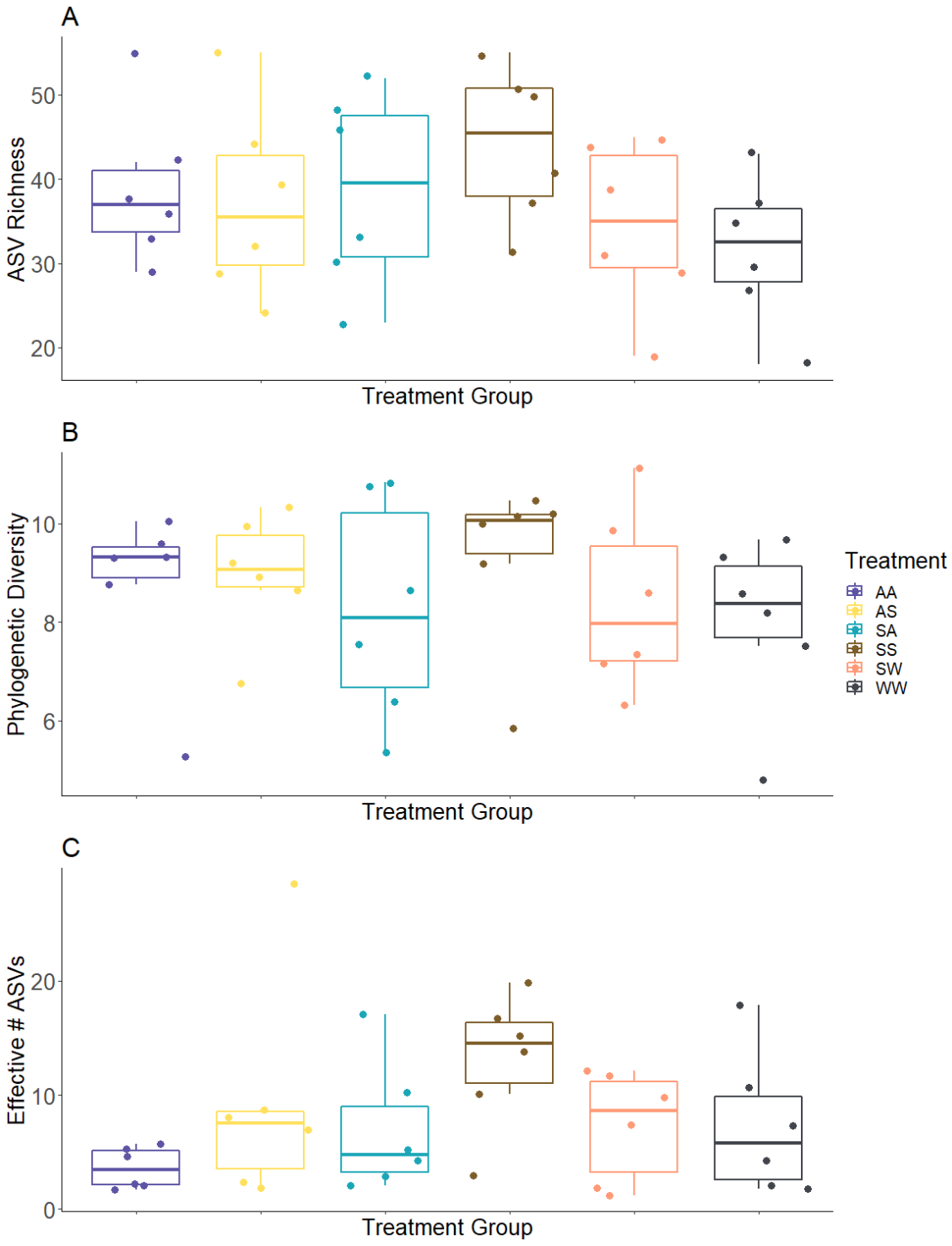
For the second experiment, five females and seven males from the F2 generation were placed within the breeding enclosure at midday. That night, we moved amplexed pairs to individual tanks (10W x 10L x 15H cm) containing a single potted plant (*Epipremnum aureum*) and artificial pond water. We collected eggs from one pair the following morning using sterilized forceps and relocated them to the laboratory.

Co-culture amplification:

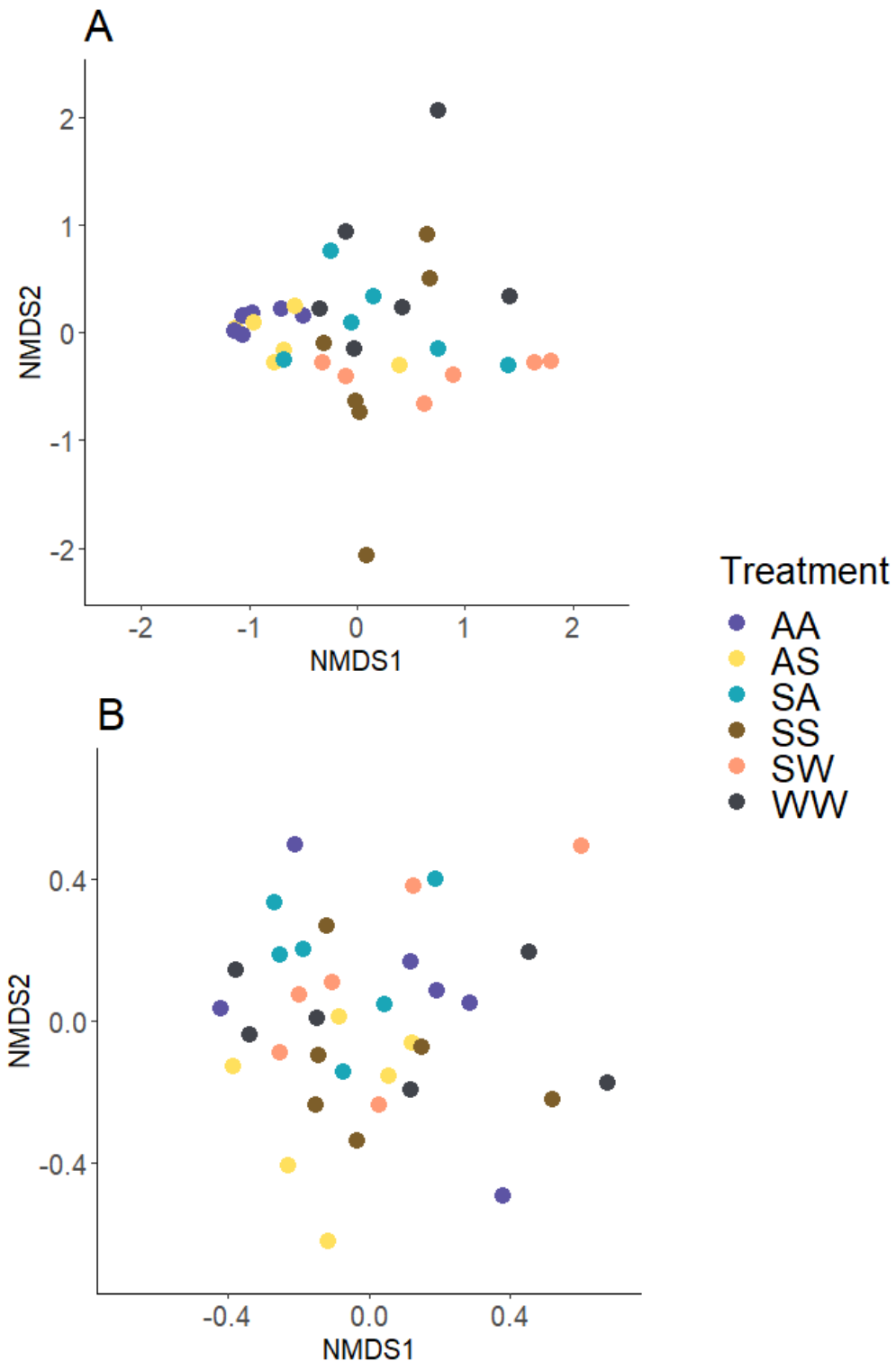
The 25 µl PCR reaction consisted of 2.5 µl DNA, 5 µl of each primer, and 12.5 µl 2x KAPA HiFi HotStart ReadyMix. Thermocycler conditions were as follows: 95 °C for 3 minutes, then 25 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds; and then final extension for 5 minutes at 72 °C. PCR product was cleaned using AMPure XP beads according to manufacturer's instructions. As we sequenced these at Vassar College on an Illumina iSeq 100, we completed a second PCR reaction to add Illumina index sequences and sequencing adapters from the Nextera Index kit to our amplicons. For that, the 25 µl PCR reaction consisted of 12.5 µl 2x KAPA HiFi HotStart ReadyMix, 2.5 µl Nextera XT Index 1 primer, 2.5 µl Nextera XT Index 2 primer, 5 µl PCR grade water and 2.5 µl DNA. A unique set of indices was used for each sample. Thermocycler conditions were 95 °C for 3 minutes, then 8 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds; and final extension at 72 °C for 5 minutes.



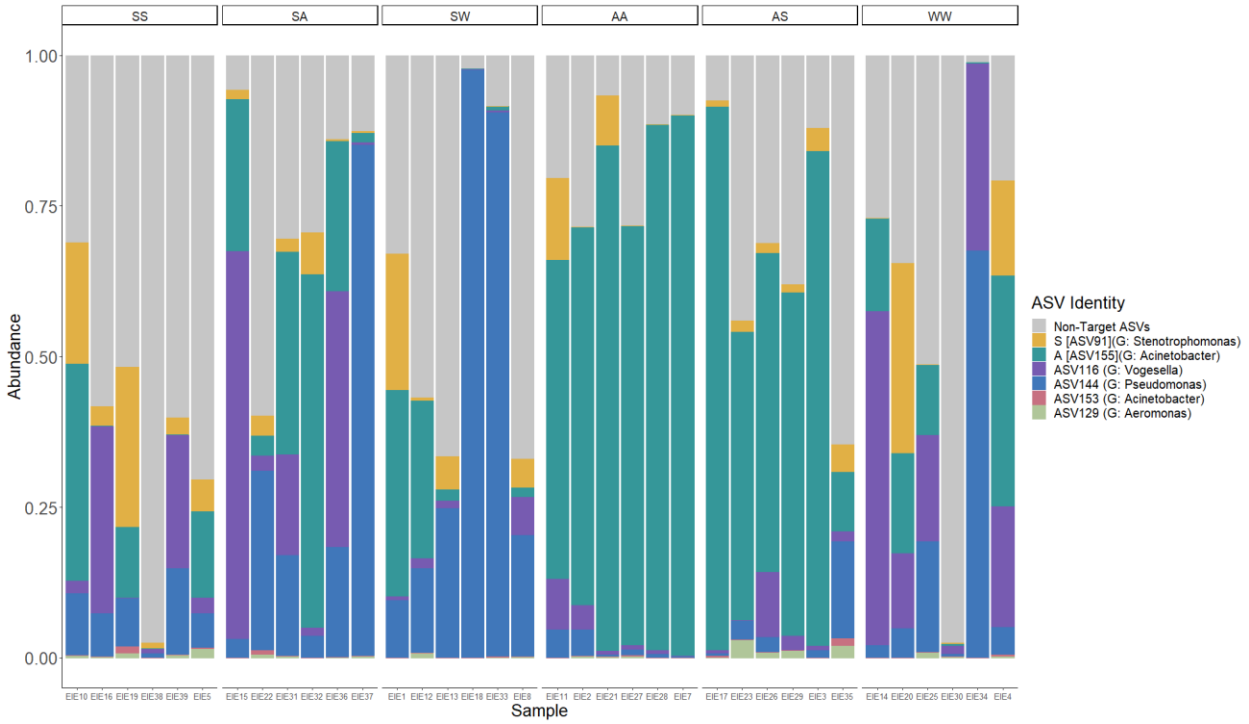
Supplemental Figure 1: Prior field survey data from the skin bacterial community of adult *D. ebraccatus* suggests an inverse relationship in relative abundance between the genera *Acinetobacter* and *Stenotrophomonas*. The plot shows the relative abundances of *Acinetobacter* (teal) and *Stenotrophomonas* (mustard) with all other genera in gray. Each bar represents a single, wild-caught frog in Panama.



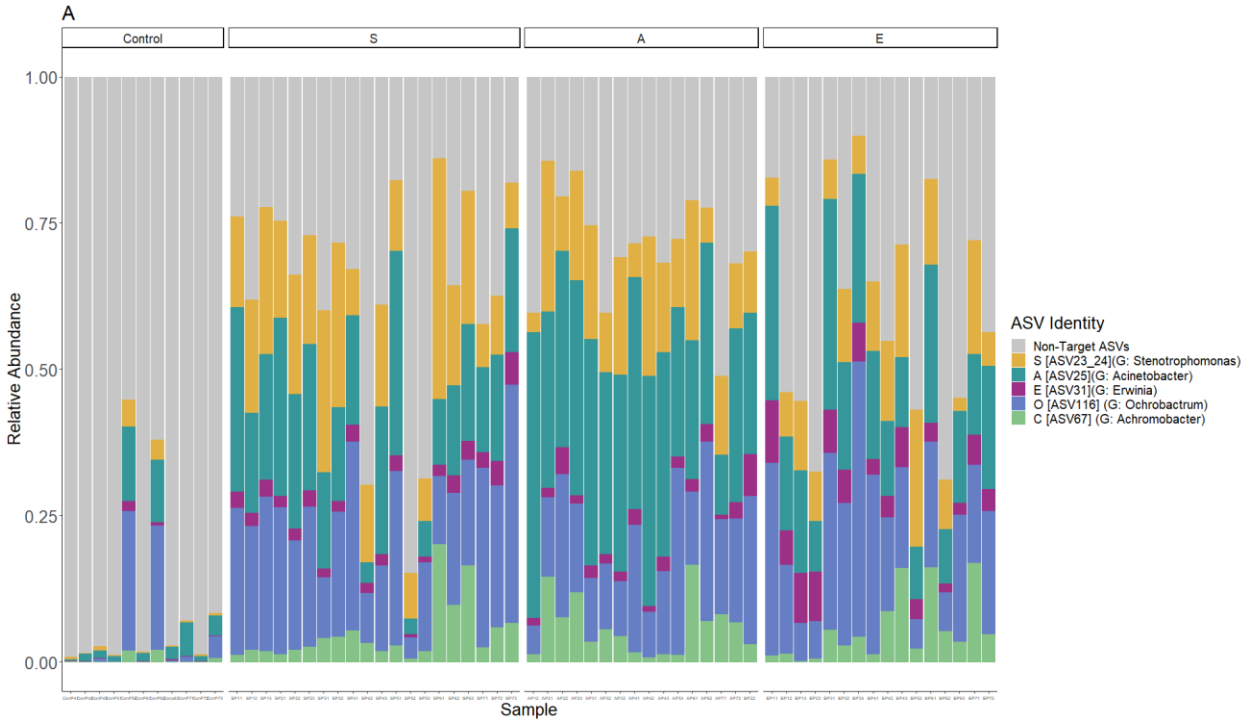
Supplemental Figure 2: Alpha diversity metrics across treatments (exp 1). Neither (A) ASV richness, B) phylogenetic diversity nor (C) the effective number of species differed between treatments.



Supplemental Figure 3: NMDS ordinations of overall community differences between our treatments (exp 1). (A) Relative abundances of ASVs within communities differed in response to our treatments, however (B) the presence/absence of taxa within communities did not.



Supplemental Figure 4: Relative abundance of target isolates from individual tadpole samples (exp 1).



Supplemental Figure 5: Relative abundance of target isolates from individual tadpole samples (exp 2).

1. Belden LK, Hughey MC, Rebollar EA, Umile TP, Loftus SC, Burzynski EA, Minbiole KPC, House LL, Jensen RV, Becker MH, Walke JB, Medina D, Ibáñez R, Harris RN. 2015. Panamanian frog species host unique skin bacterial communities. Front Microbiol 6.

2. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet C, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope E, Silva RD, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley G, Janssen S, Jarmusch AK, Jiang L, Kaehler B, Kang KB, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciolek T, Kreps J, Langille MG, Lee J, Ley R, Liu Y-X, Loftfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV, Metcalf JL, Morgan SC, Morton J, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E, Rasmussen LB, Rivers A, Michael S Robeson II, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, Hooft JJ van der, Vargas F, Vázquez-Baeza Y, Vogtmann E, Hippel M von, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CH, Willis AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R, Caporaso JG. 2018. QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science. e27295v2. PeerJ Inc.

3. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods 13:581–583.

4. Pedregosa F, Varoquaux G, Gramfort A, Michel V, Thirion B, Grisel O, Blondel M, Prettenhofer P, Weiss R, Dubourg V, Vanderplas J, Passos A, Cournapeau D. 2011. Scikit-learn: Machine Learning in Python. Mach Learn PYTHON 6.

5. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41:D590–D596.

6. Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312–1313.

7. R Core Team. 2019. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.