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Dispersal and drift drive diversity and structure in symbiont populations

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Abstract

Dispersal and drift drive diversity and structure in symbiont populations
By Jason Chen

Many hosts are colonized by specific microbial symbionts which they rely on for proper development. Thus, host preference for optimally beneficial strains and competition between strains are usually considered the predominant drivers of symbiont population structure. However, these cannot fully account for the high symbiont strain diversity and between-host heterogeneity observed in many symbioses. Understanding how symbioses assemble as ecological communities of microbes can provide insight into the population structure of symbionts.

The insect *Anasa tristis* relies on environmentally acquired bacterial symbionts (*Caballeronia* spp.) for normal growth and development. Although *A. tristis* imposes selection on environmental microbes to favor gut colonization by *Caballeronia*, how other drivers of community assembly contribute to *Caballeronia* community structure in this specialized host-microbe symbiosis is unclear. **In my dissertation, I demonstrate how dispersal and ecological drift contribute to naturally observed patterns of symbiont population structure.**

First, I demonstrate that *A. tristis* nymphs are attracted to the feces of conspecific adults, which constitutes a behavioral adaptation for symbiont acquisition. Symbiont dispersal via fecal transmission results in strain-level specificity in symbiont acquisition, allowing for host-symbiont fidelity across generations without any apparent coevolutionary history or strain-specific functional differences for the insect host.

Next, I demonstrate that the composition of symbiont communities is determined by the order in which strains disperse into and colonize a host, or a priority effect. Within 24 hours, ingestion of one symbiont strain prevents superinfection by subsequently ingested strains. While colonization elicits tissue remodeling in the gut in a manner that prevents superinfection, this mechanism occurs too slowly to impose the priority effect on its own.

Finally, I demonstrate how ecological drift, in the form of transmission bottlenecks, maintains strain diversity and generates community heterogeneity. Drift generates heterogeneous colonization outcomes, mirroring those observed in nature, with inoculated strains segregating between different host individuals even when the strains are isogenic. Ecological drift also separates less competitive symbiont strains into different hosts than more competitive strains, even at high inoculation doses. Microscopy reveals that host anatomy also imposes single cell bottlenecks on isogenic bacteria as they colonize different gut crypts, resulting in within-host heterogeneity.

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Acknowledgments

While I get to take credit for the work on microbial community ecology which I present in this dissertation, I could not have completed it without a community of my own which I found here at Emory University. First, I need to thank my co-advisors, Nic Vega and Nicole Gerardo, who guided me in the completion of these chapters from conception to publication. Their mentorship fostered my intellectual and professional growth as a scientist, and without their joint cooperation this work would not have been possible. Nic and Nicole are both versatile scientists open to somewhat unconventional projects (though luckily they always knew to rein me in when my ideas got too wild). During the PhD, a graduate student is expected to become a world-class expert in a particular aspect their field. While I think there have been just a few ways in which I have managed to grow my interests beyond Nicole's ample expertise, I have yet to grow out from under Nic's long intellectual shadow in any meaningful way. As department chair Steven L'Hernault put it to me, the final semester of a PhD is the most grueling of all, and their support and encouragement were essential for getting me over the finish line. I aspire to their examples that they set as mentors, scientists, and people.

I also need to thank my dissertation committee members: Daniel Weissman, Minsu Kim, and Sam Brown. Though I selected them for a particular vision of my dissertation that never materialized, I value the interest they showed in my work for the past four years and the feedback they gave me every committee.

I need to thank my labmates and mentees in the Vega and Gerardo labs for their support over the years. In the Vega lab, Megan Taylor, Megan Woods, Satya Spandana Boddu, and Carmen Alvarez have given me valuable feedback and encouragement, even when I stopped

working with the worms. In the Gerardo lab, the membership on the Council of Coreid Concerns has shifted considerably but includes Anthony Junker, Laura Avila, Scott Villa, Kayla Stoy, and Sandra Mendiola, plus Jacoby Robinson and Quimi Vidaurre Montoya working on the attine-fungal cultivar-fungal parasite system. In particular, Sandra was instrumental in her reliable maintenance of the squash bug lab colony that made my work possible. Besides my peers, I have had the pleasure of mentoring gifted undergrads at Emory University without whose assistance the graduate students' and postdocs' ideas would have been entirely unfeasible. Zee Kwong, Alice Acosta, Sherry Tsui, and Iris Zheng have contributed in different ways to my understanding of the workings within the microbial world around us, as well as an understanding of my own strengths and shortcomings as a mentee. My success is also their success, but their success has also often come about in spite of me. Overall, it has been gratifying to see my work, Kayla's, and Sandra's work, along with those of our respective mentees, complementing each other across time scales and spatial scales of ecology and evolution, in a way that will uniquely elevate the tractability and utility of the squash bug model system years in the future.

I have had the privilege to work in the Department of Biology during the entirety of my PhD, during which I got to know the fantastic folks who make this a uniquely productive and collegial department. I have benefitted strongly from the support of the staff in the Biology office, especially Tonya Woolcock, Sonia Perez Hayden, Jan Hawes, Scottye Davis, and James Allen. Erik Edwards, our greenhouse manager, deserves special mention as his reliable expertise and readiness to take on new horticultural challenges has made possible my work as those of my labmates. Ms. Judy, one of the resident custodians, has been a reliable source of encouragement during the grueling all-nighters necessary for meticulous dissections or tedious coding.

On the second floor, the Deal lab and Rieder labs have been excellent neighbors. It has been a pleasure to share my passion for insects with Leila and with the members of her lab, and through them I have learned (by osmosis, at least) a small amount of *Drosophila* biology. Although my interactions with the Deal lab are often silent, I am always reassured by our non-verbal acknowledgments of each other's existence as we pass each other by in the hallway as a sign of normalcy. To paraphrase Ron Swanson from the sitcom Parks & Recreation, I consider them good friends: We still never talk sometimes.

On the first floor, my neighbors in the Levin lab in particular are a continued source of, by turns, fellowship, mild consternation, and amusement (Figure 1). I am grateful to Brandon Berryhill and Andrew Smith, in particular, for engaging conversations about science and beyond, both in the People's Room and on various excursions throughout Decatur.

Figure 1. Selected members of Bruce Levin's research group. From left to right: graduate student Brandon Berryhill, now-medical student Joshua Manuel, now-postdoctoral researcher Teresa Gil-Gil, and laboratory manager Andrew Smith.

In the Population Biology, Ecology, and Evolution (PBEE) graduate program at Emory, I have found a talented group of colleagues whose interests and techniques span fields. My time in PBEE has expanded my outlook on ecology and evolution as not just a discipline in its own right, but as a framework for thinking about living systems that is applicable (and occasionally misapplied) across multiple levels of biology organization. Our cohort of 2018 has produced a bevy of gifted scientists, and I cannot wait to see how their work will not only advance their budding careers, but also public health and social well-being. Within our cohort, I want to specifically spotlight Lynda Bradley as one of my closest partners-in-crime. Lynda is one of the most empathetic people I have ever met, and she works hard to make PBEE a better place for present and future trainees. In addition to that, I appreciate her sarcastic sense of humor and her companionship at work throughout the day, including what we depressingly term ‘cockroach hour’.

Along the way to attaining my PhD, I have also met many, many people who have shaped my character and intellectual interests. There have been too many of these people to count, but I will point out a few notable standouts. Alison Slinsky-Legg served at the University of Pittsburgh as the outreach coordinator in the Department of Biological Sciences for many years, and being part of the Gene Team summer research program for high school students and teachers in 2008 provided the spark that motivated my interested in academic research in biology. I remember telling Alison at one time afterwards that I would very much like to combine my interests in insect life with what I was learning in microbiology and genetics—I’m happy to say that I accomplished this goal. From Jonathan Pruitt, I inherited an interest in the quantification of social behavior and its implications for the functional ecology of groups across scales. Their willingness to let me try new things was important for my intellectual growth as a scientist

beyond that topic. In the Pruitt lab, I also met for the first time other young scientists who shared my interests, including Donna McDermott, and their career paths in part influenced my decision to pursue a PhD rather than medical school. While Jonathan also negatively influenced the careers of other ecologists during his time in academia, I must acknowledge his influence on my own development as a scientist. Finally, from Nathan Morehouse, I learned about the myriad ways by which animals perceive their environments, and gained my first experience working collaboratively on scientific storytelling. While I no longer work in the field of animal behavior *per se*, my work carries a germ of their influence at its core.

I also need to thank Sandra Bobick, Katherine Mosley-Turner, and Caroline Evans for their guidance and opportunities they afforded me during the time I returned to community college to continue my education during a difficult turning point in my life. My participation in the BioMAS program also marked an intellectual turning point in my life, during which I found the confidence to shift into a new field. Working with Wook Kim showed me that the ideas that I was thinking about in social insects could be modelled mathematically and studied experimentally in microbial systems, and introduced me to the importance of spatial constraints in microbial populations.

Finally, no thanks to the reviewers of several graduate fellowships I applied for. Although I lacked a lot of grantsmanship skills at the time (and still need to work on them), it has been gratifying to see the progress I have made even when others did not see my potential at the time. Looking back, with the growing that I have done over the last 6 years, I am confident in saying that I am ready to embark on a proper PhD in ecology and evolutionary biology.

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Chapter 1

Introduction

Host-associated microbial communities and transmission mode

Across the tree of life, healthy hosts commonly harbor teeming numbers of microbes in intimate contact with their tissues without any ill effects. Some of these microbes form symbioses in which the association is both repeatable (the partnership is reconstituted every host generation) and persistent (the partnership is maintained with the host for multiple microbial generations) [1]. The earliest work on host-microbe symbioses discovered the beneficial nature of these associations by painstaking microscopic observation of microbial populations within their hosts and by experimental removal of these microbes with treatments such as heat and antibiotics [2]. The advent of sequencing technologies then enabled the identification of these microbes, reconstruction of their evolutionary histories and metabolic capabilities, and elucidation of how these metabolic capacities related to those of their hosts. The picture that emerged from these studies, based on model systems with pairwise interactions, was that of mutual co-dependence and co-speciation (e.g. [3,4]).

The benefits conferred by symbiosis are necessarily dependent upon microbial transmission. Transmission is therefore under selection in the host, and in turn, symbiont transmission mode impacts evolution of microbial symbionts. The necessity of inheriting obligate symbionts, for example, has resulted in the evolution of strategies by some hosts, such as aphids [5–7], to maintain strict vertical transmission of their symbionts across generations. Because, in such cases, symbiont fitness is permanently tied to that of the host, strictly vertically transmitted symbionts are thought to evolve to maximize host benefit [8]. In addition, when

symbionts are permanently sheltered by their hosts from encounters with other microbes and mobile genetic elements, symbiont genomes erode due to the accumulation of deleterious mutations and the loss of opportunities for genetic recombination [9].

Microbes can also be transmitted horizontally between host lineages. Here, symbionts can disperse and admix with other, unrelated microbes, either in the environment or during colonization of new hosts, maintaining gene flow and recombination between symbiont lineages [10]. As a result, symbiont fitness is no longer tied to that of a single host lineage; instead, horizontal transmission can select for pathogens and "cheater" symbionts [8,11]. Hosts that acquire symbionts horizontally, such as squids [12] and legume plants [13] are thought to experience strong selection to prevent colonization by non-beneficial taxa.

Microbial community assembly

Transmission mode has been studied as a driver of the evolution of symbiont genomes and host-microbe interactions over long timescales. However, transmission mode also has important effects at shorter, ecologically relevant timescales, because it describes the processes by which symbiotic communities assemble. Unlike some macroscopic symbiotic interactions between two single individuals of different species (e.g. [14,15]), host-microbe symbioses are not composed of a single individual microbial cell or microbial genome. Instead, hosts are often associated with large microbial populations or communities that can contain functional and taxonomic diversity. Differences in community assembly result in heterogeneous symbiont community structure among populations of hosts, among hosts within a population, and even within a single host.

Vellend described how communities assemble by drawing parallels with how populations of a single species evolve [16]. The four fundamental drivers of evolutionary change in finite

populations composed of discrete, interacting individuals of a single species are natural selection (in which heritable alleles are favored to increase in frequency from one generation to the next by conferring a selective advantage on individual phenotypes), dispersal (change in allele frequencies as they are carried by individuals moving into and out of a population), genetic drift (random fluctuation in allele frequencies), and mutation (*de novo* generation of new alleles). In Vellend's synthesis [16], ecological communities are finite collectives of interacting individuals of multiple species, and these species may differ in the degree to which they overlap in niche space. Just as evolutionary change in populations occurs by natural selection, dispersal, genetic drift, and mutation, ecological change in communities occurs by ecological selection, dispersal, ecological drift, and speciation. Below, I overview each of these factors in turn.

Ecological Selection

Habitats can vary in available niche space due to factors such as resource availability, the presence of competitors, and abiotic conditions. Ecological selection drives community assembly by favoring the establishment of species that are adapted to occupy the niches that are present. In the context of host-associated microbial communities, the host imposes ecological selection on its resident communities depending on both the life history of the host and the location of these communities. For example, in the human microbe, different communities on a single person arise due to differences in the conditions associated with these sites, such as hygiene, immunity, temperature, nutrient availability, etc., and a site can also differ between individuals due to differences in lifestyle.

The presence of other taxa already present in a habitat can determine whether a species is more or less likely to colonize and establish within that community. Thus, the order and timing by which taxa arrive in a habitat can fundamentally alter how ecological selection shapes

community assembly. This is known as a priority effect. While priority effects can be facilitative, with species colonizing habitats more efficiently if others have already established (e.g. [17]), most are competitive and result in mutual exclusion [18]. In host-associated microbial communities, priority effects can be due to direct competition for space or host resources, as in conventional communities, but interactions between established microbes and the host can also induce host responses, such as behavior, physiology or immunity, that indirectly facilitate (e.g. [19]) or limit (e.g. [20]) colonization by later arriving symbionts. As a result of priority effects, the order and timing of colonization can drive divergent community outcomes that cannot be predicted by selection alone.

Diversification

Taxa are not just fixed, unchanging categorizations; they are populations of similar organisms that experience diversification into distinct lineages among and within communities. These lineages provide the raw material upon which ecological selection can act, for example by adaptation to utilize new niches. In communities of complex, multicellular eukaryotes such as plants and animals, diversification, in the form of speciation, is a slow process that is rarely observable at ecological timescales. However, in microbes, this process can occur rapidly due to their short generation times, error prone replication, and the exchange of mobile genetic elements such as plasmids, transposons, and phages. The rapidity and prevalence of these mutational events generates eco-evolutionary feedbacks that drive not just the evolutionary trajectories of microbial populations but also the assembly of the communities that they make up.

There are many examples of rapid diversification within populations or communities of microbial symbionts that impact their fitness or function within the host [21–23]. Diversification can be especially important given the complexity of symbiont lifecycles, which alternate

between stages of residence within the host and transmission between hosts. However, although diversification can be an adaptive response to selection, the actual mutational events that generate these adaptions (i.e. gene duplication, transposition, conjugation) occur randomly [24]. The probability that beneficial mutations will experience positive selection depends not just on how advantageous they are, but also on the environmental context in which they emerge. Moreover, even beneficial mutations that emerge may disappear simply because they are low-frequency and lost stochastically [25].

However, many mutations have no impact on fitness, i.e. they are neutral. While selection, in the form of host-mediated processes and microbial competition, and how it shapes symbiont community assembly has been studied across many systems, the importance of stochastic processes has been less explored. We now discuss how two drivers of community assembly that continue to apply even under neutral conditions.

Dispersal

Ecological selection undoubtedly plays a critical role in determining how widely individuals of a given species are distributed. Nonetheless, the absence of a species from a community cannot necessarily be explained by its incompatibility with the environmental conditions imposed on that community. Notably, many plant and animal species inadvertently introduced from one area of the world into another are so successful that they become invasive, posing problems in their introduced ranges.

Dispersal between similar, interconnected communities is expected to homogenize these communities through the eventual admixture of taxa between them, allowing selection to act strongly upon all of them. This is the assumption behind the old maxim in microbial ecology by Baas-Becking and Beijerinck [26], that “everything is everywhere, but the environment selects”:

environmental microbes are homogenously admixed over the Earth's surface, and selection acts on different habitats in the biosphere which favors the survival and growth of only some of these microbial propagules. However, the extent to which microbial communities are truly interconnected in this manner is a subject of historical debate. Dispersal between communities is not necessarily this high, which means that communities can be more or less isolated from each other and from source communities. The fact that microbial communities are not uniformly connected, and that only some are more connected to each other than others by dispersal, is known as dispersal limitation.

Dispersal limitation is likely to be quite common in the assembly of host-associated microbial communities. Regardless of transmission mode, hosts have a limited pool of symbionts to be colonized by- they are either colonized from their immediate environment or via their parents or other social interactions [27]. On the symbiont side, microbes residing within hosts have large population sizes, especially relative to the few hosts that are available for colonization at any one time. Thus, environmentally transmitted microbes become dormant or live in relatively low densities in the environment, while socially or vertically transmitted microbes that cannot colonize a new host die with their old host. Dispersal limitation also has important consequences for microbial community structure: Low rates of dispersal can permit heterogeneity between hosts in the same population [28] and between sites on the same host [29].

Ecological Drift

As collectives of self-reproducing individuals belonging to different species, communities are subject to random fluctuations in the relative abundance of each of their component taxa. This is called ecological drift, and because it happens regardless of the selective advantage that different species have in a habitat, it is considered to operate neutrally on

communities. Just as genetic drift is more important in driving evolution in small populations than in large ones, ecological drift is more important in driving the composition of small communities than in large communities.

Hubbell's neutral theory of biogeography [30] posits that multispecies communities can be modeled as if all species with the same trophic position are functionally equivalent, lacking any differences in relative fitness in an environment. This has served as a useful null hypothesis to test the roles of dispersal and selection in community assembly [31]. Among a set of discrete communities that experience the same environmental conditions, dispersal and selection will both drive these communities to converge at similar states. On the other hand, because drift is stochastic and neutral, it drives these communities to divergent outcomes, countering dispersal and selection [32,33].

Ecological drift has important impacts on the ecology and evolution of host-microbe symbiosis. Over millions of years of drift, obligate, vertically transmitted, intracellular microbial symbionts accumulate loss-of-function mutations and experience gradual loss of fitness due to their insulation from opportunities to recombine with other genomes [9,10]. However, symbiosis is more strongly impacted by drift due to its effect on community assembly.

Drift is likely to play a strong role in symbiosis because of transmission bottlenecks. Transmission bottlenecks from one generation to the next can occur naturally as a consequence of low prevalence of symbionts in the environment [34,35], out of necessity due to limited carrying capacity for symbionts in small host zygotes/eggs/juveniles [36], or may function adaptively as a means of assessing symbiont quality or to minimize the accidental uptake of pathogens [37]. Regardless of how they emerge, drift creates local conditions of low taxonomic

or even genetic diversity in host-associated microbiomes (e.g. [28]), which can affect the evolution of cooperation and competition in microbial systems [38].

Model System

Insects have emerged as important invertebrate models for understanding the ecology and evolution of host-associated microbial communities[39]. Insects are both extremely diverse and extremely abundant across terrestrial biomes, making them both ecologically and economically important. This success can be attributed in part to the partnerships they form with beneficial microbes, which furnish them with benefits such as enhanced development, defense from predators and pathogens, and nutritional supplementation. As a result, many insects have evolved highly specialized mechanisms to control symbiont populations[40] and extract benefits from them[41,42] . In addition, the obligate and specialized nature of many insect-microbe symbioses means that insects have also evolved to maintain vertical symbiont transmission[7,43–45], guaranteeing their offspring are born with the beneficial microbial partners necessary for their survival.

As a result of frequent vertical transmission, many symbionts have eroded genomes that are unable to carry out functions otherwise essential in free-living bacteria. This has tied many obligate symbiont lineages to the lineages of their hosts, with their only means of dispersal to new hosts being through the offspring of their current host.

But bacteria can also colonize insect hosts via the environment. A host is likely to encounter many different microbes in the environment, representing a range of identities and functional capacities. This presents the host with the challenge of locating and retaining a suitable symbiont while avoiding infection by pathogens.

One group of model insect systems for studying the assembly of beneficial microbial communities are the herbivorous bugs in the superfamily Coreoidea. These bugs have specialized midguts, in which one section has been highly modified into an organ containing two or four rows comprising hundreds of sacs, called caeca or crypts [46–48]. This section, often called the M4, houses dense microbial communities comprised primarily of beta-proteobacteria in the genus *Caballeronia*, and *Caballeronia* colonization in the M4 benefits host survival and development [49]. Although *Caballeronia* bacteria are clearly beneficial, their hosts, unlike other insects that rely on specific microbes for host fitness, are born symbiont-free. Instead, these insects must acquire their symbionts from the environment. As a result, free-living species of *Caballeronia* do not have degraded genomes, and *Caballeronia* isolates from different bug species do not co-speciate with those of their hosts [20,50].

The squash bug, *Anasa tristis*, is a bug in the family Coreidae that is a major pest of cucurbit crops in North America. Like other coreids, *A. tristis* does not hatch from the egg with its *Caballeronia* symbiotns, but must acquire them from the environment. *A. tristis* houses *Caballeronia* at high population densities in M4 midgut crypts, and requires them for proper survival, growth, and development. *A. tristis* is one of several *Anasa* species native to North America, but it also ranges widely from Canada south to Brazil, co-occurring with other subtropical and tropical *Anasa* [51]. Of these species, *A. tristis* is the most notorious in its damage to cucurbits [52], both through direct feeding damage and through the vectoring of select plant-pathogenic strains of *Serratia marcescens*, the causative agent of cucurbit yellow vine disease [53,54].

Given the prevalence of multiple *Anasa* species in sympatry with *A. tristis* [51] that also harbor *Caballeronia* [50], as well as the diverse climates that *A. tristis* populations can be found

in [51], it has been thought that hosts and/or symbionts might exhibit signatures of coevolution either among *A. tristis* populations or among *Anasa* spp. Nonetheless, under lab conditions, *A. tristis* benefit from symbiont strains from across diverse clades of *Caballeronia*, isolated from different parts of its North American range, and from different host species [50].

Summary of Dissertation Chapters

Caballeronia-associated insects, like *A. tristis*, are tractable models to study processes fundamental to the ecology and evolution of animal-host associations. Many of the insect species can be reared in the lab. Because their nymphs are symbiont free upon hatching, they can be maintained *Caballeronia*-free or given a bacterial strain of interest. *Caballeronia* can be grown aerobically under standard lab conditions and fluorescently labelled to facilitate studies of colonization and transmission. Here, exploiting these properties, in Chapter 2, I explore how behavior can shape host contact with symbionts and thus symbiont acquisition. In Chapter 3, I study how priority effects limit symbiont establishment, and, finally, in Chapter 4, I study how drift shapes symbiont community assembly. Taken together, these studies elucidate the importance of taking a broad perspective as to how evolutionary and ecological processes shape the maintenance of animal-microbe symbioses.

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Chapter 2

Specialized foraging behaviors maintain reliable environmental transmission in an insect-microbial mutualism.

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Abstract

Understanding how horizontally transmitted mutualisms are maintained is a major focus of symbiosis research[1–4]. Unlike vertical transmission, hosts that rely on horizontal transmission produce symbiont-free offspring that must find and acquire their beneficial microbes from the environment. This transmission strategy is inherently risky since hosts may not find or acquire the right symbiont every generation. Similarly, microbes run the risk of being picked up by suboptimal hosts, leading to a transmission dead end. Despite these potential costs, horizontal transmission underlies stable mutualisms involving a large diversity of both plants and animals[5–9]. One largely unexplored way horizontal transmission is maintained is for hosts to evolve sophisticated mechanisms to consistently find and acquire specific symbionts from the environment. Here, we examine this possibility in the squash bug *Anasa tristis*, an insect pest that requires bacterial symbionts in the genus *Caballeronia*[10] for survival and development[11]. We conduct a series of behavioral and transmission experiments that track strain-level transmission *in vivo* among individuals in real-time. We demonstrate that nymphs can accurately find feces from adult bugs in both the presence and absence of those adults. Once nymphs locate the feces, they deploy feeding behavior that results in nearly perfect symbiont

acquisition success. We further demonstrate that nymphs can locate and feed on isolated, cultured symbionts in the absence of feces. Finally, we show this acquisition behavior is highly host specific. *Anasa tristis* nymphs fail to acquire their own beneficial symbiont if these microbes are within the feces of a closely related squash bug species, *Anasa andresii*. Taken together, our data describe not only the evolution of a reliable horizontal transmission strategy, but also a potential mechanism that drives patterns of species-specific microbial communities among closely related, sympatric host species. Our study provides some of the first direct evidence that behavior does, in fact, maintain horizontally transmitted insect-microbial mutualisms.

Results and Discussion

We aim to elucidate how host behavior is critical for maintaining specificity in a horizontally transmitted mutualism. We leverage a tractable system consisting of the widespread agricultural pest, *Anasa tristis* DeGeer (Heteroptera: Coreidae), and its beneficial microbial symbiont *Caballeronia* (formerly classified in *Burkholderia sensu lato*)[10,12–15] (Figure 1). *A. tristis* is a squash bug that relies on *Caballeronia* for growth, development, and survival[11,16]. Because *Caballeronia* is culturable and genetically manipulable, the introduction of bright, stable green and red fluorescent protein expression (Figure 1D) into different *Caballeronia* strains facilitates rapid confirmation of strain-specific patterns of colonization in live insects.

Figure 1. The tractability of the squash bug-*Caballeronia* system.
Strains of *Caballeronia* symbionts can be tagged with fluorescent proteins and experimentally fed to lab-reared *A. tristis*.

A) Inoculated adults defecate on host plants or surrounding soil.
B) Fecal deposits contain large quantities of fluorescently tagged symbionts in their feces.
C) Symbiont-free nymphs can be exposed to *Caballeronia* strains within feces harvested from adults.
D) A second instar nymph with GFP-tagged symbionts (left), and two early third instar nymphs with RFP-tagged symbionts (middle) or no symbionts (right), illustrate that fluorescent signal from symbionts established established in the host are visible through the abdominal cuticle, providing non-destructive confirmation of symbiont establishment**.** This insect-microbe system provides the unique ability to not only track strain-level transmission *in vivo* among *A. tristis* in real-time, but also provides a way to directly link symbiont availability, host behavior, and transmission success.

The obligate dependence of *A. tristis* on *Caballeronia* should favor selection for direct, vertical symbiont transmission from parent to offspring[17–20], a strategy employed by many other insects[21–27]. However, *A. tristis* instead relies on horizontal transmission, where adults produce symbiont-free offspring that must acquire their mutualistic bacteria from the environment[11,12]. This strategy is inherently risky, as some nymphs may not find the symbionts they need or may accidently acquire a less beneficial strain[28]. Indeed, recent data now suggests *A. tristis* symbionts are much rarer in the environment than previously realized (Garcia et al., *in prep*), making it even more puzzling that they would rely on environmental transmission. Yet, despite these risks, *A. tristis* nymphs consistently obtain *Caballeronia* every generation both in the lab and the field[11], suggesting they have somehow evolved robust pathways to ensure transmission.

Previous experiments have reported a high frequency of symbiont transmission across generations[11]. It was assumed that symbiont-free nymphs randomly pick up *Caballeronia* through incidental contact with soil or feeding on plants[11], as reported for related insects[5,29]. We demonstrate that nymphs instead use directed homing behavior to actively seek out their symbionts from adult feces. When adults defecate, nymphs appear to flock to the feces and deploy behavior consistent with coprophagy (Video S1, available at https://doi.org/10.1016/j.

cub.2023.05.062). Moreover, nymphs appear to sense fecal spots from a distance and move directly toward them when available (Video S2, available at https://doi.org/10.1016/j.

cub.2023.05.062). Because food was readily available to nymphs during these observations, it is unlikely this response is due to starvation or a misdirected attraction to food-based cues. Given that *A. tristis* adults frequently co-occur with nymphs in natural populations,

the ability to home in on feces as a source of *Caballeronia* would be highly beneficial for these nymphs, as it could greatly increase the likelihood of successful transmission.

We therefore conducted a series of choice assays to put *A. tristis'* symbiont finding capabilities (Figure 2A) to the test. We filmed groups of symbiont-free nymphs placed in arenas with different combinations of attractants (Figure 2B).

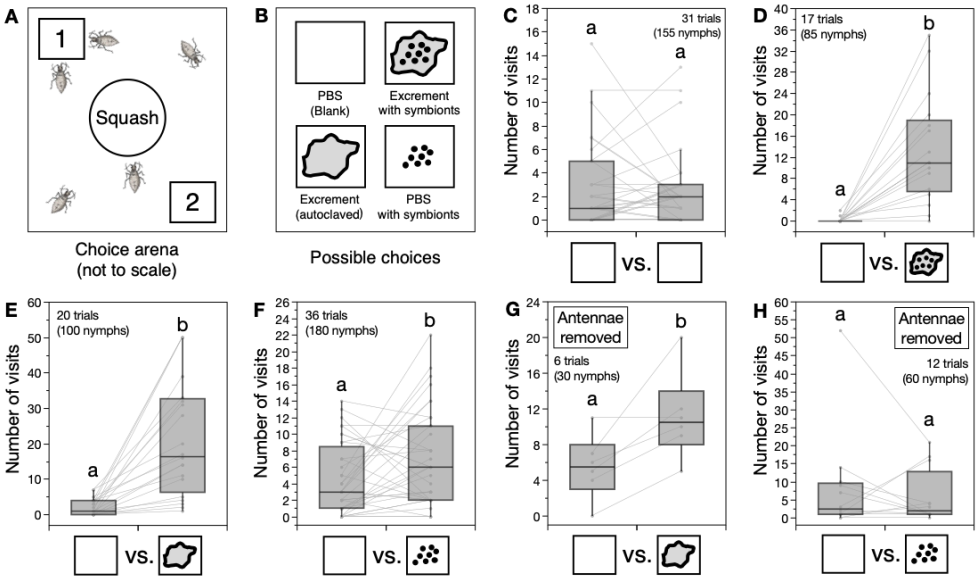

Figure 2. Influence of symbiont availability on nymph behavior.

A) *A. tristis* nymphs were placed in a series of choice trials. Each trial consisted of five symbiont-free nymphs placed in an arena with a slice of squash (food source) and two choices of attractants (boxes 1 and 2).

B) Each choice contained one of four attractants: 1) a pure PBS solution (*i.e.,* a blank), 2) adult excrement with live, GFP-tagged symbionts, 3) autoclaved adult excrement void of live symbionts, or 4) PBS solution with live, GFP-tagged symbionts.

C) -H) Nymphs were placed in trials with different combinations of attractants and recorded continuously with hi-definition cameras. Each grey line represents the total number of visits by all five nymphs to each choice in a given trial. In two scenarios (G and H), the distal segment of both antennae for each nymph was removed prior to the start of trials. Different letters indicate a significant ($P < 0.05$) bias between choices.

In the control arenas, when both choices were PBS solution (*i.e.,* no symbionts), nymphs showed no significant bias to either choice (Figure 2C; Paired t-test; n = 31; df = 30; t = 0.29; $P = 0.77$).

In arenas where one of the choices was instead a single fecal spot with *Caballeronia*, nymphs displayed extreme bias toward the fecal spot (Figure 2D; n = 17; df = 16; t = -5.38; $P < 0.0001$). Strikingly, of the 229 visits observed among these trials, 226 (98.7%) were directed toward the *Caballeronia*-positive feces. We followed up on these results with a series of experiments to decouple attraction to the feces from the symbiont itself. We presented nymphs with a choice between PBS and autoclaved feces (Figure 2E). Autoclaving the feces destroys any live symbionts, suggesting that fecal attractiveness is not reliant on ongoing symbiont metabolic activity. Nymphs in these trials still displayed significant bias toward the *Caballeronia*-negative feces (n = 20, df = 19, t = -5.41, $P < 0.0001$). Surprisingly, nymphs also displayed significant visitation bias toward just the symbiont in PBS solution (Figure 2F; n = 36, df = 35, t = -2.39, $P = 0.02$). These results indicate that *A. tristis* nymphs are strongly attracted to both fecal matter and symbionts, and that this attraction is a behavioral trait whose adaptive function is fundamental for symbiont acquisition.

We next tested the mechanisms nymphs use to detect feces and symbionts in the environment. Like other heteropterans[30,31], including the closely related alydids[32,33], squash bugs primarily use their antennae to detect contact or volatile chemicals[34] associated with food[33,35–37] or conspecific pheromones[30,35,38–42]. It is therefore likely that nymphs also rely on olfactory cues to navigate toward both the symbiont and fecal matter. We repeated the feces-only and symbiont-only choice assays described above, but this time, we ablated the distal flagellomere (segment IV) of the nymphs' antennae. The high density of sensilla on the distal flagellomere (Figure S1), which is observed across Heteroptera[30–33], suggested its removal might impede detection of olfactory cues from the excrement and symbiont.

We found that while nymphs with ablated antennae showed diminished, albeit significant, bias toward the autoclaved feces (Figure 2G; n = 6, df = 5, t = -3.24, *P* = 0.02), they completely lost preference for the symbiont by itself (Figure 2H; n = 12, df = 11, t = 0.70, *P* = 0.49). These results suggest that nymphs rely solely on olfaction to find the symbionts themselves but might integrate other cues, likely visual or social, to find feces. While it is unknown how frequently nymphs acquire symbionts that are not encapsulated in feces, our choice assays reveal behaviors unlike any previously reported in this group of pests[43,44] and show how *A. tristis* use fecal matter to maximize their chances of finding their symbiont in the environment. Taken together, these results are consistent with accumulating evidence that environmental microbes engage in chemical dialogues with animal hosts that modulate host behavior and effect their own transmission[45–49].

The fecal transmission behaviors of *A. tristis* are similar to related insects, such as stinkbugs, that transmit specific microbial partners to their offspring via frass or other anal secretions[50–58]. In these taxa, however, symbionts are deposited in association with eggs, rendering transmission effectively vertical. Instead, our data counter many comparable studies of horizontally transmitted mutualisms that suggest hosts randomly find their beneficial partners (but see references below[32,33]). For example, the alydid bean bug *Riptortus pedestris*, which also hosts *Caballeronia* symbionts, does not exhibit directed search behavior during symbiont acquisition, either for symbiont cultures[44] or feces[59]. Instead, *R. pedestris* appears to acquire symbionts opportunistically from the host plant rhizosphere[59], and employs sophisticated mechanisms that enrich for *Caballeronia* within the midgut itself[60–63]. This exclusive reliance on a physiological post-acquisition mechanism (*i.e.*, partner choice) has been well characterized in a diversity of insect[59,64–66] and non-insect mutualisms, including those in the well-studied

legume-rhizobial nitrogen-fixing symbiosis[67–71] and the squid-*Vibrio* luminescent symbiosis[8,72–74]. However, these post-acquisition filtering strategies are still predicated on opportunistic encounters with the right symbiont. Such mechanisms are theoretically problematic when host and microbial community compositions are patchy[3]. Moreover, indiscriminate uptake of microbes from the environment leaves the host vulnerable to parasites, pathogens, and "cheaters"[28,62,75]. By implementing sensitive pre-acquisition detection behaviors, *A. tristis* nymphs can mitigate these risks by homing in on specific sources of their primary symbiont. Such a strategy should theoretically reduce the time, energy, and risk associated with combing the environment for opportunistic encounters[28].

Since *A. tristis* nymphs presumably incur substantial energetic cost and predation risk to forage for feces, a complementary mechanism should evolve to halt this behavior upon successful symbiont uptake. Indeed, preliminary observations indicated that symbiont-positive nymphs spend most of their time feeding and very little time wandering around their enclosures. Nymphs that were deprived of symbionts displayed the opposite behavior, where a vast majority of their time was spent walking around and very little time feeding (Video S3, available at https://doi.org/10.1016/j.cub.2023.05.062). We therefore revisited the videos of our choice assays, rewatching a subset of arenas to determine if symbiont availability influenced levels of *A. tristis* activity. We re-analyzed two types of choice assays: 1) arenas where both choices were PBS (*i.e.*, no symbionts available to nymphs; Figure 2C) and 2) arenas where one choice was PBS and the other was feces with symbionts (*i.e.*, symbionts were available to nymphs; Figure 2D).

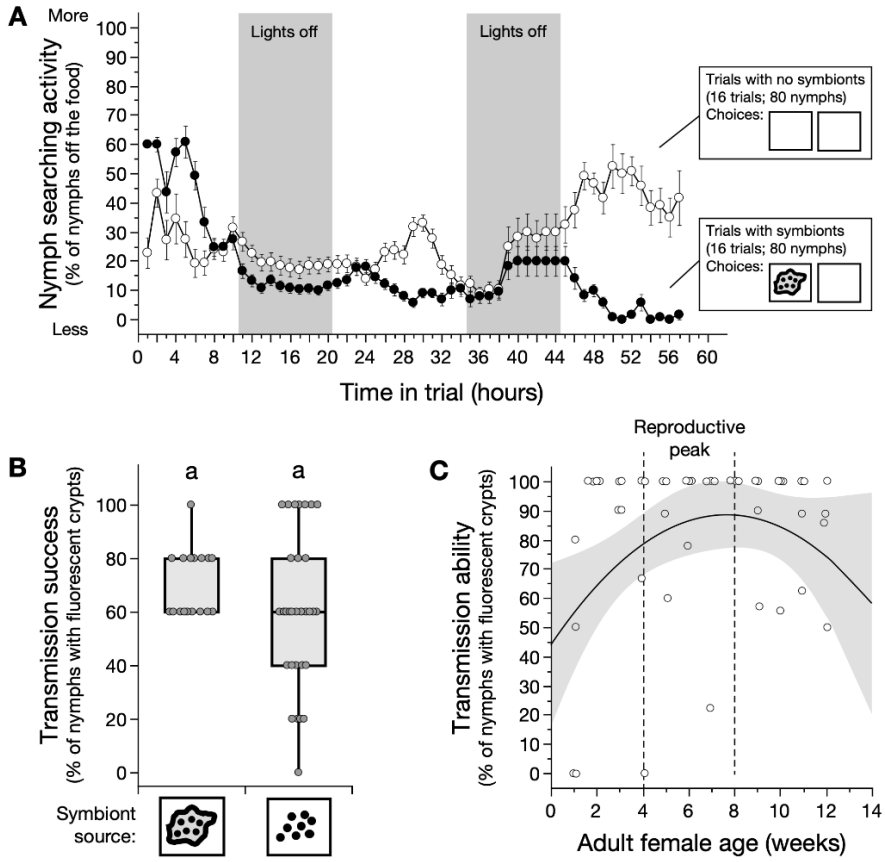

Figure 3. Symbiont transmission dynamics.

A) Nymph movement was quantified in a subset of choice trials from Figure 2C,D. Searching behavior was assessed every 10 mins in boxes that had no symbionts (*i.e.,* both choices were blanks, Figure 2C) and those that had excrement with symbionts (Figure 2D).

B) In two types of choice trials where symbionts were available (Figure 2D,F), all nymphs were removed from arenas and examined for GFP fluorescence. Transmission was considered successful if nymphs had fluorescent bacteria in their midgut (see Figure 1D for example). Each point represents the percentage of nymphs (out of 5) that were positive for symbionts for a given trial. Different letters indicate significant differences ($P < 0.05$).

C) Ability of adult females to transmit symbionts throughout their lifetime. Groups of four adult females (n = 4 groups) were exposed to a new set of 10 symbiont-free nymphs every week for 12 weeks, which is the approximate lifespan for *A. tristis*. The percentage of nymphs that acquired symbionts was assessed each week (white points). Reproductive peak of females lies within the dashed lines and indicates when females are most fecund (from Villa et al. 2021[76]).

We found that nymphs without access to symbionts displayed significantly more searching behavior than those with symbionts (Figure 3A; Table 1; GLMM, $P < 0.0001$).

Table 1. Summary of GLMM results comparing searching behavior of nymphs in choice trials either with or without symbionts (Figure 3A), with 6,372 observations from 32 trials (16 with symbionts, 16 without symbionts). The intercept is set to choice trials with symbionts at the last timepoint.

Random effects	***Variance***	***Std. Dev.***			
Trial	1.04	1.02			
Fixed effects	***Estimate***	***Std. Error***	***z value***	***P***	
(Intercept)	-2.81	0.27	-10.38	<0.0001	****
Treatment	1.99	0.38	5.27	<0.0001	****
Time	-0.01	0	-25.72	<0.0001	****
Treat x Time	0.01	0	16.61	<0.0001	****

Amazingly, this behavioral difference became more exaggerated the longer nymphs were deprived of *Caballeronia*. At the beginning of the trials, nymphs in both treatments spent similar amounts of time searching the arena. However, following this initial acclimation period, most nymphs in the trials that contained symbionts successfully acquired *Caballeronia*, ceased wandering, and consistently stayed on the food for the remainder of the experiment. Nymphs without access to symbionts displayed the opposite behavioral patterns (Figure 3A). These results confirm that successful *Caballeronia* colonization induces a behavioral cues in nymphs to switch from searching to feeding, presumably to fuel the growth and development that can only progress with the help of their symbiont[11]. Our data highlight how *A. tristis* can shut down

searching behavior when it is no longer needed, further reducing the risks associated with environmental transmission.

In addition to symbiont-seeking behavior, we also explored the mechanisms and efficacy of symbiont acquisition. We confirmed that when nymphs successfully find a source of symbionts, they insert their stylets into the solid fecal material and appear to use a lacerate-and-flush feeding technique that liquifies the feces. This behavior presumably facilitates extraction of the embedded beneficial microbes (Video S4, available at https://doi.org/10.1016/j.cub.2023.05.062). To our knowledge, this feeding strategy, which is typical of seed-feeding Heteroptera[34,77], has not been described in Coreidae before and leads to effective uptake of symbionts. In the choice trials that contained symbiont-positive feces (Figure 2D), 70.6% (60/85) of nymphs harbored fluorescent bacteria in their abdomens. Acquisition success was similarly high when the symbiont was not embedded in feces (Figure 3B; Table 2; GLMM, $P = 0.18$). In these trials, 61.1% (110/180) of nymphs acquired *Caballeronia*, indicating that the feces itself is not necessary for successful colonization. Rather, we hypothesize that the fecal matter could protect symbionts from desiccation, UV radiation, and other environmental factors until they can be taken up by a host. In this way, the fecal matrix would be functionally similar to anal secretions produced by other symbiont-reliant bug species[78]**.**

Table 2. Summary of GLMM results comparing symbiont acquisition success of nymphs in choice trials (Figure 3B) with 53 observations. (I) indicates the intercept for the model

Random effects	***Variance***	***Std. Dev.***
Trial length	0.29	0.54

Fixed effects	*Estimate*	*Std. Error*	*z value*	*P*	
Symbionts in Excrement (I)	0.92	0.28	3.29	0.001	**
Symbionts in PBS	-0.44	0.34	-1.29	0.198	

While nymphs have clearly evolved the ability to find and consume *Caballeronia*, they still depend on the symbiont being sufficiently available in the environment. The frequency and timing of adult defecation therefore becomes critical for transmission success. In many systems, adults only shed symbionts when they are ready to reproduce[57,79]. Females will often deposit symbionts on or near eggs to immediately be ingested by newly hatched offspring [54,57,58,80]. *A. tristis* nymphs, however, do not ingest their symbionts as soon as they hatch. Instead, hatchling nymphs wait two days until they molt into their second instar to acquire symbionts[61]. We therefore tested if adult *A. tristis* defecated more when second instar nymphs were present. Females housed with and without nymphs produced similar amounts of symbiont-rich feces (T-test; n = 34, DF = 29.0, t = 0.40, $P = 0.69$). This suggests that adults shed symbionts independently of the presence or absence of the next generation. We then conducted a complementary experiment to assess how squash bug age influences their ability to transmit symbionts. By tracking transmission dynamics over the course of 12 weeks, which is the typical lifespan of *A. tristis* females [76], we found that adult females can pass *Caballeronia* to nymphs throughout their entire lives (Figure 3C). Interestingly, data also show that while these females maintain high overall transmission potential as they age, this ability peaks when previous studies

have shown females are most fecund[65] (Figure 3C; Table 3; GLMM, $P = 0.0007$). This pattern may correspond with some physiological aspect of host aging, such as feeding rate and metabolism, which could impact fecal excretion rate, or alternately, it may reflect changing symbiont titers being excreted. Either way, the consistent output of symbionts in feces ensures that if *A. tristis* adults are around, symbionts should be readily available for nymphs regardless of when and where they hatch.

Table 3. Summary of GLMM results testing the lifetime ability of adult female *A. tristis* to transfer symbionts to nymphs (Figure 3C), with 48 observations from 4 groups of females.

Random effects	***Variance***	***Std. Dev.***			
Group	0.19	0.43			
Fixed effects	***Estimate***	***Std. Error***	***z value***	***P***	
(Intercept)	2.18	0.55	3.96	<0.0001	****
poly (Female age, 2) 1	3.35	1.01	3.32	0.001	**
poly (Female age, 2) 2	-3.53	1.04	-3.4	0.0007	***

Lastly, we explored the fidelity of *A. tristis'* horizonal transmission strategy in complex, semi-natural communities where nymphs are exposed to different combinations of host species and symbiont strains. Although *Anasa* species in North America have broadly overlapping ranges[81], a previous study found different squash bug species at a single field site harbor closely related but distinct strains of *Caballeronia*[82]. Traditionally, this pattern of phylosymbiosis might be interpreted as evidence of host-symbiont co-evolution[83], where interactions between certain lineages of host and microbe are sustained by reciprocal selection

for increasing compatibility. Paradoxically, however, transplant experiments show no negative consequences for *A. tristis* that are inoculated with *Caballeronia* strains isolated from other squash bug species[82]. This raises the possibility that mere selection for optimal host-symbiont combinations is not sufficient, on its own, to explain *Anasa*-*Caballeronia* phylosymbiosis; instead other host-related factors, such as those driving symbiont acquisition, drive host-symbiont co-specificity.

We used experimental inoculations to create four types of hosts: 1) *A. tristis* adults harboring the *A. tristis* symbiont strain, 2) *A. tristis* adults harboring the *A. andresii* symbiont strain, 3) *A. andresii* adults harboring the *A. tristis* symbiont strain, or 4) *A. andresii* adults harboring *A. andresii* symbiont strain (Figure 4). After confirming that both host species can deposit both strains of *Caballeronia* in their feces (Supplemental Figure S2), we exposed symbiont-free *A. tristis* to one of the four host types. Our design allowed us to decouple the influence of host species and symbiont strain on transmission to *A. tristis*. Nymphs had the highest acquisition success when housed with *A. tristis* adults that shed *A. tristis*-derived symbionts (Figure 4). Among these 10 replicated enclosures, 88.8% (80/90) of nymphs took up the symbiont. However, acquisition success significantly dropped to 71.9% (64/89) when nymphs were instead exposed to *A. tristis* adults that passed an *A. andresii*-derived symbiont (Figure 4; Table 4; GLMM, $P < 0.0001$). These results were surprising given this strain of *Caballeronia* is no less beneficial than the *tristis*-derived strain for *A. tristis*[82]. Despite the drop in acquisition success, these data confirm that conspecific transmission among *A. tristis* is stable even when passing different *Caballeronia* strains.

Amazingly, however, transmission success dropped precipitously when nymphs were housed with *A. andresii* symbiont donors. Nymphs were significantly less successful at picking

up not only the *A. andresii* symbionts (Figure 4; Table 4; GLMM, $P < 0.0001$) but their own *A. tristis* symbionts (GLMM, $P < 0.0001$) as well. Of the 190 nymphs involved in the 20 heterospecific transmission cages, only 7 (3.6%) obtained symbionts from *A. andresii* adults. Of these, 5 were from a single cage.

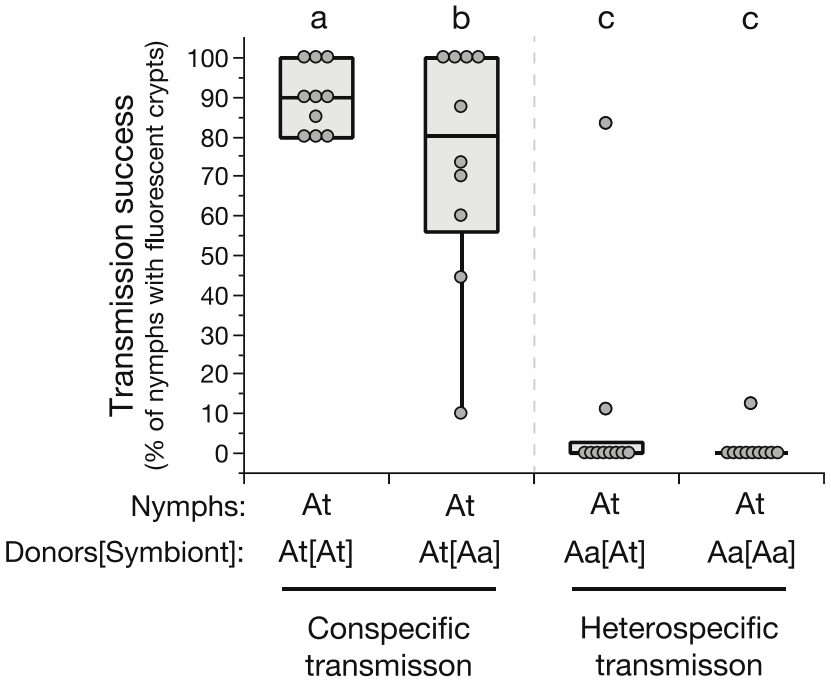

Figure 4. Transmission specificity. Groups of symbiont-free *A. tristis* (At) nymphs were placed on plants with either: 1) *A. tristis* adults harboring *A. tristis* symbiont strains (At[At]; n = 10 groups), 2) *A. tristis* adults harboring *Anasa andresii* (Aa) symbiont strains (At[Aa]; n = 10 groups), 3) *A. andresii* adults harboring *A. tristis* symbiont strains (Aa[At]; n = 10 groups), or 4) *A. andresii* adults harboring *A. andresii* symbiont strains (Aa[Aa]; n = 10 groups). Letters indicate significant differences (ANOVA with Tukey post-hoc tests, $P < 0.05$).

The paucity of heterospecific transmission indicates that patterns of host-symbiont specificity in this group may be a by-product of host behavior. In other words, narrow preferences for conspecific feces may increase the likelihood that nymphs acquire *Caballeronia* deposited by their species, even though they may be compatible with many other microbial strains in the environment. Consistent with theoretical predictions based on individual mammalian gut

taxa[84], but contrary to conventional assumptions[85], our empirical results show that species-specific transmission pathways alone can promote phylosymbiosis. Future studies should concentrate on the mechanisms hindering heterospecific transmission. We suggest the inability of *A. tristis* nymphs to either: 1) find *A. andresii* feces, or 2) extract the symbiont from heterospecific fecal matter. Indeed, it would be interesting to compare the chemical composition and physical characteristics of *A. tristis* and *A. andresii* feces to determine if *A. tristis* nymphs respond preferentially to the chemical signature of their own species' feces. Finally, additional choice and feeding assays should be run to examine *A. andresii* acquisition behaviors to determine how widespread these behaviors are among this group of pests.

Table 4. Summary of GLM model results comparing symbiont acquisition success of *A. tristis* nymphs exposed to adults that vary in host species and symbiont origin (Figure 4). n = 40, $c^2 = 274.14$, $P < 0.0001$. (I) indicates the intercept for the model; *Group* indicates significance after Tukey post-hoc tests.

Coefficients	***Estimate***	***Std. Error***	***z value***	***P***	***Group***
At donor with *At* symbionts (I)	2.08	0.34	6.2	<0.0001	A
At donor with *Aa* symbionts	-1.14	0.41	-2.78	0.0055	B
Aa donor with *At* symbionts	-4.86	0.53	-9.04	<0.0001	C
Aa donor with *Aa* symbionts	-6.53	1.06	-6.16	<0.0001	C

Our study provides insight into a longstanding paradox concerning the origin and maintenance of specificity in horizontally transmitted insect-microbial mutualisms. Unlike many studies that have relied on indirect inferences of transmission events, we incorporate direct behavioral observations to reveal how hosts can systematically reduce the risks of obtaining

beneficial symbionts in the environment every generation. These data run counter to previous assumptions that symbiont transmission in these types of insect-microbe systems takes place randomly through the environment. Overall, our study reveals how specialized behavior not only drives the evolution of a reliable horizontal transmission strategy, but also provides mechanisms that drive patterns of species-specific microbial communities among closely related, sympatric host species.

Methods

Table 5. Reagents, organisms, and other materials used in this study.

Reagent or resource	**Source**	**Identifier**
Bacterial and virus strains		
Caballeronia sp. strain GA-OX1	Acevedo *et al.* (2021)[11]	n/a
Caballeronia sp. strain AAF182	Stoy *et al.* (2021)[82]	n/a
Escherichia coli SM10(λpir)	Miller & Mekalanos 1988[86] via Wiles *et al.* 2018[87]	LMBP 3889
Experimental Models: Organisms/Strains		
Anasa tristis	Acevedo *et al.* (2021)[11]	n/a
Anasa andresii	Villa *et al.* (2021)[76]	n/a
Oligonucleotides		
Primer: glmS-1434F AGGCGCGTTGAAGCTCAAGG	This study	n/a
Primer: aacC-83F GTATGCGCTCACGCAACTGG	This study	n/a
Primer: lacI-3644F TCGCAGAGTATGCCGGTGTC	This study	n/a
Recombinant DNA		
pTn7xKS-sfGFP	Wiles *et al.* (2018)[87]	Addgene plasmid 117394

pTn7xKS-dTomato	Wiles *et al.* (2018)[87]	Addgene plasmid 117395
pTNS2	Choi *et al.* (2005)[88] via Wiles *et al.* (2018)[87]	Addgene plasmid 64968
Software and algorithms		
Olympus cellSens Standard software ver. 1.13	Olympus Corporation	https://www.olympus-lifescience.com/en/software/cellsens/
LAS X ver. 3.4.2.18368	Leica Microsystems GmbH	https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/
BORIS ver. 7.12.2	Friard & Gamba (2016)[89]	https://www.boris.unito.it/

Resource and Materials Availability. Send requests for resources and reagents to Scott Villa and Nicole Gerardo. This study did not generate any new, unique reagents.

Data and code availability. All data are available in the figures, tables, and data files associated with this manuscript. This study did not result in any unique code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Experimental Model and Subject Details. Squash bugs (*Anasa tristis* and *A. andresii*) are reared as described in Acevedo et al. (2021)[90]. The squash bug lab colonies are descended from bugs collected in Gainesville, Florida, to which wild bugs from the original source populations are added every year to maintain genetic diversity. Bugs are maintained at 27-30°C with a day/night cycle of 16 hours light/8 hours darkness. Five to ten adults are reared in 12 × 12 × 12 inch cubic mesh cages, each containing a potted summer squash (*Cucurbita pepo* "Goldstar") plant, which is changed out as needed. Egg clutches are collected every three to four days, surface sterilized with successive washes of 70% denatured ethanol, 20% bleach, and DI

water, and air dried in plastic rearing containers in a laminar flow hood. First instar nymphs, which do not need to feed but require moisture, are maintained on pieces of organic zucchini squash wrapped in Parafilm, on which they molt to the second instar in two days. Second instars to be used in experiments are drawn from this pool and are maintained on zucchini for no more than one week.

Caballeronia sp. strain GA-OX1 was previously isolated from homogenized M4 crypts dissected from an adult squash bug (*Anasa tristis*) collected in Oxford, Georgia[90]. *Caballeronia* sp. strain AAF182 was previously isolated in the same manner from a female squash bug in the community organic garden at the University of Florida[82]. For experiments, strains are streaked out onto nutrient agar plates from 25% glycerol stocks stored at -80°C, and incubated at 30°C for two days. To prepare liquid cultures, 3 mL salt-free LB (Luria-Bertani) broth cultures are inoculated with a single colony from each plate streak and grown to log phase at 30°C shaking at 225 rpm overnight.

Method Details

Fluorescent-labelling of *Caballeronia* strains. The mini-Tn7 system[88] enables site- and orientation-specific insertion of recombinant DNA in an intergenic region 20-25 base pairs downstream of the glmS gene in eubacteria. We used modified mini-Tn7 vectors[87] to label *Caballeronia* strains with superfolder GFP[91] (henceforth GFP) and dTomato[92] (henceforth RFP). Crucially, the tac promoter[93] and an efficient ribosomal binding site (iGem part BBa_K2306014) in these mini-Tn7 constructs drives stronger fluorophore expression than constructs previously utilized in *Caballeronia* (i.e. Koch et al. 2001[94]), enabling rapid screening of live or whole bugs up to adulthood for symbiotic infection without dissection.

Furthermore, the gentamicin resistance marker, combined with inducible toxic peptides on the plasmid backbone[87], enables efficient selection for transconjugants without relying on lab-evolved antibiotic resistant mutants as target strains[95]. The modern standard helper plasmid that provides the genomic integration function *in trans* contains only the TnsABC+D pathway for *att*Tn7-specific integration, unlike obsolete plasmids that encode the additional TnsABC+E non-specific integration function[96].

Triparental conjugations were conducted as follows. *Escherichia coli* SM10(λpir) carrying the donor plasmids pTn7xKS-sfGFP and pTn7xKS-dTomato, as well as the helper plasmid pTNS2, were a generous gift from Dr. Travis Wiles[87]. *Caballeronia* liquid cultures were grown as described above. Donor and helper SM10(λpir) were grown overnight in LB broth with 150 µg/mL ampicillin for selection at 37°C shaking at 225 rpm. Donor and helper cultures were washed at least twice to remove residual ampicillin by repeatedly pelleting at 10,000 xg for 2 minutes at 4°C, decanting the supernatant, and resuspending in the same volume of LB. These were combined with washed *Caballeronia* on LB agar containing 1% NaCl to mate for 24-50 hours at 30°C. Matings were harvested into salt-free LB broth, subjected to selection with lytic T7 phage for 4 hours to eradicate *E. coli*, then plated on low-salt LB or M9 + 0.4% glucose[97] agar plates, both containing 1mM IPTG and 5-10 µg/mL gentamicin for selection. Fluorescent colonies were streaked on nutrient agar to confirm stability of fluorophore expression, and correct insertion of the mini-Tn7 construct was confirmed by PCR amplifying the fragment bridging the glmS gene and the gentamicin resistance marker aacC, using primers glmS-1434F and aacC-83F. The absence of the plasmid backbone in these strains was confirmed by PCR amplifying the fragment bridging aacC and the lacI gene, using primers aacC-83F and lacI-3644F.

Preparation of donor insects. Eggs were collected from lab colonies of *A. tristis* and *A. andresii*, surface sterilized, and air dried as described above. To maintain the sterility of the bugs prior to infection, hatchlings were maintained on droplets of DI water until they molted to the second instar, which takes place two days after hatching. Second instar nymphs were subsequently maintained on ~100 µL of 2% glucose in 10% PBS solution, which was provided as droplets every 24 hours, until symbiont inoculation. Nymphs were not maintained on this sterile diet for more than a week.

To prepare symbiont inocula, overnight liquid cultures were started from single colony picks as described above. 200 µL of log-phase culture was then washed twice in phosphate-buffered saline (PBS), by pelleting for 2 min at 10,000 ×g and decanting the supernatant. Inocula were prepared by diluting these washed cells 100-fold in 400 µL of feeding solution (2% glucose in 10% PBS), and spotting droplets of these inocula in plastic rearing boxes that housed second instar nymphs that had been starved for ~24 hours. Nymphs typically imbibed these droplets readily, but were exposed to inocula for 24 hours before being transferred to parafilmed cubes of surface-sterilized zucchini. Bugs that did not feed typically starved to death, but colonization was verified by anesthetizing second instars after 2-3 days with carbon dioxide and checking nymphs individually for fluorescence. Successfully infected bugs were reared, segregated by host species and symbiont strain, to adulthood on summer squash plants in mesh cages, as described above.

Microscopy. Images of bacterial colonies and fecal spots, as well as videos of feeding, were taken on an Olympus SZX16 stereomicroscope with an Olympus XM10 monochrome camera and Olympus cellSens Standard software ver. 1.13. For rapid phenotyping of nymphs for GFP and RFP symbiont colonization, nymphs were immobilized with carbon dioxide or killed

with 70% ethanol, then individually examined at the SZX16 stereomicroscope for GFP and RFP signal with the camera set to autoexposure.

Brightfield images of second instar *A. tristis* antennae were taken using whole nymphs were killed in 70% ethanol and mounted on depression slides. Images were taken on a Leica DMi8 inverted widefield light microscope with a Leica DFC9000 GT fluorescence camera and Leica Application Suite X ver. 3.4.2.18368 software.

Choice assay. Our initial observations showed that second instar nymphs kept in plastic rearing boxes exhibited a strong attraction to feces in the bedding used to line adult rearing cages. To characterize the magnitude of this attraction and the cues that elicited this response, we set up choice assays in bleach- and ethanol-sterilized 11.2 cm × 11.2 cm × 3.9 cm plastic rearing boxes with a piece of parafilm covered organic zucchini squash in the middle of the arena and two choices (fecal spot vs. PBS, autoclaved fecal spot vs. PBS, cultured symbiont vs. PBS, and PBS vs. PBS) in opposing corners of the boxes. The symbiont strain used as the autoclaved or untreated fecal spot and as the cultured bacterial treatment in all these choice assays was always GA-OX1 sfGFP-V10, to control for strain-specific effects. For cultured symbiont vs PBS, a black mark was placed on the side of the box with the cultured symbiont to distinguish between the two treatments. The control filter paper was cut to a size similar to the paper towels containing fecal spots. Five second instar nymphs were placed onto the squash piece, and boxes were recorded with two boxes under each camera. Orientation and order of each box was decided using a random number generator. To prevent interactions between nymphs in adjacent boxes, white tape was applied to the adjoining side of the boxes. All trials were recorded using overhead HD cameras for 24 or 48 hours in a humidity and light-controlled room. Using second instar nymphs with ablated antennae to specifically test the role of olfaction, we conducted additional

cultured symbiont vs PBS, fecal spot vs PBS and autoclaved fecal spots vs PBS. Assays were recorded continuously using high-definition Owl AHD10–841-B cameras, equipped with infrared bulbs to film in complete darkness. Videos were exported as separate ~17 minute files that were stitched together using MacX Video Converter Pro. After assays were completed, nymphs were allowed to develop for several more days and checked for GFP colonization **by visual inspection of whole and dissected nymphs.**

Fecal spot collection and sterilization. Paper towels were used to line the bottom of cages where we maintained donor *Anasa tristis* adults infected with GA-OX1 sfGFP-V10. We checked these paper towels every one to two days for dark, semisolid, fecal spots, which are distinct from the clear, watery feces excreted more frequently by bugs during feeding. As soon as fecal spots were identified, they were cut out from the surrounding paper towel and immediately used in a choice assay or sterilized. Collected paper towel cutouts with fecal spots were sterilized by autoclaving in a glass beaker on a gravity cycle (sterilization cycle of 121°C for 30 minutes with a dry cycle of 10 min.).

Cultured bacteria. Two and one-half mL of overnight culture were washed twice in phosphate-buffered saline (PBS), by pelleting for 2 min at 10,000 xg and decanting the supernatant. This pellet was then resuspended in 2.5 mL of PBS, bringing the washed cells back to their original concentration. For assays comparing PBS to cultured bacteria, 150 µL of washed cells were spotted onto a quartered qualitative filter paper disc (diameter 55 mm) in one corner of each arena, and the same amount of PBS was spotted onto a quarter filter paper disc in the opposite corner. Serial dilution of the inocula *post hoc* confirmed that cells had high viability at the time they were used in the assays.

Antennal ablation. We chose to ablate only the fourth antennal segment, rather than the whole antenna, because 1) nymphs with completely ablated antennae exhibited extremely poor survival, impaired mobility, and reduced activity; and 2) microscopy showed that the fourth antennal segment is covered in an unusually high density of sensilla relative to the rest of the antennal segments, in line with observations from related insects[32]. Nymphs were briefly anesthetized with carbon dioxide then placed into a glass dish with sterile DI water to immobilize them. The fourth antennal segment was removed using pairs of fine metal forceps. To minimize damage to the rest of the antennae, one set of forceps gently grasped the fourth antennal segment, or the distal part on the third antennal segment, while the second set of forceps was run across the second forceps to sever the fourth segment cleanly at the intersegmental membrane. Control bugs were treated in the same way as ablated bugs, but antennal segments were gently manipulated without being severed. Ablated and control bugs were returned to rearing boxes lined with paper towels to dry off and allowed to recover for two days before being used in choice assays.

Video analysis. We measured searching behavior in the video-recorded choice assays using Behavioral Observation Research Interactive Software (BORIS) ver. 7.12.2, an open-source event logging software for live observations of animal behavior[89]. Because each arena contained 5 nymphs, the ethogram was set up with 6 point events, ranging from 0 bugs to 5 bugs. Additionally, each event had 2 modifiers corresponding to the 2 attractants in each arena (feces vs. PBS, cultured bacteria vs. PBS, PBS vs. PBS). Video frames were then evaluated at 10-minute intervals for the entire 24-hour period and the number of bugs sitting on filter paper laden with each attractant was recorded. To quantify searching behavior over time, the number of bugs

wandering off the squash was counted at 10-minute intervals for the entire 48-hour time period, with 0 indicating that all bugs were on the squash in each frame.

Fecal spot elicitation. We wanted to test if adult fecal production is socially influenced by exposure to nymphs. To test this, same-sex pairs of adult donors infected with the same symbiont were placed in plastic rearing boxes, to which either five second instar nymphs were added or no nymphs were added. Each box was lined with paper towels, which were monitored daily for 11 consecutive days for the appearance of dark fecal spots.

Age-dependent transmission. We sought to determine whether ability to transmit symbionts changed during the lifespan of adult squash bugs. Four replicate cages were set up each with four *Anasa tristis* donors infected with RFP symbionts (due to the higher intensity RFP signal) as described above. To prevent mating and nymphal recruitment within these cages, and to control for sex, donors were sexed as fifth instar nymphs, just prior to adulthood, and only female nymphs were kept to be used in experiments. Groups of donors were passaged to new autoclaved cages with new plants and clean water on a weekly basis to prevent symbiont transmission via accumulated biological material over the course of the experiment. The experiment was terminated after 12 weeks, the typical lifetime of unmated *A. tristis* females[76]. Cohorts of nymphs were assessed for colonization rate within the four replicate cages on a weekly basis. Ten aposymbiotic L1 or L2 nymphs were introduced and allowed to develop. During the weekly passaging of adult donors, live nymphs were harvested, euthanized with 70% alcohol, and checked for RFP under a NightSEA RFP filter attached to a dissecting microscope.

Interspecific transmission mesocosm assays. Cages surrounding a single potted summer squash plant were set up containing either four adult donor *A. tristis* or four adult donor *A. andresii*, reared as described above and colonized with either GFP- or RFP-labelled GA-OX1 or

AAF182. After 5 days, 10 first to second instar nymphs were introduced into the cages; adults continued to be maintained on the plant. (First instars are a two-day long non-foraging stage, but were included for use in these assays because eggs are only synchronized every three to four days.) After 10 days, surviving nymphs were collected from the cages, killed in 70% EtOH, and individually assessed for presence/absence of GFP and RFP. Experiments were started in blocks to ensure that we could compare across treatments.

Statistical analyses. Preferences in choice assays (Figure 2) were assessed using a series of paired t-tests, where the total number of visits to each choice was quantified for every trial. We analyzed symbiont transmission dynamics using a series of generalized linear mixed models (GLMMs) (Figure 3; Tables 1-3). First, we used a GLMM with Poisson distribution to model searching behavior as a function of treatment (with or without symbionts), time in the trial, and their interaction (Figure 3A; Table 1). We included trial as a random effect to account for the repeated measures of each trial over the course of the experiment. The intercept of the model was set to the choice trials at the end of the experiment. Next, we model transmission success as a function of symbiont availability (Figure 3B; Table 2). Because transmission success was scored as “yes” or “no” for each nymph, we used a GLMM assuming a binomial distribution and logit link function. Trial length was included as a random effect to account for any influence of time spent in the trial on likelihood of picking up a symbiont. Finally, we used a GLMM with binomial distribution and logit link function to model transmission success as a function of female age (Figure 3C; Table 3). We evaluated both a linear and quadratic relationship between transmission and female age. We included group as a random effect to account for repeated measures of transmission success in each cage over time.

We analyzed transmission specificity using a generalized linear model (GLM) with binomial distribution (Figure 4; Table 4). We assessed the likelihood that a nymph would pick up symbionts as a function of the donor type. Doner type consisted of a combination of host species and symbiont strain. Post hoc tests were run to account for multiple comparisons.

All models were fit in R using the 'lme4' library package[98,99] (Bates et al. 2015); R Core Team 2016). Degrees of freedom and resulting *P*-values were estimated with the Satterwhite approximation using the lmerTest library[100] (Kuzentsova et al. 2016). When post hoc comparisons between treatments were performed, we used a Tukey's honesty significant difference test (HSD) using the R library package 'emmeans'. The distribution that best fit the data for each model was determined using the 'fitdisplus' package v.1.1e12[101] (Delignette-Muller and Dutang, 2015).

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Supporting Information

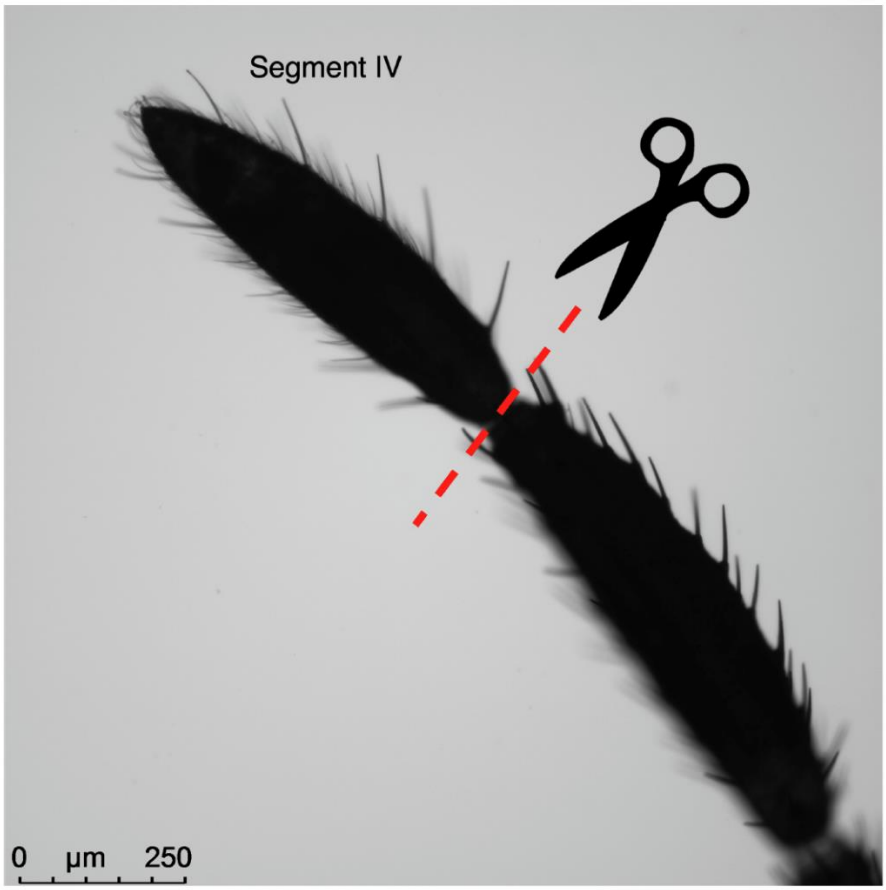

Figure S1. Antennal ablation of distal flagellomere, related to Figure 2. The sensilla rich distal segment (IV) of the *A. tristis* nymph antennae were clipped off to determine how host find symbionts. Nymphs with this segment removed where placed in choice assays shown in Figure 2 F,H.

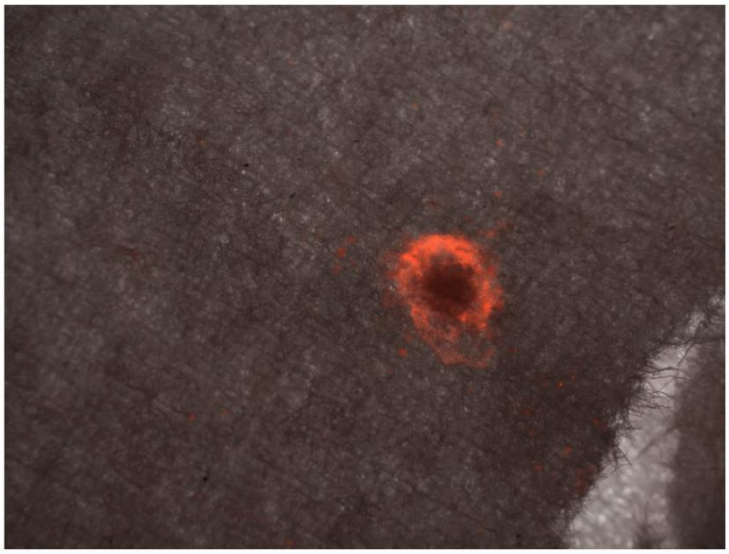

Figure S2. Symbiont rich feces of *Anasa andresii*, related to Figure 4. A fecal spot deposited by *A. andresii* colonized with the *A. andresii* symbiont AAF182 tagged with RFP, displaying RFP signal. *A. andresii* were used as symbiont donors to determine species-specific patterns of transmission among *Anasa* squash bugs (see Figure 4).

Chapter 3

Strong priority effects without tissue remodeling in the assembly of an insect-microbe symbiosis.

Abstract

Microbial community assembly is determined in part by interactions between taxa that colonize ecological niches available within habitat patches. The outcomes of these interactions, and by extension the trajectory of community assembly, can display priority effects - dependency on the order in which taxa first occupy these niches. The underlying mechanisms of these phenomena vary from system to system and are often not well resolved.

Here, we characterize priority effects in colonization of the squash bug (*Anasa tristis*) by bacterial symbionts from the genus *Caballeronia*, using pairs of strains that are known to strongly compete during host colonization, as well as strains that are isogenic and thus functionally identical. By introducing symbiont strains into individual bugs in a sequential manner, we show that within-host populations established by the first colonist are extremely resistant to invasion, regardless of strain identity and competitive interactions. By knocking down the population of an initial colonist with antibiotics, we further show that colonization success by the second symbiont is still diminished even when space in the symbiotic organ is available and physically accessible for colonization. We propose a paradigm in which established symbionts exclude subsequent infections by manipulating the host environment, partially but not exclusively by eliciting tissue remodeling of the symbiont organ.

Introduction

The composition of microbial communities is determined not just by colonization and extinction events among its constituent members, but also by the timing of these events. Cases where colonization ability of later-arriving taxa are conditional on the taxa already established in that habitat are referred to as showing *priority effects*. Microbial community assembly across different systems may exhibit priority effects for many reasons[1,2]. Most simply, early colonizers can simply occupy a physical or nutritional niche, leaving fewer resources for later colonizers. In addition, early colonizers can modify the physical or chemical environment in a way that facilitates or hinders colonization by immigrating taxa [3,4]. In particular, microbes that establish within a community first can deploy density-dependent strategies to combat less numerically dominant competitors. These strategies include socially-mediated transcriptional regulation (i.e., quorum sensing [5]) and collective activity in spatially structured habitats [6–10]. The net effect of such processes is that outcomes of community assembly are contingent on the history and timing of past colonization events.

Microbial communities are frequently associated with multicellular hosts [11], which can be considered discrete, island-like habitat patches open for colonization by competing microbes [12–14]. However, hosts also undergo immunological, physiological, developmental, and behavioral transitions in response to microbial colonization, which can affect the probability of subsequent colonization. As a result, while host-associated microbial communities follow the same rules of assembly as other ecological communities, the host is a unique contributor to the ecology of host-associated microbiota due to the communication between host and microbe. Characterizing the exact mechanisms from which priority effects may [3,15,16] or may not [17,18] emerge is of interest not only for understanding priority effects and their functional

consequences as widespread and striking ecological phenomena[1], but also potentially for the engineering of these communities.

The squash bug *Anasa tristis* hosts bacterial symbionts of the genus *Caballeronia*, and it relies on these symbionts for proper growth and development[19]. Bacteria are housed in a specialized region of the midgut, the M4, in which *Caballeronia* are the dominant taxa. The M4 in *A. tristis* consists of two rows of numerous crypts whose primary function appears to be the maintenance of symbiont biomass, and its presence and morphology are strongly conserved among related insects (the Pentatomomorpha)[20], which maintain beta- and gamma-proteobacterial symbionts such as *Caballeronia* [21–28]. Although this symbiosis is highly specific, genetically distinct *Caballeronia* symbiont isolates are capable of host colonization, with no apparent difference in the fitness benefits that they confer to the host [30,31]. There is also striking heterogeneity between individual squash bugs in the strain-level taxonomic composition of their symbiont communities [31], raising the question of how community structure and strain diversity emerges and is maintained [32].

The bean bug *Riptortus pedestris* of the family Alydidae (Kikuchi et al., 2005), which last shared a common ancestor with *A. tristis* over 100 million years ago [20,33,34], has yielded remarkable insights into the establishment and maintenance of specialized *Caballeronia* symbioses in insects. Symbiotic infection occurs through a physical bottleneck in the midgut at the anterior entrance to the M4, called the constricted region. In *Riptortus*, this constricted region imposes selectivity on the bacterial cells that can access the symbiotic organ [35]. Once symbionts have passed through the constricted region and colonized the M4, the constricted region of *Riptortus* closes off entirely, preventing food intake into the M4 for the duration of host development and rendering the midgut physically discontinuous in nymphs [35,36]. The closure

of the constricted region within 12-15 hours after establishment of early-arriving symbionts has been implicated in preventing subsequent symbionts from colonizing the M4 [37]. Thus, at least in *Riptortus*, gut anatomy is remodeled in a way that imposes a priority effect on community assembly, preventing compatible symbiont strains from invading established symbiont populations within the host. While the presence of the constricted region is conserved among host insects in this clade [35], the degree to which it plays similar roles in *Caballeronia*-associated insects other than *R. pedestris* is unknown.

Here, we evaluate the evidence for priority effects, and the mechanisms underlying them, in symbiont colonization of the *A. tristis* symbiotic organ. Using two strains that exhibit an asymmetrically competitive interaction, we show that a priority effect exists that allows the first symbiont strain to completely exclude establishment of a second strain regardless of competitive ranking. This phenomenon emerges even when two strains are isogenic, suggesting that the priority effect takes place regardless of strain identity or the nature of between-strain interactions. By depleting symbiont populations with antibiotics and by examining the timing of gut development after colonization, we rule out the role of spatial occupancy and symbiont-elicited tissue remodeling as mechanisms governing priority effects in the squash bug-*Caballeronia* system.

Results

Priority effects override between-strain competition.

To assess whether priority effects play an important role in the establishment of symbiont populations in *A. tristis*, we sequentially inoculated cohorts of nymphs with two unrelated bacterial strains, *C. zhejiangensis* GA-OX1 and *C. sp. nr. concitans* SQ4a (Figure 1A), that

represent different clades within the bacterial genus *Caballeronia*. In pairwise competition, GA-OX1 tends strongly to exclude SQ4a [32]. If priority effects play a role in community assembly in this symbiosis, the early-arriving colonist (referred to here as the resident or established strain) should dominate symbiont communities regardless of which strain or fluorophore is used. Conversely, if community assembly is driven primarily by competition between colonizing strains, then the order of colonization should not be significant, and GA-OX1 should tend to exclude SQ4a as observed when both strains are presented simultaneously [32]. When bugs were inoculated first with GA-OX1 sfGFP, and then with SQ4a RFP, all bugs were colonized exclusively with GA-OX1 sfGFP (Figure 1B, top); SQ4a RFP was not recovered from any of these nymphs (Figure S1A, left). Conversely, when bugs were inoculated first with SQ4a RFP, and then with GA-OX1 sfGFP, all bugs were colonized exclusively with SQ4a RFP (Figure 1B, bottom; Figure S1A, right). Thus, symbiont composition between these two treatments significantly differed according to colonization order but not according to strain identity (Figure S1A, $W = 0$, p-value $= 9.975\times10^{-13}$). We observed this effect regardless of which fluorescent marker was associated with which strain (Figure 1B, 1C). Our results demonstrate a strong priority effect producing mutual exclusion.

Priority effects exclude superinfection by isogenic strains.

To further explore the possible effects of inter-strain competition, we repeated these experiments using a neutral competition scenario where hosts were colonized sequentially with counter-labeled, isogenic isolates of GA-OX1. If asymmetric competitive interactions between colonizing symbionts are necessary for priority effects, then colonization order should not be important in this case, and individual hosts should be co-colonized with GFP+RFP isogenic symbionts. Conversely, if priority effects operate independently of competitive interactions

between symbiont strains, then priority effects should manifest as in the previous experiment. As before, outcomes differed based on colonization order (Figure S1B, W = 23.5, p-value = 1.53×10^{-9}). Individual hosts were colonized almost exclusively with the first colonist, regardless of whether GFP (Figure 1D, top; Figure S1B, left) or RFP (Figure 1D, bottom; Figure S1B, right) GA-OX1 was presented first. In each of these treatments, exactly one bug was exclusively colonized with the counter-labelled second strain rather than the first-inoculated strain. Because these two nymphs did not establish mixed infections, we hypothesize that the first strain failed to successfully establish, allowing the second strain to colonize as if no other strains had come before it; this is consistent with prior results indicating a non-zero failure rate for initial colonization even by beneficial microbes within their native hosts [38,39]. Our results demonstrate that the strong priority effect in symbiont colonization that we observed in the previous experiment cannot be explained as a result of direct conflict between genetically distinct microbial competitors.

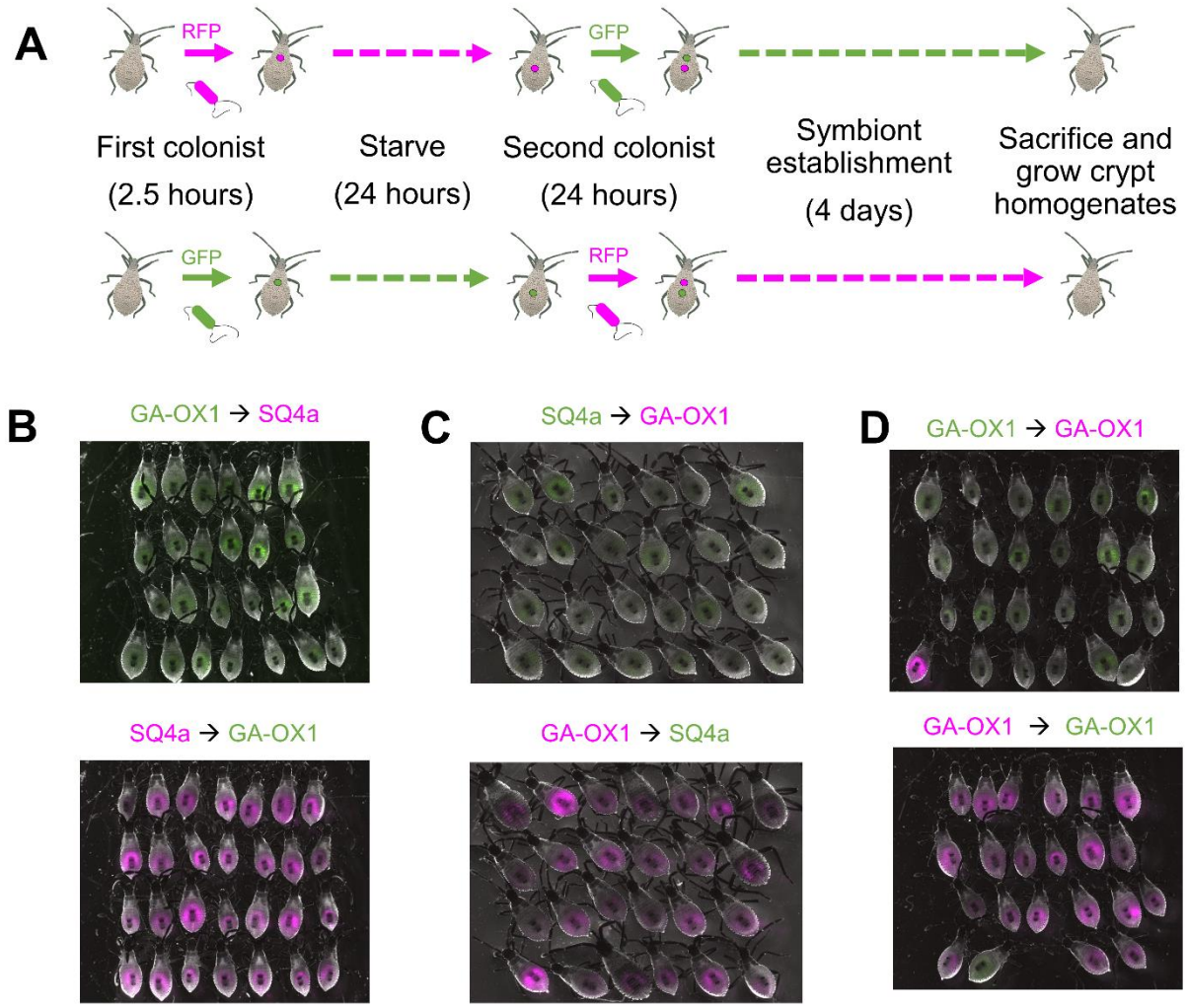

Figure 1. Random colonization order mediates the outcome of colonization.

A) Experimental design. Nymphs were starved overnight, then allowed to feed on a 1:100 dilution of either a GFP-expressing or RFP-expressing symbiont strain (first colonist) for two hours before being starved for 24 hours. The next day, nymphs were fed with the strain of the opposite color *ad libitum* for 24 hours (second colonist). Infections were allowed to establish for four days before nymphs were sacrificed.

(B-D) Sequential colonization by counter-labeled symbiont strain pairs. Strain identity and fluorophore of the first and second colonists are shown above each plot.

B) Sequential colonization by GA-OX1 sfGFP and SQ4a RFP.
C) Sequential colonization by GA-OX1 RFP and SQ4a sfGFP
D) Sequential colonization by isogenic GA-OX1 sfGFP and GA-OX1 RFP.

Priority effects are not due to pre-emption of space within the gut.

Following the example of Kikuchi et al. 2020 [37], we next interrogated some possible mechanisms that might be responsible for the priority effect we observed. One possible mechanism for priority effects is competitive exclusion as a result of spatial occupancy of the symbiotic organ, in which full occupancy of the crypts by the first microbe could reduce the physical space available for colonization by the second strain. This has been demonstrated in other systems [39], including those in which microbes colonize analogous crypt-like spaces consisting of a small, confined lumen with only one opening [40,41]. This hypothesis implies that purging the symbiotic organ of previously established residents should restore efficient establishment by the second colonizer.

To knock down intra-host populations of a colonizing symbiont, we adapted the protocol from Kikuchi et al. 2020 [37] to clear hosts of symbionts with trimethoprim (Tmp) (Figure 2A), an antibiotic to which GA-OX1 is sensitive *in vitro.* We tested the efficacy of Tmp *in vivo* by treating nymphs pre-colonized with GA-OX1-RFP with the antibiotic, then dissecting the symbiotic organs to determine clearance of the symbiont. After Tmp treatment was over, we moved the nymphs to their normal, antibiotic-free diet to purge as much Tmp as possible and to permit recovery of any symbionts which still might be in the symbiotic organ (Figure 2A). As in [37], we found that even after an extended recovery period of 3 days, many nymphs exhibited diminished fluorescence compared to colonized nymphs not treated with antibiotics (Figure 2B). However, closer examination found individual RFP puncta inside of symbiotic organs dissected from all six individuals we chose for microscopy (Figure 2C). These results indicated that Tmp treatment cleared and/or suppressed the bulk of the symbiont population, but that low-level occupancy of the symbiotic organ by viable RFP symbiont cells persisted.

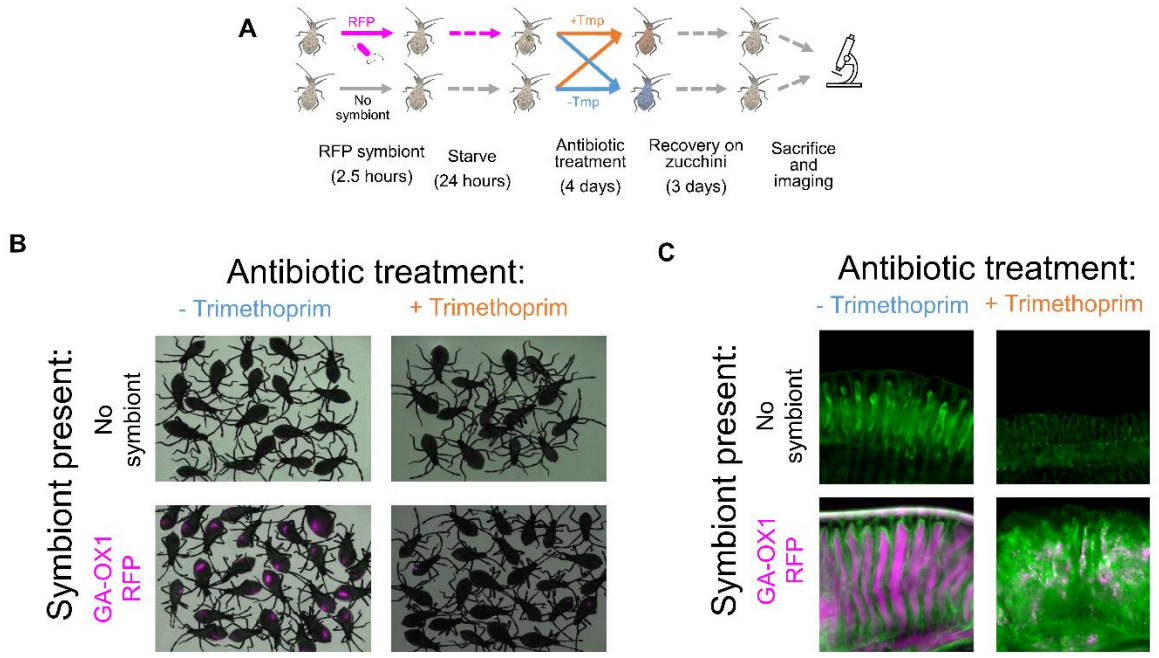

Figure 2. Trimethoprim suppresses symbiont proliferation in squash bug nymphs after colonization.

A) Experimental design. Nymphs were fed either sterile inoculation medium or inoculation medium containing either GA-OX1 RFP for only 2.5 hours, before being starved for 24 hours. The next day, aposymbiotic or inoculated nymphs were fed either sterile inoculation medium or inoculation medium containing >100 µg/mL trimethoprim. Bugs were maintained on this diet for 4 days before being moved to squash to allow potential recovery of any surviving symbionts. On the last day of recovery, whole nymphs were imaged to confirm successful depletion of RFP symbionts, and the individuals with the dimmest RFP signal dissected for widefield microscopy.

B) Colonization of an antibiotic sensitive strain after dietary administration of trimethoprim. Top: Symbiont-free nymphs never exposed to antibiotics (left) or treated with trimethoprim for 4 days (right). Bottom: Nymphs previously colonized with GA-OX1 RFP that were either never exposed to antibiotics (left) or treated with trimethoprim for 4 days (right).

C) Dietary trimethoprim does not totally eliminate antibiotic-sensitive symbiont strains. Top: Crypts from symbiont-free nymphs never exposed to antibiotics (left) or treated with trimethoprim for 4 days (right). Bottom: Crypts from nymphs previously colonized with GA-OX1 RFP that were either never exposed to antibiotics (left) or treated with trimethoprim for 4 days (right).

Despite our inability to fully eradicate symbionts with Tmp, symbiont density remained suppressed in the M4 for many days after Tmp administration ceased, suggesting lingering post-treatment effects of the drug. We tested the hypothesis that this vacant space in the symbiotic organ could be available for colonization. To ensure that drug remaining after Tmp treatment, if any, would not interfere with inoculation, we used an RFP-expressing, Tmp-resistant (dsRed-TmpR) symbiont as the second colonist (Figure 2C, lower right). We inoculated bugs with GA-OX1 sfGFP, treated them with Tmp to knock down the first colonist, and then exposed them to a high density of GA-OX1 dsRed-TmpR for 24 hours [42] (Figure 3A). As we expected, GA-OX1 dsRed-TmpR consistently colonized symbiont-free nymphs regardless of whether they were previously treated with Tmp (Figure 3B, top row); this provided a baseline for expected colonization by this strain. In line with our previous results, the second strain never colonized nymphs in which GA-OX1 sfGFP was never suppressed with Tmp (Figure 3B, bottom left). Surprisingly, GA-OX1 dsRed-TmpR nearly always failed to colonize even after curing insects of the Tmp-sensitive sfGFP strain; instead, almost all the nymphs either recovered sfGFP or remained non-fluorescent (Figure 3B, bottom right) a full week after their last antibiotic exposure. In these experiments, trimethoprim treatment did not produce a significant increase in colonization by a second strain, despite providing it an ostensible fitness advantage over the susceptible established strain (binomial regression: p_{GFP_first} = 6.58e-07; p_{Tmp_cure}= 0.37250). Our results clearly indicate that the vacant space left in the M4 by antibiotic suppression of resident symbionts is not accessible for colonization by a second, incoming strain.

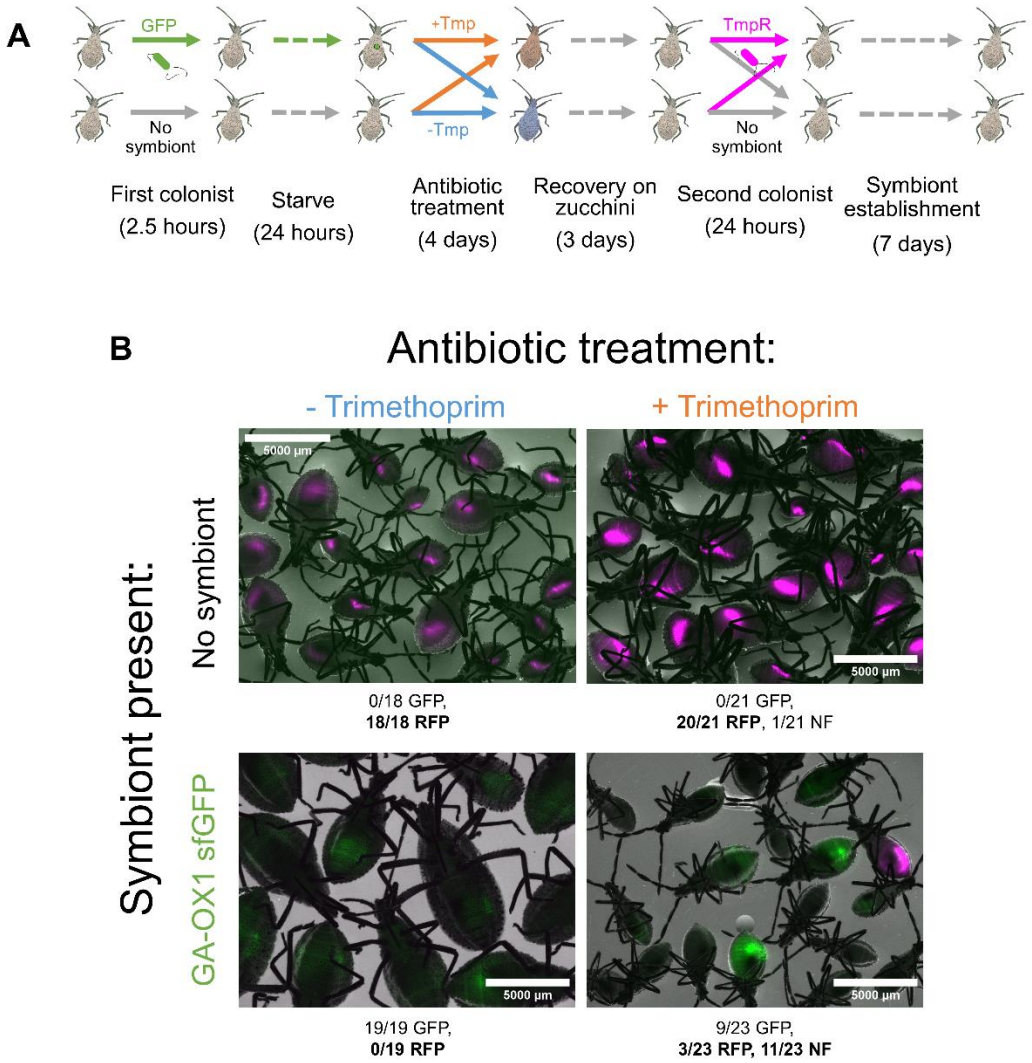

Figure 3. Priority effects after antibiotic depletion of the first colonizing symbiont strain.

A) Experimental design. Nymphs were fed either sterile inoculation medium or inoculation medium containing GA-OX1 GFP for 2.5 hours, before being starved for 24 hours. The next day, aposymbiotic or inoculated nymphs were fed either sterile inoculation medium or inoculation medium containing >100 µg/mL trimethoprim. Bugs were maintained on this diet for 4 days before being moved to squash to allow recovery of any surviving symbionts. On the last day of recovery, nymphs were imaged to determine depletion of RFP symbionts. Next, bugs in each treatment were fed either sterile inoculation medium or inoculation medium containing an isogenic trimethoprim-resistant RFP symbiont for 24 hours. Nymphs were thereafter maintained on squash to permit symbiont population growth until sacrifice and imaging.

B) Colonization by a Tmp-resistant symbiont strain after antibiotic clearance of a susceptible established symbiont population. Top: GA-OX1 dsRed-TmpR colonization of aposymbiotic

nymphs never exposed to trimethoprim (left) or treated with trimethoprim for 4 days (right). Bottom: GA-OX1 dsRed-TmpR colonization of nymphs, inoculated previously with GA-OX1 GFP, that were either never exposed to antibiotics (left) or treated with trimethoprim for 4 days (right). Bugs that exhibited neither RFP nor GFP fluorescence were scored as nonfluorescent ("NF").

Priority effects cannot be explained by symbiont-elicited tissue remodelling of the symbiotic organ.

In *Riptortus* [37], ingestion of the first symbiont strain only prevents colonization of the second strain after a 15-hour interval between inoculations. This accompanies the physical closure of the entrance to the symbiotic organ, known as the constricted region, at around nearly the same time. This closure seems to depend on the presence of a colonizing symbiont in the host [37]. The constricted region is highly conserved in pentatomomorphan insects with beta- and gamma-proteobacterial gut symbionts [35]. The constricted region is likewise present in squash bug nymphs (Figure 5A) and forms a closed plug without an apparent lumen after successful *Caballeronia* colonization (Figure 5B and 5C).

We reasoned that the closure of the constricted region could explain the inability of the second strain to colonize the host, despite the availability of physical niche space in the symbiotic organ after antibiotic treatment. Indeed, the continued presence of small bacterial populations in all symbiont-colonized nymphs fed with Tmp (Figure 2C, bottom right) suggested that these few viable cells might be sufficient to signal their presence to the host, triggering the closure of the constricted region. Therefore, this hypothesis states that the priority effect we observed in all our experiments might result not from direct microbe-microbe interactions, but rather from microbial modulation of host physiology. A specific prediction of this hypothesis is that the constricted region must be completely closed in nearly all squash bug nymphs within 24

hours to explain the prevalence and magnitude of the priority effects in our experiments (Figure 1).

To ascertain whether the closure of the constricted region could impose a physical barrier to subsequently ingested symbionts, we measured the diameter of the lumen within the constricted region dissected from symbiont-colonized nymphs every 24 hours for three days without exposing them to antibiotics, and compared these with the constricted region in aposymbiotic nymphs over the same time period. Contrary to our expectations, we observed no significant differences in the luminal diameter of the constricted region 24 hours post infection, although the robust priority effect we observed in our previous experiments (Figure 1) is in place sometime before this point (Figure 5D, Wilcoxon signed-rank test; p_{24hpi}= 0.3913). Instead, the constricted region appears to fully close between 48 to 72 hours post infection (p_{48hpi}=0.1939; p_{72hpi}= 0.005741). We conclude that, at least in our model system, the timescale at which closure of the constricted region takes place is not sufficiently rapid to explain the complete exclusion of the second symbiont from colonization.

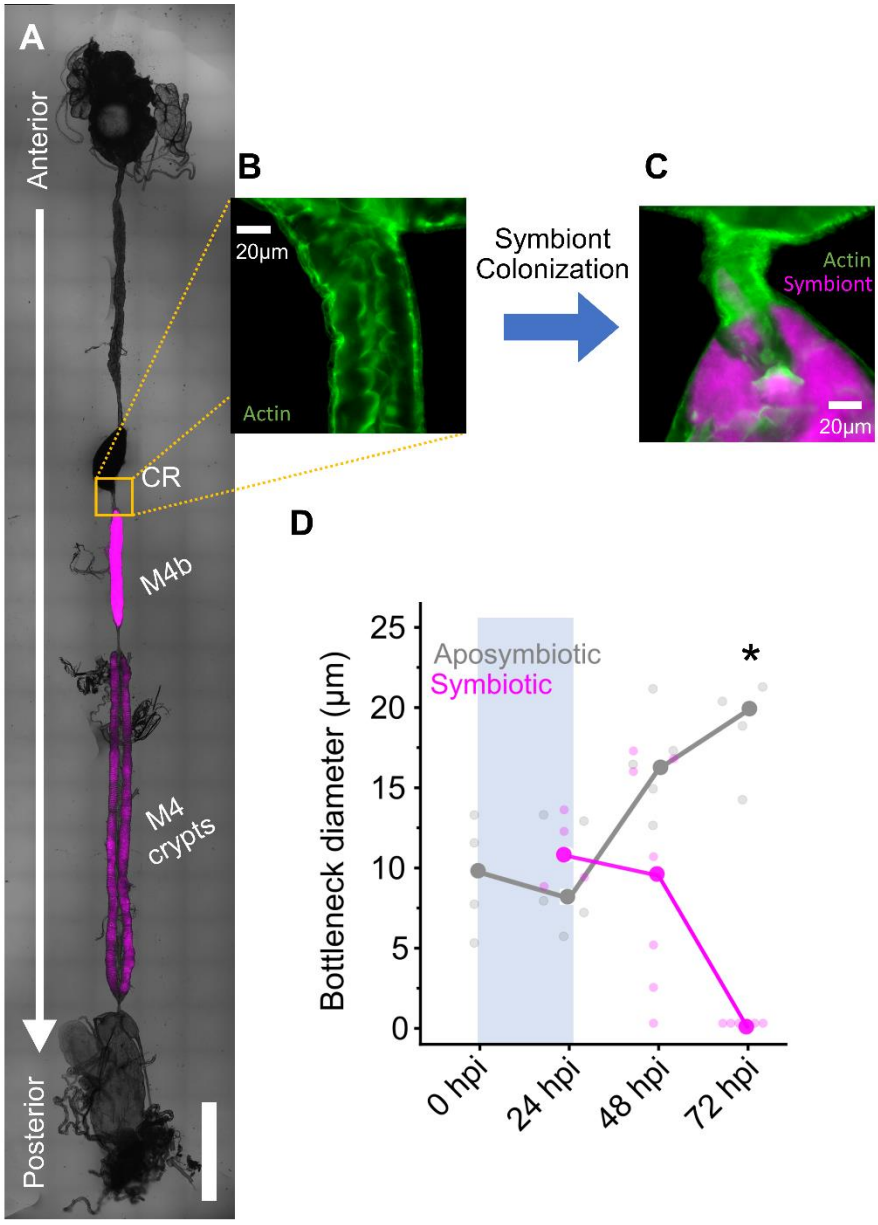

Figure 4. Closure of the constricted region (CR) within the gut is not necessary for the priority effect.

A) Tilescan of the entire midgut of a pharate (unmolted) L5 squash bug nymph, linearized to depict the sections of the midgut. The magenta signal localized to the M4 crypts and the M4b is indicative of colonization with GA-OX1 RFP. The CR is indicated in the orange box.

B) (inset) The CR of an aposymbiotic second instar nymph, with an open bottleneck. Host actin is stained in green.

C) Closed CR of a second instar nymph colonized with GA-OX1 RFP (magenta).

D) Changes in the diameter of the bottleneck within the CR of second instar nymphs kept symbiont-free (gray) vs. colonized with GA-OX1 (magenta), over the course of 72 hours. Bugs colonized with symbionts exhibit a reduction in the size of the bottleneck between 24 and 72 hours post infection (hpi), while aposymbiotic bugs do not, consistent with a slow tissue-remodeling response to symbiont colonization. However, this reduction in the diameter of the lumen does not take place within the 24 hour period in which the priority effect emerges (shaded in light blue-gray) in our experiments. Smaller faded points indicate exact measurements, while large points and lines indicate averages for each treatment.

Discussion

In this work, we demonstrate a strong, rapid priority effect in colonization of the squash bug *Anasa tristis* by symbiotic bacteria in the genus *Caballeronia*. By manipulating the order by which colonization takes place within a 24-hour time window, we show that the first strain to colonize a host prevents colonization of the second. This effect is agnostic to symbiont strain and cannot be reversed by making physical space available for colonization. These results corroborate previous work in *Riptortus pedestris*, which also exhibits strong priority effects in symbiont colonization [37].

The mechanism(s) responsible for priority effects in this system are not clear. We first showed that strain identity and relatedness are not factors; two different host-beneficial strains mutually prevented each other from subsequent colonization, and isogenic lineages of the same strain showed the same effect. Next, we demonstrated that the availability of space within the M4 does not mean that this space can be colonized by a subsequent symbiont, implying that the open space in the symbiont organ is somehow inaccessible. Further, we determined that host tissue remodeling and closure of the M4, which is normally elicited by symbiont colonization, does not occur rapidly enough after colonization to exclude subsequent symbionts within the timeframe of our experiments.

There are many potential mechanisms we could not explore due to the limitations in the experimental tractability of our system. First, we were unable to completely eliminate resident symbionts in the symbiotic organ, contrary to previous reports [37]. The surviving initial colonists may have interactions with subsequent colonists, including niche interactions and warfare [43] [5], that might affect populations of a second colonizer. However, the bacterial populations remaining after Tmp treatment were visibly small and sparse, and it is highly unlikely that these interactions would be sufficient to entirely prevent entry of a second colonist.

Other mechanisms on the part of the host might mediate the priority effect. First, although microscopy did not reveal a reduction in the physical diameter of the constricted region, the entrance to the M4, it is possible that changes in the secretions in this region might block off access to subsequent colonization. Such modifications would be invisible to the actin stain we used to visualize the lumen of the constricted region. Furthermore, we suspect that colonization likely causes shifts in immunity or the nutritional environment throughout the gut, which may pose a more rapidly erected barrier to superinfection than tissue remodeling in the constricted region. Finally, previous data from our lab suggest that under natural pathways of transmission, a rapid behavioral switch from foraging for symbiont-laden feces to a more inactive feeding stage may further mitigate the probability of ingesting potential symbionts [44]. Future studies should address both microbial and host contributions to the priority effect we observed in this study.

These results have applied implications. Strong priority effects could be exploited to permanently colonize squash bugs with symbiont strains that express desirable phenotypes in the field. This is a long-term goal of many such so-called “paratransgenic” approaches in insect livestock and pests [45–48]. Possible applications include use of engineered symbionts that reduce pest fitness[49], suppress pathogen vectoring [50], or induce abortive host infection.

Engineered symbionts in this system may be useful as part of a broader strategy for integrated pest management even if they have decreased fitness relative to wild strains, as long as they are the first to colonize their hosts. Further studies to determine the feasibility of symbiont-mediated pest management using this strategy should investigate the mechanism(s) behind priority effects in this system, as a potentially important component of within- and between-host and between-host ecology of symbionts in natural environments.

Acknowledgements

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Methods

Study system

Adult insects were maintained at 28-30°C on yellow crookneck squash plants (*Cucurbita pepo* 'Goldstar') housed in 30 cm^3 mesh cages. Eggs were routinely collected and surface-sterilized by rinsing sequentially in 70% ethanol, 20% bleach, and sterile water. Upon hatching, symbiont-free nymphs were maintained on surface-sterilized, parafilmed organic zucchini slices until they were ready to be used in inoculation experiments. Symbiont-free nymphs molt to the second instar two days after hatching from eggs, whereupon they become competent for symbiont colonization. Nymphs used in inoculation experiments were never more than one week old.

Caballeronia zhejiangensis GA-OX1 and *Caballeronia sp. nr. concitans* SQ4a are isolates from wild-caught squash bugs in northeast Georgia [19,31,51]. These strains represent two different clades (*sensu* [52]) within the genus *Caballeronia* but exhibit no distinguishable differences in the degree of host benefit conferred [19,31]. For experiments, both GA-OX1 and SQ4a were cultivated in the laboratory at 25°C on nutrient agar (NA) plates or in low-salt Luria-Bertani Lennox broth (LB).

Sequential inoculation experiments

The generalized experimental design is depicted in Figure 1A. *Caballeronia* strains were previously labelled with superfolder GFP (sfGFP) or dTomato (RFP) ([32,44]) using the mini-Tn7 system [53,54]. Fluorescent proteins are genomically integrated at a specific, neutral intergenic site and are thus stably maintained with minimal effects on phenotype and fitness *in vitro* [55–57] and with minimal opportunity for horizontal gene transfer.

Fluorescently labelled *Caballeronia* strains were streaked out on NA plates and grown for two days at 25°C. Liquid cultures were started by picking single colonies into 2 mL LB and incubated at 25°C overnight in glass tubes shaking at 200 rpm. Three hundred µL of each culture was diluted into 1000 µL 1X phosphate buffered saline (PBS) in a 1.5 mL microcentrifuge tube and washed by pelleting at 10,000 x g for two minutes at 4°C, removing the supernatant, and resuspending in 1000 µL PBS. After a second wash step, the pellet was resuspended in 300 µL PBS.

In preparation for the first round of inoculation, nymphs were starved for 20-24 hours in a surface-sterilized plastic rearing box, supplied with only 100 µL sterile water spotted as droplets across the lid of the box with a 1000 µL tip for hydration. The next day, the starved nymphs were transferred into another clean, surface-sterilized plastic rearing box. For each *Caballeronia* strain, an inoculum was prepared by diluting 2 µL of washed live cells into 200 µL of a defined inoculation medium, an aqueous solution containing 2% w/v glucose and 10% v/v PBS. Droplets totaling 150 µL of inoculum were spotted onto the plastic surface of the box, and nymphs were allowed to feed *ad libitum* on the inoculum for 2.5 hours at 28-30°C. An inoculum containing viable bacteria typically contains 10^3-10^4 colony forming units per µL (CFUs/µL), which was confirmed by dilution plating the inoculum on NA just before and just after the inoculation period. After 2.5 hours, nymphs were transferred to another sterile rearing box and starved for an additional 20-24 hours. This second starvation period was necessary not only to synchronize bouts of feeding activity among all nymphs in each experimental cohort but also to eliminate any nymphs that may not have ingested the inoculum.

For the second round of inoculation, liquid cultures were grown and washed as described above, using the same bacterial colonies that were used for the first round of inoculation. Inocula were

prepared by diluting 5 µL of washed cells into 495 µL of inoculation medium, then dilution plated to confirm cell density, as above. To maximize exposure to the second strain, nymphs were exposed to this second inoculum for 24 hours before being individually isolated in 24-well cell culture plates with pieces of organic zucchini.

Nymphs were allowed to feed and develop on zucchini for 4-6 days after the second round of inoculation before sacrifice. Nymphs were killed with 70% denatured ethanol, and intact nymphs were imaged on an Olympus SZX16 stereomicroscope with an Olympus XM10 monochrome camera and Olympus cellSens Standard software ver. 1.13, as described in [32]. Nymphs were immersed in a shallow volume of PBS in 6 cm plastic petri dishes, and images were taken in darkfield (30 ms exposure 11.4 dB gain), brightfield (autoexposure, 11.4 dB gain), a GFP channel (autoexposure, 18 dB gain), and a RFP channel (autoexposure, 11.4 dB gain). Darkfield and brightfield images were merged in FIJI version 1.54f using the Image Calculator plugin, and the result was then merged with the GFP channel, RFP channel, or both.

To confirm imaging data, relative strain abundance was measured within each individual. After imaging, cadavers were surface sterilized in 10% bleach for 5-10 minutes, washed off again in 70% ethanol, and immersed in ~20 µL droplets of PBS. M4s were individually dissected and stored in 300 µL PBS. Once all M4s from a set of nymphs were collected, samples were crushed with micropestles and dilution plated on NA. Plates were incubated at 30°C for 22-24 hours, then stored at 4°C until GFP and RFP colonies were enumerated.

Antibiotic clearance experiments

The protocol for curing nymphs of symbionts is depicted in Figure 2A. Second instar nymphs were either inoculated with trimethoprim (Tmp)-sensitive GA-OX1 RFP or with sterile

inoculation medium for 2.5 hours, following the protocol as described above. The next day, nymphs were moved to a clean rearing box and given 200 µL of sterile inoculation medium amended either with 167 µg/mL Tmp, a canonically static antibiotic against which *Caballeronia* strains are extremely sensitive, or 20 µL of DMSO (as a control). This procedure was repeated every ~24 hours for the next two days with a lower dose of 111 µg/mL Tmp due to antibiotic toxicity. After three days of Tmp administration, all nymphs were allowed to feed on surface-sterilized organic zucchini for 5 hours to replenish their food reserves before being fed sterile inoculation medium containing 111 µg/mL Tmp or DMSO for one more day.

After four days of Tmp administration, nymphs were transferred to parafilmed, surface-sterilized organic zucchini pieces, where they were allowed to recover for the next three days. At the end of the third day of recovery (one week after GA-OX1 was first administered), bugs were anesthetized with dry ice, immersed in sterile DI H_2O, and their ventral aspects imaged to confirm that GA-OX1 RFP was successfully depleted. Nymphs were imaged at the

To qualitatively demonstrate the degree to which GA-OX1 RFP densities were reduced within the hosts, we also dissected the symbiotic organs from a subset of these insects and examined them under a widefield scope.

Symbiont replacement experiments

To generate a trimethoprim-resistant, RFP symbiont (dsRed-TmpR), electrocompetent *C. zhejiangensis* GA-OX1 were first prepared by inoculating 1 mL of LB with a single colony and growing at 25°C overnight shaking at 200 rpm. The log phase culture was transferred in its entirety into 100 mL of yeast glucose (5 g/L yeast extract, 4 g/L glucose, 1 g/L sodium chloride) and grown at 30°C for 6 hours shaking at 225 rpm. Cells were gently pelleted and washed in ice-cold 10% glycerol four times before being frozen as 80-120 µL aliquots. The broad host range,

multicopy plasmid pIN63 [42], which carries a trimethoprim-resistant dihydrofolate reductase and a dsRed.T3 fluorescent protein, was introduced into electrocompetent GA-OX1 by electroporation of 40 µL of cells with 100 ng of plasmid DNA in a 0.2 cm cuvette on a Bio-Rad Micropulser (1 pulse at 3.0 kV voltage). Cells were recovered in 1 mL of yeast glucose broth and incubated at 30°C for 2 hours before plating on NA containing 0.4 µg/mL trimethoprim for selection.

The protocol for curing nymphs of symbionts is depicted in Figure 4A. Second instar nymphs were either inoculated with the Tmp-sensitive GA-OX1 sfGFP or with sterile inoculation medium for 2.5 hours, then either fed with a Tmp-laced or a control inoculation medium before recovering on organic zucchini as previously described. At the end of the third day of recovery, nymphs were starved for 12 hours before being fed GA-OX1 TmpR, which is Tmp-resistant and expresses the red fluorescent protein DsRed.T3. Nymphs were then allowed to develop for a further week before imaging to confirm GA-OX1 TmpR colonization.

Statistical analyses.

Differences in the relative abundance of RFP CFUs recovered from homogenized M4s were evaluated with a two-sided Wilcoxon test. The effect of Tmp on GA-OX1 dsRed-TmpR colonization of nymphs that had previously been inoculated with GA-OX1 sfGFP was assessed with a binomial regression, with Tmp administration and GA-OX1 sfGFP presence as factors. To assess the change in the minimum diameter of the lumen within the constricted region, we used a one-sided Wilcoxon test.

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Supporting Information

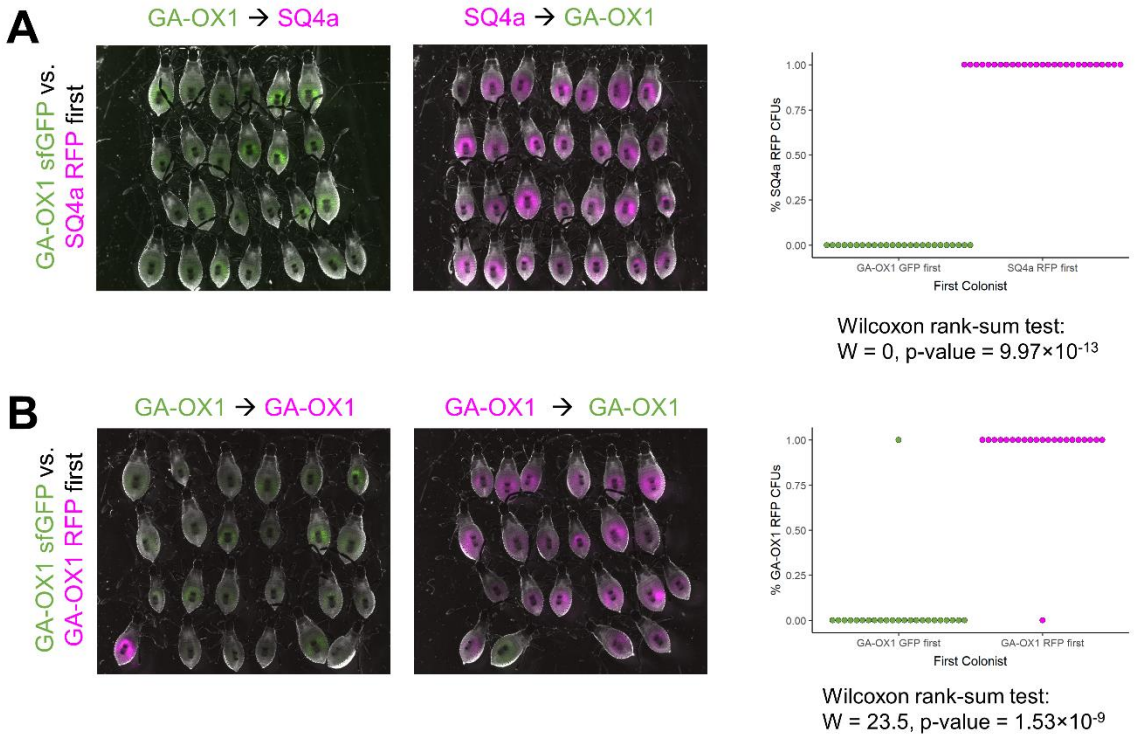

Figure S1. Relative abundances of sfGFP and RFP strains after sequential colonization.

A) Relative abundance of GA-OX1 sfGFP and SQ4a RFP after sequential colonization of nymphs from Figure 1B. Nymphs were infected with either *C. zhejiangensis* GA-OX1 sfGFP first (left) or *C. sp. nr. concitans* SQ4a RFP first (middle), then SQ4a RFP or GA-OX1 sfGFP second, respectively. Homogenates from dissected symbiotic organs of GA-OX1-colonized nymphs yielded no SQ4a, while those of SQ4a-colonized nymphs yielded no GA-OX1.

B) Relative abundance of GA-OX1 sfGFP and GA-OX1 RFP after sequential colonization of nymphs from Figure 1D. Nymphs were infected with either *C. zhejiangensis* GA-OX1 sfGFP first (left) or *C. zhejiangensis* GA-OX1 RFP first (right), then GA-OX1 RFP or SQ4a sfGFP second, respectively.

Chapter 4

Ecological drift during colonization drives within- and between-host heterogeneity in animal-associated symbiont populations.

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Abstract

Specialized host-microbe symbioses canonically show greater diversity than expected from simple models, both at the population level and within individual hosts. To understand how this heterogeneity arises, we utilize the squash bug, *Anasa tristis*, and its bacterial symbionts in the genus *Caballeronia*. We modulate symbiont bottleneck size and inoculum composition during colonization to demonstrate the significance of ecological drift, the noisy fluctuations in community composition due to demographic stochasticity. Consistent with predictions from the neutral theory of biodiversity, we found that ecological drift alone can account for heterogeneity in symbiont community composition between hosts, even when two strains are nearly genetically identical. When acting on competing strains, ecological drift can maintain symbiont genetic diversity among different hosts by stochastically determining the dominant strain within each host. Finally, ecological drift mediates heterogeneity in isogenic symbiont populations even within a single host, along a consistent gradient running the anterior-posterior axis of the symbiotic organ. Our results demonstrate that symbiont population structure across scales does not necessarily require host-mediated selection, as it can emerge as a result of ecological drift acting on both isogenic and unrelated competitors. Our findings illuminate the processes that

might affect symbiont transmission, coinfection, and population structure in nature, which can drive the evolution of host-microbe symbioses and microbe-microbe interactions within host-associated microbiomes.

Introduction

A persistent paradox in the study of host-microbe symbioses is that, like microbes in natural environments, microbial symbionts exhibit enormous strain diversity [1–8]. This is observed even when natural selection, imposed by specialized interactions with their hosts, is expected to erode genetic variation. Different mechanisms, based on environmental selection or host variation, are typically invoked to explain the maintenance of symbiont genetic variation, often in terms of host benefit [9,10]. However, these hypotheses do not account for how host-associated consortia assemble as ecological communities, which embeds this genetic variation within patches in physical space [11,12]. This is an inherently stochastic process that generates heterogeneity [13–15]. Heterogeneity in host-associated microbial communities manifests at two scales: as heterogeneity in colonization *between hosts*, and as spatial heterogeneity across tissues and organs *within each host*. At both scales, it is critical to understand how this heterogeneity emerges during establishment of symbiosis, which drives the evolution, ecology, and physiology of both host and microbe.

While the ecological processes that create heterogeneity during community assembly have been studied with mathematical models (e.g. [16]), validation of these models in empirical studies using natural, ecologically realistic communities, including host-associated microbial communities [12–14,17], is scarce. Some of these processes are deterministic, acting on specific traits that allow or hinder establishment of a taxon in a predictable, niche-based fashion. However, community assembly is also governed by dispersal between habitats. Dispersal imparts

a stochastic element on community assembly [14]: Taxa immigrate and establish in new patches in a probabilistic manner, in part because they experience transient reductions, called bottlenecks, in population size [18]. These bottlenecks intensify ecological drift (i.e., stochastic variation in community composition). Since the proposal of Hubbell's unified neutral theory of biodiversity, the relative role of stochastic processes such as ecological drift in community assembly, compared with deterministic niche-based processes such as between-species interactions, has been a matter of continuous study [19–21].

In the context of host-microbe mutualistic symbioses, hosts impose stringent ecological selection during community assembly by filtering out or sanctioning non-beneficial and pathogenic microbes[10,22–25]. While this paradigm can explain the consistency with which hosts can acquire symbionts while excluding non-symbiotic taxa (Figure 1D), it does not explain how these symbiont communities differ between individuals (Figure 1E), nor can it account for spatial structure in communities within the host. To illustrate the importance of ecological drift during the establishment of even highly specific symbioses, we employ the squash bug, *Anasa tristis* (Figure 1A), as a model. *A. tristis* is host to specific symbionts in the β-proteobacterial genus *Caballeronia* (previously referred to in the literature as the *Burkholderia* "SBE" clade [26] or the *B. glathei*-like clade [27]), which it requires for survival and normal development to adulthood [28]. Acquisition of *Caballeronia* occurs through the environment after nymphs (immature insects) molt into the second instar. Once they successfully colonize the host, symbionts are housed in hundreds of sacs called crypts, which form two rows running along a specific section of the midgut, called the M4 (Figure 1B). Unlike many other insect symbionts, *Caballeronia* can be isolated from bugs and established in pure culture in the laboratory. Because

A. tristis nymphs hatch from their eggs symbiont-free, the symbiosis can be reconstituted anew every generation by feeding cultured symbionts to these nymphs in the laboratory [7,29].

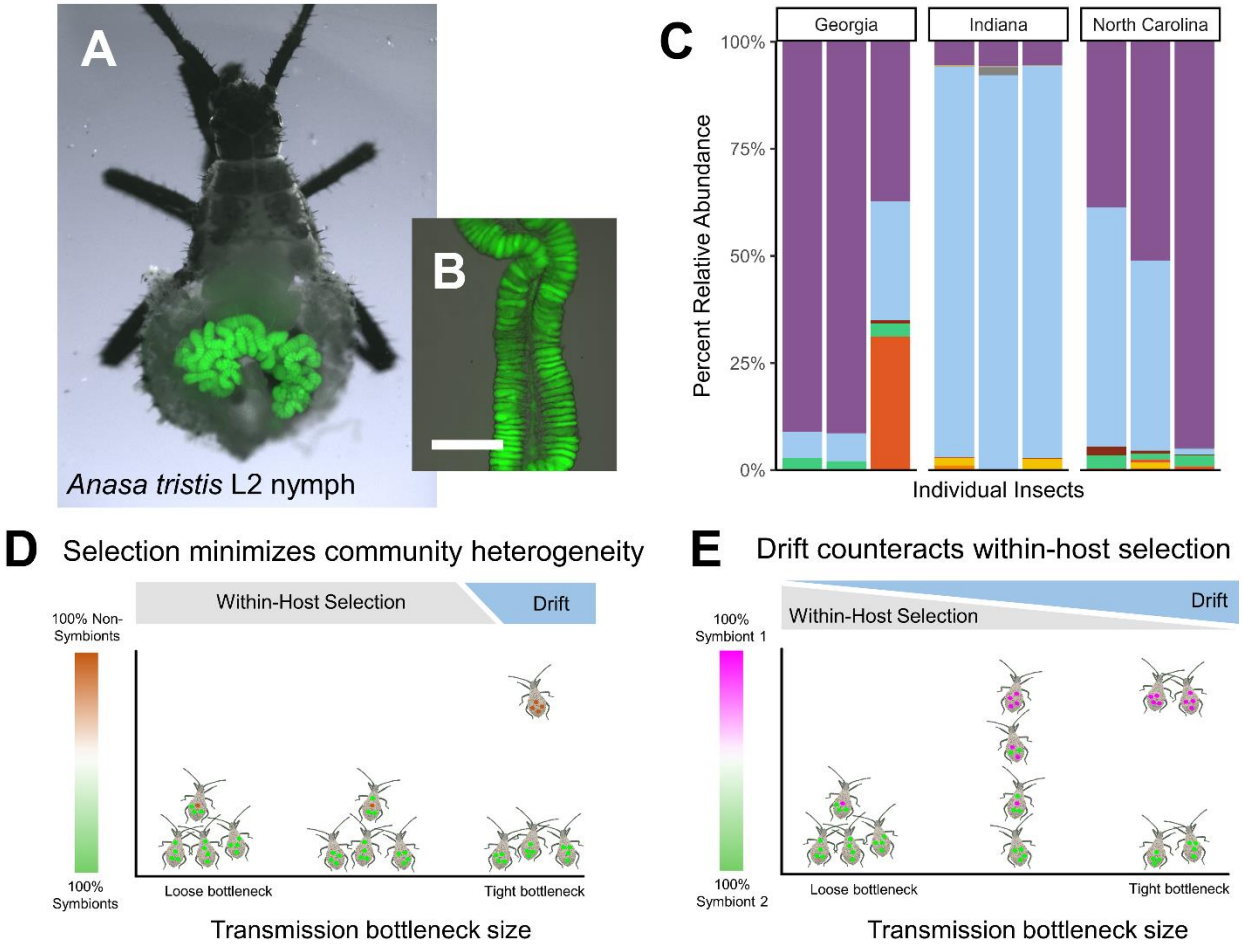

Figure 1. The squash bug, *Anasa tristis*, engages in a specialized host-microbe symbiosis with bacteria in the genus *Caballeronia*.

A) A second instar squash bug (*Anasa tristis*) dissected to reveal the M4 section of the midgut, colonized with *Caballeronia* symbionts expressing the sfGFP fluorescent protein.

B) Inset: The fine structure of the M4, consisting of two rows of hundreds of sac-like crypts lining a central lumen. Crypts are colonized at high density with *Caballeronia* symbionts expressing the sfGFP fluorescent protein. The scale bar represents 250 µm.

C) Relative abundance of the top 20 *Caballeronia* 16s V3-V4 amplicon sequence variants (ASVs) within 9 bugs across 3 field localities in Georgia, Indiana, and North Carolina, with different colors representing different ASVs. Data provided in Dataset S1, from [7].
D) A common notion is that because hosts with obligate microbial symbionts are under uniquely strong selection to transmit and maintain particular symbiotic taxa, selection (gray) rather than drift (blue) broadly governs the outcome of community assembly in these highly specialized microbiomes. In this paradigm, communities (left and middle clusters) invariably are dominated by symbionts (green) that have higher within-host fitness than non-symbionts (brown). As a result, rare, highly divergent communities (top right) containing non-symbionts might emerge only by chance (right cluster), when symbionts undergo extremely tight transmission bottlenecks. However, this paradigm does not explain compositional heterogeneity among symbiont communities, in which competing symbiont strains may be functionally identical in host benefit and ability to colonize.
E) We posit that ecological drift plays an important role in structuring symbiont strain diversity by counteracting within-host selection in symbiont populations during colonization. As in D), selection (gray) acts on differences in relative fitness between competing strains (magenta and green) to drive community convergence towards a mean composition (left cluster). However, the effect of selection is only apparent when symbiont populations undergo weak population bottlenecks during host colonization. Drift (blue) minimizes the impact of selection, such that even if competing symbiont strains differ substantially in within-host fitness, heterogeneity in community composition emerges as if this fitness difference is absent, i.e. neutrally (middle and right clusters).

Because the host relies on *Caballeronia* strains as its symbiotic partners, its digestive tract imposes strong selection to favor *Caballeronia* colonization [25,30], as in other specialized systems [22]. As a result, *Caballeronia* constitutes the vast majority of the microbial community within the M4 symbiotic organ, even though squash bug nymphs are exposed to diverse environmental microbes on squash fruit and plants [28]. However, this and similar bug-*Caballeronia* symbioses are extremely non-specific below the genus level [31], with distantly related symbiont isolates conferring nearly the same degree of host benefit [7,32]. In accordance with this apparent lack of specificity, we observe that within-host *Caballeronia* communities from wild squash bug populations vary widely in their composition [7] (Figure 1C). So, beyond the coarse ecological filter that the host insect applies against non-symbiotic taxa [25,30], little is

known about the ecological processes that maintain within- and between-host diversity of this beneficial symbiont.

Here, we explore the hypothesis that both within- and between-host diversity in symbiont populations arise stochastically as a result of ecological drift during infection [14]. First, we set out to explore a range of conditions under which this pattern might emerge, incorporating neutral competition (where all cells are isogenic, and thus functionally equivalent, individuals) [20] and interspecies competition (where cells are genetically distinct, but still equally host beneficial) between symbiont strains. By experimentally manipulating transmission bottleneck size, we show that ecological drift alone can account for heterogeneity between hosts, segregating strains between hosts and decreasing the probability of coinfection. Using isogenic coinfections, we additionally demonstrate that the symbiotic organ imposes spatial heterogeneity on within-host populations, whereby separate crypts are colonized by different strains. Our results demonstrate the role of ecological drift in the assembly of a highly specialized host-microbe system and in structuring symbiont population diversity across scales.

Results

Ecological drift is sufficient to generate variation in colonization outcome.

We reasoned that if ecological drift plays a role in generating heterogeneity in symbiont populations between hosts, it should generate greater and greater heterogeneity under smaller and smaller inoculum densities, which represent tightening transmission bottlenecks in our experiments. Specifically, the neutral model [33] implies that under tight bottlenecks, which shrink the effective size of a local community, colonization outcomes should be bimodal, with hosts dominated by clonal lineages, regardless of strain identity [16,19]. By contrast, when host control determines colonization, altering the inoculum size should have minimal impact on

community composition across hosts. Additionally, if strong competition between symbionts determines the outcomes of colonization, individual hosts should be mono-colonized across a broad range of inoculum densities. To test this, we implemented a simple experimental design (Figure 2A), previously applied to human pathogens and legume nodule symbionts, that modulates transmission bottleneck size while maintaining the relative abundance of each strain during transmission [34,35]. To minimize the involvement of selection, we used isogenic, green- and red-fluorescently labelled isolates of *C. zhejiangensis* GA-OX1, a highly beneficial strain isolated previously from *A. tristis* [7]. Because our experiments involved only two competitors, we used the bimodality coefficient [14,36] to quantify heterogeneity in community composition. The bimodality coefficient is a composite measure of skewness and kurtosis, which is maximized for a distribution with equal weight at the extrema of the data. As this measure can be sensitive to small sample sizes, we also calculated the Hartigan's dip statistic, which represents a more robust general measure of multi-modality. We inoculated second instar squash bug nymphs with approximately 1:1 mixtures of GA-OX1 sfGFP with GA-OX1 RFP, diluted to produce inocula ranging from approximately 10^6 to 10^1 CFU/μL (Figure S1A). These inoculum densities are within the natural range of variation in symbiont density that hosts might encounter, whether in freshly deposited adult feces (10^5 to 5×10^6 CFUs/μL), on which nymphs can feed to acquire symbionts [37], or in soil, in which *Caballeronia* is present at lower densities alongside many other microbes [38,39].

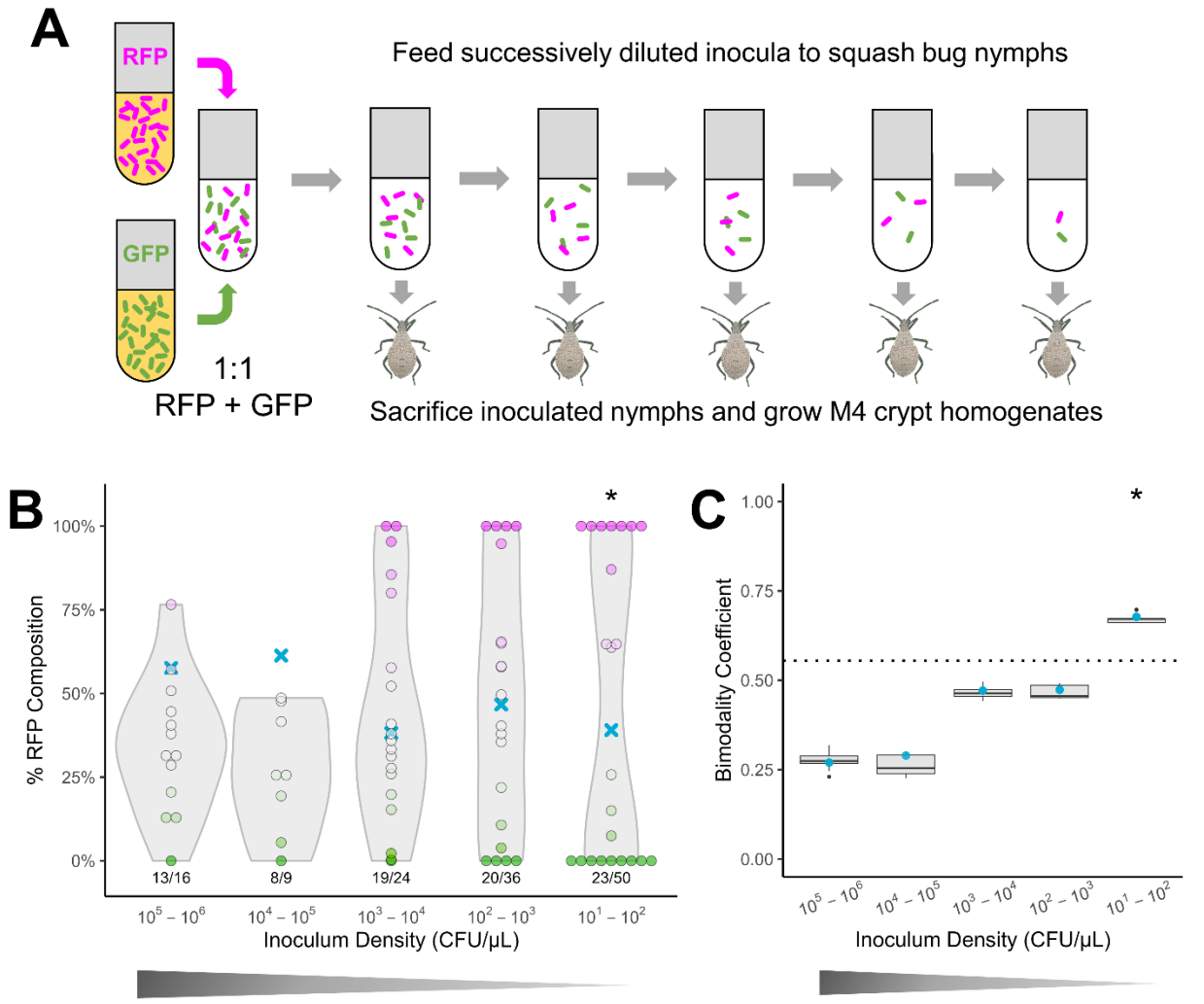

Figure 2. The strength of ecological drift mediates variability in the outcome of symbiont colonization.

A) Experimental design. Symbionts previously isolated from squash bugs, made to stably express green or red fluorescent proteins (GFP or RFP), are grown individually in liquid culture. Liquid cultures are combined at a pre-determined ratio, then the mixture is diluted at various concentrations in the inoculation medium such that the inoculum density (a proxy for transmission bottleneck size) varies over several orders of magnitude while retaining the relative abundance of each strain across inocula.

B) Variable colonization outcomes associated with different transmission bottleneck sizes in isogenic co-inoculation, using *Caballeronia zhejiangensis* GA-OX1 sfGFP and RFP. Blue X marks indicate the mean % GA-OX1 RFP associated with each inoculum treatment, ranging from 10^1 to 10^6 CFU/µL. Points represent individual nymphs, and the color of each point and its position along the y-axis represent the percent relative abundance of GA-OX1 RFP colonies among all fluorescent colonies recovered from each nymph. Magenta points represent nymphs from which only RFP colonies were recovered, green points represent nymphs from which only GFP colonies were recovered, and faded

magenta/green colonies represent coinfected nymphs. Violin plots associated with each treatment depict the shape of the distribution in relative RFP abundance. Asterisks indicate significantly multimodal infection outcomes as determined by Hartigan's dip test, at a significance value of $p < 0.05$. Below each violin plot, the success rate of colonization is indicated, as the number of nymphs that were successfully colonized with *Caballeronia* out of all nymphs sampled. Data provided in Dataset S2. Trials were aggregated across multiple runs.

C) Bimodality coefficients calculated from results in panel B. Large blue dots indicate bimodality coefficients calculated from all bugs in each treatment; boxplots indicate bimodality coefficients calculated by jackknife resampling in each treatment. The 0.555 threshold (marked with a dotted line) indicates the bimodality coefficient expected from a uniform distribution. Asterisks indicate significantly multimodal infection outcomes as indicated by Hartigan's dip test, at a significance level of $p < 0.05$. Analyses conducted on Dataset S2.

Consistent with the neutral model, under the highest inoculum densities, corresponding to the loosest bottlenecks, differences between the M4 communities of individuals are minimized, with a slight bias in favor of the sfGFP strain (Figs 2B and S1B). The slight bias towards sfGFP colonization could be due to toxic aggregation of the dTomato fluorescent protein, which has been observed in eukaryotic cells [40]. As inoculum density decreases, and thus as transmission bottlenecks tighten, individual infections become increasingly dominated by one or the other strain, causing the bimodality coefficient to increase (Figs 2C and S1C, Table S1). Below 100 CFU/μL, individual infections are comprised of mostly either GFP or RFP, manifesting as a weakly but significantly bimodal outcome (bimodality coefficient = 0.677, Hartigan's dip statistic = 0.152, $p < 2.2 \times 10^{-16}$) (Table S1). Fluorescence images of whole nymphs provided qualitative confirmation of our results, with more heterogeneity observed between nymphs at lower inoculum densities (Figure S2). Through this set of experiments, we show that ecological drift is sufficient to drive heterogeneous colonization outcomes.

Ecological drift maintains coexistence between competing strains across separate hosts.

Having illustrated the action of transmission bottlenecks on a single symbiont genetic background, we next sought to understand how they would act on genetically distinct host-beneficial strains. If ecological drift has an effect even when selection can act on competitive differences between strains, we should see a similar result to our previous experiment, with bimodality increasing with tightening transmission bottlenecks. We tested *C. zhejiangensis* GA-OX1 alongside *C. sp. nr. concitans* SQ4a [28], which represent two lineages within the *Caballeronia* genus (Figure S3) [27,41] but are nonetheless equally beneficial for host developmental time and survivorship in the laboratory [7,28]. SQ4a was previously labelled with GFPmut3 [28,42], and was additionally labelled with sfGFP and dTomato for this study using the same constructs [43] that were applied to GA-OX1 above.

First, we demonstrated that GA-OX1 and SQ4a compete under an *in vitro* approximation of natural conditions within the host midgut (Figure 3A). In trials where SQ4a sfGFP and RFP were grown together as liquid cultures in filter-sterilized zucchini squash extract, both strains were recovered at high densities after 24 hours. On the other hand, when either SQ4a strain was grown with a counter-labelled GA-OX1, SQ4a almost always went extinct (T-test, $p < 0.001$, $n = 10$). Labelled GA-OX1 strains grew to high densities regardless of whether they were growing alongside SQ4a or the counter-labelled GA-OX1. These data suggest that GA-OX1 is the superior competitor to SQ4a under these culture conditions.

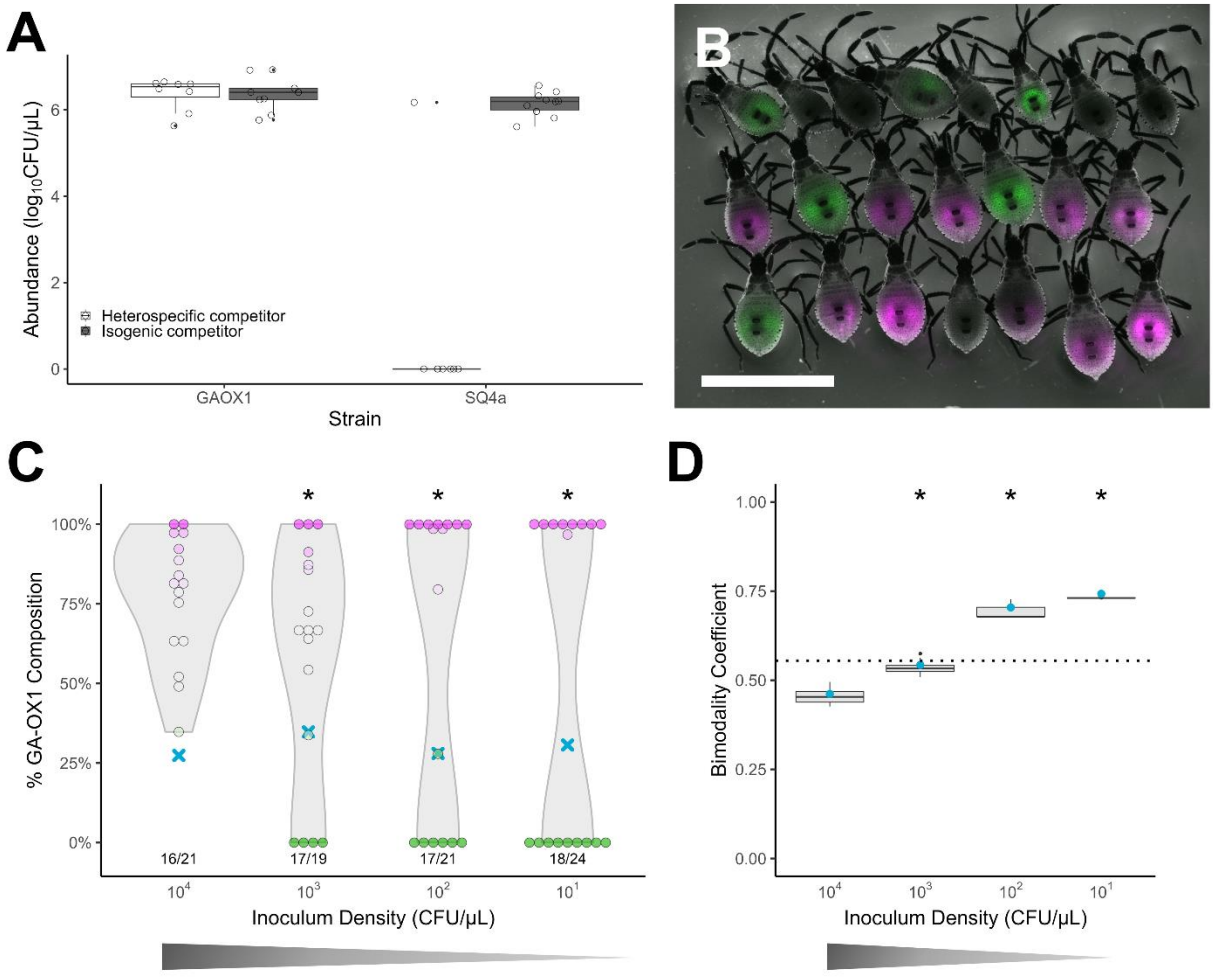

Figure 3. Ecological drift mediates heterogeneity in host infection by competing strains.

A) Competitive interactions between two symbiont strains in filter-sterilized zucchini squash extract. GA-OX1 and SQ4a differentially labelled with sfGFP or RFP were cocultured with a strain with the opposite fluorescent marker, either of an isogenic background or of the other species, then assayed after 24 hours for abundance. Data are pooled across fluorophore reciprocal swaps. White boxplots represent growth with a heterospecific competitor, while gray boxplots represent growth with an isogenic competitor. Data provided in Dataset S3.

B) Fluorescence image of one cohort of squash bug nymphs fed a combination of SQ4a sfGFP and GA-OX1 RFP, at a combined density of ~5000 CFU/µL. Green indicates the presence of SQ4a sfGFP in nymphs, and magenta indicates the presence of GA-OX1 RFP. Nymphs without fluorescence were not successfully colonized with either symbiont strain. Scale bar indicates 5 mm.

C) Variable colonization outcomes associated with different transmission bottleneck sizes in two-species co-inoculation, using *C. sp. nr. concitans* SQ4a GFPmut3 and *C. zhejiangensis* GA-OX1 RFP. Blue X marks indicate the percent GA-OX1 RFP associated

with each inoculum treatment, ranging from 10^1 to 10^4 CFU/μL. Points represent individual nymphs, and the color of each point and its position along the y-axis represent the percent relative abundance of GA-OX1 RFP colonies among all fluorescent colonies recovered from each nymph. Magenta points represent nymphs from which only GA-OX1 RFP colonies were recovered, green points represent nymphs from which only SQ4a GFPmut3 colonies were recovered, and faded magenta/green points represent coinfected nymphs. Violin plots associated with each treatment depict the shape of the distribution in relative GA-OX1 RFP abundance. Below each violin plot, the success rate of colonization is indicated, as the number of nymphs that were successfully colonized with *Caballeronia* out of all nymphs sampled. Asterisks indicate significantly multimodal infection outcomes as determined by Hartigan's dip test, at a significance level of $p < 0.05$. Data provided in Dataset S4.

D) Bimodality coefficients calculated from results in panel C. Large blue dots indicate bimodality coefficients calculated from all bugs in each treatment; boxplots indicate bimodality coefficients calculated by jackknife resampling in each treatment. The 0.555 threshold (marked with a dotted line) indicates the bimodality coefficient associated with a uniform distribution. Asterisks indicate significantly multimodal infection outcomes as indicated by Hartigan's dip test, at a significance value of $p < 0.05$. Analyses conducted on Dataset S4.

We next co-colonized hosts with mixtures of GA-OX1 RFP and SQ4a GFPmut3, using the same experimental design as in single-strain colonization. Moderately high to low inoculum densities all resulted in strong bimodality in infection outcomes, where individual hosts were dominated either by GA-OX1 or by the competitor SQ4a (Figs 3B, 3C, and Table S2). Only at high inoculum density ($\geq 10^4$ CFUs) were infections biased in favor of GA-OX1 (Figure 3C). This bimodality was qualitatively reproducible across different combinations of SQ4a and GA-OX1 expressing different fluorophores from different synthetic constructs (Figs 3B and S4, Table S3 and S4). Bimodality coefficients were consistently higher at high colonization densities in interspecific competition experiments than neutral competition experiments (Figs 3D, S4B, and S4D, Tables S2-S4); this is expected, as neutral competition should produce unimodal populations when colonization rates are high.

Ecological drift during colonization generates within-host spatial heterogeneity.

The squash bug symbiotic organ, called the M4, contains hundreds of crypts (Figs 1A and 1B). Because each crypt is filled with its own population of symbionts, we asked whether symbiont composition might exhibit between-crypt heterogeneity within the host, consistent with previous unquantified observations from related insect-*Caballeronia* models [25,44]. If crypts indeed contain heterogeneous populations, we would expect crypts to contain mostly RFP- and mostly GFP-expressing symbionts, as opposed to highly similar populations composed of one or both types. We also asked if within-host heterogeneity might be sensitive to inoculum density in the same manner as between-host heterogeneity. If so, we would expect greater heterogeneity among crypts within a host when symbionts are subjected to tighter transmission bottlenecks during host colonization.

We systematically characterized within-host spatial heterogeneity by co-inoculating nymphs with 1:1 mixtures of counter-labeled GA-OX1 at approximately 10^6 and 10^2 CFU/μL, as above. Co-infected nymphs were selected by screening whole insects under fluorescence prior to dissection. By imaging freshly dissected whole guts from coinfected nymphs, we observed that the M4 does impose spatial heterogeneity on symbiont populations, with individual crypts varying in GFP and RFP intensity even at colonization with 10^6 symbiont CFU/μL (Figure 4A). However, there is a clear gradient in the degree of heterogeneity among crypts along the length of the M4, with anterior crypts being co-colonized and posterior crypts being singly infected (Figure 4B). We quantified this gradient by measuring the variance in RFP intensity relative to GFP along the length of the M4 (Figure 4C). Contrary to our expectations, we saw that nymphs colonized with just 10^2 symbiont CFU/μL also exhibited this gradient, with anterior crypts being co-colonized despite a 10,000-fold reduction in inoculum density (Figs 4B and 4C). Thus, patterns of heterogeneity within the host are consistent over four orders of magnitude in

inoculum density. Even when microbe-microbe competition is nearly neutral, host anatomy appears to impose spatial structure on symbiont populations.

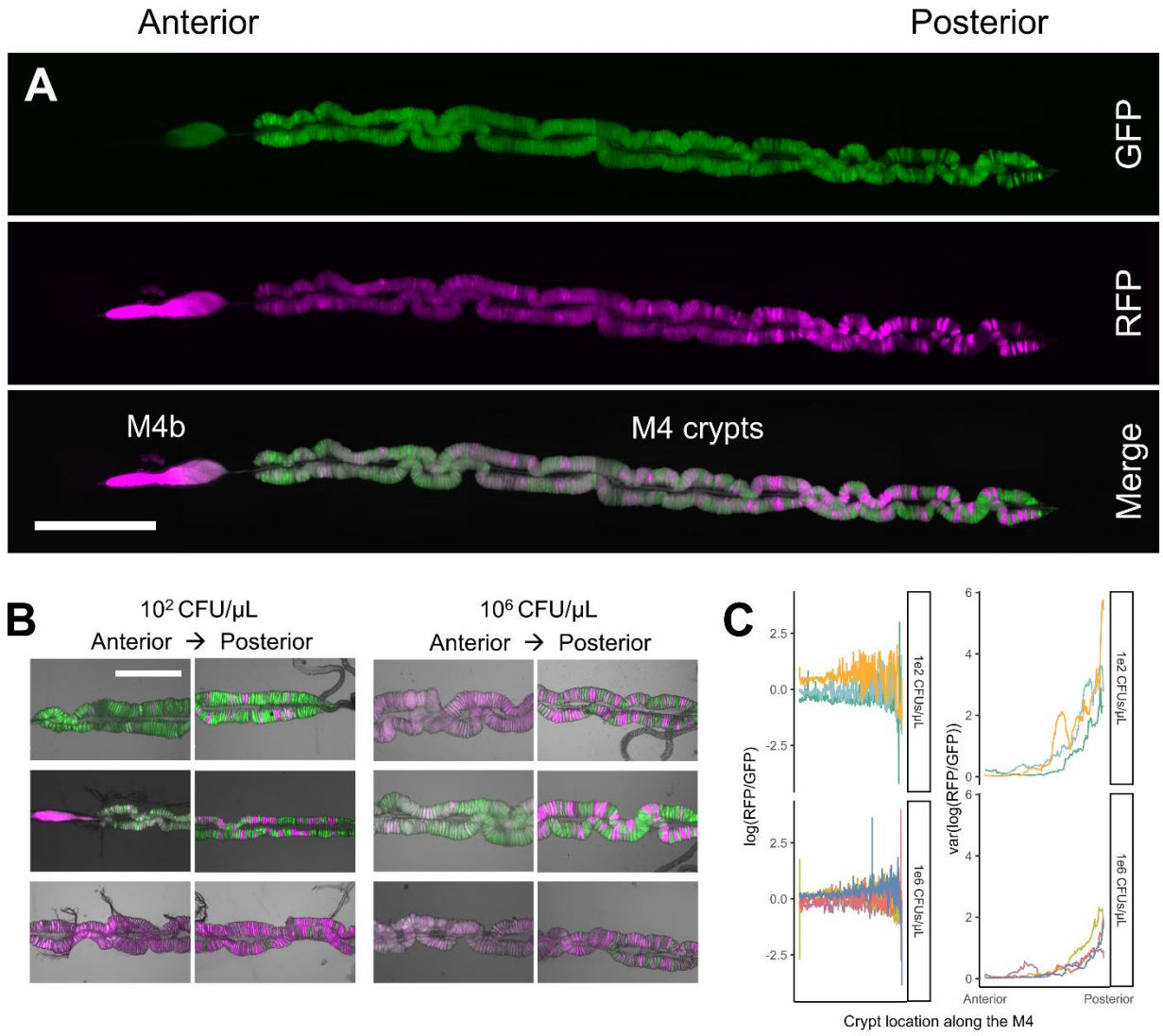

Figure 4. The squash bug symbiotic organ (the M4) imposes spatial heterogeneity on *Caballeronia* populations within the host.

a) Tilescan of the entire M4 of a representative second-instar nymph fed combined 10^6 CFU/µL GA-OX1 GFP and RFP, dissected and linearized to illustrate symbiont colonization along its length. Individual panels represent the merged GFP and RFP channels (top), only the GFP channel (middle), and only the RFP channel (bottom). In each panel, the anterior end of the M4 is oriented to the left and the posterior end is oriented to the right. The intensely magenta, spindle-shaped organ is the M4b, which is functionally distinct from the crypts that house the symbiont population. The scale bar represents 1 mm.

b) Anterior and posterior crypts from 3 nymphs fed 10^2 CFU/μL (left) and 3 other nymphs fed 10^6 CFU/μL (right) GA-OX1 GFP and RFP, dissected and prepared as in a). Panels represent the merging of GFP, RFP, and DIC (differential interference contrast) channels. For each specimen, the anterior crypts are on the left and the posterior crypts are on the right. The scale bar represents 500 μm.
c) Ratio of normalized RFP intensity relative to normalized GFP intensity (left) and variance in this ratio within a sliding window (right) along a transect from the anterior to the posterior of the M4. Nymphs were either inoculated with 10^2 CFU/μL (n = 3, top) or 10^6 CFU/μL (n = 4, bottom), and different colored lines represent the trajectories of these values associated with each nymph.

Discussion

Previous research on a suite of closely related insect-*Caballeronia* symbioses has demonstrated both heterogeneity in symbiont composition and low diversity of symbiont populations within hosts [7,31,45,46]. In the present study, we reveal the processes that underlie these patterns are consistent with stochastic colonization, which results in strong ecological drift as symbionts establish in their host insects. By modulating transmission bottleneck sizes of inocula containing isogenic, nearly neutrally competing strains, we show that ecological drift alone can generate heterogeneity in colonization outcome between different hosts, consistent with the neutral theory of biodiversity [20,21]. The transmission bottlenecks in our experiments are likely to be within the range of natural variation in transmission bottleneck size through natural routes [37,38] of symbiont transmission in *Anasa tristis* and related insects [47], suggesting that our results pertain to how drift affects symbiont population structure in wild bug-*Caballeronia* assemblages.

Next, by manipulating bottleneck sizes of inocula containing different symbiont species, we not only experimentally demonstrate the role of ecological drift in maintaining genetic diversity but also highlight the role of between-symbiont competition. Ecological drift generates variation in founding populations between hosts, while competition drives homogeneity in

symbiont populations during subsequent proliferation inside a single host. The effect is bimodality in symbiont colonization, even when transmission bottlenecks are loose. Our results mirror findings from similar studies using plant communities, where competitive asymmetries between species also exaggerate the effect of ecological drift [19], and call attention to the role that inter-symbiont competition might play during the early stages of colonization in other host-microbe systems [13,48–50].

Generality of drift in generating heterogeneity in symbiotic systems

Although we implemented our experiments using horizontal transmission, many symbioses exhibit elaborate, host-controlled mechanisms that ensure vertical transmission [51–55]. Despite host control, vertically transmitted symbionts, including obligate insect mutualists [56], are not immune to the effects of ecological drift, which acts on communities regardless of how they disperse. Indeed, some vertically transmitted symbionts undergo extreme transmission bottlenecks [57–59], exaggerating the intensity of drift, and vertically transmitted symbionts also compete for host colonization [59–62]. Thus, we should expect drift to generate heterogeneity in vertically transmitted infections [63,64] in a similar manner as we observed in our horizontal transmission experiments.

Based on our findings, we argue that the role of ecological drift is inadequately considered in host-microbe associations [13,14,34,35,65]. Notably, as long as ecologically overlapping microbes are capable of colonizing a within-host niche, host benefit, partner choice and coevolutionary history may be unnecessary to explain between-host variation in microbiomes [2,5,15,49,64,66–69]. This is of course not to say that niche-based ecology does not affect these communities or their evolution (e.g., [70]). In our system, there is limited diversity in the microbial symbiont community, and interactions between strains are likely dominated by

competition [5,44,59,71,72]. By contrast, multispecies communities may contain facilitative interactions, such as signaling crosstalk, cross-feeding, metabolic division of labor, and complementary host-provisioning [8,73–80], all of which could fundamentally alter ecological dynamics. Nonetheless, the neutral model often performs surprisingly well in explaining patterns in multispecies communities [21], suggesting that drift and other stochastic processes should at least be considered when attempting to explain patterns of diversity within more specialized symbioses as well.

The pervasive effect of ecological drift suggests it may also play a key but undervalued role in the evolution of specialized host-microbe symbioses. First, ecological drift can override selection and maintain strain variation within a host population, by providing refugia for suboptimal or less competitive symbionts. In addition, by driving compositional variation between host-associated microbiomes, ecological drift can expose taxonomically or functionally distinctive strains and communities to selection [61,81–85]. If a distinctive microbiome can maintain its association with a particular host lineage, coevolution with the host may eventually occur. By simultaneously maintaining genetic variation among symbionts and generating heterogeneity in symbiont community composition, we argue that ecological drift could provide another explanation for the paradox of variation in host-microbe mutualisms [9].

Beyond its role in generating between-host heterogeneity, ecological drift also generates heterogeneity in symbiont populations within a host. In the squash bug, we found that gut crypts, a unique anatomical feature of the M4 symbiotic organ, generate heterogeneity by segregating strains into discrete compartments within the same host. This has parallels in other symbiotic organs, including the crypts in the light organs and accessory nidamental glands of sepiolid squid [86–88], coralloid roots and root nodules in cycads and legumes [35,89,90], and pores in human

skin [91]. Because we show that such compartmentalization acts even on isogenic cells, we propose that within-host population spatial structure, as with between-host population structure, is not adequately explained by either host selection or microbial competition, and is instead characterized by stochastic colonization of different crypts [35]. While spatial heterogeneity frequently emerges as a result of between-strain interactions within *in vitro* communities [92–96], here, the anatomy of a host forcefully imposes it even in the apparent absence of such interactions. How squash bugs and other multicellular hosts benefit from subjecting their symbiont populations to such elaborate compartmentalization remains an open question [97,98].

Although we have discussed how ecological drift results in segregation of genetic variation within the host, we were surprised to find that population diversity within individual crypts is apparently independent of inoculum density. We expected that the degree of population admixture within the crypts would depend on inoculum density, with crypts being predominantly coinfected at high inoculum density and predominantly singly infected at low inoculum density, as has been demonstrated *in vitro* systems on plates and in microfluidics experiments [99,100]. However, we instead observed an anterior-posterior gradient of admixture for all co-colonized bugs, consistent across four orders of magnitude in initial inoculum density. This suggests that *in vivo* colonization processes impose distinct conditions that generate structure in *Caballeronia* populations. We know almost nothing about symbiont colonization at the single-cell level in the squash bug. However, we speculate that the host permits colonization of individual crypts by only a limited number of symbiont cells, and that inoculation of individual crypts continues to some extent after the initial colonization event by movement of propagules within the symbiont organ. Further study is necessary to ascertain whether coinfection within single crypts affects

within-host symbiont evolution and host fitness, as predicted by others [101], or creates opportunities for horizontal gene transfer [102].

In this work, we illustrate the role of ecological drift in shaping symbiont host populations at multiple scales. Our findings highlight the effect of ecological drift during colonization by maintaining heterogeneity in symbiont populations both within and between hosts. We posit that ecological drift can weaken selection from microbe-microbe interactions within the host, while also setting the stage for the evolution of these same processes. These results contribute to our understanding of the role that stochastic dynamics play in the assembly of ecological communities, even in ancient, highly specific host-microbe associations subjected to extensive host control [21].

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Methods

Study system

Squash bugs (*Anasa tristis*) were maintained on yellow crookneck squash plants (*Cucurbita pepo* 'Goldstar') in 1 ft^3 mesh cages. Hatchlings were maintained on pieces of surface-sterilized organic zucchini in plastic rearing boxes, where they remain aposymbiotic (i.e., *Caballeronia*-free, though not necessarily free of other microbes). Hatchlings molt to the second instar, the life stage competent for symbiont colonization, after two days of feeding. Nymphs utilized in this experiment were typically one week old or less.

Caballeronia symbionts *C. sp.* SQ4a and *C. zhejiangensis* GA-OX1 were originally isolated from wild squash bugs at different localities in northeastern Georgia, USA. SQ4a and GA-OX1 form phenotypically very distinct colonies on nutrient agar (NA; 3 g/L yeast extract, 5 g/L peptone, 15 g/L agar) and are not closely related within the genus *Caballeronia* [41] (Figure S3). Cultures were typically grown on NA plates or in Luria Bertani (LB) Lennox broth with low salt (Sigma-Aldrich L3022), at 25°C. Unless otherwise stated, 2 mL broth cultures were initiated from colony picks of three to four day old colonies grown on NA at 25°C, and grown overnight with shaking at 200 rpm at 25°C.

Strain construction

The mini-Tn7 system [103] facilitates the stable, orientation-specific introduction of foreign DNA into bacterial genomes at a neutral intergenic site, *attTn7*, with minimal effects on phenotype and fitness *in vitro* [104–106]. To make readily distinguishable but otherwise isogenic symbiont strains, we genomically integrated a green fluorescent protein (sfGFP; henceforth GFP) and a red fluorescent protein (dTomato; henceforth RFP) into SQ4a and GA-OX1 using improved versions of previously developed mini-Tn7 vectors (Table 1) [103]. The conjugative

Escherichia coli K12 strain SM10(λpir) harboring pTn7xKS-sfGFP or pTn7xKS-dTomato (Table 1), which were a generous gift from Travis Wiles [43], as well as an *E. coli* parent of the same strain harboring helper plasmid pTNS2 [103], were plated with SQ4a and GA-OX1 at high density on LB plates with salt (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar). After 24-48 hours of incubation at 30°C, matings were harvested into LB Lennox low-salt broth with a lytic coliphage, T7, to eradicate *E. coli*. After further incubation for four hours at 30°C shaking at 200-225 rpm, cultures were plated on NA amended with 1 mM isopropyl-β-D-1-thiogalactoside (IPTG) and 10 μg/mL gentamicin to select for successful integrants. Colonies on selective plates were screened for fluorescence and frozen at -80°C as 20% v/v glycerol stocks.

Table 1. Strains and plasmids used in this study.

Strains	Description	Source/Reference
Escherichia coli SM10(λpir)	recA::RP4-2-TcR::Mu, pir+ conjugative strain	Travis Wiles [107,108]
Caballeronia zhejiangensis GA-OX1	Wild *Anasa tristis* isolate, Oxford College Organic Farm	[7]
Caballeronia zhejiangensis GA-OX1 sfGFP	Mini-Tn7T-GmR-sfGFP derivative of GA-OX1; high fluorophore expression driven by a tac promoter	[37]
Caballeronia zhejiangensis GA-OX1 RFP	Mini-Tn7T-GmR-dTomato derivative of GA-OX1; high fluorophore expression driven by a tac promoter	[37]
Caballeronia sp. nr. concitans SQ4a	Isolated from a wild-caught *Anasa tristis* held in captivity; originally collected at Oakhurst Community Garden	[28]
Caballeronia sp. nr. concitans SQ4a GFPmut3	mini-Tn7-KmR-GFPmut3 derivative of SQ4a; low fluorophore expression driven by a $P_{A1/04/03}$ promoter	[28,109]

Caballeronia sp. nr. concitans SQ4a sfGFP	Mini-Tn7T-GmR-sfGFP derivative of SQ4a; high fluorophore expression driven by a tac promoter	[37]
Caballeronia sp. nr. concitans SQ4a RFP	Mini-Tn7T-GmR-dTomato derivative of SQ4a; high fluorophore expression driven by a tac promoter	[37]

Plasmids	**Description**	**Source/Reference**
pTNS2	R6K γ oriV, AmpR, tnsABCD	Travis Wiles [103]
pTn7xKS-sfGFP	colE1 oriV, mini-Tn7T-GmR-sfGFP, AmpR, lacI P_{tac} hokB ghoT tisB	Travis Wiles [43]
pTn7xKS-dTomato	colE1 oriV, mini-Tn7T-GmR-dTomato, AmpR, lacI P_{tac} hokB ghoT tisB	Travis Wiles [43]

To confirm stability of fluorophore expression, newly constructed strains were streaked on NA plates and visually assessed for fluorescence after two days. To confirm site- and orientation-specificity of mini-Tn7-GmR integration, we ran PCR to amplify the fragment between the endogenous *glmS* gene (GA-OX1: 5' AGGCGCGTTGAAGCTCAAGG 3'; SQ4a: 5' CGCTGGAGCCGCAAATCATC 3') and the inserted *aacC* gentamicin resistance marker (aacC-83F: 5' GTATGCGCTCACGCAACTGG 3'). We did not screen for insertion at additional sites, as to our knowledge the strains of *Caballeronia* we used have only one *attTn7* site.

Competition assays *in vitro*

During the summer growing season, squash bugs feed on macerated cell contents, xylem, and phloem in tissues from squash plants and fruits [110,111]. To replicate microbial competition in this environment, competition assays in liquid culture were conducted in filter-sterilized zucchini squash extract. In short, juice from organic zucchini fruits was extracted in a juicer, combined, and filtered to remove large suspended particles. This filtrate was then centrifuged at

10,000 xg for 3 hours to pellet suspended particles, then filter-sterilized through a 0.2 µm filter and stored at -20°C.

To initiate competition assays, GA-OX1 and SQ4a, labelled with sfGFP or dTomato as described above, were initially streaked from frozen 20% glycerol stocks onto NA plates and incubated at 30˚C for 48 hours. Individual colonies from each plate were inoculated into 2 mL of LB media and incubated in a shaking incubator (New Brunswick Scientific Excella E25) at 25˚C for 12 hours with shaking at 225 rpm. All cultures were equalized to an optical density (OD) of 1.0 by adding 100 µL of each culture to a 96 well plate and taking readings with a Synergy HTX multimode plate reader. The equalized cultures were spun down with an Eppendorf centrifuge 5424 R and washed with one mL of 1X phosphate buffered saline (PBS) three times.

Monocultures of GA-OX1 and SQ4a were combined to form counterlabelled self vs. self and self vs. competitor co-cultures, for a total of four combinations. Self vs. self co-cultures contained the same *Caballeronia* strain, differing only in the fluorescent protein, while self vs. competitor co-cultures contained different *Caballeronia* strains, also differing in the fluorescent protein. All co-cultures were set up in 500 µL of a 1:1 mixture of filter-sterilized zucchini squash extract and PBS, then incubated for 24 hours at 30˚C with shaking at 225 rpm. As described above, co-cultures were dilution plated on NA and incubated a further 20-24 hours, and single colonies containing each fluorophore were distinguished and counted under a dissecting scope. We confirmed that SQ4a and GA-OX1 do not appear to inhibit each other intensely on NA plates, suggesting that plating co-cultures on NA provides an unbiased count for both competitors (Figure S5).

Competition assays *in vivo*

The generalized protocol for competition assays with varying transmission bottlenecks *in vivo* is presented in Figure 2A. GFP- and RFP-labelled *Caballeronia* strains were streaked out from glycerol stocks onto NA and incubated at 25°C for at least three days. To initiate liquid cultures, single colonies were picked into two mL LB in glass tubes and incubated at 25°C with shaking at 200 rpm; to account for different growth rates between SQ4a and GA-OX1, a glass inoculating loop was used to pick up an entire colony of SQ4a, while a p10 micropipettor tip was used to extract a small plug from part of a single GA-OX1 colony.

To prepare inocula for feeding, cultured bacterial cells were washed to remove LB. Two hundred µL of culture was spun down at 10,000 x g at 4°C for 2 min. The supernatant was removed, and the pellet was resuspended in 1000 µL 1X PBS. After a second centrifugation, the pellet was resuspended in 200 µL 1X PBS to bring the cells to their original culture density. For quality assurance, tenfold serial dilutions were carried out using 30 µL washed cells in 270 µL PBS in a 96-well plate. Fifty µL each of the washed GFP- and RFP-labelled strains were then diluted into 400 µL of a complex feeding solution (a 1:1 mixture of filter-sterilized zucchini squash extract and PBS; for neutral competition trials) or a defined feeding solution (2% m/v glucose 10% v/v PBS; for interspecies competition trials), in each case containing 20 µL of a nontoxic blue dye (1 mM erioglaucine disodium). We found that the defined feeding solution improved nymphal feeding response and better prevented bacterial population growth during the inoculation time window, with minimal impact on our experimental results (Figure S6). Nymphs previously starved overnight for 15-25 hours in clean plastic rearing boxes (7 cm X 7 cm X 3 cm) were supplied with 120 µL of a single inoculum treatment blotted on quartered sectors of 55 mm-diameter qualitative filter paper (Advantec MFS N015.5CM). Nymphs were then allowed to wander and feed *ad libitum* for 2-3 hours. After this brief inoculation period, nymphs were

housed singly in 24 well plates with small pieces of organic zucchini to develop for three days. Just before and after the inoculation period, inocula were serially diluted as above to quantify the concentration of each strain and ensure that no substantial growth or death of either strain occurred during the inoculation period (Figure S6).

On the fourth day after inoculation, nymphs were killed in 70% denatured ethanol, surface sterilized in 10% bleach for 5-10 minutes, washed off again in 70% ethanol, and immersed in ~20 µL droplets of 1xPBS. Whole nymphs, when applicable, were imaged on a Olympus SZX16 stereomicroscope with an Olympus XM10 monochrome camera and Olympus cellSens Standard software ver. 1.13. Nymphs were immersed in a shallow volume of PBS in 6 cm plastic petri dishes, and images were taken in darkfield (30 ms exposure 11.4 dB gain), brightfield (autoexposure, 11.4 dB gain), a GFP channel (autoexposure, 18 dB gain), and a RFP channel (autoexposure, 11.4 dB gain). Darkfield and brightfield images were merged in FIJI version 1.54f using the Image Calculator plugin, and the result was then merged with the GFP channel, RFP channel, or both. M4s were individually dissected from nymphs, and the degree of green and red colonization was qualitatively estimated under a fluorescent microscope. Each M4 was then held in 300 µL 1x PBS in Eppendorf tube and crushed with a sterile micropestle. Thirty µL of homogenate was serially diluted in 270 µL PBS and immediately dilution plated onto NA. Plates were then incubated at 30°C for about 24 hours. Counts of GFP and RFP fluorescent colonies were recorded after refrigeration at 4°C for at least 24 hours to enhance fluorescent protein expression. The count data of GFP and RFP colonies yielded by our sampling procedure almost always reflected our qualitative observations of GFP and RFP colonization inside the M4, suggesting that our data accurately represent the colonization state within live insects.

Microscopy of Within-Host Symbiont Populations

Bugs were inoculated and allowed to develop for four days as described above with ~60 and 931,000 CFU/µL inocula containing GA-OX1 GFP and RFP. We then intentionally screened individual insects for co-colonization, and only these insects were selected for dissection and microscopy. From each bug, the whole gut was dissected in a 20-30 µL droplet of PBS in a 30 mm diameter plastic dish. The M4 was stretched out to its full length, and straightened out as much as possible by severing tracheoles associated with the crypts and flipping the M4 over to minimize the number of twists in the M4. This was critical to minimize aberrations in fluorescence intensity and colocalization due to overlap between multiple crypts. The M4 was anchored at the posterior end by the tip of the bug abdomen and at the anterior end by the M1-M3 sections of the midgut, and cleaned several times by pipetting off debris, fat body, and hemocytes with clean PBS. Finally, the whole preparation was re-immersed in 2550 µL of PBS, to which 1 µL of M9 buffer containing 1% Triton-X100 was added to aid the spreading of the droplet.

Gut preparations, which degrade or dry rapidly, were imaged as soon as possible. Tilescan images were taken using a Leica DMi8 inverted widefield light microscope with a Leica DFC9000 GT fluorescence camera and Leica Application Suite X ver. 3.4.2.18368 software. Automated tilescans were taken with a 10X objective lens with brightfield, DIC, GFP, and RFP channels. Fluorescent channels were established by filter sets. The GFP channel was set to: bandpass filter 470/40 nm emission, dichroic mirror 495 nm, emission 525/50 nm. The dsRed channel was set to: 546/11 nm excitation, dichroic mirror 560 nm, 630/75 nm emission. As each sample is unique, care was taken to set GFP and RFP channel exposure times manually according to the most intense pixels in the entire M4 (usually in the posterior crypts), to minimize signal saturation in any part of the preparation. Due to the convoluted shape of the M4,

images were taken with and without autofocus, and stitched images were visually assessed to determine which images were more useful. The repetitive structure of the M4, composed of nearly identically sized, regularly spaced crypts, also necessitated a lower overlap value between tiles for tilescans, as low as 2%. LAS X software was used to merge tiles from tilescans without smoothing for quantitative analysis.

Statistical analysis

All statistical analyses were conducted in R version 4.1.1, and the R package ggplot2 (version 3.4.2) was used for all data visualization. Because multiple trials were run for inoculation experiments, and some trials recovered very low numbers of infected nymphs, we binned nymphs from multiple trials into discrete treatment groups, based on inoculum size, for analysis. For neutral competition experiments, which utilized isogenic GA-OX1 GFP and RFP, we measured the proportion of GA-OX1 RFP extracted from each host. For interspecies competition experiments, utilizing different combinations of SQ4a and GA-OX1, we measured the proportion of GA-OX1 compared to the sum of all green and red fluorescent colonies extracted from each host.

Raw colony counts of each fluorescent strain recovered from each individual insect were converted into proportions for analysis and visualization. To quantify between-host heterogeneity in symbiont colonization for each inoculation treatment, we calculated a bimodality coefficient [36] using the R package mousetrap (version 3.2.0), as well as the population variance, for each inoculation treatment. Using the R package diptest (version 0.76-0), we also implemented Hartigan's dip test [112], which calculates a dip statistic (Tables S1-S4) based on the shape of the cumulative distribution function of a dataset. We considered a distribution to deviate from unimodality if p-values from the dip test repeatedly fell below the threshold of 0.05.

To quantify within-host spatial structure, a linear region of interest (ROI) was sampled from one complete row of crypts from each sample to obtain GFP and RFP intensities. Crossover of the ROI from one side of the M4 to the next was occasionally necessary to follow that row through each twist of the M4. The identical ROI was translated to obtain GFP and RFP intensities from the empty background immediately adjacent to the crypts. GFP and RFP intensities at each point along the M4 were normalized by subtracting the background signal from the same point outside the M4. For the RFP channel, the difference between crypt and background signal was occasionally less than 0; in these rare cases the normalized RFP intensity at that point was assigned a value of 1. The log-transformed ratio of RFP to GFP intensity was measured from each pixel along the ROI. In addition, the variance in this value was calculated by iteratively sampling pixels from within a sliding interval 10% of the length of the ROI.

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Supporting Information

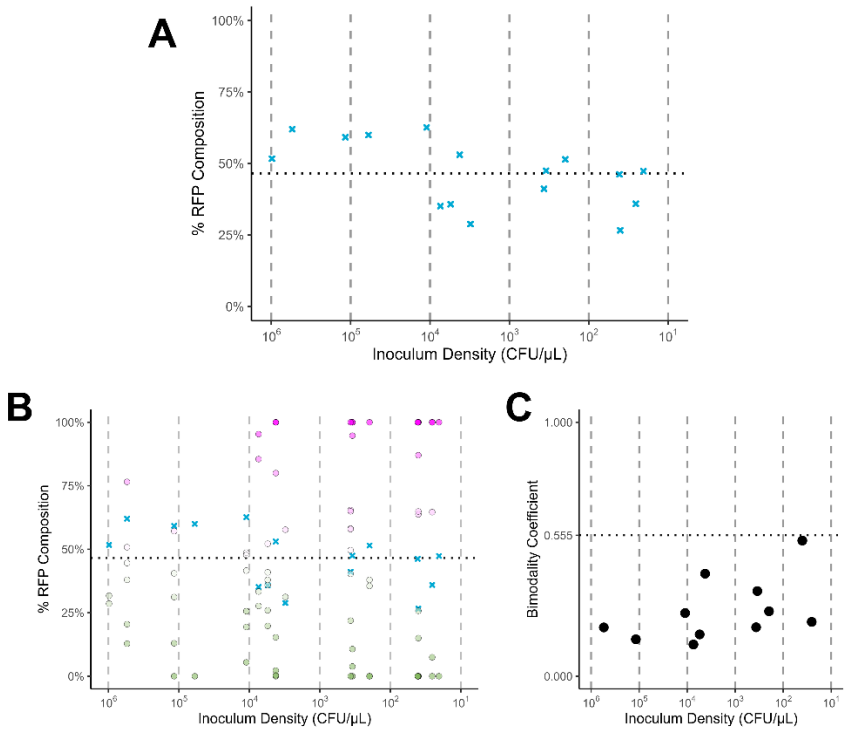

Figure S1. Unaggregated infection outcomes resulting from neutral competition during isogenic co-colonization.

A) Relative GA-OX1 RFP abundance within inocula used in isogenic colonization trials. Vertical lines demarcate which trials were aggregated for analysis in Figure 1. Blue X marks indicate the inoculum density, and the percent relative abundance of GA-OX1 RFP, in each trial. The dotted horizontal line represents the average relative abundance of GA-OX1 RFP (46.5%) across all trials.

B) Variable colonization outcomes associated with different transmission bottleneck sizes in isogenic co-inoculation, disaggregated from Figure 2B. Blue X marks indicate the inoculum density, and the percent relative abundance of GA-OX1 RFP, in each trial. Points indicate successfully colonized nymphs associated with each inoculation trial, and the color of each point and its position along the y-axis represent the percent relative abundance of GA-OX1 RFP colonies among all fluorescent colonies recovered from each nymph. Magenta points represent insects containing only RFP colonies, green points represent insects containing only GFP colonies, and faded magenta/green colonies are co-colonized. Note that multiple points overlap, particularly at the extremes of 0% and 100% RFP composition, due to the absence of jittering. Data provided in Dataset S2.

C) Bimodality coefficients calculated from unaggregated trials. Bimodality coefficients (black points) calculated from data in panel B. The 0.555 threshold (marked with a dotted line) indicates the bimodality coefficient expected from a uniform distribution. Analyses conducted on Dataset S2.

Table S1. Bimodality coefficients, population variances, species-level Fst, and dip statistics calculated from competition trials between GA-OX1 sfGFP and GA-OX1 RFP.

Inoculum Density	Sample Size	Bimodality Coefficient	Variance	Fst (GA-OX1 RFP)	Dip Statistic
10^1—10^2	23	0.68	0.20	0.79	0.15
10^2—10^3	20	0.47	0.14	0.55	0.11
10^3—10^4	19	0.47	0.11	0.43	0.07
10^4—10^5	8	0.29	0.03	0.15	0.13
10^5—10^6	13	0.27	0.04	0.17	0.08

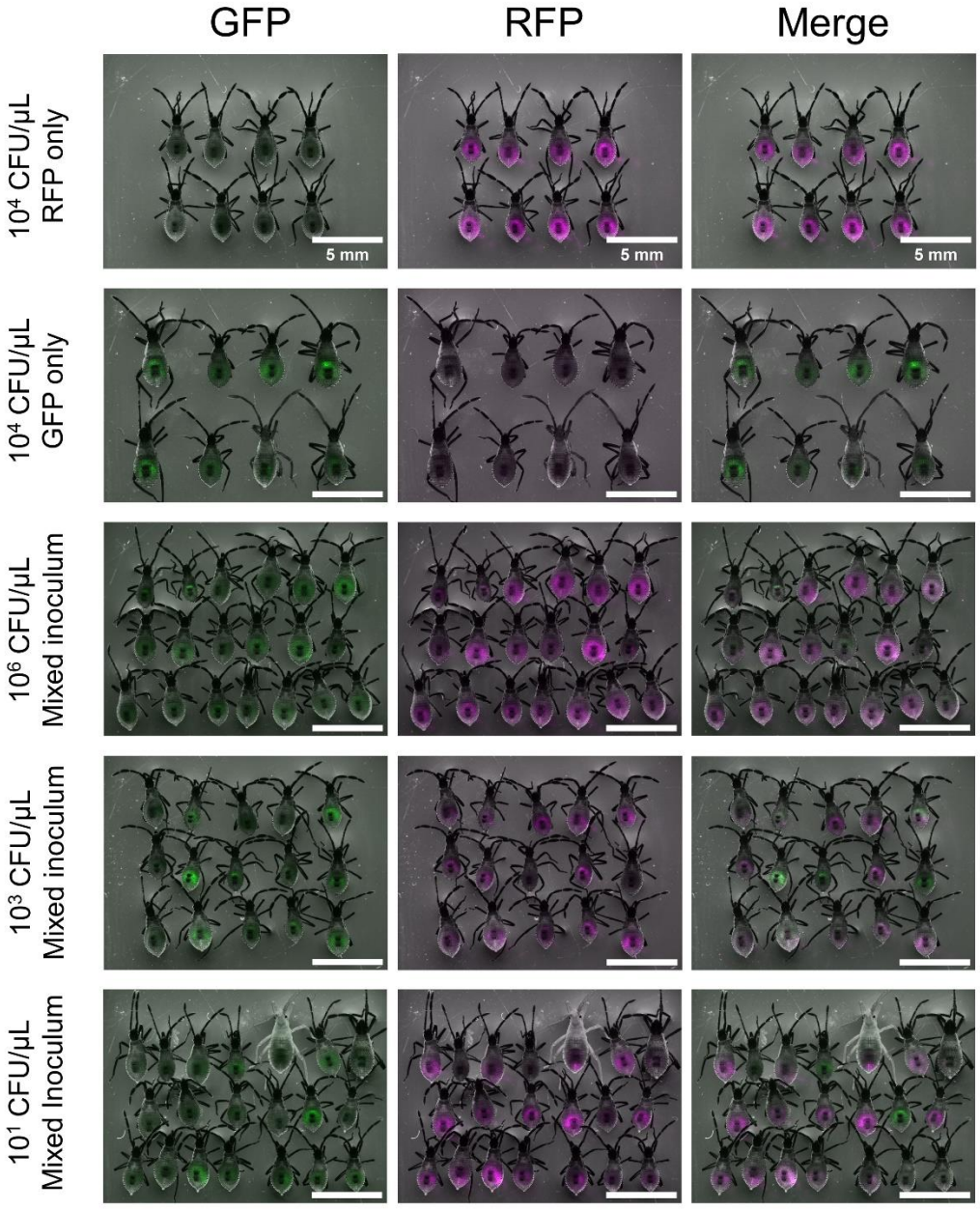

Figure S2. Isogenic coinfections of *A. tristis* nymphs over five orders of magnitude in inoculum density.

Fluorescence images of nymphs from a single cohort colonized with different densities of GA-OX1 sfGFP and GA-OX1 RFP, ranging from 10^1 to 10^6 CFU/µL. Nymphs inoculated with only GA-OX1 sfGFP or only GA-OX1 RFP serve as controls (top 2 rows); the bottom three rows show nymphs from mixed inoculation trials (GA-OX1 GFP + RFP). Note that the red fluorescent protein dTomato is brighter in whole-body preparations of nymphs than the green fluorescent protein sfGFP, due to increased absorbance of green light by living tissue [113] and the high stability of free dTomato under physiological conditions.

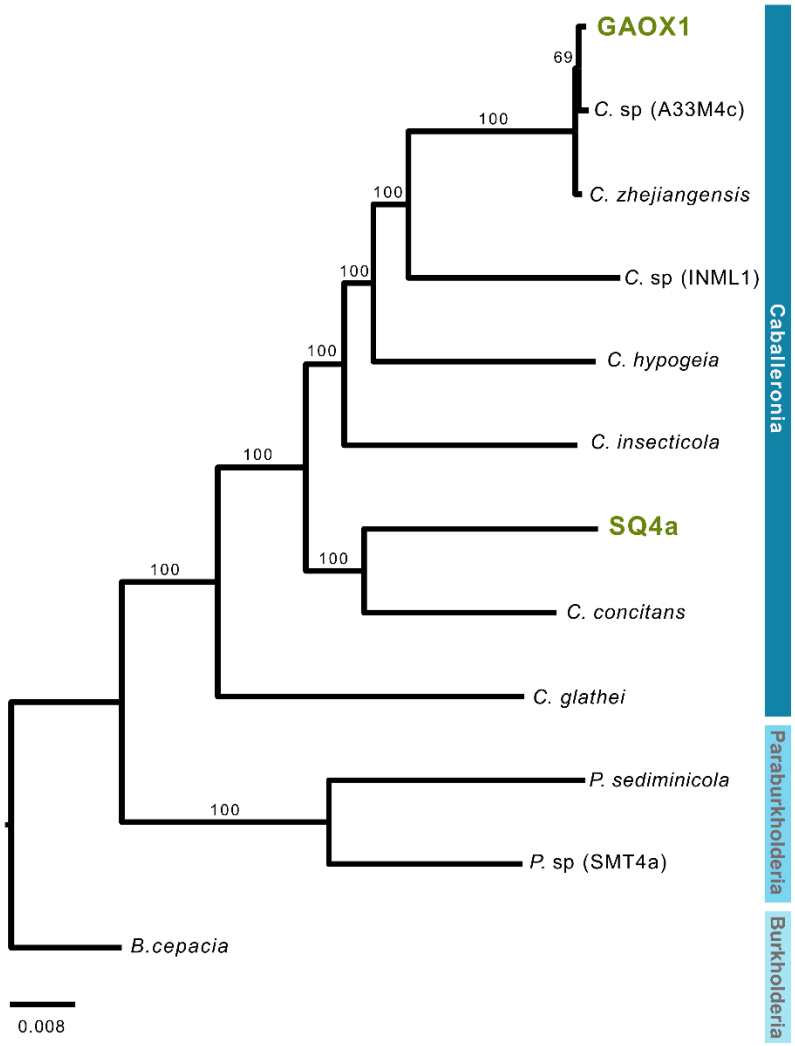

Figure S3. Symbiont strains SQ4a and GA-OX1 represent distinct clades within the genus *Caballeronia*.
A whole genome-based phylogeny of selected species and previously isolated *Anasa tristis* symbionts, representing major clades within the genus *Caballeronia* as defined by Peeters *et al.* (2016) [27], including the experimental strains *C. sp. nr. concitans* SQ4a and *C. zhejiangensis* GA-OX1. The phylogeny was constructed using RealPhy [114], with *Burkholderia cepacia* as the reference genome, using default settings except for a gap threshold of 0.1 and setting the model of evolution to GTR. Support values are bootstrap values based on 100 replicates. In addition to SQ4a and GA-OX1, *Caballeronia* strains A33M4c and IN-ML1 were also previously isolated from *A. tristis* [7,28]. SMT4a is a *Paraburkholderia terricola* soil isolate that can colonize *A. tristis* [28,41]. GenBank assemblies are as follows: GCF_023631065.1 (GA-OX1), GCF_022879815.1 (A33M4c), GCF_022627895.1 (*C. zhejiangensis*), GCF_023631085.1 (INML1), GCF_001544875.2 (*C. hypogeia*), GCF_000402035.1 (*C. insecticola*), GCF_023170545.1 (SQ4a), GCF_001544615.1 (*C. concitans*), GCF_902833485.1 (*C. glathei*), GCF_902859805.1 (*P. sediminicola*), GCF_022879555.1 (SMT4a) and GCA_009586235.1 (*B. cepacia*).

Table S2. Bimodality coefficients, population variances, species-level Fst, and dip statistics calculated from competition trials between SQ4a GFPmut3 and GA-OX1 RFP.

Inoculum Density	Sample Size	Bimodality Coefficient	Variance	Fst (GA-OX1 RFP)	Dip Statistic
10^1	18	0.74	0.25	0.99	0.24
10^2	17	0.70	0.22	0.90	0.18
10^3	17	0.54	0.13	0.55	0.12
10^4	16	0.46	0.04	0.21	0.07

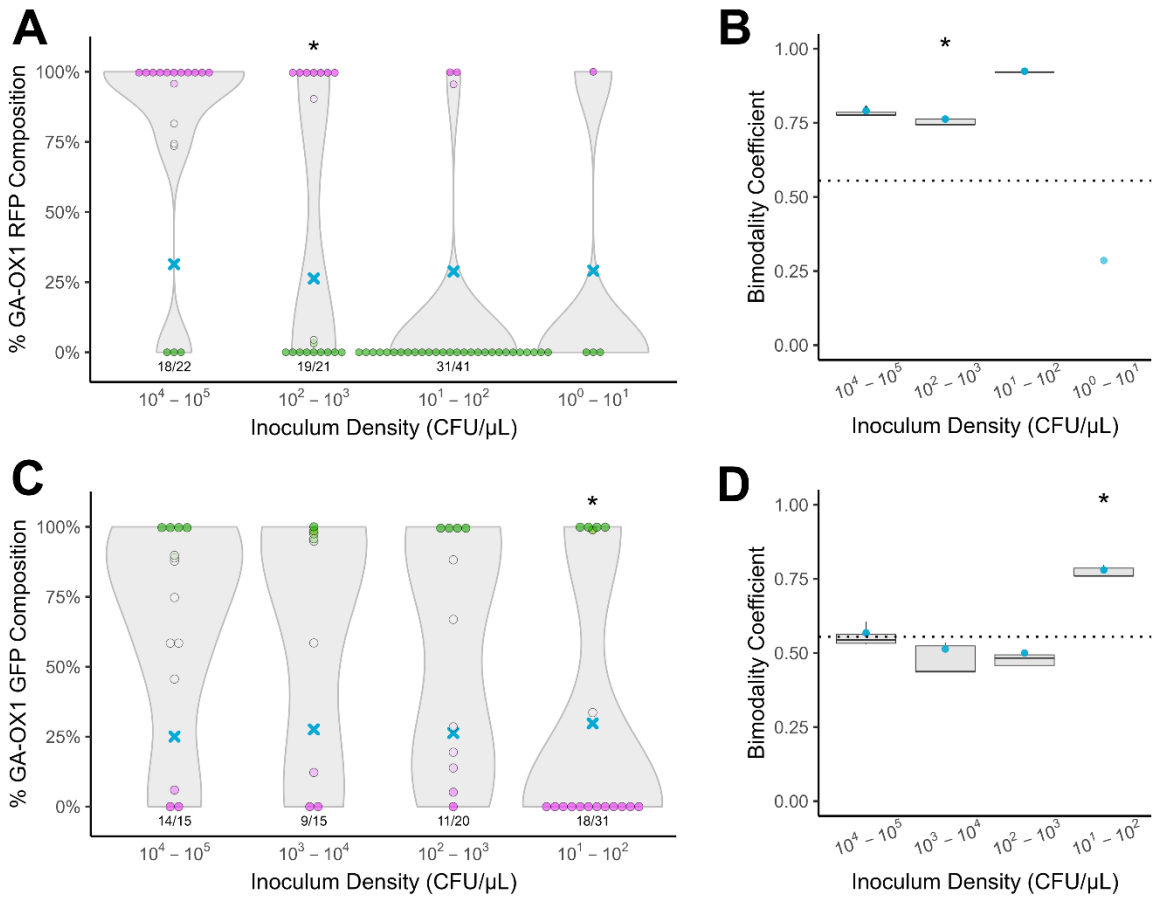

Figure S4. Different combinations of competing GA-OX1 and SQ4a fluorescent strains yield qualitatively similar responses to increasing stochasticity in transmission.

A) Variable colonization outcomes associated with different transmission bottleneck sizes in two-species co-inoculation, using *C. sp. nr. concitans* SQ4a sfGFP and *C. zhejiangensis* GA-OX1 RFP. Blue X marks indicate the mean percent GA-OX1 RFP associated with each inoculum treatment, ranging from 10^0 to 10^5 CFU/µL. Points represent individual nymphs, and the color of each point and its position along the y-axis represent the percent relative abundance of GA-OX1 RFP colonies among all fluorescent colonies recovered from each nymph. Magenta points represent nymphs from which only GA-OX1 RFP colonies were recovered, green points represent nymphs from which only SQ4a sfGFP colonies were recovered, and faded magenta/green points represent coinfected nymphs. Violin plots associated with each treatment depict the shape of the distribution in relative GA-OX1 RFP abundance. Below each violin plot, the success rate of colonization is indicated, as the number of nymphs that were successfully colonized with *Caballeronia* out of all nymphs sampled. These values were not recorded for the 10^0-10^1 treatment and thus omitted. Asterisks indicate significantly multimodal infection outcomes as determined by Hartigan's dip test, at a significance level of $p < 0.05$. Data provided in Dataset S5.

B) Bimodality coefficients calculated from results in panel A. Large blue dots indicate bimodality coefficients calculated from all bugs in each treatment; boxplots indicate coefficients calculated by jackknife resampling in each treatment. Colonization is bimodal (bimodality coefficient > 0.555) across several orders of magnitude of inoculum density. The 0.555 threshold (marked with a dotted line) indicates the bimodality coefficient associated with a uniform distribution. Analyses conducted on Dataset S5. Note that for the 10^0-10^1 treatment, the sample size was insufficient for jackknife resampling.

C) Variable colonization outcomes associated with different transmission bottleneck sizes in two-species co-inoculation, using *C. sp. nr. concitans* SQ4a RFP and *C. zhejiangensis* GA-OX1 sfGFP. Blue X marks indicate the mean percent GA-OX1 GFP associated with each inoculum treatment, ranging from 10^1 to 10^5 CFU/µL. Points represent individual nymphs, and the color of each point and its position along the y-axis represent the percent relative abundance of GA-OX1 GFP colonies among all fluorescent colonies recovered from each nymph. Magenta points represent nymphs from which only SQ4a RFP colonies were recovered, green points represent nymphs from which only GA-OX1 sfGFP colonies were recovered, and faded magenta/green points represent coinfected nymphs. Violin plots associated with each treatment depict the shape of the distribution in relative GA-OX1 GFP abundance. Below each violin plot, the success rate of colonization is indicated, as the number of nymphs that were successfully colonized with *Caballeronia* out of all nymphs sampled. Asterisks indicate significantly multimodal infection outcomes as determined by Hartigan's dip test, at a significance level of $p < 0.05$. Data provided in Dataset S6.

D) Bimodality coefficients calculated from results in panel C. Large blue dots indicate bimodality coefficients calculated from all bugs in each treatment; boxplots indicate coefficients calculated by jackknife resampling in each treatment. Colonization is bimodal (bimodality coefficient > 0.555) across several orders of magnitude of inoculum density. The 0.555 threshold (marked with a dotted line) indicates the bimodality coefficient associated with a uniform distribution. Analyses conducted on Dataset S6.

Table S3. Bimodality coefficients, population variances, species-level Fst, and dip statistics calculated from competition trials between GA-OX1 RFP and SQ4a sfGFP.

Inoculum Density	Sample Size	Bimodality Coefficient	Variance	Fst (GA-OX1 RFP)	Dip Statistic
10^0 - 10^1	4	0.29	0.19	1.00	0.13
10^1 - 10^2	31	0.92	0.08	0.98	0.05
10^2 - 10^3	19	0.76	0.23	0.96	0.19
10^4 - 10^5	18	0.79	0.13	0.80	0.08

Table S4. Bimodality coefficients, population variances, species-level Fst, and dip statistics calculated from competition trials between GA-OX1 GFP and SQ4a RFP.

Inoculum Density	Sample Size	Bimodality Coefficient	Variance	Fst (GA-OX1 sfGFP)	Dip Statistic
10^1 – 10^2	18	0.78	0.19	0.94	0.14
10^2 – 10^3	11	0.50	0.17	0.68	0.14
10^3 - 10^4	9	0.51	0.18	0.77	0.14
10^4 - 10^5	14	0.57	0.14	0.60	0.09

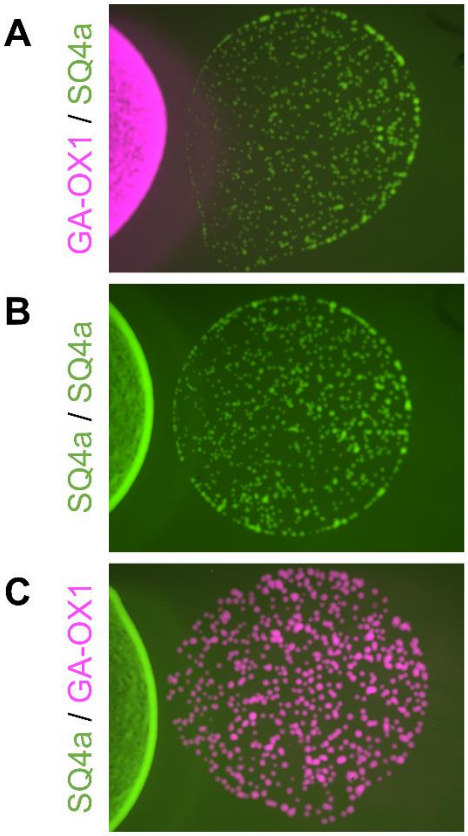

Figure S5. GA-OX1 and SQ4a do not exhibit strong inhibition on nutrient agar.
Spots of GA-OX1 RFP and SQ4a sfGFP plated side-by-side at high and low densities on nutrient agar.
A) A dense culture of GA-OX1 RFP spotted adjacent to single SQ4a sfGFP colonies.
B) A dense culture of SQ4a sfGFP spotted adjacent to single SQ4a sfGFP colonies.
C) A dense culture of SQ4a sfGFP spotted adjacent to single GA-OX1 RFP colonies.

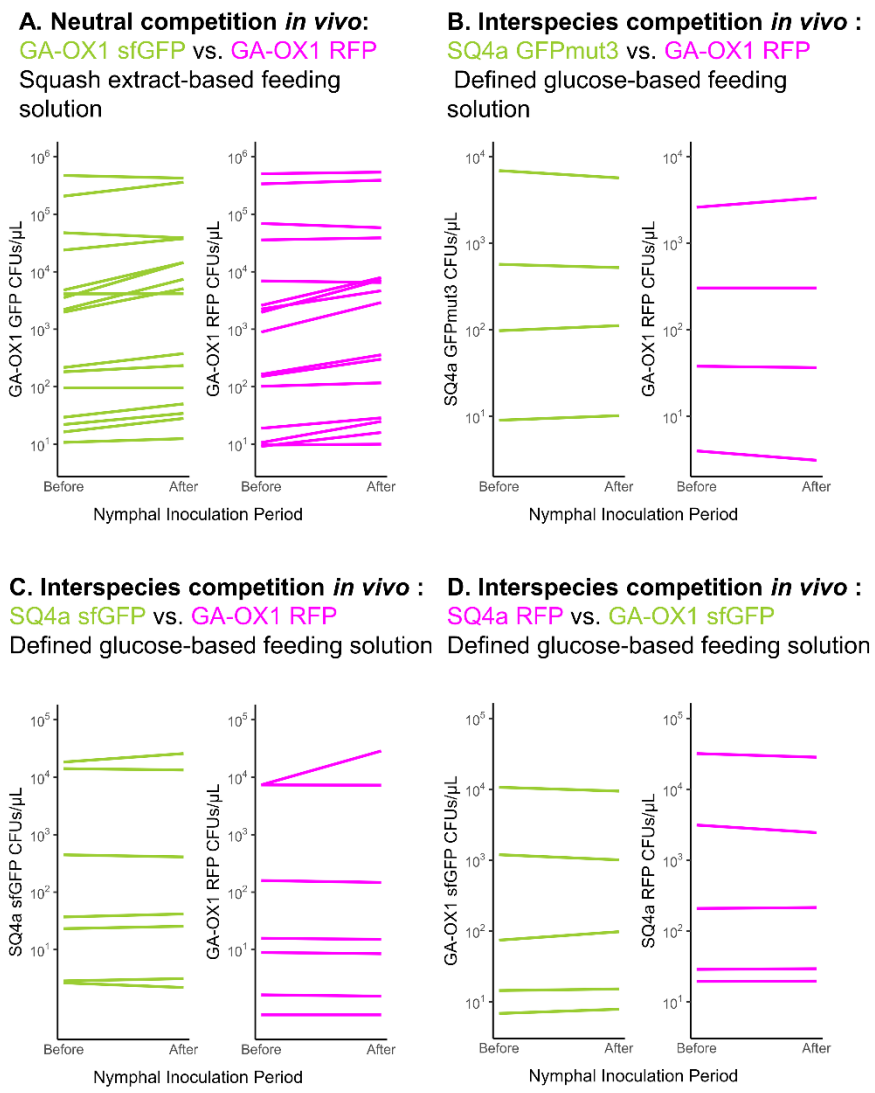

Figure S6. Changes in titer of each strain during all inoculation trials.
Bacterial strain titers before and after inoculation trials.

A) GA-OX1 GFP and RFP (cf Figs 2B, 2C, and S1)
B) SQ4a GFPmut3 and GA-OX1 RFP (cf Figs 3C and 3D)
C) SQ4a sfGFP and GA-OX1 RFP (cf Figs S4A and S4B)
D) GA-OX1 sfGFP and SQ4a RFP(cf Figs S4C and S4D)

Chapter 5

Conclusion

Host-microbe symbioses are beneficial for hosts. This has resulted in the evolution of manifold mechanisms to maintain these relationships every generation. One way in which many host-microbe symbioses are maintained is through vertical transmission - host lineages maintain fidelity with symbiont lineages by passaging them in intimate contact with the host for many generations. Hosts can also use horizontal transmission, in which symbionts migrate between host lineages. This can result in the co-colonization of individual hosts by multiple symbiont lineages and can break down fidelity between host and symbiont lineages.

To understand how symbiosis evolves under different modes of transmission, it is necessary to think of transmission as a process of microbial community assembly, in which hosts are not just partners, but discrete patches of habitat that may or may not be available or accessible for colonization. Hosts impose filters on incoming microbes, resulting in ecological selection that favors colonization by beneficial taxa. However, other drivers of community assembly in host-microbe symbioses are not always accounted for: microbes also disperse, and differential rates or probabilities of dispersal can bias community composition among hosts. Moreover, the order by which dispersing microbes arrive can dictate the outcomes of interactions among community members, modifying the trajectory of community assembly. Finally, ecological drift can stochastically generate variation in community sizes, with particularly potent effects during sharp reductions in community size.

In this dissertation, I demonstrated these principles using the squash bug *Anasa tristis* and its bacterial symbionts in the genus *Caballeronia*. The squash bug symbiotic organ, called the

M4, is a specialized section of the midgut containing two rows of numerous crypts, each filled with *Caballeronia* at high density [1]. Because *Caballeronia* are culturable and can be genetically manipulated with conventional tools, we can isolate symbionts from insects, grow them in pure culture, modify their genomes to express antibiotic resistance genes and fluorescent proteins, and study their population dynamics *in vitro* and *in vivo* with manipulable experiments.

Behavioral adaptions for symbiont acquisition and effects on symbiont community structure

In chapter 2, I demonstrated a natural mechanism for environmental transmission of *Caballeronia* symbionts from *A. tristis* adults to nymphs. Previous reports in *Riptortus pedestris*, a related herbivorous hemipteran from east Asia that also utilizes *Caballeronia*, suggested that nymphs acquire their symbionts from the soil, but without clear evidence that these nymphs can also detect volatiles from at least one symbiont strain [2]. In contrast, I show that: 1) nymphs are attracted to fecal spots deposited on environmental surfaces by adults, likely via olfactory cues; 2) nymphs also are attracted somewhat to symbionts cultured *in vitro*; and 3) symbiont acquisition mediates a behavioral shift from wandering and foraging for feces to a state of relative inactivity and feeding on the squash plant. These findings are significant for understanding how hosts that acquire their symbionts from the environment do not only rely on filtering mechanisms to discriminate beneficial symbionts from other non-beneficial taxa. Instead, the possibility exists that hosts can evolve sophisticated behavioral adaptations to facilitate encounters with symbionts - even before symbionts are ingested and subjected to filters imposed by host physiology, including immunity.

I next show that host behavioral adaptations for symbiont acquisition have profound effects for symbiont community assembly. The feces of both *A. tristis* and its close relative, *A. andresii*, are potential sources of symbionts for *A. tristis* nymphs. Yet, while *A. tristis* nymphs are able to acquire *Caballeronia* from *A. tristis* adults with high success, they only do so infrequently from *A. andresii* adults. This results in a pattern of host-microbe specificity that is primarily driven by biases in dispersal, rather than selection. Notably, while different symbiont genotypes are essentially equivalent in the benefits that they confer to *A. tristis* nymphs in our experimental conditions, the rates at which they are successfully transmitted to *A. tristis* nymphs from *A. tristis* adults vs. *A. andresii* adults starkly differ. To our knowledge, this is one of the few demonstrations that natural pathways of transmission, rather than differences in host species or symbiont genotype, can generate specificity in host-microbe associations.

Future directions. This unique behavioral ecology of symbiont transmission merits further study. First, and most broadly, the mechanism of strong host attraction to both symbiont-laden and autoclaved feces, as well as weak host attraction to cultured symbionts, remains unknown. Future studies should characterize the chemical cues responsible for attraction, the nature and location of chemical receptors sensitive to these cues, and the origin (host, plant, or microbe) of the synthesis of these chemical cues. In addition, studies should evaluate the role of proteins or other chemical species present in the feces in stabilizing desiccated symbiont cells on exposed environmental surfaces for extended periods.

An understanding of the proximate mechanisms underlying natural *Caballeronia* transmission will facilitate comparative studies on the evolution of transmission. Although Chapter 1 highlights the evolution of behavioral adaptations mediating symbiont transmission in *A. tristis*, we know little about the prevalence of behavioral attraction in nymphs of other

Coreidae [3], including even the closely related *A. andresii*, or the precursors of these behaviors from which they were elaborated. Tantalizing reports from the Old World dock bug *Coreus marginatus*, of nymphal development in captivity requiring the presence of adults, suggest this behavior could have evolved or been lost multiple times in coreid evolution. Future studies should replicate these studies in other Coreidae, including *A. andresii* and the putative sister species of *A. tristis*, the Mexican species *A. uhleri*.

Colonization order governs the outcome of symbiont community assembly

In Chapters 3 and 4, I address the emergence of heterogeneity in microbial community composition between hosts at the strain level, which is apparent in the squash bug [4] as well as in other insect-microbe systems [5]. The appearance of this heterogeneity is often attributed to priority effects, because the maintenance of this heterogeneity implies that established communities somehow resist homogenization by microbial dispersal between hosts [6].

In chapter 3, I demonstrate how priority effects impact establishment of *Caballeronia* communities within squash bug populations. By feeding nymphs sequentially with two strains, distinguishable by the expression of red and green fluorescent proteins, with an intervening period of 24 hours, I show that the strain that is ingested first dominates the symbiotic organ, to the complete exclusion of the second. This effect is agnostic of *Caballeronia* strain identity or relatedness between *Caballeronia* strains; isogenic symbionts differing only in the expression of the fluorescent marker also exclude each other. I also show that this priority effect is only slightly alleviated even after heavy treatment of colonized nymphs with antibiotics. While the host does remodel the midgut to create a barrier to subsequent colonization by *Caballeronia*, this

remodeling does not occur rapidly enough, as it does in *Riptortus*, to explain the occurrence of the priority effect.

Priority effects are common in host-associated microbiomes [6,7], and in complex and/or less specialized microbiomes, can be driven by facilitative (e.g. [8]) as well as competitive [9] interactions between strains. However, in highly specialized host-microbe mutualisms, such as the squash bug-*Caballeronia* symbiosis, in which the host depends on a particular taxon from which it draws symbiotic benefits, the ecological niche space is tightly constrained due to the dependence of the microbial community on host-provisioned metabolites and due to selective filters imposed by host immunity. As a result, only a narrow range of often closely related taxa are capable of host colonization, and priority effects are most likely to operate by superinfection exclusion, in which different strains are competitors [10]. In turn, priority effects allow different strains to establish permanently within a host regardless of genotype, maintaining strain diversity across a population of individual hosts due to stochasticity in the order by which strains are ingested.

Different *Caballeronia* strains are similarly beneficial to their insect hosts [4,11]. This suggests that hosts may not experience strong selection to favor particular *Caballeronia* symbionts in natural populations. The frequent occurrence of individual bugs with mixed infections alongside those with mostly single-strain infections also suggests that the bugs may not experience a fitness penalty if their *Caballeronia* communities are susceptible to regular turnover in strain-level composition. Thus, while priority effects may be important for symbiont fitness, it is unclear if they have adaptive value for the squash bug, and it may be a byproduct of mechanisms by which the host prevents pathogens and non-beneficial environmental microbes from establishing within the gut [12]. An answer to this question requires a clear understanding

of the mechanisms that generate the priority effect, which I was unable to elucidate here. Nonetheless, the ability of offspring to acquire diverse symbiont communities that harbor accessory functions [13] may allow hosts to maximize their own fitness [14]. Moreover, at the group level, populations that experience fluctuating environments may better persist when their members have more diverse symbiont communities.

Future directions. Priority effects in microbial communities, though common, emerge from interactions between their component taxa through diverse molecular and ecological mechanisms that vary idiosyncratically from one model system to the next. The involvement of a living host adds further complexity, as the underlying interactions can also occur indirectly through host-microbe communication. In this work, I have not ruled out the numerous possible changes in host anatomy, physiology, and immunity that are invisible to microscopy that occur prior to the physical closure of the constricted region. Similar findings in host-pathogen systems [15], on the other hand, indicate that microbes are able to sustain priority effects that exclude isogenic strains, presumably without the intervention of the host. Future work should address the contribution of host and microbe to the emergence of superinfection exclusion. This can also inform the utility of using engineered strains to control squash bugs in the field [16].

Further work should also address the manifestation of priority effects in natural fecal transmission. Second instar nymphs are able to acquire symbionts successfully in the laboratory by feeding on a single fecal spot, and it is possible that the behavioral switch to plant feeding and inactivity that occurs after one fecal spot feeding event can represent a behaviorally mediated priority effect that takes place even earlier than the physiological priority effects that I describe in Chapter 3. Whether this is the case, or if nymphs are likely to sample multiple fecal spots before symbiont establishment, is unknown. Finally, it is unknown if non-beneficial taxa that are

occasionally capable of infecting the symbiotic organ are also able to initiate a priority effect that excludes *Caballeronia* from host colonization. If so, this may have important consequences for using microbes as an applied strategy to lessen squash bug damage in the field.

Transmission bottlenecks govern the outcome of symbiont community assembly

Ecological drift describes random fluctuations in the relative abundance of taxa within a community over time as a result of demographic stochasticity. Microbiomes commonly undergo drastic reductions in size as a result of transmission [17–19], and in communities that exhibit strong priority effects, these reductions can have lasting effects on community composition. In Chapter 4, by feeding nymphs with GFP- and RFP-tagged strains simultaneously but varying inoculum densities, I reveal how ecological drift impacts community composition and structures strain-level diversity across scales in the squash bug-*Caballeronia* symbiosis. Although selection favors the GFP-tagged strain over its isogenic RFP strain only at high inoculum densities, drift is able to generate heterogeneity in symbiont communities as transmission bottlenecks tighten. This pattern was repeated using two strains that differed in their competitive ability. Taken together, I show that drift is strongest in communities and populations of small size, and can override the deterministic effects of selection. My results mirror those from other host-microbe systems, ranging from pathogens to commensals to specialized mutualists [20–22], underscoring commonalities in the population structure of microbial symbionts irrespective of their relationship with the host.

By feeding isogenic strains to squash bugs and examining the contents of the symbiotic organ, I next show that mixed infections are spatially structured within the host. Single crypts situated in the posterior of the M4 are dominated either by the GFP strain or the RFP strain,

rather than both. Anterior crypts, on the other hand, always contain a mixture of both GFP and RFP symbionts. This anterior-posterior gradient in the degree of between-crypt heterogeneity is robust to a nearly 10,000-fold difference in inoculum size, suggesting it may be a common feature of within-host *Caballeronia* populations. Significantly, because the symbionts in these experiments were isogenic, spatial structure within the host could only have emerged by population bottlenecks during the colonization of posterior crypts, rather than selection, mirroring the effect of drift on structuring communities between hosts.

By generating heterogeneity in community composition between hosts and between crypts within hosts, drift has important consequences on the evolution of microbe-microbe and host-microbe interactions in the context of symbiosis. Drift segregates strains into different compartments, and the persistent formation of clonal groups might facilitate the evolution of public goods cooperation in these spatially structured communities [23]. By generating heterogeneous communities of skewed composition, ecological drift can expose rare strains or communities with unique accessory functions [24] to natural selection, providing the raw material for symbiont switching and other innovations in long-term evolution. In contrast, drift can also decompose communities in which symbionts are interdependent on each other, which may force hosts to adapt by controlling bottleneck size [25].

Future directions. The results of this experiment suggest that symbiont colonization under certain conditions proceeds neutrally (i.e., distinct symbiont strains are functionally identical). This assumption could be violated in two respects. First, different symbionts may behave non-neutrally by having different colonization efficiencies at different inoculum sizes. Future studies should investigate the possibility of trade-offs in competitive ability at high inoculum density vs. ability to infect at low inoculum density. Second, I was only able to test

within-host selection in the form of microbe-microbe competition within the host, not host choice. While, under laboratory conditions, most symbiont strains in the squash bug system are functionally identical in the host benefit [4], in *Leptoglossus*, another coreid that relies on *Caballeronia*, strains have been discovered that confer a smaller degree of benefit than others [11]. Whether hosts can discriminate between more or less beneficial strains in a manner that modifies the response of community heterogeneity to increasingly tight transmission bottlenecks remains to be studied.

In light of what is now understood about natural *Caballeronia* transmission in the environment, it would be important to understand how drift and selection have opposing effects through fecal transmission. Because the bacteria are in a desiccated, and likely dormant state, it is possible that the microbe-microbe competition that I observed in my experiments (using metabolically active, growing bacteria) may not reflect how symbionts behave when ingested via feces. Moreover, it is unknown how many symbiont cells participate population establishment after fecal transmission, and this is likely to vary depending on fecal spot quality and duration of feeding.

The between-crypt heterogeneity in symbiont populations elucidated here suggests patterns of symbiont dispersal during the early stages of establishment that generate within host spatial structure. Future studies should incorporate competition between unrelated strains to understand the contribution of top-down (host factors) and bottom-up (microbial interactions) factors constraining the spatial distribution of symbionts in the host. This is especially pertinent for anterior crypts, which tend to receive both strains in isogenic infections. It is unclear whether or how unrelated strains would compete in these co-colonized crypts and whether there would be fitness costs to hosts associated with such competition. This warrants further investigation.

Moreover, it remains unclear how stable spatial structure is throughout the life of the host, and how mutations that may arise within these large, long-lived population may spread throughout the population.

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